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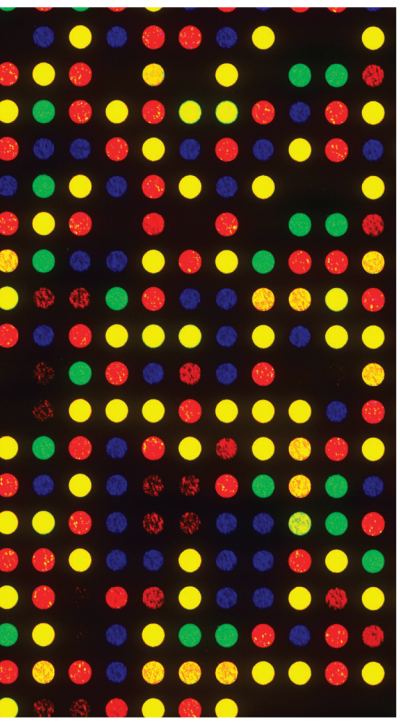
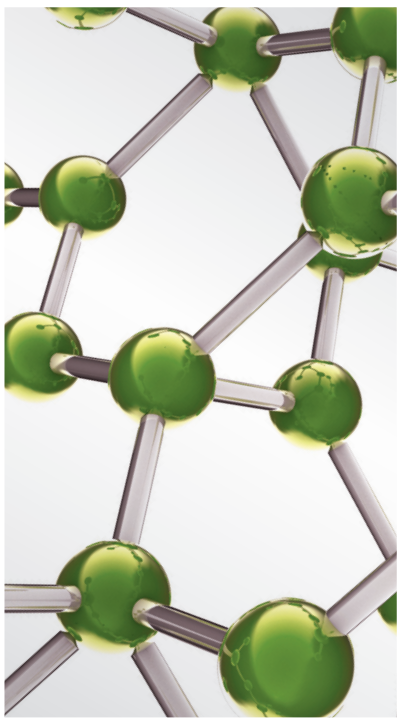
Acupuncture and Herbal Medicine for Cancer Patients

Guest Editors: S. Schröder, S. Lee, T. Efferth, and Y. Motoo



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Editorial Board

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Editorial

Acupuncture and Herbal Medicine for Cancer Patients

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1. Complementary and Alternative Medicine (CAM) in Cancer Care

In recent decades, cancer treatment has made remarkable progress with targeted therapy applied on targeted subpopulation [1]. Though the life expectancy of the general population is increasing, cancer is still one of the most common causes of death worldwide [2]. The sophisticated treatments are often accompanied with increased adverse events, and the survival rate is still unsatisfactory in some cancers. In advanced or recurrent cancers, the goal of cancer treatment is often not curing the disease but prolonging survival time with good quality of life. The health-related quality of life is getting value as a cancer outcome, and the effect of early palliative care is emphasized importantly [3]. Nowadays medical information is easily accessed by patients, and most of patients are looking for treatments with less side effects and all available information to prolong survival time and to improve quality of life. Therefore, complementary and alternative medicine (CAM) for cancer is increasingly being demanded by patients, and more physicians are getting interested in the use of CAM in cancer therapies.

The use of CAM in cancer therapies differs from country to country, but up to 80% of cancer patients use some kinds of CAM to support their conventional cancer treatments [4, 5]. In Southwestern China, the prevalence of Chinese herbal medicine use is up to 53.0% during cancer treatment [6], and it seems similar to that of Hong Kong [7]. In Taiwan, up to 98% of the cancer patients use any kind of CAM [8]. In Korea, 78.5% of cancer patients use CAM [9]. A Japanese study revealed that 44.6% of cancer patients use CAM [10], while 83% of cancer patients or cancer survivors use CAM treatments in Australia [11]. An European survey in 13 countries showed 35.9% of average CAM prevalence in cancer patients (range among countries 14.8% to 73.1%) [12]. In other parts of the world similar results were obtained [13]. CAM is also used by 31–84% of children with cancer [14]. This usage of CAM for cancer is more common among educated people with better health behaviour [15, 16] as well as among women [17]. In the case of female cancers, more patients use CAM; for example, 60–80% of breast cancer patients (in comparison to 50% among cancer patients in general) and 75% of female colon cancer patients use CAM [18].

Patients mostly use CAM as a complement to their conventional therapy, not as an alternative treatment [19]. Only very few patients replace conventional treatment by alternative medicine [20]. Even though the use of CAM is a fact worldwide, the view on CAM varies enormously, and the integration of CAM therapies into conventional therapies is extremely complicated in different countries.

Although the governmental support on the research on CAM for cancer therapies differs among various countries, the use of CAM for cancer patients is still debated in an ideological way by supporters and opponents of CAM. One of the causes for the reluctance of Western academia towards CAM is still the insufficient number of convincing clinical studies providing evidence for the efficacy and safety of CAM therapies. The Western concept of "evidence-based

medicine" does only poorly match to the clinical practice of CAM. Nevertheless, an increasing number of clinical studies are being conducted during the past years to gain credibility and reputation of CAM. Illustrative examples on the power of CAM have been documented in cancer research with a focus on three major fields [21]:

(i) acupuncture is widely applied for treating pain, a prominent side effect encountered during cancer therapy;

(ii) reduction of severe adverse effects of standard chemotherapy;

(iii) unwanted interactions of standard therapy with herbal medicines.

Another reason for critical opinions towards CAM regards the quality of herbal products according to international quality standards [22].

Therefore, an important question for recognition and implementation of CAM into general medical practice concerns the clinical evidence for efficacy and safety of CAM treatments.

This induces confusion and fear in patients and provides a burden for the communication between patient and doctor. Only 2.4% of patients used their healthcare professional record as primary source of information on CAM in cancer care [23] and 92% of American breast cancer patients withheld information about the CAM treatment from their medical oncologists [24]. Similar results were found in a survey in Taiwan, where more than two out of three cancer patients never informed their physicians of their CAM use [8]. Reasons for this might be the expected inflexibility of medical oncologists and patients' fear of harming the relationship with their oncologists caused by the use of CAM. It is not common for patients to have a communication between Western medicine-oriented doctors and CAM practitioners in clinical practice. However, the integrative cancer care is increasingly required by patients and recently it is getting popular in cancer treatment.

2. Acupuncture and Herbal Medicine for Cancer Patients

Acupuncture and herbal medicine are the most qualified CAM treatments. Their effectiveness is still under debate, although they have been used in many fields of cancer treatment and palliative care [25, 26]. The efficacy of the acupuncture and herbal treatment is not currently well recognized by the Western academia and clinical scholars, so that advantages of these therapies in treating cancer or supporting cancer treatment are not adequately reflected. One reason of this estimation is because of the quality of clinical research. Many studies still have the level of case studies [27], which seem like an anecdotal, and the generalization of CAM results might be questionable for scholars who are familiar with well-designed clinical trials.

Acupuncture and herbal medicine have been used for the treatment of cancer pain [25, 26] and for attenuation of side effects of cancer treatments. Acupuncture has been shown to be effective for chemotherapy-induced nausea and vomiting [28], as well as acupressure [29] and herbal medicine [30]. However, acupuncture has not been systematically evaluated with well-designed clinical trial for its effect on it, while new drugs got approvals from FDA for the relief of chemotherapy-induced nausea. Other studies showed an effect of acupuncture on xerostomia after radiation therapy [31, 32]. There are reports that acupuncture was effective on the chemotherapy-induced peripheral neuropathy [33] and herbal medicine was effective on the oral stomatitis [34], which lead patients to higher acceptance of Western cancer treatment with less interruption or discontinuation of therapy. Many studies were focused on the relief of cancer-related symptoms such as fatigue [35–37] and the improvement of quality of life [38].

Recently, randomized controlled trials (RCTs) have been conducted to seek evidences on acupuncture and herbal medicine [39–41]. These therapies are usually considered as supportive for major treatment, in order to attenuate side effects of surgery, radiotherapy, and chemotherapy.

One of the issues of RCTs on acupuncture and herbal medicine is how to deal with the individualized approach of treatments based on the Asian tradition. Asian herbal therapy is commonly a combination of multiple herbs; sometimes approximately up to 15 herbs are in one prescription, while a single herb already contains multiple tentative active compounds. The daily practice in acupuncture and herbal medicine with its individualized approach cannot be easily transferred into standardized controlled trials. The conclusion of systematic review studies that better qualified studies are necessary [42–44] is not so surprising. The study design to solve these issues should be developed.

Even though the effects of acupuncture and herbal medicine are still under debate and further clinical research is necessary, the clinical use of acupuncture and herbal medicine has already been recommended to control cancer-related symptoms in some of the clinical practice guidelines. According to the evidence-based guidelines of the *American College of Chest Physicians for Lung Cancer*, acupuncture has been recommended as a complementary therapy for lung cancer when pain is poorly controlled or when side effects such as neuropathy or xerostomia are clinically significant [45].

Acupuncture and herbal treatment have been used as a complementary treatment in combination with highly effective and partly aggressive Western medicine such as chemotherapy or hormonal therapy. But interactions are quite unknown, underestimated, or under debate. For instance, the herbal treatment with hormone-active herbs in patients with hormone-sensitive cells of breast or ovarian cancers is an important topic of ongoing debates [46, 47]. The interaction of acupuncture and especially herbal medicine with conventional treatments is not all known. Guidance on the safety of herbal medicine to prevent potential risks to cancer patients is necessary, but data have not yet been collected systematically in Mainland China but are now being established in Hong Kong.

In Japan, Kampo, traditional Japanese medicine, is extensively used for cancer patients as supportive measures, covered by National Health Insurance. Japanese medical doctors

can prescribe both Western and Kampo drugs, knowing the natural history of diseases and the indication and limitation of Kampo. But, there are no strong recommendations on the Kampo use based on high-quality evidence in clinical practice guidelines in Japan [48]. The Japanese medical system is a unitary one, and Kampo is practiced in this system. From this point of view, the system of Japanese traditional medicine is different from those of China and Korea, where traditional medicine is generally practiced in a dual system, but in the recent years integrative approaches were developed. Japanese Kampo practitioners take advantage of this unitary system, conducting high-quality clinical practice and research. This situation consequently leads to the integrative medicine by a single doctor, whereas the integrative medicine in other countries is usually done by a Western medicine doctor and a traditional medicine practitioner. The system of Japanese Kampo medicine well fits the methodologies of modern medicine, and many clinicians utilize Kampo, accumulating evidence data. This unified situation might be an inspiring example for countries with a unitary medical system.

In Korea, the government health insurance covers only acupuncture treatment for cancer patients. In Western countries, acupuncture and herbal medicine, in spite of frequent use, for years were not in the main focus of the medical academic society. So, research in this field was limited leading in consequence to a situation that especially treatments with Asian herbs often had a lack of scientific controls. But due to increasing interest of patients and practitioners, acupuncture had partly become an integrative therapy in pain management, and, for example, in Germany the use of Western herbs as a complementary medicine is common and Asian herbs are increasingly used.

Since herbal therapy is the most commonly used CAM treatment [49], in recent years, the search for active compounds has mainly focused on Asian herbs, whereby the emphasis has been on classical product-based leads for Western drug discovery, usually performed by screening the extracts or compounds from diverse biological sources. Many *in vivo* experiments showed effectiveness on cell cultures and in animal models [50–53], but translation from bench to bedside is still a difficult challenge. This research, mainly focusing on single active compound, has been done often without regard to preexisting knowledge of the therapeutic utility of the plant source [54]. While interactions of ingredients during the preparation procedure are sometimes essential to the therapy, an extraction of the active ingredients is often not a simple task, and evidence shows that single components extracted from plants are less potent than the crude extract [55]. Scientists of many countries worldwide have tried to apply modern experiment-based research methods to isolating active compounds from herbs, characterizing their pharmacodynamic and pharmacokinetic properties and defining their molecular modes of action with limited success. This reductionist paradigm of a "single chemical entity" is not easily applicable to the multidimensional complexity of Asian herbal prescriptions. Researchers often do not use any concepts of traditional theory as the basis for their investigations on these compounds [56].

Studies on the influence of single herbs or their components on different microbiological pathways of human physiology are necessary and important, but this research is not likely to lead to single-component treatments for multifactorial diseases such as cancer. Cancer is a systemic disease of the entire body. A single-target approach has limited effectiveness, and there is evidence that a multitarget approach might be more effective [57, 58]. It seems only rational to apply a multitargeted therapy to a multifactorial disease. The realization that multicomponent medicines may have advantages over single-component drugs has a scientific foundation. The pharmacological advantages of mixtures may lie in the potentiating action of their multiple bioactive components and the advancement of individualized therapy [59, 60].

Modern research methods on a single herb aimed at isolating active compounds from herb have to be the fundament for future researches in herbal medicine. But when basic information is found and made available, experiments with herbal combinations might be the productive direction for further research to control cancer. While cancer is a multifactorial disease with diverse heterogeneous mechanisms, a combination of components might provide a promising opportunity to focus on multiple targets. Furthermore, these efforts may eventually offer an individualized approach to the treatment. Basic research on single herbs and their active compounds is still essential for the scientific understanding of traditional herbal medicine. But research should not stop at this level but continue with research on multicompounds, their interactions, and increasing or decreasing activity in combinations. Gaining knowledge from tradition might be helpful, not ending in a dead end. This approach is ambitious and time-consuming but has a chance not to fail like conventional drug discovery procedure in the field of herbal medicine in recent years.

While it is difficult to get a patent on natural products, the further interest of pharmaceutical companies might be limited. Progress in this research area can only be found in intense national as well as international cooperation, founding international joint working groups to overcome the obstacles of this sophisticated challenge.

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Review Article

A Survey of Chinese Medicinal Herbal Treatment for Chemotherapy-Induced Oral Mucositis

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Oral mucositis is one of the common side effects of chemotherapy treatment with potentially severe implications. Despite several treatment approaches by conventional and complementary western medicine, the therapeutic outcome is often not satisfactory. Traditional Chinese Medicine (TCM) offers empirical herbal formulas for the treatment of oral ulceration which are used in adaptation to chemotherapy-induced mucositis. While standard concepts for TCM treatment do not exist and acceptance by conventional oncologists is still low, we conducted a review to examine the evidence of Chinese herbal treatment in oral mucositis. Eighteen relevant studies on 4 single herbs, 2 combinations of 2 herbs, and 11 multiherbal prescriptions involving 3 or more compounds were included. Corresponding molecular mechanisms were investigated. The knowledge about detailed herbal mechanisms, especially in multi-herbal prescriptions is still limited. The quality of clinical trials needs further improvement. Meta-analysis on the existent database is not possible but molecular findings on Chinese medicinal herbs indicate that further research is still promising for the treatment of chemotherapy-induced oral mucositis.

1. Introduction

Oral mucositis is one of the most common side effects of chemotherapy treatment with potentially severe implications. According to the American National Cancer Institute, ulcerative oral mucositis occurs in approximately 40% of patients receiving standard-dose chemotherapy [1]. Medical interventions are required in about 50% of these patients, including changes of medication or chemotherapy dose reduction. Severe mucositis symptoms occur in up to 80% in high dose chemotherapy treatments of leukaemia or in stem cell transplant regimens [1].

Mucosal damages may be induced for example, by antimetabolites such as methotrexate, 5-fluorouracil, anthracyclines such as doxorubicin and bleomycin, alkylating antineoplastic agents such as cyclophosphamide and busulfan, taxanes and the platinum complexes, including cisplatin and carboplatin [1, 2]. All of them may have possible toxic effects on rapidly dividing mucosal cells, partly related to drug secretion in the saliva. Saliva volume and consistence as well as the oral microbial flora may be altered, affecting the mucosal metabolism [3]. Several molecular mechanisms are involved in the pathogenesis of mucositis, such as oxidation and apoptosis mediated by nitric oxide (NO), cyclooxygenase

(COX), protein kinases, cytokines, and nuclear factors [4]. The research field involves also genetic-based risk factors [5]. Epigenetic changes of DNA methylation are discussed as being responsible for inflammatorial precancerous conditions [6]. A cancer diagnosis itself may lead to posttraumatic stress disorder (PTSD), causing depression and anxiety as well as an increased level of biomarker expression, such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), cortisol, and high-reactive sensitive C-reactive protein. The incidence of oral ulceration was associated with the level of PTSD. These effects were observed to be significantly higher in malignant than in benign diagnosed breast tumor patients [7].

Typical manifestations of oral mucositis are soreness, edema, erythema, ulcerations, bleeding, pain, difficulties in swallowing and possible alteration of taste, and they may severely affect the patient's quality of life. Impaired nutrition and complications by viral, bacterial, or mycotic infections may additionally increase the risk of anticancer treatment delay [8]. Mucositis grading is based on clinical aspects and the nutritional state, as seen in Table 1. Grades III and IV are considered as severe mucositis [9, 10] and in more than one-third of these patients the next chemotherapy cycle needs to be delayed leading to a possible deterioration of treatment [11].

When uncomplicated by infections, mucositis may be self-limiting in about 2 to 4 weeks [1]. Mucosal damages and local factors such as periodontitis and suboptimal oral hygiene increase the risk of infections. Systemic exacerbation is facilitated by the commonly decreased immunological status [12, 13]. Consequences are an increased mortality risk as well as a prolonged hospitalisation, including the necessity of fluid replacement and parenteral nutrition, causing an increase of costs [9].

1.1. Preventive and Treatment Methods by Conventional Medicine. The detailed guidelines for prevention and treatment of mucositis are depending on the chemotherapy regimens used in each case. General prevention instructions include prior dental examinations and treatment and optimal oral care [10, 16], as well as avoidance of spicy, hard and hot foods and saline-peroxide mouthwashes [16].

There are numerous western experimental preventive and therapeutical interventions for oral mucositis. Updated clinical practice guidelines for the prevention and treatment of mucositis were published by the Mucositis Study Section of the Multinational Association of Supportive Care in Cancer and the International Society for Oral Oncology (MASCC/ISOO) in 2007 [10], suggesting the use of keratinocyte growth factor-1 (KGF-1) for preventing mucositis in high-dose chemotherapy regimens. Cryotherapy was suggested for melphalan, 5-fluorouracil, and etidronate. Systemic glutamine was not recommended because of severe toxicity. Mouthwashing with granulocyte-macrophage-colony stimulating factor (GM-CSF) did not show consistent effect. Updated results are frequently published by MASCC/ISOO [17]. According to a Cochrane review from 2010, nine interventions for prevention and treatment of mucositis showed statistical benefit: allopurinol, amifostine, cryotherapy, intravenous glutamine, honey, KGF-1, laser, aloe vera, and polymixin/tobramycin/amphotericin (PTA) antibiotic pastille or paste compared to either placebo or no treatment [18]. The updated Cochrane review, published in February 2013, came to the conclusion that cryotherapy and keratinocyte growth factor had some benefits in preventing mucositis and sucralfate showed effects in reducing the severity of mucositis. Aloe vera, amifostine, granulocyte growth factor, honey, laser, and PTA did not show consistent effects [19].

Even concerning the progress achieved during the last years, chemotherapy-induced oral mucositis continues to be a challenge for a positive cancer treatment outcome [2, 18, 19]. The development of further treatment options for oral mucositis remains an important research objective.

1.2. Complementary Medicine with Western Herbs. In western complementary medicine several herbal treatment approaches are existent, including *Salvia officinalis, Camomilla matriciana, Calendula officinalis, Hamamelis virginiana, Tormentilla rhizome, Commiphora molmol, Rhataniae radix, Myrtilli fructus, Althaea, Malva, Cetraria islandica, Linum usitatissimum, Caryophylli flos, Hippophea rhamnoides, Aloe vera, Carica papaya, Centaurii herba, Gentianae radix, Menyanthis folium, Eriodictyon crassifolium, Oleum olivae, and Citrus limon*. They are applied as single infusions for gargling or topical application [2]. Of these, Salvia officinalis, Chammomilla matriciana, Aloe vera, and Gentianae radix have also been used in the tradition of TCM.

Up to 80% of cancer patients use some kinds of Complementary and Alternative Medicine (CAM) therapies to support their conventional cancer treatments [20, 21]. Herbal treatment is the most frequently used CAM therapy and many of the used herbs originate from TCM [22, 23]. TCM offers empirical herbal formulas for treating mouth ulcers and stomatitis which have frequently been used in complementary treatment of oral mucositis in the last decades, but the evidence of these therapies is unclear. While standard concepts for this kind of treatment do not exist and acceptance by conventional oncologists is still low, we conducted this review to critically examine the evidence of Chinese herbal treatment in oral mucositis.

2. Methods

2.1. Objective. The objective of this article is to examine the role of Chinese herbal medicine approaches to oral mucositis in search of adjuvant treatment options for minimizing a painful and risky side effect of chemotherapy as a potential cooperation of western and Chinese medicine.

2.2. Search Strategy and Selection Criteria. Electronic searches of PubMed, MEDLINE via OVID, EMBASE via OVID, Cochrane Database, CNKI and reference lists of relevant articles were undertaken. The mesh-terms used were chemotherapy, chemotherapy-induced, oral ulcer, mouth ulcer, oral mucositis, stomatitis, Chinese herbal

Table 1: WHO oral toxicity scale [14, 15].

Grade 0	Grade I	Grade II	Grade III	Grade IV
(None)	(Mild)	(Moderate)	(Severe)	(Life-threatening)
None	Oral soreness, erythema	Oral erythema, ulcers, solid diet tolerated	Oral ulcers, liquid diet only	Oral alimentation impossible

medicine, medicinal herbs, Chinese herbs, traditional Chinese medicine, antioxidant, anti-inflammatory. All interventional clinical trials concerning chemotherapy-induced oral mucositis treated by Chinese herbal medicine that offered an English or Chinese abstract were reviewed. Articles investigating herbal or animal products used in the tradition of Chinese medicine were included. Articles investigating radiochemotherapy-induced oral mucositis were excluded, unless the chemotherapy-related results were separately processed.

2.3. Data Analysis. No meta-analysis was effected since there were reservations with regard to the high risk of bias due to inadequate study designs and a diversity of herbal formulas. We limited the discussion to the comparison of single herbs and herbal formulas and to the quality of studies which have to date been set up with regard to this topic.

3. Results

A total of 686 articles were retrieved from electronic searches and from examination of reference lists of clinical and review articles. After screening titles and/or abstracts, 632 articles were excluded since the focus was either on an intervention rather than on oral mucositis and Chinese herbal treatment or they were duplicated studies or not relevant. From a total of 54 articles which were retrieved for detailed evaluation, 18 studies representing 1,476 patients met the selection criteria and were included in the review, focusing on 4 single herbs, 2 combinations of 2 herbs, and 11 multi-herbal prescriptions involving 3 or more components. For a summary of the investigated studies, see Table 2.

4. Single Herbs or Single Herbal Compounds

4.1. Evodiae fructus. In terms of TCM theory, *Evodiae fructus* has been used for nausea and pain caused by cold exposition [24–26]. Evodiamine, a major compound of *Evodiae fructus*, was found to inhibit inducible nitric oxide synthase (iNOS) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, as well as the cyclo-oxygenase-2 (COX-2) expression, hypoxia-inducible factor 1-alpha (HIF-1-α) accumulation and prostaglandin E-2 (PGE-2) synthesis, and interferon-gamma (INF-γ) mediated processes in murine 264.7 macrophage-like cell lines [27].

Application of *Evodiae fructus* extract on acupoint Yongquan (KID1) was reported to have positive effects on mucositis symptoms in 92% of chemotherapy patients in an uncontrolled case study ($n = 50$) measured by subjective scales [28]. Ulcer grading has not been mentioned [28]. The result requires further investigation with higher-quality study designs.

4.2. Rhodiola algida. *Rhodiola* subspecies are used as common tonics in various Asian regions. In Tibetan medicine, it has an ancient tradition for its antifatigue effects, as well as for cardiovascular diseases, pneumonia, and hemoptysis [29]. *Rhodiola algida* is externally used for injuries, burns, and scalds [29] and has shown immunomodulatory effects by interleukin 2 (IL-2) regulation in Th-1 cells and interleukin 4, 6, and 10 (IL-4, IL-6, IL-10) regulation in Th-2 cells [30]. In a randomised, controlled, two-armed clinical study on breast cancer patients ($n = 130$; treatment: $n = 65$; control: $n = 65$) [31], oral administration of *Rhodiola algida* resulted in faster recovery of white blood cell count as well as fewer and smaller ulcers compared to the non-treatment control group, judged by clinical measurement. Furthermore, positive effects of *Rhodiola algida* on lymphocyte proliferation was reported while animal feeding with *Rhodiola algida* did not show toxic effects [31]. These findings are promising, but so far no further trials confirming the results have been found.

4.3. Catechu from Acacia catechu. *Catechu* is an extract of *Acacia catechu* which is clinically used for tissue regeneration, wound healing, sores and abscesses as well as mouthwash for oral ulcers [26, 32]. In combination with *Scutellariae baicalensis radix*, anti-inflammatory effects are reported in animal and human immortalized cell lines [33]. Local application of Catechu powder had a superior effect on oral mucositis compared to local norfloxacin application in a randomized, controlled, two-armed clinical study on chemotherapy patients ($n = 60$; treatment: $n = 30$; control: $n = 30$), judged by clinical measurement [34]. Amelioration occurred in all patients (100%) treated with *Catechu* and in 73.3% of the norfloxacin group. Further trials confirming this result have not been found.

4.4. Kangfuxin from Periplaneta americana. Kangfuxin is an ethanolic extract of *Periplaneta americana*, used for its anti-inflammatory and wound healing qualities in ulcerative and inflammatory diseases, including recurrent aphthous ulcers [35]. Regulating effects of the cytokine expression IL-4, interleukin 5 (IL-5), IFN-γ, and TNF-α by decreased gene expression have been reported [36]. In a randomized, controlled, two-armed clinical study on chemotherapy patients ($n = 64$; treatment: $n = 26$; control: $n = 38$), topical Kangfuxin application showed an oral mucositis rate of 36%, compared to saline gargling with a mucositis rate of 84% [37]. This result as well requires verification by more trials with high-quality study designs.

5. Herbal Combinations

5.1. Lonicerae flos Plus Glycyrrhizae radix. Lonicerae flos and *Glycyrrhizae radix* are two important ingredients of the traditional herbal formula *Yin Qiao San (Chinese) (Honeysuckle and Forsythiae Powder (English), Pulvis Lonicerae et Forsythiae (Latin))* from *Wen Bing Tiao Bian* (Systematic Differentiation of Warm Diseases) [38]. It is used in inflammatory conditions including those of skin and mucosa [39, 40]. In TCM tradition, *Lonicerae flos* is used for abscesses, swelling, ulcers and erysipelas [24–26]. It offers antioxidant properties, suppressing interleukin 1-beta (IL-1β), IL-6, and COX-2 gene expression in human umbilical vein cells [41]. In traditional treatment, *Glycyrrhizae radix* is known to be useful for abscesses, scores, and ulcer treatment [24–26]. Anti-inflammatory effects are transmitted by variant compounds. Glycerol inhibits lipopolysaccharide (LPS)-induced NF-κB, IL-1, and IL-6 mRNA activation, while liquiritigenin was found to inhibit the activation of NF-κB in macrophages, decreasing iNOS and proinflammatory cytokines by inhibition of LPS-induced NF-κB DNA binding activity [42, 43].

This herbal combination has been investigated by two authors. In one randomized, controlled, two-armed clinical trial, the combined topical and internal use was compared to borax mouth washing in chemotherapy patients ($n = 190$; treatment: $n = 97$; control: $n = 93$). Mucositis symptoms were found in 19.4% of treatment group patients and in 85.2% of control group patients, measured by the WHO scale for mucositis [44]. The study did not establish a control for the internal use of herbal medicine, comparing a dual-mode treatment to a single control. In another clinical trial on chemotherapy patients ($n = 86$; treatment: $n = 43$; control: $n = 43$), herbal gargling solution was compared to hydrogen peroxide mouth washing [45]. Positive effects on mucositis symptoms were found in 95.3% of the herbal treatment group and 76.7% of the control group, measured by a subjective scale. Further investigation by high-quality studies is required.

5.2. Lonicerae flos Plus Glycyrrhizae radix Plus Astragali membranacei radix. In this formula, *Astragali membranacei radix* is used in addition to the herbs described above, *Lonicerae flos* and *Glycyrrhizae radix*. In traditional use, *Astragali membranacei radix* clears toxicity and is used for abscesses and promoting skin regeneration [24–26]. It has anti-inflammatory and immunoregulatory effects due to IL-2 and IFN-γ release and IL-4 and iNOS suppression [46].

Topical use of this triple combination on oral mucositis was effected in a randomized, controlled, two-armed clinical trial ($n = 97$; treatment: $n = 50$; control: $n = 47$), with positive results compared to *Dobell's solution* (sodium borate, sodium bicarbonate, phenol and glycerol) [47]. The herbal treatment group showed an overall lower grade of ulcers. 30% did not show any mucositis symptoms while only 2% suffered from severe ulcers. In the *Dobell's solution* control group, 5% had remained without ulcers while 21.3% suffered from severe ulcers. There was no statistical difference in the duration of healing period. In addition to the investigated drugs, all patients received a basic treatment by intravenous injections of the Chinese herbal preparations *Shengmai* (*Ginseng radix, Ophiopogonis radix, Schisandrae chinensis fructus*), *Shenqi Fuzheng* (*Codonopsis radix, Astragali membranacei radix*), *Ai Di* (*Acanthopanacis radix, Astragali membranacei radix, Ginseng radix*) and *Cantharidin*, as well as vitamin supplements not further described, and tetracaine solution for local application. In total, this is a strongly tonifying herbal regime. Regarding this treatment by applying several intravenously administered herbs, significant conclusions on the investigated herbs of the gargle solution cannot be drawn due to various potential interactions between the single components.

5.3. Lonicerae flos Plus Ophiopogonis radix Plus Platycodonis radix. Lonicerae flos and *Ophiopogonis radix* are part of the classical herbal combination called *Qing Ying Tang (Chinese), Clear the Ying Level Decoction (English), Decoctum Refrigerationis Qi Constructivi (Latin)* from *Wen Bing Tiao Bian* (Systematic Differentiation of Warm Diseases) [38], which is composed by *Rhinoceri cornu, Rehmanniae viridae radix, Scrophulariae radix, Lophatheri folium, Lonicerae flos, Forsythiae fructus, Coptidis rhizoma, Salviae miltiorrhizae radix* and *Ophiopogonis radix*. It is used for certain forms of fever accompanied by a dry mouth [39, 40]. *Ophiopogonis radix* and *Playcodonis radix* are combined in *Bai He Gu Jin Tang (Chinese), Lily Bulb Decoction To Preserve The Metal (English), Decoctum Firmans Metallum cum Lilio (Latin)* from *Yi Fang Yi Jie (Analytic Collection of Medical Formulas)* [57], used for dry pharyngitis and bronchitis [39, 40]. Both formulas have other principal herbs whose effects are assisted by *Lonicerae flos, Ophiopogonis radix* and/or *Platycodonis radix* [39, 40].

In TCM theory, *Ophiopogonis radix* is used for sore throats and dry coughs [24–26]. There are scarce reports about single molecular mechanisms of *Ophiopogonis radix*. Opaw-2, a compound from *Ophiopogonis radix*, showed dose-dependent stimulation of lymphocyte proliferation in vitro [58]. *Platycodonis radix* had been traditionally used for relieving soreness, expelling pus and for treating general skin and mucosal diseases [24–26]. *Platycodonis radix* Saponins derived from *Platycodonis radix* showed anti-inflammatory effects via inhibition of COX-2, TNF-α, and PGE2 expression, as well as reduction of inflammatory markers like the number of leukocytes and neutrophiles and edema [59].

For *Lonicerae flos*, see above.

This triple combination was investigated in a randomised, controlled, two-armed clinical trial on chemotherapy patients ($n = 65$; treatment: $n = 30$; control: $n = 35$). The treatment group patients received oral herbal administration while the control group patients used furacilin mouth washing and received oral administration of vitamins B1 and C and daily intravenous infusions of metronidazol. Basic treatment was performed by saline gargling accompanied by diet instructions. The herbal treatment group showed improvement of ulceration in 93%, compared to 73.8% in the control group, classified by subjective scales of ulcer size [48]. In this trial, diversified control interventions impede the comparability to the treatment intervention.

Table 2: Clinical trials for chemotherapy-induced mucositis.

No.	Author/year	Formula	Drug application	Study design	Ulcer classification	Control group	Random/blinding	Effect/control, case number
4.1	Xu and Han 2006 [28]	*Evodiae fr.*	Acupoint application	Case study	No	No	No/No	92% improved/no control $N = 50$
4.2	Loo et al. 2010 [31]	*Rhodiola algida*	Oral administration	Randomized group comparison	WHO scale	Basic treatment	Yes *5/No	Improved (ulcer size and number) $N = 130$
4.3	Shi and Shan 2009 [34]	*Catechu*	Topical application	Randomized group comparison	Clinical judgement	Topic norfloxacin powder	Yes *5/No	100%/73.3% improved $N = 60$
4.4	Wang et al. 2010 [37]	*Periplaneta americana*	Gargle solution	Randomized group comparison	WHO scale	Saline gargle	Yes *5/No	64%/16% improved $N = 64$
5.1a	Ma and Song 2005 [44]	*Lonicerae fl. Glycyrrhiza r.*	Oral administr. + gargle solution	Randomized group comparison	WHO scale	Boxax gargle	Yes *5/No	80.6%/14.8% improved $N = 190$
5.1b	Zeng 2005 [45]	*Lonicerae fl, Glycyrrhiza r.*	Gargle solution	Randomized group comparison	Clinical judgement	Peroxide gargle	Random number table/No	95.3%/76.7% improved $N = 86$
5.2	Bao et al. 2008 [47]	*Lonicerae fl. Ophiopogonis r. Astragali memb. r.*	Gargle solution	Randomized group comparison	WHO scale	Dobell's solution gargle	Yes *5/No	30%/5% improved for ulcer grading, no effect on healing time $N = 97$
5.3	Chen et al. 2005 [48]	*Lonicerae fl. Ophiopogonis r. Platycodonis r.*	Oral administration	Randomized group comparison	Ulcer size	Furacilin gargle oral vit B1 + C metronidazol infusion	Yes *5/No	93%/73.8% improved $N = 65$
5.4	Wu Zhu YF et al. 2009 [49]	*Lonicera fl. Glycyrrhiza r. Menthae hapl. h. Chrysanthemi fl. Ganoderma luc.*	Topical ice cube application	Randomized group comparison	WHO scale	Dobell's solution gargle	Yes *5/No	86.9%/60.9% improved $N = 217$
5.5	Wang et al. 2006 [50]	Sheng Mai San *1	Intravenous injection	Randomized group comparison	Clinical judgement	Dexamethasone MCP ondansetr. injection	Yes *5/No	94.5%/71.5% improved $N = 71$
5.6	Jin et al. 2009 [51]	*Bubali cornu, Callicarpae mac. fol.*	Oral administer. + gargle solution	Randomized group comparison	WHO scale VAS QOL	Saline, gentamycin, tetrahydrofolate gargle/oral oryzanol	Yes *5/No	93.3%/93.3%/7.8% improved $N = 88$
5.7	Hou et al. 2001 [52]	*Phellodendri c. Forsythiae fr. Galla chin. Verbenae h. Catechu* Borneolum + saline	Gargle solution	Randomized group comparison	Clinical judgement	No	Yes *5/No	96.2%/76.1% improved $N = 101$

Table 2: Continued.

No.	Author/year	Formula	Drug application	Study design	Ulcer classification	Control group	Random/blinding	Effect/control, case number
5.8/5.9	Zhu and Zhang 1993 [53]	*Yu Nu San*[*]2/*Qing Wei San*[*]3	Oral administr.	Case study, clinical allocation to group 1 or 2	Clinical judgement	No	No/No	83.9 improved/no control $N = 31$
5.10/5.11	Zhou et al. 2005 [54]	*Chrysanthemi fl. Gardeniae fr Hypericum perf. Scrophulariae r. Sophora tonk. r./Borax, Borneolum, Indigo naturalis*	Oral administration + gargle solution	Randomized group comparison	Ulcer size, quantity	Vit B12, gentamicin and sodium bicarbonate gargle	Yes [*]5/No	96.7% improved, shorter healing period/86.7% improved $N = 60$
5.12	Chen and Zheng 2005 [55]	*Codonopsitis r. Atractylodis mac. rh. Agastachis h. Glycyrrhiza r. Dioscoreae rh Astragali memb. r. Angelica sin. r. Alismatis rh. Lopatheri h. Rehmannia vir. r.* + *Centrum Wyeth*	Oral administr. + gargle solution	Randomized group comparison	WHO scale	Vit B2	Envelope lottery/No	78.8%/33.3% improved $N = 66$
5.13	Sun 2007 [56]	Modified *Xie Huang San*[*]4 + added modules	Oral administr.	Randomized group comparison	WHO scale	Vit B2 and Vit C	Yes [*]5/No	98%/72% improved $N = 90$

[*]1: *Ginseng radix, Ophiopogonis radix, Schisandrae chinensis fructus.*
[*]2: *Gypsum fibrosum, Rehmanniae viridae radix, Anemarrhenae rhizoma, Ophiopogonis radix, Achyranthis bidentatae radix.*
[*]3: *Coptidis rhizoma, Cimicifugae rhizoma, Rehmanniae viridae radix, Moutan cortex, Angelicae sinensis radix, Achyranthis bidentatae radix.*
[*]4: *Gypsum fibrosum, Saposhnikoviae radix, Gardeniae fructus, Agastachis herba, Glycyrrhizae radix, Astragali membranacei radix, Atractylodis macrocephalae rhizoma, Dendrobii herba, Lophatheri herba, Hedyotidis herba, Taraxaci herba, Coptidis rhizoma, Ginseng radix*, plus added modules.
[*]5: Randomisation process not described.

5.4. Lonicerae flos Plus Glycyrrhizae radix Plus Chrysanthemi flos Plus Ganoderma lucidum Plus Menthae haplocalycis herba. A *Lonicerae flos*, *Glycyrrhizae radix* and *Menthae haplocalycis herba* combination is found in *Yin Qiao San* [39, 40] as described above. A *Chrysanthemi flos*, *Glycyrrhizae radix* and *Menthae haplocalycis herba* combination is found in *Sang Ju Yin (Chinese), Clear Wind Heat Tea (English)i Potio Mori et Chrysanthemi (Latin)* from *Wen Bing Tiao Bian* [38] which contains *Mori folium*, *Chrysanthemi flos*, *Armeniacae semen*, *Forsythiae fructus*, *Menthae haplocalycis herba*, *Platycodi radix*, *Glycyrrhizae radix*, and *Phragmitis rhizome* and is used in beginning fever and coughing [39, 40]. *Ganoderma lucidum* has not been found in these classical combinations.

In TCM theory, *Chrysanthemi flos* is used for septic wounds and abscesses [24–26]. *Chrysanthemi flos* compounds inhibit NO, PGE-2, TNF-α, and IL-1β production, as well as iNOS and COX-2 expression in LPS-induced macrophages [60]. *Ganoderma lucidum* showed positive effects on intestinal epithelium healing [61] and refractory diabetic wounds [62] and was found to decrease NO, PGE-2, and proinflammatory cytokine production, including IL-1β, TNF-α and NF-κB in microglia [63]. *Menthae haplocalycis herba* in TCM theory is used for mouth sores, exanthema, and itching [24–26]. It showed antimicrobial activity against streptococcus mutans [64] and attenuated histamine release and PGD-2 synthesis in mast cells [65].

For *Lonicerae flos* and *Glycyrrhizae radix*, see above.

The decoction of *Lonicerae flos, Glycyrrhizae radix, Ganoderma lucidum, Chrysanthemi flos*, and *Menthae haplocalycis herba* was prepared for local ice cube application and compared to ambient tempered *Dobell's gargle solution* (sodium borate, sodium bicarbonate, phenol, and glycerol) in a randomised, controlled, two-armed clinical trial on gynaecological tumor patients receiving 5-FU chemotherapy ($n = 217$; treatment: $n = 84$; control: $n = 133$) [49]. The mucositis incidence resulted in 13.1% in the herbal treatment group and in 39.1% in the control group. The study design did not consider the fact that single ice application is known to have a preventive effect on oral mucositis in patients treated with 5-FU [1, 66], as there was no similar application design for both groups.

5.5. Shengmai San (Chinese), Generate the Pulse Powder (English). Shengmai San contains *Ginseng radix, Ophiopogonis radix,* and *Schisandrae chinensis fructus* and originates from *Yu Xue Qi Yuan (Expounding on the Origins of Medicine)* [67]. It is used in multiple clinical patterns including cardiovascular and neurologic disorders, diabetes, and cancer for its tonifying and yin nourishing properties [39, 40]. Regarding the single compounds, there are multiple reports about anti-inflammatory effects of ginsenosides by inhibition of proinflammatory cytokines and other mediators of inflammation including iNOS, NO, IF-γ, COX-2, NF-κB, and TNF-α [68]. Ginsenoside Rd showed wound healing effects on skin level, increasing the proliferation and migration of keratocyte progenitor cells and dermal fibroblasts by cyclic adenosine monophosphate (cAMP) induction via 9-β-d-arabinofuranoside attenuation [69]. *Schisandrae chinensis fructus* in terms of TCM theory is astringent and preserving fluids [24–26]. Schisandrin B was found to inhibit ataxia telangiectasia and Rad3-related (ATR) protein kinase activity following DNA damage by inhibition of phosphorylation processes [70]. A hydrophobic fraction of dried *Schisandrae chinensis fructus* was found to suppress IL-β-induced NO and iNOS expression, as well as the transcription of IL-1β and inflammatory cytokines [71].

For *Ophiopogonis radix*, see above.

In a randomised, controlled, two-armed clinical trial ($n = 71$; treatment: $n = 36$; control: $n = 35$) on acute chemotherapy toxicity regarding primarily white blood cell and platelet counts as well as nausea, vomiting, and oral mucositis, intravenous *Shengmai* injection was compared to dexamethasone, metoclopramide, and ondansetron injection (which targeted nausea and vomiting rather than mucositis due to the ampler study design). Oral mucositis occurred in 5.5% of the treatment group and in 28.5% of the control group [50]. The control intervention of this trial was not specific for oral mucositis.

5.6. Bubali Cornu and Callicarpae macrophyllae folium. Bubali cornu is frequently found as modern substitute for *Rhinoceri cornu* (forbidden due to the Convention on International Trade in Endangered Species of Wild Fauna and Flora, also known as Washington Convention) in the classical formula *Qing Ying Tang* (see above). There are no classical formulas combining these two drugs, but this is a modern empirical combination called *Shui Zhong Cao Tang Ji (Chinese), Water Grass Decoction (English)*. In terms of TCM theory, *Bubali cornu* has effects similar to *Rhinoceri cornu*, used for febrile diseases, exanthema, and convulsions [24–26]. It has shown antipyretic and antioxidant effects on proteins and inhibition of TNF-α-induced PGE2 production, as well as protection against hydrogen peroxide (H_2O_2)-induced injuries in rat cerebral microvascular endothelial cells [72]. *Callicarpae macrophyllae folium* is reported to be used in TCM tradition for bleedings, hematoptysis, and hematemesis [26]. Anti-inflammatory, antimicrobial, and analgesic effects of *Callicarpae macrophyllae folium* are reported but not yet fully investigated [73].

This formula was topically and internally administered in a randomised, controlled, three-armed clinical trial ($n = 88$; treatment: $n = 30$; first control: $n = 30$; second control: $n = 28$) to chemotherapy patients, compared to topical application of gentamycin, tetrahydropholate, and saline gargling (first control) and to oral oryzanol administration (second control) [51]. There was a mean better outcome of curative voted cases from herbal treatment to the gentamycin/tetrahydropholate/saline control group (53.3% to 50.0%). General improvement of oral mucositis symptoms was seen in 93.3% of both groups. Oral mucositis symptoms remained in 92.2% of the oryzanol group, showing an advantage of the two former regimens compared to oxyzanol administration alone. The comparison of the combined

topical and internal use to a single control intervention does not allow significant conclusions.

5.7. Huang Wu Shu Kou Ye (Chinese), Yellow Five Decoction (English) (Phellodendri Chinensis Cortex, Forsythiae fructus, Verbenae officinalis herba, Borneolum, Galla chinensis, and Catechu). The name of this empirical prescription may be borrowed from *Dang Gui Liu Huang Tang, Angelica Six Yellows Decoction (English), Decoctum Angelicae Sinensis et sex Luteorum (Latin)* from *Lan Shi Mi Cang* (Sectrets from the Orchid Chamber) [74], as *Phellodendri chinensis cortex* is one of the contained 6 yellow coloured drugs (*Rehmanniae viridae radix, Rehmanniae preparata radix, Coptidis rhizoma, Scutellariae baicalensis radix, Phellodendri chinensis cortex, and Astragali membranacei radix*). The combination of *Phellodendri chinensis cortex, Forsythiae fructus, Verbenae officinalis herba, Borneolum, Galla chinensis*, and *Catechu* has not been found in classical prescriptions.

Phellodendri chinensis cortex is traditionally considered as anti-toxic and is used for abscesses and sores [24–26]. It was found to inhibit TNF-α, IL-1β, and iNOS production, as well as phosphorylation of extracellular-signal regulated kinases (ERK) and NF-κB activation in microglia cells [75]. *Forsythiae fructus* has a traditional use as anti-toxin as well as for erysipelas and abscesses [25–27]. A *Forsythiae fructus* compound, arctiin was found to decrease proinflammatory cytokine production including IL-1β, IL-6, TNF-α, and PGE-2, as well as NF-κB and co-stimulating molecules such as peripheral membrane protein B7-1 and B7-2 in mouse leukaemic monocyte macrophage cell line (RAW 264.7) cells [76]. *Verbenae officinalis herba* in TCM terms removes toxicity and is used for sores and boils as well as pharyngitis [26, 77]. *Verbenae officinalis herba* extractions were found to possess antioxidant, anti-inflammatory, and wound-healing properties [78]. Borneolum is a crystal steam distilled product of *Cinnamomum camphora* [26]. It is used externally for mouth sores, ulcerations, and wounds [24–26] and showed anti-inflammatory and antioxidant cell protective effects by decreasing iNOS expression, NO, and inflammatory factor release, as well as NF-κB translocation and caspase-related apoptosis in an ischemic/reperfusion neuron model [79]. *Galla chinensis* in TCM theory astringes, promotes wound healing and is used for ulcers and edema [24–26].

For *Catechu*, see above.

A gargle solution composed by these 5 herbs as well as saline gargle solution was administered in a randomised, controlled, two-armed clinical trial ($n = 101$; treatment: $n = 53$; control: $n = 48$) to chemotherapy patients, compared to *Borax* solution gargling alone. All patients received basic treatment with antibiotics and vitamin supplements not further described. Improvement on mucositis symptoms was seen in 96.2% of the treatment group and 76.1% of the control group, judged by subjective clinical scales [52]. Significant conclusions on this result cannot be drawn as antibiotic regimens may have interfered with possible anti-inflammatory effects of the herbal solution used at the same time.

5.8. Yu Nu Jian (Chinese), Jade Woman Decoction (English). This classical TCM formula containing *Gypsum fibrosum*, *Rehmanniae viridae radix*, *Anemarrhenae rhizoma*, *Ophiopogonis radix*, and *Achyranthis bidentatae radix*, is described in *Collected Treatises of Zhang Jing Yue* [80]. It is used for inflammatory diseases including oral ulcerations [39, 40]. *Gypsum fibrosum* is traditionally used for burns and ulcers [24, 25] as well as for fever [24–26], and it showed antipyretic activity demonstrated against LPS-induced pyrexia in rats, while calcined Gypsum and $CaSO_4$ did not have this effect [81]. *Rehmanniae viridae radix* had been traditionally used for exanthema, abscesses, and sore throats [24–26]. Compounds of *Rehmanniae viridae radix* were found to inhibit NO production and iNOS, PGE-2, IL-6, and COX-2 expression in RAW 264.7 macrophages [82]. *Anemarrhenae rhizoma* had been traditionally used for dry coughs and infectious diseases [24–26]. Nyasol, a compound of *Anemarrhenae rhizoma*, was found to reduce NO and PGE-2 production as well as mRNA-levels of TNF-α and IL-1β in LPS-stimulated microglia cells. P38 mitogen-activated protein kinase (p38MAPK) was inactivated and LPS-induced I-κBα degradation was suppressed [83]. *Achyranthis bidentatae radix* calms bleeding from the mucosa in TCM theory and is used for mouth soreness, epistaxis, and hematemesis [24–26]. *Achyranthis bidentatae* polysaccharides derived from *Achyranthis* were found to positively modulate murine dendritic cell maturation by cell surface molecules CD86 and CD40 and major histocompatibility complex II (MHC II) enhancement and increase IL-12 production, indicating a possible immune boosting effect [84].

As *Yu Nu Jian* was investigated with *Qing Wei San (see below)* in one study, oral mucositis related results are stated below.

5.9. Qing Wei San (Chinese), Clear the Stomach Powder (English). This classical TCM formula contains *Coptidis rhizoma*, *Cimicifugae rhizoma, Rehmanniae viridae radix, Moutan cortex, Angelicae sinensis radix*, and *Achyranthis bidentatae radix*. It is described in *Lan Shi Mi Cang* (Secrets from the Orchid Chamber) [74] and used for gingivitis and inflammations of tongue and lips.

In terms of TCM theory, *Coptidis rhizoma* is regularly used in inflammatory and septic processes [24–26]. It was found to inhibit IL-1α, IL-6, and granulocyte macrophage colony-stimulating factor (GM-CSF) secretion, iNOS expression, and NO production in RAW 264.7 macrophages [85]. *Cimicifugae rhizoma* in TCM terms removes toxicity and is used in exanthema, mucosal inflammation, and ulceration [24–26]. It was found to reduce LPS-induced release of IL-6, TNF-α, IFN-γ, and stimulation of IL-8 in LPS-induces human blood cells [86]. *Moutan cortex* in TCM terms is used in cases of inflammation and mucosal bleeding [24–26]. It was found to inhibit the activation of several inflammation-related genes in gingival fibroblasts [87]. *Angelica sinensis radix* is used for sores, ulcers, and abscesses in TCM theory. Ligustilide, an *Angelica sinensis radix* compound, was found to suppress NO production, PGE-2, and TNF-α in LPS-stimulated RAW 264.7 macrophages, to decrease activator

protein-1 (AP-1), iNOS and NF-κB activation, phosphorylation of IκB kinase (IKK), MPAKs, ERK1/2, and c-Jun-N-terminale kinase (JNK) and to downwardly regulate intracellular reactive oxygen species (iROS) [88].

For *Rehmanniae viridae radix* and *Achyranthis bidentatae radix*, see above.

Both *Yu Nu Jian (see above)* and *Qing Wei San* were investigated in an uncontrolled case study on leukaemia patients ($n = 31$), clinically allocated into two groups according to the criteria of exuberance or deficiency by terms of TCM theory. *Yu Nu Jian* was administered to the exuberance group while *Qing Wei San* was administered to the deficiency group. Additional topical medication was effected in all patients without further description. Ulcer grading was clinically judged. Reported without group differentiation, 7 patients obtained very good results and 19 patients offered good results on mucositis symptoms, indicating an improvement of 83.9% in summary [53]. In the context of a scientific study, the simultaneous use of different formulas precluded significant results. The findings require further investigation by higher-quality trials.

5.10. Chrysanthemi flos Plus Gardeniae fructus Plus Hyperici perforati herba Plus Scrophulariae radix Plus Sophorae tonkinensis radix Combination. Classical combinations of these herbs have not been found. Obviously, the formula was empirically composed with the purpose of obtaining anti-inflammatory and wound-healing effects. In terms of TCM theory, *Gardeniae fructus* had traditionally been externally used for wounds and contusions [24–26]. Geniposide from *Gardeniae fructus* were found to inhibit TNF-α, IL-6, and IL-1β, to block the phosphorylation of IκBα and transcription factor p65 and p38, as well as extracellular-signal-regulated kinases (ERK) and c-Jun N-terminal kinases (JNK), and to decrease the toll-like receptor-4 (TLR4) expression in LPS-simulated macrophages. They were also found to decrease the LPS-induced IL-8 production in human embryonic kidney cells (HEK293-mTLR4/MD-2 cells) [89]. *Hyperici perforati herba* is astringent and expels toxins in TCM theory [26]. *Hyperici perforati herba* compounds were found to inhibit LPS-induced PGE-2, COX-2, and NO through the suppression of cytokine signaling 3 (SOCS3) activation in 264.7 macrophages [90]. *Scrophulariae radix* in TCM theory is classified as removing toxicity and moistening tissues; it is used for sores and exanthema [24–26]. *Sophorae tonkinensis radix* had been traditionally used for tonsillitis, pharyngitis, and mouth sores [26, 91]. It is toxic in higher doses. In vitro, it showed antiviral activity on Coxsackie-, Echo-, and Poliovirus [92].

For *Chrysanthemi flos*, see above.

As this prescription was investigated in one study with Qing Dai San (see below), mucositis related results are stated below.

5.11. Qing Dai San. Borax and *Borneolum* combination is found in *Bing Peng San (Chinese), Borneol and Borax Powder (English), Pulvis Borneoli et Boracis (Latin)*, as a locally used prescription for oral inflammation and aphthous ulceration from *Yi Zong Jin Jian* (Golden Mirror of the Medical Tradition) [93]. An alternative to this prescription, *Qing Dai San* contains *Borax*, *Borneolum*, and *Indigo naturalis* [39, 40]. *Borax* is an extract of natural borax mineral. In terms of TCM theory, it removes toxicity from skin and mucosa upon external application [24, 25]. Borax compounds were found to reduce genotoxic effects of heavy metal exposure of human blood cell cultures by arsenic, bismuth, cadmium, mercury, and lead, normalising decreased antioxidant enzyme activities as well as sister chromatid exchange and micronuclei and plasma malondialdehyde (MDA) levels [94]. High *Borax* doses showed toxic cellular effects, decreasing human lymphocyte proliferation and increasing sister chromatid exchange in chromosomes [95].

Indigo naturalis is the fermented and chalked extract of *Strobilenthes flaccidifolius, Indigo tinctoria, Isatis oblongata* or *Polygonaum tinctorium*. It is used for exanthema and ulcers in TCM [24–26] and was found to to inhibit superoxide anione generation as well as MPAK phosphorylation and calcium mobilisation in formyl-methionyl-leucyl-phenylalanine (FMLP)-activated human neutrophils [96].

For *Borneolum*, see above.

The compositions 5.10 and 5.11 were investigated together in a randomised, controlled, four-armed clinical trial on both chemotherapy- and radiation-induced oral mucositis. This article refers only to the two-armed part concerning chemotherapy patients ($n = 60$; treatment: $n = 30$; control: $n = 30$). The decoction of *Chrysanthemi flos, Gardeniae fructus, Hyperici perforate herba, Scrophulariae radix*, and *Sophorae tonkinensis radix* was orally administered to the treatment group who also used a gargle solution composed of *Borax*, *Borneolum*, and *Indigo naturalis*. The control group patients received vitamin B12 administration and used a gentamycin/sodium//bicarbonate gargle solution. Ulcer grading was defined by size and quantity of ulcers. Improvement on oral mucositis symptoms were found in 96.7% of the herbal treatment group, compared to 86.7% in the control group. An overall shorter healing period for the herbal treatment group was reported [54]. In this trial, the investigation of two formulas at the same time compared to disparate controls impeded clear results.

5.12. Codonopsitis radix Plus Atractylodis macrocephalae Rhizome Plus Glycyrrhizae radix Plus Angelicae sinensis radix Plus Rehmanniae viridae radix Plus Astragali membranacei radix Plus Dioscoreae oppositae rhizoma Plus Alismatis rhizoma Plus Agastachis herba Plus Lophatheri herba. This formula may be regarded as a modified incomplete *Ba Zhen Tang (Chinese)*, Eight Treasure Tea (English), Decoctum octo Gemmarum (Latin), from *Zhen Ti Lei Yao* (Catalogued Essentials for Correcting the Body) [97], which is a strong formula for devitalised patients, composed *by Ginseng radix* (or alternatively *Codonopsitis radix*), *Atractylodis macrocephalae rhizoma*, *Glycyrrhizae radix*, *Angelicae sinensis radix*, *Rehmanniae radix*, *Poria alba*, *Paeoniae alba radix,* and *Ligustici chuangxiong rhizoma* [39, 40]. In prescription 5.12, the last three herbs of Ba Zhen Tang were replaced by *Astragali membranacei radix, Dioscoreae oppositae radix,*

Alismatis rhizoma, Agastachis herba, and *Lophatheri herba*, directing the prescription to immune consolidating effects [24–26].

Agastachis herba, Atractylodis macrocephalae radix, and *Glycyrrhizae radix* are found in *Huo Xiang Zheng Qi San* (Chinese), Agastache Powder to Rectify the Qi (English), *Pulvis Agastachis pro Qi Orthopathico (Latin)* from *Tai Ping Hui Min He Ji Ju Fang* (Imperial Grace Formulary of the Tai Ping Era) [98] with *Magnoliae cortex, Citri reticulatae pericarpium, Perillae folium, Angelica dahuricae radix, Pinelliae rhizoma, Arecae pericarpium, Poria alba,* and *Platycodonis radix*. It is used for endemic infections and gastritis [39, 40]. *Alismatis rhizoma, Codonopsitis radix, Atractylodis macrocephalae rhizoma, Angelicae sinensis radix, and Glycyrrhizae radix* are also found in *Dang Gui Nian Tong Tang (Chinese), Decoction to Lift the Pain (English) from Nei Wai Shang Bian Huo Lun* (Clearing Doubts about Injury from Internal and External Causes) [99] with *Atractylodis radix*, *Ledebouriellae radix, Puerariae radix, Scutellariae radix, Anemarrhenae rhizoma, Artemisiae herba, Polyporus, Sophorae radix, Notopterygii radix*, and *Cimicifugae radix*. It is used for inflammatory diseases such as arthritis, impetigo, and eczema [100].

Codonopsitis radix extract was found to inhibit NO, TNF-α, IL-3 IL-6, and the ERK signalling pathway as well as LPS-induced phagocytic uptake and CD29-mediated cell-cell-adhesion in RAW 264.7 macrophages [101]. *Dioscoreae oppositae rhizoma* was found to decrease the NO and proinflammatory cytokine production including IL-1β, Il-6, TNF-α, and PGE-2, as well as iNOS, and the COX-2, and NF-κB activation in RAW 264.7 macrophages [102]. *Alismatis rhizoma* is diuretic in TCM theory [24–26]. It is not typically used for oral diseases but was found to suppress NF-κB, COX-2, IL-1β and iNOS, as well as induced nuclear factor-like 2 (Nrf2)-regulated gene expression in RAW 264.7 cells [103]. *Agastachis herba* in TCM theory has antiedematous effects and is used for nausea and fever [24, 25]. *Agastachis herba* extract showed antioxidant effects increasing heme oxygenase-1 (HO-1) enzyme activity by way of the protein kinase G (PKG) signalling pathway in RAW 264.7 macrophages [104]. *Lophatheri herba* has been traditionally used for mouth and tongue sores [26]. Glycosides derived from *Lophatheri herba* were found to possess an anti-respiratory syncytial virus (RSV) effect in vitro [105].

For *Atractylodis macrocephalae rhizoma, Glycyrrhizae radix, Astragali membranacei radix, Angelicae sinensis radix,* and *Rehmanniae viridae radix*, see above.

The decoction of *Codonopsitis radix, Atractylodis macrocephalae rhizoma, Glycyrrhizae radix, Angelicae sinensis radix, Rehmanniae viridae radix, Astragali membranacei radix, Dioscoreae oppositae rhizoma, Alismatis rhizoma, Agastachis herba,* and *Lophatheri herba* was administered in a randomised, controlled, two-armed clinical trial ($n = 66$; treatment: $n = 33$; control: $n = 33$) to chemotherapy patients during 4 chemotherapy cycles. The treatment group received not only the herbal decoction but also a multi vitamin, mineral, and micronutrient supplement (Centrum Wyeth). The control group patients received only vitamin B2 administration. Oral ulcer incidence grew from the first to the fourth chemotherapy cycle up to 21.2% in the treatment group and 66.7% in the control group, judged by WHO scale for Oral Mucositis. The increase was not only higher but also faster in the control group [55].

In this trial, the diversified vitamin supplement application confused the effect of the herbal medicinal treatment. Vitamin B2 is not a valuable control to any multivitamin supplement and/or Chinese medicinal herbs. Basic conditions should be equal in both groups in order to achieve a measurable effect. The effect of vitamin supplements should be investigated independently from herbal medicine.

5.13. Modified Xie Huang San (Chinese), Drain the Yellow Powder (English). The classical prescription *Xie Huang San contains Gypsum fibrosum, Saposhnikoviae radix, Gardeniae fructus, Agastachis herba*, and *Glycyrrhizae radix*. It is described in *Xiao Er Yao Zheng Zhi Jue* (Craft of Medicines and Patterns for Children) [106] and used for inflammatory diseases of stomach and mouth [39, 40].

Saposhnikoviae radix is used for affections of skin and mucosa [24–26]. It was found to inhibit NO production through iNOS and its mRNA expression in LPS-induced RAW 264.7 cells [107].

For the further herbs of this formula, see above.

Modifying standard prescriptions by herb addition related to syndrome patterns or individual symptoms is common in TCM tradition [39, 40]. In a clinical trial on 90 chemotherapy patients [56], *Xie Huang San* was administered amended by addition of *Ginseng radix, Astragali membranacei radix, Atractylodis macrocephalae rhizoma, Coptidis rhizoma, Taraxaci herba, Dendrobii caulis, Hedyotidis herba*, and *Lophatheri herba* which offer additional anti-inflammatory qualities [24–26].

In terms of TCM theory, *Taraxaci herba* is used for swelling, abscesses, and sore throat [24–26]. Taraxacosterol a flavonoid, isolated from *Taraxaci herba*, was found to inhibit NO, PGE-2, TNF-α, IL-1β, and IL-6 production as well as LPS-induced NF-κB translocation in RAW 264.7 macrophages [108]. *Dendrobii caulis* had been used for dry mouth in TCM theory [26]. Anti-inflammatory and saliva secretion increasing effects of *Dendrobii caulis* were indicated in a Sjögren's mouse model [109]. *Hedyotis herba* in TCM theory has been used for abscesses and ulcers [24, 25].

For *Ginseng radix, Atractylodis macrocephalae rhizoma, Coptidis rhizoma*, and *Lophatheri herba*, see above.

Astragali membranacei radix, Atractylodis macrocephalae rhizome, and *Ginseng radix* are found in *Bu Zhong Yi Qi Tang* (Chinese), *Tonify the Middle and Augment the Qi Decoction (English), Decoctum Suppleens Centrum et Augmentans Qi (Latin)* from *Nei Wai Shang Bian Huo Lun* (Clearing Doubts about Injury from Internal and External Causes) [99] with *Glycyrrhizae radix tosta, Angelicae sinensis radix, Aurantii pericarpium, Cimicifugae rhizoma, and Bupleuri radix.* Furthermore, *Yu Ping Feng San (Chinese), Jade Windscreen Powder (English), Pulvis Paraventi Jaspidis (Latin)* from *Shi Yi De Xiao Fang* (Effective Formulas from Generations of Physicians) [110] composed of *Astragali membranacei radix, Atractylodis macrocephalae radix*, and *Saposhnikoviae radix*,

traditionally considered as consolidating the body's immune defence, is present in this multiple decoction.

This complex formula was investigated in the above-mentioned randomised, controlled, two-armed clinical trial on chemotherapy patients ($n = 90$; treatment: $n = 50$; control: $n = 40$) by oral administration to the treatment group [56]. In addition, several of their individual symptoms were considered in each case by adding herbal modules. In case of diarrhoea, neutropenia, thrombopenia, petechiae, lymphadenopathy, fever, insomnia, night sweat, or increased ministerial fire (which is a specific term of TCM theory), two different herbs were added to the recipe.

This total of herbal medicine was compared to oral administration of vitamin B2 and vitamin C in the control group. All patients received dental care instructions and diet advices. Ulcer degree was classified by a subjective clinical scale.

Though positive effects on mucositis symptoms were seen in 98% of the treatment group compared to 72% of the vitamin control group, the formula complexity does not allow congruent conclusions due to several variances in the treatment group. The multiple interactions between single herbal components do not allow a clear view on the (formula's) effects. Furthermore, vitamin application may be useful for oral mucositis patients but does not represent a valid control to any complex herbal prescription of this size.

6. Discussion

Chemotherapy-induced oral mucositis continues to be a challenge for anticancer treatment [8, 9, 12–15], representing one of the most common problems for chemotherapy patients [1]. Despite of the dedicated research on this field [1–7] and some resulting guidelines for prevention and treatment of oral mucositis [9, 10, 16, 17], therapeutic results are not yet satisfactory. Chemotherapy side effects in general represent an additional physical and psychological burden to patients diagnosed with cancer, reducing their quality of life and leading to the risk of anticancer treatment delay with fatal consequences [7, 8]. On the other hand, posttraumatic stress reactions as seen in cancer patients lead to a decreased defence against oral mucositis, determined by the alteration of several biomarkers [6, 7].

Medicinal herbs are commonly used for complementary treatment when there is no sufficient western treatment concept. A Chinese review identified diverse approaches regarding oral mucositis by applying herbal medicine, including various gargling preparations, sprays, formulas for oral administration, acupoint application or intravenous injection, resulting in a generally positive effect [111], but the evidence of herbal use on oral mucositis still remains unclear.

Generally, systematic review studies on Chinese herbal medicine come to the conclusion that better qualified studies are necessary [112–114]. At the clinical level, study designs used to be mostly suboptimal but even on pharmacological level, the study quality is criticized as being sufficient. This is not surprising, because classical pharmacological research is generally focusing on single active compounds and this method of approach is not easily transferred to the multi-dimensional complexity of Asian herbal prescriptions. But in some aspects this view is short sighted, as a single-target approach can have limited effectiveness, and there is some evidence that a multi-target approach might be more effective [115, 116] and mixtures may have potentiating actions of their multiple bioactive components [117].

In this review, on the one hand we tried to summarize the state of knowledge of Chinese herbal treatment for chemotherapy-induced oral mucositis based on clinical trials. On the other hand, we tried to examine the TCM tradition based rationality of the particular herbs used for mucositis.

While aggressive treatments like chemotherapy have not been used in the history of TCM, application of traditional Chinese herbal treatment to these side effects of modern therapy requires an intentional transfer of historical concepts to modern treatment procedures. In daily practice, Chinese herbal medicine has an individualized approach that cannot be easily transferred into standardized controlled trials due to the uniform treatment concepts usually required by controlled trials.

For a full understanding of the mechanisms of herbal prescriptions, the effects of every single herb must be known on a molecular basis. Based on these data, herbal combinations should be investigated for detecting synergisms that may result in molecular effects which are not found in the single herb components [118, 119]. Further elaborated research is necessary for clearing multiple questions about single as well as combined herbal use, resulting in the aim of rational prescription rather than application based only on empirical knowledge.

Even though all reviewed clinical trials reported positive effects of Chinese herbal treatment, they did not show adequate study designs proportionately with regard to the investigated questions. Some studies used complex multi-herbal formulas that lead to difficulties in understanding the detailed effects. Formula complexity should be well elaborated in order to achieve significant results. Monoherbal applications offer clear results on the basis of well elaborated study designs. Herbal combinations may be even more effective in clinical results [115–117], while under clinical study conditions the evidence of exaggerated multi-herbal application is narrowed by numerous interferences between the single compounds, especially in combined prescriptions of variant formulas (see 5.2, 5.10, 5.11, 5.12, and 5.13).

The use of individualized herbal combinations has a long tradition in TCM on an empirical basis. One of the evaluated trials tries to take this classical approach into account by additional prescriptions depending on the accompanying symptomatology, resulting in extremely complex treatment procedures (see 5.13). In consequence, the general overview gets lost. In other trials treatment procedures of topical application and internal or even intravenous administration were combined (see 5.1a, 5.3, 5.6, and 5.10 and 5.11), making it impossible to draw applicable conclusions about one of the interventions. One trial used a formula mixed with vitamins (see 5.12), while others used vitamin B2, B12, and/or vitamin C application for controls without discussing the rationale (see 5.3, 5.6, 5.11, 5.12, and 5.13). In the same manner, it is

difficult to judge the efficacy on herbal ice application versus ambient tempered gargle solution (see 5.4). Antibiotics were used for control groups in some studies (see 4.3, 5.6, and 5.11) while basic use of antibiotics challenged the result of one study (see 5.7).

Only in one study a non-treatment group had been established (see 4.2) and in only 6 studies the same type of application was used for treatment as well as for control groups (see 4.3, 4.4, 5.1b, 5.2, 5.5, and 5.7). Two authors reported uncontrolled case studies (see 4.1, 5.8, and 5.9).

In summary it is almost impossible to evaluate which parts of the treatment concepts are responsible for the measured effects in the reviewed trials.

Another problem for judging study results is the fact that oral mucositis is a severe but short term side effect of chemotherapy, typically self-limiting in about 2 to 4 weeks if not complicated by infections [1]. Study designs have to consider that even without any specific treatments, symptoms possibly improve in this time.

In general, the investigated trials showed low-quality designs. Control groups were established in most investigated trials but control interventions did not represent any standards. It has to be admitted that the mucositis guidelines so far existing are limited, so it is not easy to find valid control interventions for some treatment concepts. But in general, clinical studies should offer a standard basic treatment for all patients or placebo non-treatment groups in order to gather valid data on the investigated intervention. In the case of mucositis, establishing non-treatment groups could cause an ethical dilemma for having possible severe consequences [9, 12, 13] but it is possible to establish basic treatment conditions for all patients taking part of the study which do not interfere with the investigated intervention. This approach includes placebo administration for control groups in order to achieve valid data. In the case of Chinese medicinal herbs, the use of capsules containing herbal extractions or placebo is a good option. In the case of gargling solutions, fabricating a valid placebo may be more complicated but should not be impossible. Patients and medical practitioners should be blinded regarding the applied intervention in order to minimize placebo effects. The blinding technique has not been reported in any of the investigated studies. Randomisation has been reported in all controlled studies though the process has not been described except very briefly in two publications (5.1b and 5.12). For proving a strict randomisation protocol so as not to create bias, it is necessary to provide a detailed description.

Summarizing the collected data so far, results of Chinese medicinal herbal administration for chemotherapy-induced oral mucositis are potentially promising, but poor study designs do not allow valid conclusions. Conducting a meta-analysis is not possible with the present database. Further investigations are necessary on molecular mechanisms of multi-herbal formulas and the corresponding single herbs as well as in well designed clinical trials. Providing adequate study designs are developed, traditional Chinese herbal medicine has the potential of complementing western regimens such as chemotherapy in order to achieve lower levels of side effects, thus enabling patients to better resist chemotherapy impacts.

7. Conclusion

All evaluated trials in this review reported positive effects about Chinese herbal treatment for chemotherapy-induced oral mucositis, but the value of these treatments remains unclear. Study designs are generally poor, some herbal prescriptions are far too complex and adequate controls are missing. Mechanisms of action are rarely described.

While basic research provides data about anti-inflammatory and protective effects of some herbs or herbal compounds, further research is still promising, but study designs need considerable improvement. So future research should start with mechanism based studies first. The following clinical studies should reduce the complexity of the treatment procedures in order to produce clear results, before Chinese herbal medicine can become an evidenced based part of the treatment of chemotherapy induced mucositis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Potential Therapeutic Role of Hispidulin in Gastric Cancer through Induction of Apoptosis via NAG-1 Signaling

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Gastric cancer is one of the most common malignant cancers due to poor prognoses and high mortality rates worldwide. However, an effective chemotherapeutic drug without side effects remains lacking. *Saussurea involucrata* (SI) Kar. et Kir., also known as snow lotus, grows in mountainous rocky habitats at 2600 m elevation in the Tian Shan and A'er Tai regions of China. The ethyl acetate extract of SI had been shown to inhibit proliferation and induce apoptosis in various tumor cells. In this study, we demonstrated that Hispidulin, active ingredients in SI, inhibits the growth of AGS gastric cancer cells. After Hispidulin treatment, NAG-1 remained highly expressed, whereas COX-2 expression was downregulated. Flow cytometric analysis indicated that Hispidulin induces G1/S phase arrest and apoptosis in time- and concentration-dependent manners. G1/S arrest correlated with upregulated p21/WAF1 and p16 and downregulated cyclin D1 and cyclin E, independent of p53 pathway. In addition, Hispidulin can elevate Egr-1 expression and ERK1/2 activity, whereas ERK1/2 inhibitor markedly attenuated NAG-1 mediated apoptosis. Taken together, Hispidulin can efficiently activate ERK1/2 signaling followed by NAG-1 constitutive expression and trigger cell cycle arrest as well as apoptosis in cancer cell. It can be a potential compound for combination therapy of gastric cancer in the future.

1. Introduction

Gastric cancer is one of the most common causes of cancer-related mortality in China and other Asian countries [1, 2]. Surgery and chemotherapy are the standard treatment modalities for gastric cancer [3–5]. The 5-year survival of gastric cancer patients is currently estimated at approximately 30%; therefore, the development of novel treatment strategies to improve patients' prognoses is urgently required. The majority of gastric adenocarcinomas express high levels of cyclooxygenase-2 (COX-2) [6–9]. Houghton et al. reported that angiogenesis and *Helicobacter pylori* infection are both associated with COX-2 expression in gastric cancer patients [10]. The knockdown of COX-2 in a SGC-7901 gastric adenocarcinoma cell line by RNA interference inhibited proliferation and induced apoptosis, indicating that suppression of COX-2 might represent an effective approach for the treatment of gastric cancer. The majority of selective COX-2 inhibitors exert pronounced side effects that limit their administration. Based on this clinical phenomena, AGS gastric adenocarcinoma cell line which constitutively expressed both COX-1 and COX-2 and additional COX-2 induced by IL-1β to potentially make it an excellent model for assessing gastrointestinal toxicity of COX-2 inhibitors was an ideal model to evaluate the potential compound for adjuvant therapy in gastric cancer [11]. In this study, we observed that

Hispidulin inhibited the proliferation of AGS gastric adenocarcinoma cells; therefore, we aimed to establish if the mechanism underlying the antiproliferative effects of Hispidulin on AGS cells is the downregulation of COX-2 expression.

In our previous study, isochaihulactone markedly upregulated the expression of the nonsteroidal anti-inflammatory-drug- (NSAID-) activated gene-1 (*NAG-1*; also known as *MIC-1*, *GDF-15*, placental *TGF-β*, and *PLAB*). NAG-1 is a transforming growth factor-*β*-like secreted protein, initially characterized as a p53-regulated gene [12–14]. Overexpression of NAG-1 in breast cancer cells resulted in growth arrest and apoptosis *in vitro* and *in vivo*. Previous studies observed similar results in colon cancer cells [15–17] and following the treatment of prostate cancer cells with purified NAG-1 [18]. These findings suggested that NAG-1 is associated with apoptosis and that the downregulation of NAG-1 expression might promote tumorigenesis. In our previous investigation, we identified that isochaihulactone, a novel lignan isolated from the root of *Bupleurum scorzonerifolium*, induced microtubule depolymerization, cell cycle arrest, and proapoptotic activity in A549 human lung cancer cells [19]. We also observed that NSAIDs upregulated the expression of several isochaihulactone-induced genes and that isochaihulactone upregulated NAG-1 protein expression in a time-dependent manner. The upregulation of NAG-1 by isochaihulactone was associated with the upregulation of EGR-1 expression; therefore, the silencing of EGR-1 expression by siRNAs could also be associated with downregulated NAG-1 RNA and protein expression. In our analyses, the MEK1/2 inhibitor PD98059 reduced the inhibitory effects of isochaihulactone significantly; however, the p38 inhibitor SB203580 and the JNK inhibitor SP600125 did not limit isochaihulactone-induced growth inhibition. The MEK1/2 inhibitor PD98059 reduced isochaihulactone-induced upregulation of EGR-1 and NAG-1 protein expression, whereas SB203580 and SP600125 had nonsignificant effects on EGR-1 and NAG-1 expression. These data supported the concept that isochaihulactone-induced ERK1/2 activity is critical for the regulation of EGR-1 and NAG-1 expression. The induction of ERK1/2 activity and subsequent induction of *EGR-1* and *NAG-1* contributes to the growth inhibitory and apoptosis-promoting effects of antitumor compounds in cancer cells.

Saussurea involucrata Kar. et Kir., or the snow lotus, grows in mountainous rocky habitats at 2600 m elevation or higher in the Tian Shan and A'er Tai regions of China. Because of excessive harvesting of the wild plants for use in pharmaceutical preparations and their remarkably slow growth, the wild population of *S. Involucrata* has depleted in recent years. *S. Involucrate* is currently close to extinction and, therefore, listed as a second-grade national protected wild plant in China [20, 21]. According to the theories of traditional Chinese medicine, *S. Involucrata* has the effects of warming the kidney, activating "yang," expelling wind, eliminating dampness, inducing menstruation, and promoting blood circulation [22]. Hispidulin (40,5,7-trihydroxy-6-methoxyflavone) is a naturally occurring flavone in *S. Involucrata* [20]. Several studies have shown its potent antioxidative, antifungal, anti-inflammatory, antimutagenic, and antineoplastic properties *in vitro* [23–25]. A recent study identified Hispidulin as a potent ligand of the human central benzodiazepine receptor *in vitro* [26]. Hispidulin also acts as a partial positive allosteric modulator at GABAA receptors, penetrates the blood-brain barrier, and possesses anticonvulsant activity in the central nervous system (CNS) [27]. Lin et al. further reported that in Hispidulin-treated glioblastoma (GBM) cells, the activation of AMP-activated protein kinase (AMPK) suppressed protein synthesis, lipogenesis, and cell cycle progression. Their results suggested that Hispidulin might be useful as a chemopreventive or therapeutic agent for GBM. Subsequent observations indicated that Hispidulin is a potential modulator of CNS activity, prompting our own investigation of its antineoplastic activity against GBM [28].

In this study, we identified that Hispidulin treatment markedly upregulated NAG-1 protein expression and downregulated COX-2 protein expression significantly, in AGS gastric cancer cells. After various durations of exposure of cells to Hispidulin, the expression of EGR-1 and that of NAG-1 was upregulated in a time-dependent manner. Hispidulin treatment also increased ERK1/2 activity, and an ERK1/2 inhibitor markedly downregulated the expression of NAG-1 and the growth inhibitory effects of Hispidulin in AGS cells. These results suggested that the apoptotic effects of Hispidulin in human gastric cancer cells might be directly associated with the upregulation of NAG-1 expression through ERK1/2 activation. Our findings indicate that Hispidulin exerts therapeutic effects on human gastric cancer cells through the activation of NAG-1 through the ERK1/2 signaling pathway.

2. Materials and Methods

2.1. Preparation of Fractions. The wild plant of *S. involucrata* used in this study was a gift from Biopure Biotechnology (Changhua, Taiwan). Twenty grams of dried and powdered aerial parts, including flower, of *S. involucrate* was extracted with 100 mL of methanol three times under reflux for 2 h, respectively. The methanol extracts (SI-1) were combined, and the solvent was evaporated in vacuum to give a deep brown syrup. The syrup was resuspended in water and then partitioned successively with pentane, ethyl acetate (SI-2), and n-butanol (SI-3) to leave a water layer (SI-4). The solvents were evaporated, respectively, and the residues were used throughout this study.

2.1.1. Reverse-Phase High-Performance Liquid Chromatography (HPLC) Analysis of Flavonoids in S. involucrate. The determination of flavonoids from *S. involucrata* was carried out by HPLC with a photodiary detector. The HPLC system consisted of a Shimadzu LC-20AT solvent delivery system, equipped with a SPD-M20A photodiode array detector, set at 270 nm. Samples were injected with SiL-20A autosample to separate on the TSK-Gel ODS-100S column. The column was maintained at an ambient temperature of 25°C. The flow rate of the system was 1.0 mL/min. The mobile phase consisted of solvent A (0.3% formic acid) and solvent B (acetonitrile). The elution profile for A was 0–10 min, with a linear gradient change of 0–5%; 10–40 min, with a linear gradient change to 55%; and maintained for another 10 min with a postrun time

to equilibrate the column and for the baseline to return to the normal and initial working conditions.

2.2. Chemicals and Reagents. Rutin was dissolved in DMSO to a concentration of 50 mM and stored in −20°C as a master stock solution. Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), Hoechst 33342, thiobarbituric acid (TBA), hydrogen peroxide (H_2O_2), trichloroacetic acid,(TCA), malondialdehyde (MDA), propidium Iodide (PI), and actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). NuPAGE Bis-Tris Electrophoresis System (precast polyacrylamide minigel) was purchased from Invitrogen (Carlsbad, CA, USA). COX-2 antibody was purchased from Thermo scientific (Waltham, MA, USA). PARP antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were purchased from Cell signaling (MA, USA). Polyvinyldene fluoride (PVDF) membranes, BSA protein assay kit, and Western blot chemiluminescence reagent were purchased from Amersham Biosciences (Arlington Heights, IL). Superoxide dismutase activity assay kit was purchased from biovision (Mountain View, CA). Glutathione peroxidase assay kit was purchased from Cayman Chemical (MI, USA). DNA Fragmentation Assay Kit was purchased from Clontech Laboratories (Mountain View, CA). Nonradioactive Cytotoxicity Assay was purchased from promega (Madison, WI, USA).

2.3. Cell Lines and Cell Culture. AGS human gastric adenocarcinoma cell line (ATCC, CRL-1739) were obtained from American Type Culture Collection (Manassas, VA) and propagated in culture dishes at the desired densities in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.4. Growth Inhibition Assay. The viability of the cells after treatment with various chemicals was evaluated using the MTT assay preformed in triplicate. Briefly, the cancer cells (3×10^3) were incubated in 96-well plates containing 200 μL of the culture medium. Cells were permitted to adhere for 12–18 h then washed with phosphate-buffered saline (PBS). Solutions were always prepared fresh by dissolving 0.2% DMSO or drugs in culture medium and then were added to AGS cells. For inhibitor treatment experiments, cells were treated with 50 μM Hispidulin and preincubated for 1 h with 25 and 50 μM ERK1/2 inhibitor PD98059. After 48 h of exposure, the drug-containing medium was removed, washed with PBS, and replaced by fresh medium. The cells in each well were then incubated in culture medium with 500 μg/mL MTT for 4 h. After the medium was removed, 200 μL of DMSO and 25 μL of glycine buffer (0.1 Mglycine and 0.1 M NaCl, pH 10.5) were added to each well. Absorbance at 570 nm of the maximum was detected by a PowerWave X Microplate ELISA Reader (Bio-Tek Instruments, Winooski, VT). The absorbance for DMSO-treated cells was considered as 100%. The results were determined by three independent experiments.

2.5. IC50 Determination. MTT assay was according to the paper [29]. Briefly, the pale yellow redox indicator 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced to a dark blue end product, MTT-Formazan, by the mitochondrial dehydrogenases of living cells. MTT reduction can be measured spectrophotometrically at a wavelength of 570 nm. According to the dosage dependent curve, the MTT reduction half was indicated IC50. In our study, Hispidulin is resolved on DMSO agent with final concentration 0.2%. Therefore, we used 0.2% DMSO as a control agent. The absorbance for DMSO treated cells without adding any drugs was considered as 100%.

2.6. Cell Cycle Analysis. The cell cycle was determined by flow cytometry with DNA staining to reveal the total amount of DNA. Approximately 5×10^5 of cells were incubated in various concentrations of Hispidulin for the indicated time. Cells were harvested by treating the cells with trypsin/EDTA. The cells were collected, washed with PBS, fixed with cold 70% ethanol overnight, and then stained with a solution containing 20 μg/mL PI and 0.1% Triton X-100 for 1 h in the dark. The cells will then pass through FACScan flow cytometer (equipped with a 488-nmargon laser) to measure the DNA content. The data was obtained and analyzed with Cell Quest 3.0.1 (Becton Dickinson, Franklin Lakes, NJ) and Mod FitLT V2.0 software.

2.7. Western Blot Analysis. Approximately 5×10^6 cells were cultured in 100 mm^2 dishes and then incubated in various concentration of Hispidulin for 48 h. The cells were lysed on ice with 150 mL of lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 0.5 mol/L NaCl, 5 mmol/L $MgCl_2$, 0.5% nonidet P-40, 1 mmol/L phenylmethylsulfonyl fluoridefor, 1 mg/mL pepstatin, and 50 mg/mL leupeptin) and centrifuged at 13000 ×g at 4°C for 15 min. The protein concentrations in the supernatants were quantified using a BSA Protein Assay Kit. Electrophoresis was performed on a NuPAGE Bis-Tris Electrophoresis System using 50 mg of reduced protein extract per lane. Resolved proteins were then transferred to polyvinyldene fluoride (PVDF) membranes. Filters were blocked with 5% nonfat milk overnight and probed with appropriate dilution of primary antibodies for 2 h at room temperature. Membranes were washed with three times with 0.1% Tween 100 and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. All proteins were detected using Western Lightning chemiluminescence reagent plus and quantified using a densitometer.

2.8. Detection of Apoptosis. The viability of the cells after treatment with various chemicals was evaluated using an MTT assay preformed in triplicate. Briefly, the cancer cells (5×10^3) were incubated in 96-well plates containing 200 μL of serum-containing medium. Cells were permitted to adhere for 12 to 18 h and then were washed with phosphate-buffered saline (PBS). Solutions were always prepared fresh by dissolving 0.2% DMSO or drugs in culture medium and added to AGS cells. For inhibitor treatment experiments, cells were treated with 50 μM Hispidulin and preincubated for 1 h

with ERK1/2 inhibitor PD98059. After 24 h of exposure, the drug-containing medium was removed, washed with PBS, and replaced by fresh medium. The apoptosis was analyzed according to the method described by van Engeland et al. [30] to detect the integrity of cellular membrane and the externalization of phosphatidylserine (Cytometry 1998; 31 : 1–9). In brief, approximately 1×10^6 cells were grown in 10 mm diameter plates. The cells were incubated in various concentrations of K8 for the indicated time and then labeled with FITC Annexin V and PI prior to harvesting After labeling, the cells were washed with binding buffer and harvestied. by scraping. Cells were resuspended in binding buffer at a concentration of 2×10^5 cells/mL before analysis by flow cytometry (FACScan). The data was analyzed on WinMDI V2.8 software. The percentage of cells undergoing apoptosis was determined by three independent experiments.

2.9. Caspase Activity Assay. Activity of caspase-3 was detected by using a fluorometric assay kit (Promega) according to the manufacturer's protocol. In brief, 2×10^6 control or treated cells were lysed in 50 μL of cold lysis buffer and incubated in ice for 10 min. Fifty microliters of cell lysates was added to 50 μL of reaction buffer and 5 μL of fluorogenic report substrates specific for caspase-3 in a 96-well microplate. After incubation at 37°C for 1 h, the fluorescence from the cleaved C-terminal side of the aspartate residue of DEVD-7-amino-4-trifluoromethyl coumarin was detected by a fluorescence microplate reader (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA), with excitation at 400 nm and emission at 505 nm.

2.10. Statistical Analysis. The data was shown as mean with standard deviation. The statistical difference was analyzed using the Student's t-test for normaly, distributed values and by nonparametric Mann-Whitney U test for values of nonnormal distribution. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Hispidulin Inhibits Human Gastric Cancer Cell Growth. To test the inhibitory effect of Hispidulin (40,5,7-trihydroxy-6-methoxyflavone) on cancer cell, we treated human gastric cancer AGS cells with Hispidulin. Twenty-four, 48, and 72 hours after treatment followed by MTT cell viability assay, we observed that Hispidulin displayed significantly higher cytotoxicity to AGS cells than other drugs such as rutin and Aspirin (Figure 1). The IC50 of Hispidulin to AGS cell was 50 μM at 48 h after treatment and 20 μM at 72 h after treatment (Figure 1(a)). On the other hand, the IC50 of rutin, a main component of *Saussurea involucrata* that can attenuate the senescence [21], was over 500 μM (Figure 1(b)). Since cyclooxygenase-2 (COX-2) was often overexpressed in gastric adenocarcinomas, we also treated AGS cells with COX-2 inhibitor (celecoxib or NS-398). The results indicated that the IC50 of celecoxib and NS-398 were around 40 μM and 50 μM 48 h after treatment and 30 μM and 40 μM 72 h after treatment, respectively, (Figures 1(c)-1(d)). However, treated AGS cells with COX-1 inhibitor, Aspirin, had less inhibitory effect on cell viability (IC50 > 1 mM) (Figure 1(e)). Taken together, Hispidulin can be more effective in gastric cancer survival inhibition than well-known COX-2 inhibitors.

3.2. Hispidulin Induces AGS Cells Apoptosis and G1/S Phase Cell Cycle Arrest. We then evaluated the association between Hispidulin-induced inhibition of cell growth and induction of apoptosis. After Hispidulin treatment, the percentage of cell apoptosis was significantly elevated in dose and time-dependent manners (Figure 2(a)). In addition, activation of caspase-3 was also significantly induced with dose-dependent phenomena (Figure 2(b)). Furthermore, caspase-3 was activated significantly 24 to 48 h after Hispidulin treatment (Figure 2(c)), and the caspase-3 mediated apoptosis was attenuated by pan caspase-3 inhibitor FMK (Figures 2(b)-2(c)). These results indicated that the apoptosis effect by Hispidulin on AGS cell line was a caspase-dependent manner. The western blot analysis also more supported that Hispidulin induced apoptosis included activation of caspase-9, cleavage of caspase-3, and poly (ADP-ribose) polymerase (PARP) with in time- and dose-dependent manners (Figures 2(d)-2(e)).

3.3. Hispidulin Induces AGS Cell Cycle Arrest during the G1/S Phase. To elucidate the mechanisms underlying the activities of Hispidulin, we evaluated its effects on cell cycle progression. Our results from flow cytometric analysis showed that Hispidulin treatment induced the accumulation of cells in the G1/S phase in a time-dependent manner (Figure 3(a)). This result suggested that Hispidulin might induce G1/S phase arrest, similar to fluorouracil (5-FU). Hispidulin also upregulated the expression of the G1/S regulatory proteins, including p53, p16, and p21, in time- and dose-dependent manners (Figure 3(b)). Hispidulin treatment downregulated the expression of the cell cyclin-associated proteins, including cyclin D1 and cyclin E, in AGS cells (Figure 3(b)).

3.4. Involvement of NAG-1 Activation and COX-2 Inhibition in Hispidulin-Induced Inhibition of AGS Cell Growth. The IC50 for Hispidulin was 50 μM in AGS cells (Figure 1(a)); therefore, we used the same concentration in this study's experiments. To evaluate the involvement of EGR-1 in the upregulation of NAG-1 expression induced by Hispidulin treatment in AGS cells, we used western blotting to analyze EGR-1 and NAG-1 protein expression. After various durations of exposure of cells to Hispidulin, the expression of EGR-1, as well as NAG-1, was upregulated in a time-dependent manner. EGR-1 was upregulated significantly after 6 h treatment and this effect remained until 24 h treatment. NAG-1 expression peaked after 48 h Hispidulin treatment. Hispidulin treatment markedly downregulated COX-2 expression and NF-kappa B subunit p65 expression in AGS cells (Figure 4).

3.5. The Involvement of ERK1/2 Signaling in the Hispidulin-Induced Upregulation of EGR-1 and NAG-1 Expression. To investigate the possible role of ERK1/2 in the regulation of EGR-1 and NAG-1, we treated AGS cells with Hispidulin in

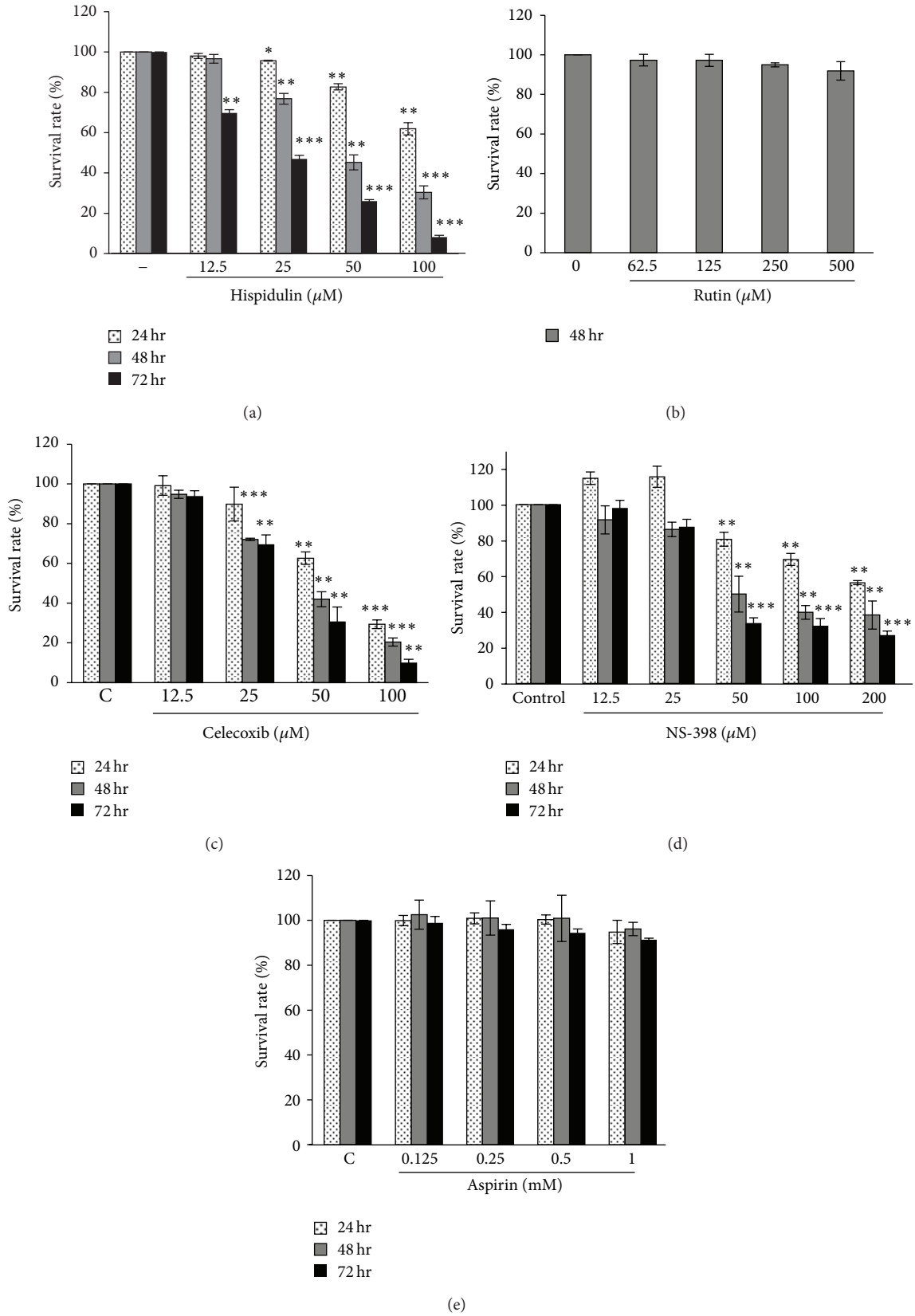

Figure 1: Hispidulin can efficiently inhibit gastric cancer cell growth. (a) Cell viability after Hispidulin treatment. (b) Cell viability after rutin treatment. (c) Cell viability after COX-2 inhibitor, celecoxib, treatment. (d) Cell viability after COX-2 inhibitor, NS-398, treatment. (e) Cell viability after COX-1 inhibitor, Aspirin, treatment. Each column represents the mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

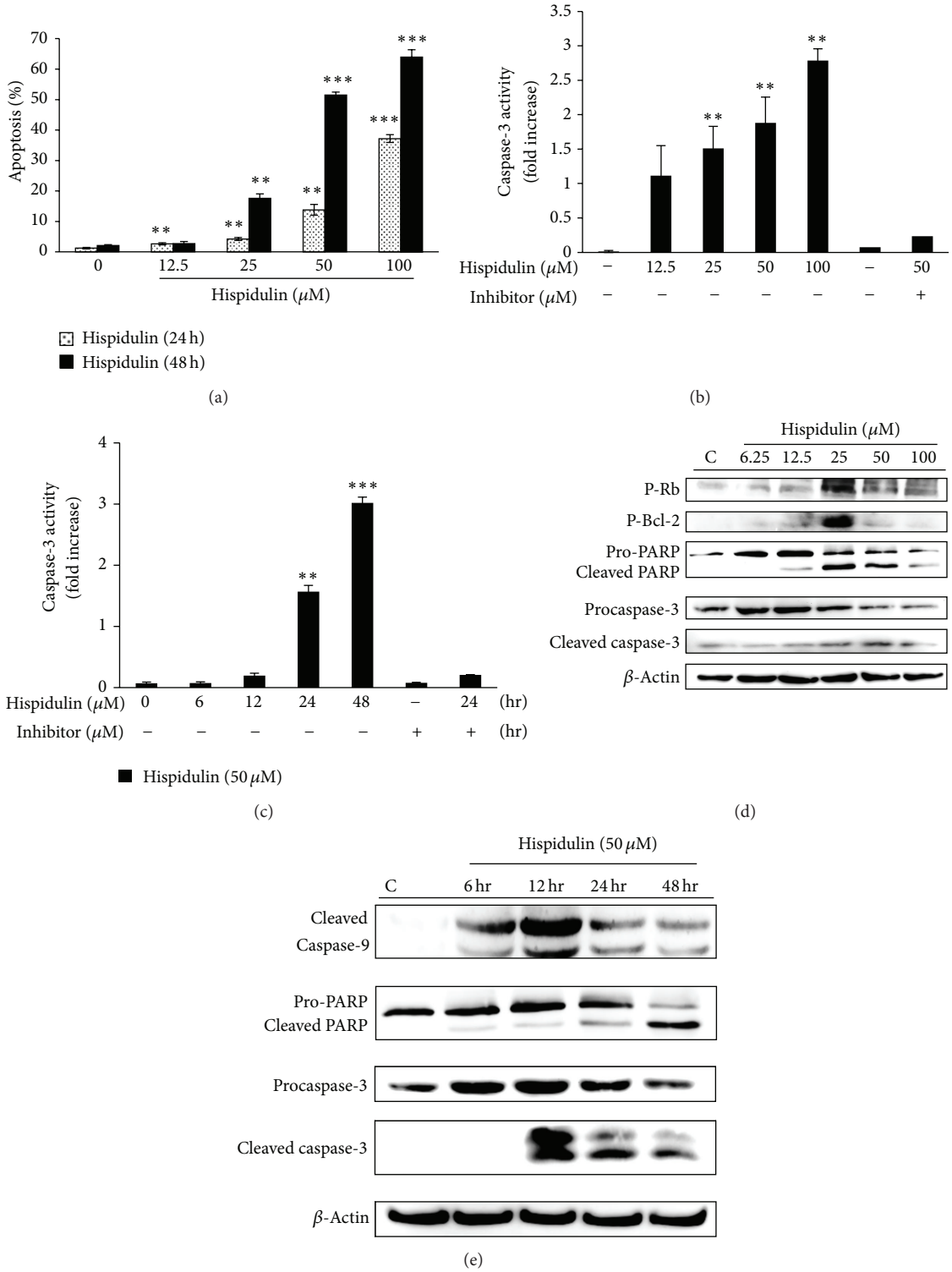

Figure 2: Hispidulin can induce cancer apoptosis by caspase-3 activation. (a) Cell apoptosis assay after treatment by flow cytometry apoptosis analysis. (b) Caspase-3 activity assay after treatment in various dosage. (c) Caspase-3 activity assay 6, 12, 24, and 48 h after 50 μM Hispidulin treatment. (d) The Western blot analysis for apoptosis related protein analysis after Hispidulin treatment. Expression of β-actin was used as an internal control. (e) The Western blot analysis for apoptosis related protein analysis 6, 12, 24, and 48 h after treatment. Expression of β-actin was used as an internal control. Each column represents the mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

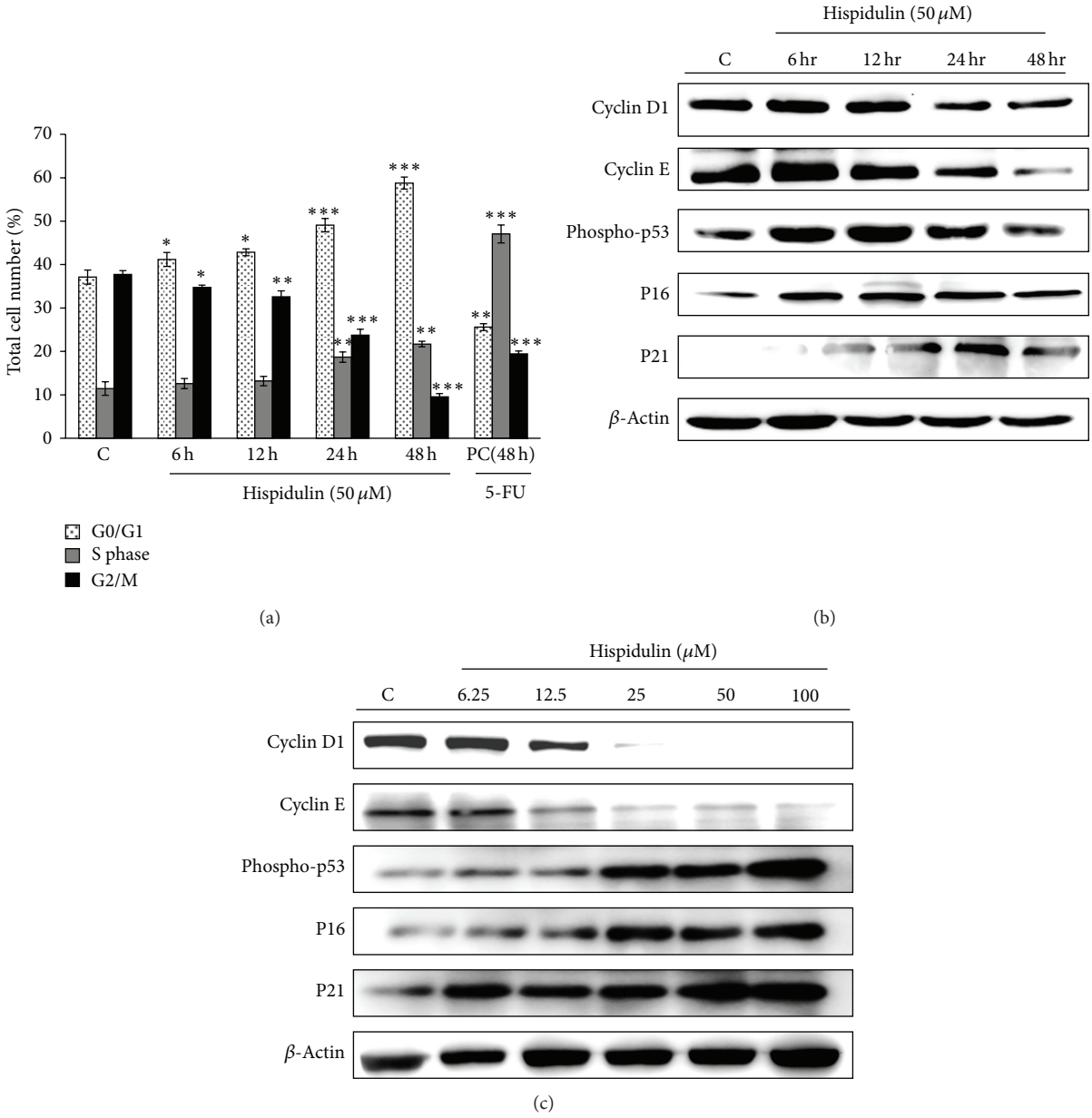

Figure 3: Effect of Hispidulin-induced G1/S phase arrest and changes expression of G1/S regulatory proteins in AGS cells. (a) The cell cycle analysis 6–48 h after Hispidulin treatment. (b) The Western blot analysis for p16, p21, cyclin D1, and cyclin E expression 6–48 h after treatment. Expression of β-actin was used as an internal control. (c) The western blot analysis for p16, p21, cyclin D1, and cyclin E expression in various dosage treatments. Expression of β-actin was used as an internal control. Each column represents the mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

the presence and absence of the MEK1/2 inhibitor PD98059 (25 μM and 50 μM), the p38 inhibitor SB203580 (10 μM and 20 μM), or the JNK1/2 inhibitor SP600125 (10 μM and 20 μM). PD98050 reduced the growth inhibitory effects of Hispidulin in a dose-dependent manner (Figure 5(a)). We then investigated the effects of Hispidulin on MAPK activation to establish which MAPK pathways are involved in its growth inhibitory effects. Following the exposure of AGS cells to Hispidulin, we observed the upregulation of phosphor-ERK protein expression (Figure 5(b)). Using western blot analysis, we then identified that the inhibition of ERK1/2 expression by PD98059 induced the downregulation of EGR-1, NAG-1, and COX-2 protein expression, and reduced growth inhibition, in a dose-dependent manner in Hispidulin-treated AGS cells (Figures 5(c) and 5(d)). In contrast, inhibition of JNK1/2 exerted minimal effects on NAG-1 expression, whereas inhibition of p38 did not affect the expression of the 2 genes. These results suggested that the activation of the ERK1/2 signaling pathway is involved in the upregulation of EGR-1 and NAG-1 expression by

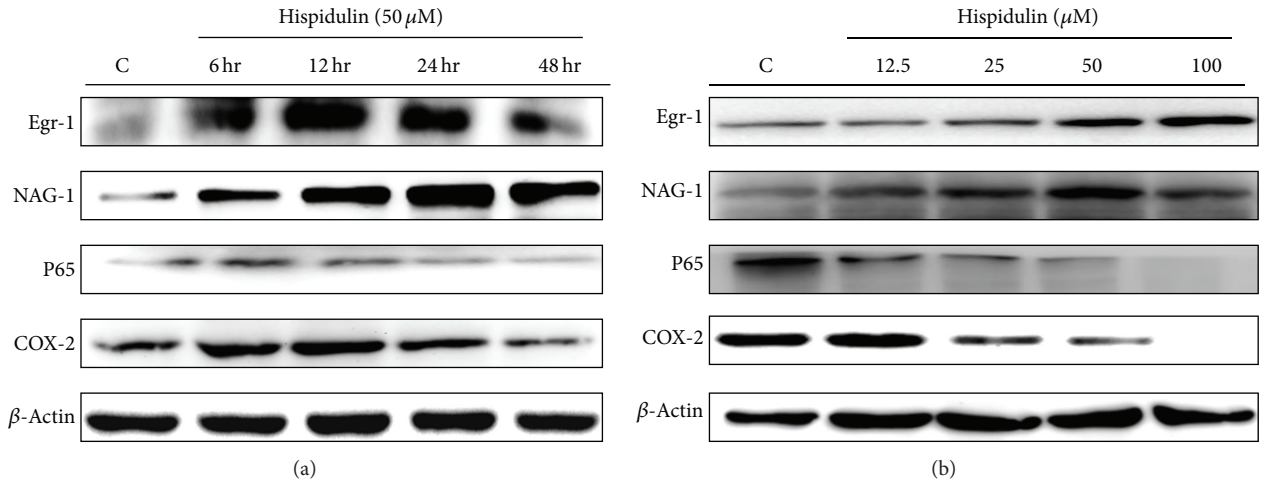

Figure 4: Effect of Hispidulin on NAG-1 signaling associated protein expression in AGS cells. (a) NAG-1 related signaling analysis 6–48 h after treatment. The expression of β-actin was used as an internal control. (b) NAG-1 related signaling analysis 48 h after various dosage treatments. The expression of β-actin was used as an internal control.

Hispidulin. Taken together, Hispidulin can efficiently inhibit cancer cell survival through apoptosis induction via ERK1/2, NAG-1 mediated pathway. Thus, it can be applied to clinically combined treatment for gastric cancer elimination (Figure 6).

4. Discussion

NSAIDs are effective chemopreventive agents for various cancers via the inhibition effect on prostaglandin synthesis. Previous studies identified the chemopreventive and antitumorigenic activities of NSAIDs against colorectal and other human cancers; however, the molecular mechanisms responsible for these properties have yet to be fully elucidated [31–33]. It has been reported that *NAG-1* is a target gene for NSAIDs and a unique member of the transforming growth factor superfamily. Increases in NAG-1 expression result in the induction of apoptosis in several cancer cell lines [14, 16]. Also, NAG-1 expression is induced not only by NSAIDs but also by several antitumorigenic compounds. These include dietary compounds, peroxisome proliferatoractivated receptor-γ ligands, and phytochemicals [34, 35] as well as resveratrol, genistein, diallyl disulfide, 5F203, and retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid [17, 36, 37]. In our previous study, we also found that a novel lignin, isochaihulactone, increased NAG-1 mRNA and protein expression and inhibited cell proliferation on human lung cancer A549 cells. In this study, we reported that the flavonoid Hispidulin upregulates the expression of the proapoptotic and antitumorigenic protein NAG-1. Our results indicated that NAG-1 is an important target gene for Hispidulin. Our study results could increase understanding of the mechanisms by which Hispidulin can affect tumor development. During our analyses, we observed the Hispidulin-induced upregulation of NAG-1 expression in the human AGS gastric cancer cell line had relatively reduced COX-2 activity repression. Our results demonstrated that Hispidulin-treated cells had NAG-1 elevated expression and reduced COX-2 expression. However, the regulatory relationship between NAG-1 and COX-2 still remained to be investigated although many anticancer drugs and compounds had effects on NAG-1 and COX-2 at the same treatment [20, 28]. Since COX-2 overexpression had relative malignance in clinical gastric cancer patients, this finding provide a potency that Hispidulin can be served as an adjuvant therapy.

Expression of iNOS and COX-2 is largely regulated by transcriptional activation. Among these transcription factors, NF-κB, which is a primary transcription factor and regulates various genes, is critical in the inflammation [38]. NF-κB is a redox-sensitive transcription factor that regulates a multitude of inflammatory genes, including cytokines, chemokines, adhesion molecules, and acute phase proteins. Under basal conditions, NF-κB is inactive and prevented from DNA binding and nuclear translocation by tight association in the cytoplasm with inhibitory proteins. Cell activation by a variety of extracellular signals, such as oxidative stress, induces a cascade of events that lead to activating NF-κB then translocating it to the nucleus where it binds to DNA elements in the promoters of numerous proinflammatory gene families [21, 39]. In our study, after treatment with hispidulin, the expression of nuclear factor κB (NF-κB)-p65 in cytoplasm extract fractions decreased, as compared to that of the control (Figure 4). Taken together, the results suggest that hispidulin may exert anti-inflammatory effects *in vitro* in AGS cells through inhibition of NF-κB signal pathway activation. Induction of EGR-1 expression by antitumorigenic compounds is known to involve members of the family of mitogen-activated protein kinases (MAPKs) or phosphatidylinositol-3-kinase (PI3 K) dependent pathways. For example, induction of EGR-1 expression by the peroxisome proliferator-γ activated receptor-γ (PPARγ) ligand troglitazone occurs by the ERK phosphorylation pathway rather than by the PPARγ pathway [15, 16].

To determine which MAPK family is involved in the major signaling pathway for Hispidulin-mediated NAG-1

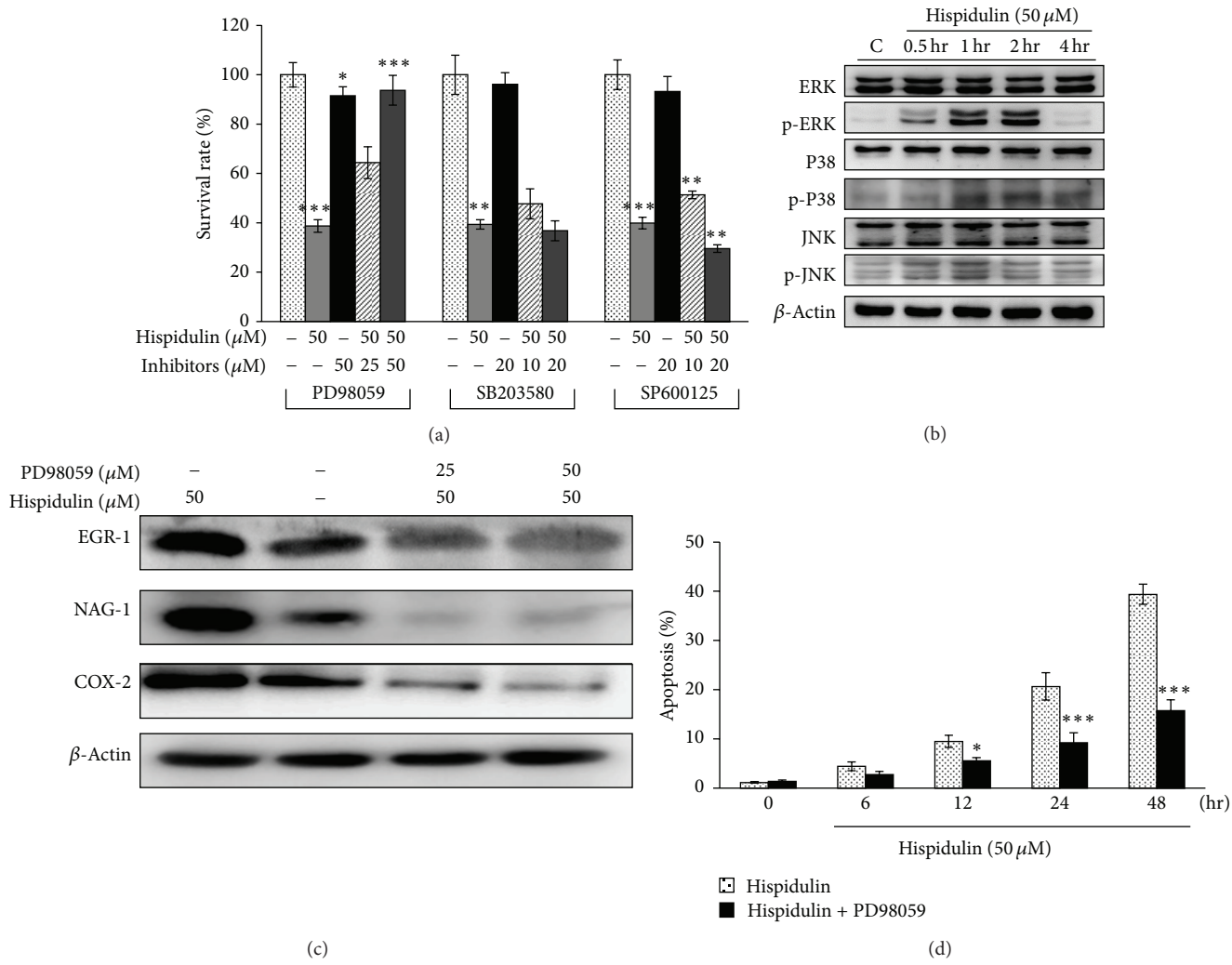

Figure 5: Inhibition of NAG-1 expression and growth inhibition by ERK1/2 inhibitor. (a) AGS cells were treated with 30 μM Hispidulin in the presence or absence of the mitogen-activated protein kinase kinase 1/2 inhibitor PD98059, p38 inhibitor SB203580, and JNK1/2 inhibitor SP600125. For 48 h incubation, growth inhibition effect was determined by MTT assay. (b) AGS cells were treated with 30 μM Hispidulin for the indicated times. Total ERK1/2, phosphor-ERK1/2(*p*ERK1/2), total p38, phosphor-p38(*p*p38), total JNK, and phosphor-JNK (*p*JNK) were detected by Western blot. (c) Attenuation of Hispidulin-induced NAG-1 upregulation in AGS cells by ERK inhibitor PD98059. AGS cells where treatment with 25–50 μM of PD98059 and NAG-1 expression was evaluated by Western blot analysis. (d) Apoptosis induced by Hispidulin was attenuated by PD98059 ERK inhibitor treatment followed by flow cytometry analysis. Each column represents the mean ± SD ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). Expression of β-actin was used as an internal control.

upregulation and growth inhibition, we applied MAPK inhibitors to Hispidulin-treated AGS cells. The ERK inhibitor PD98059 reduced the growth inhibitory effects of Hispidulin significantly (Figure 5(a)); however, the p38 inhibitor SB203580 and the JNK inhibitor SP600125 did not limit its growth inhibitory effects in AGS cells (data not shown). The ERK1/2 inhibitor PD98059 was the sole compound to reduce Hispidulin-induced upregulation of EGR-1 and NAG-1 protein expression (Figure 5(c)). These data support the concept that Hispidulin-induced ERK1/2 activity is critically involved in the regulation of EGR-1 and NAG-1 expression.

In an attempt to identify the signaling pathway through which PI3K/AKT/GSK3β is involved in receptor signal transduction through tyrosine kinase receptors for hispidulin, the effect of LY294002, a PI3K inhibitor, was examined. We found that LY294002 did not reverse tumor growth inhibition caused by hispidulin (data not shown). Compared with the effects of other phytochemical agents, this result suggests that the ability of our drug to cause tumor apoptosis might not go through this pathway. Results from analyses of COX-2 protein expression in AGS cells following their treatment with Hispidulin showed that Hispidulin induced morphological changes in the AGS cells (data not shown), inhibited AGS cell growth (Figure 1(a)), and arrested the cell cycle at the G1/S phase (Figure 3(a)). The tumor suppressor protein p53 plays a role in the molecular response to DNA damage. Acting as a DNA-binding transcription factor, it regulates specific target genes to arrest the cell cycle and initiate apoptosis. Following

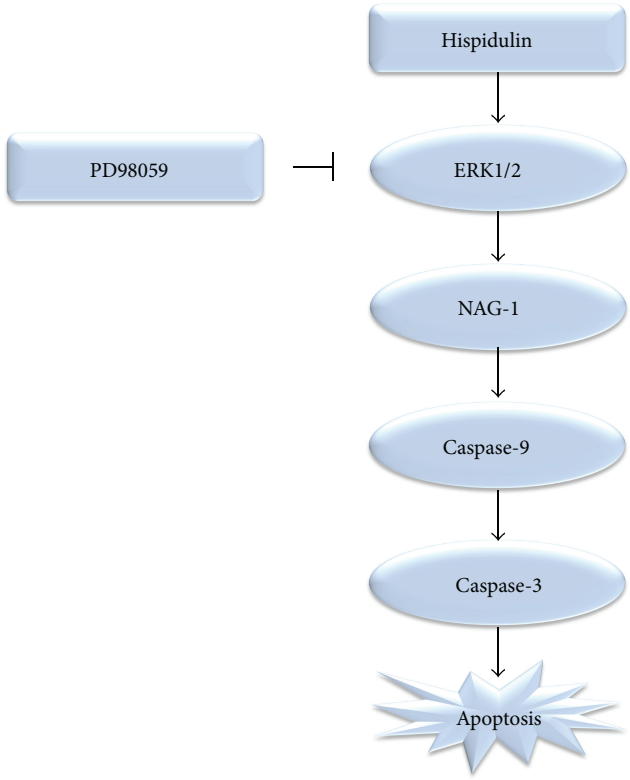

Figure 6: Hypothetic mechanism of signaling for gastric cancer inhibition by Hispidulin. Hispidulin can efficiently activate ERK1/2 signaling followed by NAG-1 constitutive expression and trigger cell cycle arrest as well as apoptosis in cancer cell.

DNA damage, the cyclin-dependent kinase inhibitor p21 is upregulated in a p53-dependent or p53-independent manner. The p21 might assist with the maintenance of G1 cell cycle arrest by inactivating the cyclin D1/cyclin E complex, leading to the phosphorylation of the Rb protein. In this study, Hispidulin treatment upregulated phsopho-p53, p16, and p21 expression in AGS cells (Figure 3(b)). Our findings indicated that Hispidulin mediates AGS cell arrest at the G1/S phase (Figure 3(a)), with cell arrest accompanied by the downregulation of cyclin D1 and cyclin E expression. These results suggested that Hispidulin treatment causes inappropriate accumulation of G1/S regulators leading to apoptosis.

Hispidulin induces apoptosis in A549 cells following mitotic arrest. The mechanism by which microtubule-damaging agents induce apoptosis is not well understood. The activation of aspartate-specific cysteine protease (caspase) represents a crucial step in the induction of drug-induced apoptosis, with the cleavage of PARP by caspase-3 considered one of the hallmarks of apoptosis. In this study, the pan caspase-3 inhibitor FMK reduced the apoptosis-promoting effects of Hispidulin (Figures 2(b) and 2(c)). These results suggested that a caspase-dependent pathway mediates Hispidulin-induced apoptosis (Figures 2(d)-2(e)) and cell death. In our experiments, Hispidulin induced caspase-9 activation, Bcl-2 phosphorylation, and cleavage of caspase-3 and PARP in a time-dependent manner (Figures 2(d)-2(e)).

In summary, Hispidulin is a novel flavonoid compound that has modulatory effects on the expression of cyclin D1/cyclin E G1/S regulatory proteins and initiates the apoptotic cascade [19, 20]. Our study findings show that NAG-1 displays anti-tumorigenic and proapoptotic activities *in vitro* and indicate that Hispidulin, regulates NAG-1 expression. Although further detailed analyses are required to fully elucidate the mechanisms underlying the antitumorigenic effects of Hispidulin these results should encourage further investigation of Hispidulin as a potential novel clinical anticancer drug.

Abbreviations

NAG-1: NSAID-activated gene-1
K8: Isochaihulactone
RT-PCR: Reverse transcription-polymerase chain reaction
DMSO: Dimethyl sulfoxide
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
MTT: 3-(4,5-dimethyl thizol-2-yl)-2,5-diphenyl tetrazolium bromide
PVDF: Polyvinyldenefluoride
MBD: Methyl-CpG binding domain
NSAIDs: Anti-inflammatory drugs
CI: Combination index
FBS: Fetal bovine serum
IHC: Immunohistochemical.

Conflict of Interests

All of the authors indicated no potential conflict of interests relevant to this paper.

Authors' Contribution

Due-Chuan Chan and Yi-Lin Sophia Chen contributed equally to this work.

Acknowledgments

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Research Article

Chemotherapy-Induced Peripheral Neuropathy in Cancer Patients: A Four-Arm Randomized Trial on the Effectiveness of Electroacupuncture

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Purpose. Chemotherapy-induced peripheral neuropathy (CIPN) is a common and dose-limiting side effect of cytostatic drugs. Since there are no proven therapeutic procedures against CIPN, we were interested to define the role of electroacupuncture (EA) from which preliminary data showed promising results. *Methods*. In a randomized trial with a group sequential adaptive design in patients with CIPN, we compared EA (LV3, SP9, GB41, GB34, LI4, LI11, SI3, and HT3; $n = 14$) with hydroelectric baths (HB, $n = 14$), vitamin B1/B6 capsules (300/300 mg daily; VitB, $n = 15$), and placebo capsules ($n = 17$). The statistical power in this trial was primarily calculated for proving EA only, so results of HB and VitB are pilot data. *Results*. CIPN complaints improved by 0.8 ± 1.2 (EA), 1.7 ± 1.7 (HB), 1.6 ± 2.0 (VitB), and 1.3 ± 1.3 points (placebo) on a 10-point numeric rating scale without significant difference between treatment groups or placebo. In addition no significant differences in sensory nerve conduction studies or quality of life (EORTC QLQ-C30) were found. *Conclusions*. The used EA concept, HB, and VitB were not superior to placebo. Since, contrary to our results, studies with different acupuncture concepts showed a positive effect on CIPN, the effect of acupuncture on CIPN remains unclear. Further randomized, placebo controlled studies seem necessary. This trial is registered with DRKS00004448.

1. Background

Chemotherapy-induced peripheral neuropathy (CIPN) is common and can be dose limiting for several cytotoxic drugs, for example, the antitubulins (paclitaxel, docetaxel, ixabepilone, and vincristine), platinum analogs (cisplatin, carboplatin, and oxaliplatin), and the proteasome inhibitors bortezomib and thalidomide. CIPN symptoms usually appear symmetrically in a stocking-glove shaped distribution pattern. Typical symptoms include numbness and tingling, whereas neuropathic pain appears less frequently. Affected patients experience considerable impairments including difficulty in walking, increased risk of falls, and weakness and restrictions in fine motor skills such as writing and other differentiated manual tasks. After the completion of chemotherapy, the symptoms frequently determine the patient's quality of life and often considerably hinder social rehabilitation, social reintegration, and return to work [1, 2].

Various substances including amifostine, glutathione, vitamin E, glutamic acids, intravenous calcium and magnesium infusions, and neurotrophic growth factors have been examined in clinical studies as a prophylaxis against chemotherapy-induced neurotoxic effects. No studies have shown convincing evidence of substantial clinical benefit [3, 4]. Anticonvulsants (e.g., carbamazepine and in particular gabapentin), tricyclic antidepressants (e.g., amitriptyline), and

selective serotonin reuptake inhibitors (e.g., venlafaxine) play a clinical role in the prevention and treatment of neuropathic pain. However, these drugs are ineffective for the treatment of the typical sensory CIPN symptoms and cannot induce neuroprotection or neuroregeneration [5–8].

A recently published survey revealed that approximately 43% of patients with chronic peripheral neuropathy use or have used complementary or alternative medicine (CAM), including high doses of vitamins, magnet therapy, herbal remedies, and chiropractic treatment. In addition, 30% of patients used acupuncture. Approximately a quarter of the patients stated that their symptoms improved after using CAM therapies [9].

The WHO and leading German acupuncture societies have long-listed neuropathy as an indication for acupuncture [10]. Few reports have published on its effect. Several studies demonstrated beneficial effects of acupuncture in diabetic neuropathy [11–14], HIV-associated neuropathy [15, 16], and in peripheral neuropathy with an unclear etiology [17]. The very limited conclusions from these studies resulted either from the small number of treated patients, the uncontrolled study design, or the publications in Chinese journals with merely an abstract in English and therefore not easy to evaluate. Acupuncture in combination with electrostimulation for treating CIPN has rarely been evaluated [18, 19]; however it has been demonstrated to improve neuropathic pain in paclitaxel-treated rats at both low (10 Hz) and high (100 Hz) frequencies [20].

In German-speaking countries, hydroelectric baths are traditionally used in many rehabilitation centers for the treatment of peripheral neuropathy [21]. However, their effectiveness has not been proven via controlled studies. The use of vitamin B supplements to treat neuropathy is also very common but also without rigorous clinical evaluation [22].

In this randomized controlled trial we compared the effects of electroacupuncture with placebo, hydroelectric baths, and vitamin B complex.

2. Methods

2.1. Study Design. The study was conducted as a randomized, placebo-controlled trial comparing electroacupuncture (EA) with hydroelectric baths (HB), high doses of vitamin B1 and B6 (VitB), and a placebo for the treatment of CIPN. Patients and physicians were blind to the VitB and placebo treatment but not to EA or HB.

The trial was planned according to a group sequential adaptive design with one interim analysis [23]. The experimental type I error rate was set at a one-sided $\alpha = 0.025$. Early cessation was planned if either EA proved to be significantly better than placebo (i.e., $P < \alpha_1 = 0.0207$) or showed no relevant superiority ($P > \alpha_0 = 0.6$).

Patients were allocated to one of the four treatment groups by a nonstratified block randomization with randomly varying block lengths. The biometrician drew numbers from the "ranuni" random number generator of the SAS/STAT software and prepared sealed, opaque, and sequentially numbered envelopes containing the treatment assignments. If a patient fulfilled all inclusion criteria the study physician opened the lowest numbered envelope to reveal the patient's assignment, that is, "EA," "HB," or "medical intervention." Patients from the latter group were given coded bottles of study capsules that were prepared prior to the beginning of the study by an impartial pharmacist. The bottles contained 63 capsules of VitB or placebo. Placebo and VitB capsules were identical in form, taste, and odour. The randomization list was kept closed by the biometrician, the pharmacist, and the principle investigator and was not accessible to the study physician.

The study was conducted according to the Declaration of Helsinki and its amendments. The study protocol was approved and accepted by the Ethics Committee of the Albert Ludwigs-University of Freiburg.

2.2. Patients. The study enrolled male and female adult cancer patients, who were in remission after chemotherapy with taxanes, platinum derivatives, or vinca alkaloids and who presented with symptoms of CIPN. Patients were recruited from a 3-4-week inpatient rehabilitation program in the Tumor Biology Center at the Albert Ludwigs University Freiburg, Germany. All patients received detailed information about the study and provided written informed consent before participation.

2.3. Treatments. Treatment protocols spanned 3 weeks and were as follows.

2.3.1. Electroacupuncture (EA). 8 ± 1 sessions of EA were scheduled to treat the affected extremities with the following point combination: LV3 (Taichong), SP9 (Xiongxiang), GB41 (Zulingqi), GB34 (Yanglingquan) (legs; in patients with CIPN symptoms in the lower extremities) and LI4 (Hegu), LI11 (Quchi), SI3 (Houxi), and HT3 (Shaohai) (arms; in patients with CIPN symptoms in the upper extremities). Patients with CIPN symptoms in the upper and lower extremities were treated with the complete point combination. According to the practices of traditional Chinese medicine (TCM) the acupuncture needles were deeply inserted bilaterally until the deqi phenomenon (sensation which spreads over the whole body part described as "aching," "soreness," "pressure," or "tingling" [24]) was triggered. Each session included 15 minutes of electrostimulation (50 Hz) consisting of a combination of rectangular currents and high amplitude waves. The stimulus strength was increased until the deqi phenomenon was triggered again. The acupuncture was carried out by two specially trained, highly experienced physicians at the University Medical Center Freiburg who had completed training in acupuncture with the German Physicians Association.

2.3.2. Hydroelectric Baths (HB). 8 ± 1 sessions of HB were scheduled to treat the affected extremities. The patients dipped their arms up to a hand's width above the elbow and their feet up to a hand's width above the ankle into a special water basin with water at a temperature of about 35°C. The water served as an electrode for the skin surface. Each treatment lasted for 15 minutes with cross-galvanisation of each

individual extremity by low-frequency (50 Hz) faradic current (direct current impulses) up to the individual's sensitive threshold (i.e., the point where the tingling feeling is considered to be just acceptable).

2.3.3. Vitamin B Complex (VitB). The treatment consisted of 3 capsules of high-dosage vitamin B1/B6 (100 mg thiamine nitrate, 100 mg pyridoxine hydrochloride) per day for three weeks.

2.3.4. Placebo. The placebo treatment consisted of 3 lactose capsules per day identical in form, taste, and odour to the VitB capsules.

3. Outcomes

3.1. Primary Outcome. By means of detailed questionnaires, patients were interviewed before the start of the therapy (day 0), after treatment on day 21, and during follow-up on day 84, about extension and intensity (non, mild, moderate, or severe) of their CIPN complaints (numbness, swelling, tingling, pain, and subjective impairment in everyday life and at work).

Finally, patients were asked to describe how heavily they suffered at the respective point in time from CIPN complaints altogether and to rate the severity of neuropathic symptoms on a numerical rating scale (NRS)—ranging from 0 (no complaints) to 10 (highest imaginable complaints). The change from day 0 to day 21 on this patient-reported numerical rating scale was the primary outcome of the study.

3.2. Secondary Outcomes. Before the start of the study (day 0) and after completion of the treatment (day 21), the patients were examined by an independent neurologist. The neurologist ascertained a neuropathy score and performed electroneurographical tests.

The neuropathy score (0–15 points) was based on sensory symptoms (0–3 points), pin sensibility (0–3 points), vibratory threshold (0–3 points), strength (0–3 points), and deep tendon reflexes (0–3 points). The electroneurographical tests included sensible nerve conduction studies of the median (upper extremities) and the sural nerve (lower extremities).

Finally, the neurologist evaluated the intensity of the CIPN complaints by classification according to the NCI common toxicity criteria (CTC) [25].

Examinations by the neurologist were only performed at day 0 and day 21, while the follow-up interview after 12 weeks, at day 84, was done in writing, via questionnaires sent to the patients' homes all over Germany.

Quality of life: the study participants' quality of life was examined at day 0, day 21, and day 84 by means of EORTC QLQ-C30 [26].

4. Statistics

This trial was planned using a group-sequential adaptive design which allowed for an adaptation (i.e., recalculation) of sample sizes after the first interim analysis. A priori power calculation showed that our test procedure had a type II error probability to stop the trial early for nonsuperiority (i.e., $P > \alpha_0 = 0.6$) of 16.7% (type II error) if EA had a moderate effect beyond the placebo of 0.5 standard deviations.

The data were analyzed using ANCOVA and modeling the treatment group and the baseline value (linear predictor) as covariates. Within this model, treatment groups were compared in pairs by one-sided t-tests following the principle of a priori ordered hypothesis [27]. All six comparisons were ordered according to a previously defined list which started with the comparison of EA and placebo. This list was processed successively and a subsequent comparison was performed if and only if the actual comparison could be rejected at the nominal level (i.e., 0.0207 at the interim analysis). This procedure ensured that the overall experimental type I error rate was maintained.

All analyses were performed based on intention to treat; that is, all randomized patients who received at least one study treatment were included in all (effectiveness or safety) analyses. Missing values were imputed using last observation carried forward [28].

5. Results

From September 2000 to February 2003 a total of 199 cancer patients with CIPN were assessed for eligibility and 60 were randomized into one of the four treatment groups (Figure 1). The main reasons for exclusion were pretreatment with vitamin B ($N = 40$), progressive cancer ($N = 22$), ongoing chemotherapy ($N = 16$), treatment with cytostatic drugs not allowed in the protocol ($N = 17$), or patient's unwillingness to take part ($N = 26$). Immediately after randomization one patient in the HB group withdrew his consent (before receiving any study treatment). 4 patients stopped treatment: 1 EA patient stated he was anxious of being needled (day 1), 1 VitB patient's tumor progressed (day 13), 1 placebo patient withdrew his consent (day 1), and 1 VitB patient found the study too much strain (day 1). Another 4 patients were lost to follow-up after day 21 without providing any reason.

(a) Baseline Data. The majority of patients were female (78%) and were on average 52.7 ± 10.0 years old, and 92% had ECOG performance status of 0. Nineteen patients were obese (BMI > 30). Four patients (3 HB, 1 placebo) presented with additional neurological problems other than CIPN, 2 patients had facial paresis, one patient had double vision, and one had diminished strength in the right hand. Overall, the groups were balanced with regard to demographic characteristics, health status, and comorbidities (Table 1).

The underlying cancer diagnoses as well as the cancer treatments were very heterogeneous within the study cohort (Table 2). Seventeen patients had a lymphoma (4 Hodgkin and 13 non-Hodgkin), and 42 patients had solid tumors, predominantly breast cancer (21) and ovarian cancer (13). Breast cancer was the most frequent disease in the placebo group and lymphoma in the HB group. Due to these imbalances there were also some differences in the use of chemotherapeutic drugs: vinca alkaloids were most often used in the VitB group and taxanes in the EA and the placebo

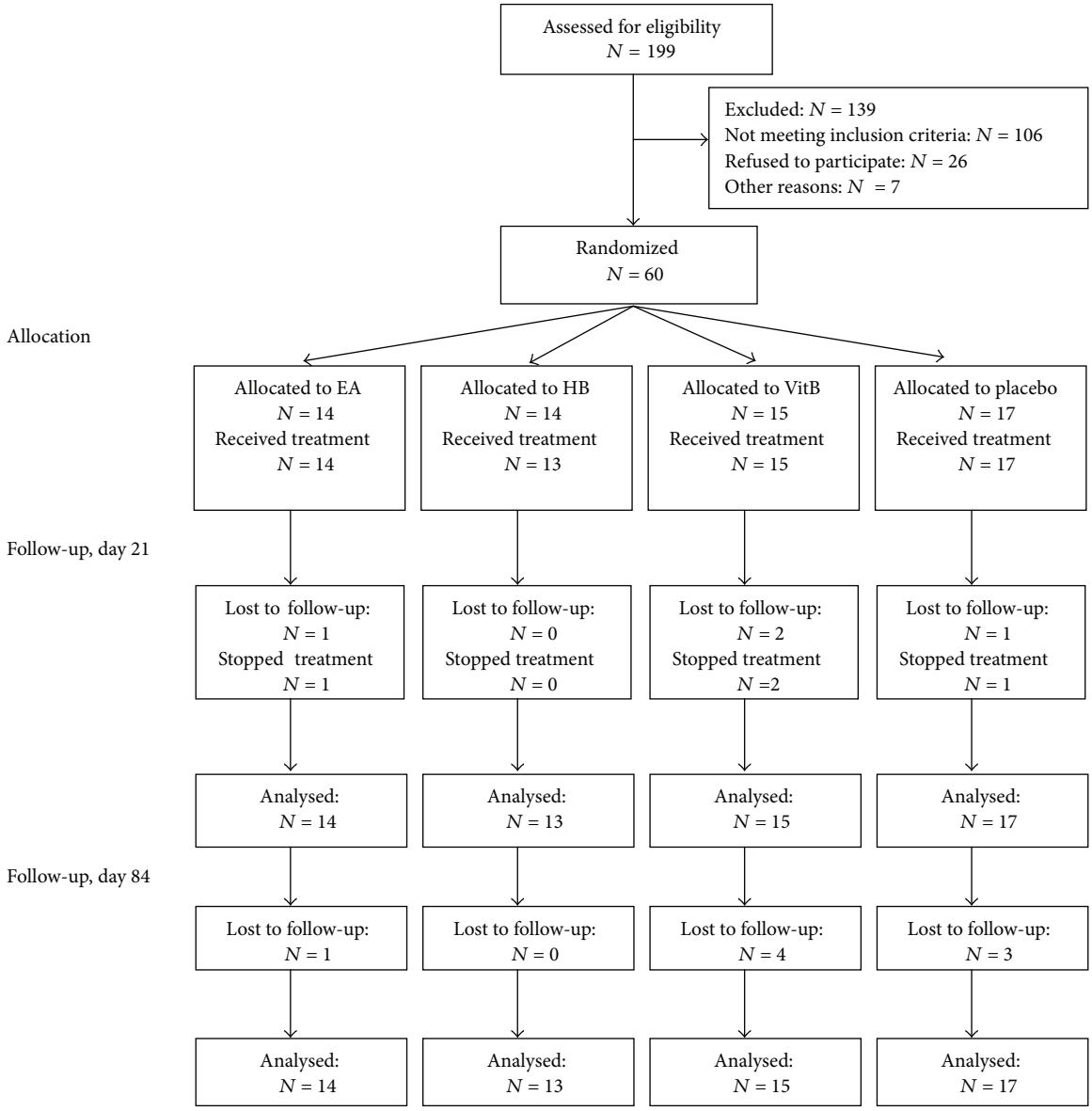

Figure 1: Flow chart.

Table 1: Basic data (no. of patients or mean ± SD).

	EA	HB	VitB	Placebo
Sex (m : f)	4 : 10	1 : 12	5 : 10	3 : 14
Age (years)	49.9 ± 9.6	52.3 ± 11.3	56.3 ± 11.1	52.0 ± 8.1
Body mass index (kg/m^2)	24.1 ± 4.1	25.8 ± 5.5	24.5 ± 3.3	26.1 ± 4.9
General condition (ECOG score 0)	14 (100%)	11 (84.6%)	14 (93.3%)	15 (88.2%)
Neurological problems (other than CIPN)	0 (0.0%)	3 (23.1%)	0 (0.0%)	1 (5.9%)

groups, but these differences were statistically not significant (all P values > 0.30, chi-square tests). Moreover, the number of chemotherapy courses in total, or with neurotoxic chemotherapy only, was comparable between the groups (P = 0.291 and P = 0.667, Kruskal-Wallis tests).

The mean time since first cancer diagnosis ranged from 12.1 months in the EA to 24.9 months in the HB group. As the latter was dominated by an extreme exception (112 months) these differences were not statistically relevant (P = 0.825, Kruskal-Wallis test). Similarly, mean times from the last chemotherapy, last surgery, or last radiotherapy were comparable between groups (all P values >0.25).

Only a few patients (N = 7; 11.9%) reported that their first CIPN complaints had occurred after the chemotherapy

Table 2: History of cancer and CIPN (no. of patients or mean ± SD).

	EA	HB	VitB	Placebo
Breast cancer	6 (42.9%)	3 (23.1%)	4 (26.7%)	8 (47.1%)
Ovarian cancer	3 (21.4)	3 (23.1%)	4 (26.7%)	3 (17.6%)
Other	1 (7.1%)	2 (15.4%)	1 (6.7%)	4 (23.5%)
Lymphoma	4 (28.6%)	5 (38.5%)	6 (40.0%)	2 (11.8%)
Time from first diagnosis (months)	12.1 ± 11.6	24.9 ± 38.6	14.0 ± 13.8	14.5 ± 13.7
Secondary cancer	2 (14.3%)	3 (23.1%)	4 (26.7%)	4 (23.5%)
Chemotherapy				
Vinca alkaloids	4 (28.6%)	5 (28.5%)	6 (40.0%)	3 (17.6%)
Platin derivatives alone	1 (7.1%)	2 (15.4%)	0 (0.0%)	3 (17.6%)
Taxanes alone	6 (42.9%)	3 (23.1%)	4 (26.7%)	8 (47.1%)
Platin derivatives and taxanes combined	3 (21.4%)	3 (23.1%)	5 (33.3%)	5 (29.4%)
Total no. of different cytostatics	2.1 ± 1.4	1.5 ± 0.9	1.7 ± 1.0	2.0 ± 0.8
No. of diff. neurotoxic cytostatics only	1.6 ± 1.2	1.1 ± 0.3	1.2 ± 0.4	1.3 ± 0.6
Time from last chemotherapy (weeks)	20.1 ± 27.4	27.8 ± 49.3	8.7 ± 5.8	14.2 ± 17.0
Cancer surgery	11 (78.6%)	6 (46.2%)	10 (66.7%)	15 (88.2%)
Radiotherapy	9 (64.3%)	8 (61.5%)	7 (46.7%)	13 (76.5%)
Duration of CIPN > 6 months	6 (42.9%)	5 (38.5%)	5 (33.5%)	4 (23.5%)
CIPN stable or worsened	9 (64.3%)	7 (53.8%)	9 (60.0%)	10 (58.8%)

Table 3: CIPN: detailed subjective complaints.

Symptoms	EA	HB	VitB	Placebo	Sum
	$N = 14$ (%)	$N = 13$ (%)	$N = 15$ (%)	$N = 17$ (%)	$N = 59$ (%)
Numbness	11 (78.6%)	10 (76.9%)	13 (86.7%)	16 (94.1%)	50 (84.7%)
Sensation of swelling	9 (64.3%)	10 (76.9%)	8 (53.3%)	12 (70.6%)	39 (66.1%)
Parasthesia	14 (100%)	13 (100%)	11 (73.3%)	15 (88.2%)	53 (89.8%)
Pain	7 (50%)	6 (46.2%)	6 (40%)	2 (11.8%)	21 (35.6%)
Subjective impairment in walking	7 (50%)	8 (61.6%)	9 (60.0%)	14 (82.4%)	38 (64.4%)
Subjective impairment in fine motor skills	7 (50%)	8 (61.6%)	10 (66.7%)	10 (58.9%)	35 (59.3%)

was finished; mostly, complaints were first noticed during chemotherapy. Symptoms usually started during the first or second chemotherapy cycle. At the start of the study, one-third of the patients had been experiencing CIPN for more than 6 months ($N = 20$; 33.9%) and another two-fifths had it for more than 3 months ($N = 25$; 42.4%). In 31 patients the CIPN had been stable over the last 4 weeks (52.5%), in 23 patients it had improved (38.9%), and in 4 patients it had worsened (6.8%). The treatment groups were balanced with respect to all these parameters (all P values >0.15, Kruskal-Wallis tests).

Although statistically not significant ($P = 0.263$, Kruskal-Wallis test), mean baseline symptoms varied considerably between groups and ranged from 4.0 ± 1.7 in the EA to 5.5 ± 2.6 in the HB group (Table 4 and Figure 2). At study entry subjective CIPN complaints of the included patients differed as shown in Table 3: while every patient in EA and HB groups suffered from parasthesia, only three-quarters in vitamin B group and 88% in placebo group did so. In the placebo group less patients described painful neuropathy compared to the treatment groups. Subjective impairment in fine motor skills was nearly equal in all four groups.

(b) Treatment Results. Symptoms improved similarly in all groups during the three weeks of treatment and remained at this lower level for another 9 weeks, except in the HB group where some deterioration was observed. At day 21, improvement was best in the HB (1.7±1.7) and the VitB group (1.6 ± 2.0). Compared to placebo (1.3 ± 1.3) EA showed worse effects (0.8 ± 1.2) resulting in a group difference of $d = -0.3$ (CI: −1.4 to 0.8; $P = 0.705$). As the P value exceeded the predefined threshold of $\alpha_0 = 0.6$, the study was stopped early at the first interim analysis. Moreover, improvements in the EA group were smaller than in the VitB group ($d = -0.5$; CI: −1.7 to 0.6) and the HB group ($d = -0.2$; CI: −1.3 to 0.9) (Table 4).

The neuropathy score decreased in all groups during treatment to a similar degree. Improvements were observed most frequently in the EA group and were smallest in the HB and placebo groups (Table 5). Group differences were not significant between any two groups; for example, the difference between EA and placebo was $d = -0.4$ (CI: −1.1 to 0.3; $P = 0.128$).

There were no statistically significant differences between the treatment groups by electroneurographical test results

Table 4: (a) CIPN perceived symptom severity (NRS) day 21–day 0 and day 84–day 0; (b) NRS—subjective CIPN complaints (ANCOVA day 21).

(a)

Group	Day	Mean ± SD	Diff. mean day 21–day 0 day 84–day 0
EA ($N = 14$)	0	4.0 ± 1.7	
	21	3.2 ± 1.9	**−0.8**
	84	3.4 ± 1.9	−0.6
HB ($N = 13$)	0	5.5 ± 2.6	
	21	3.8 ± 2.9	**−1.7**
	84	4.7 ± 3.3	−0.8
VitB ($N = 15$)	0	4.9 ± 1.8	
	21	3.3 ± 2.3	**−1.6**
	84	3.1 ± 1.6	−1.8
Placebo ($N = 17$)	0	4.9 ± 2.1	
	21	3.6 ± 1.6	**−1.3**
	84	3.1 ± 1.3	−1.8

(b)

	Difference	95% CI	One-sided t-test	Two-sided t-test
EA versus placebo	0.3	−0.8–1.4	$P = 0.705$	$P = 0.59$
HB versus placebo	−0.2	−1.3–0.9	$P = 0.35$	$P = 0.699$
EA versus VitB	0.5	−0.6–1.7	$P = 0.83$	$P = 0.34$
HB versus VitB	0.0	−1.1–1.2	$P = 0.52$	$P = 0.959$
EA versus HB	0.2	−0.9–1.3	$P = 0.65$	$P = 0.699$
VitB versus placebo	−0.2	−1.3–0.8	$P = 0.323$	$P = 0.646$

(Table 6) and NCI common toxicity criteria classification (Table 7). Sensory neuropathy symptoms improved similarly in all four groups: 32.7% with a CTC grade 2 or 3 at day 0 and 17.3% at day 21; 0% with a CTC grade 0 at day 0 and 21.2% at day 21.

Health related quality of life also moderately improved in all groups, but without any statistical group differences at day 21 (Table 8).

6. Discussion

In this randomized, placebo-controlled study, electroacupuncture, hydroelectric baths, and a high dosage of oral vitamin B1/B6 combination were studied to determine their effectiveness and safety in patients with chemotherapy-induced peripheral neuropathy. Sixty cancer patients were included within the setting of an inpatient oncology rehabilitation program.

In our study we observed no therapeutic advantage of electroacupuncture over an orally administered placebo control. In addition, no effects of hydroelectric baths and vitamin

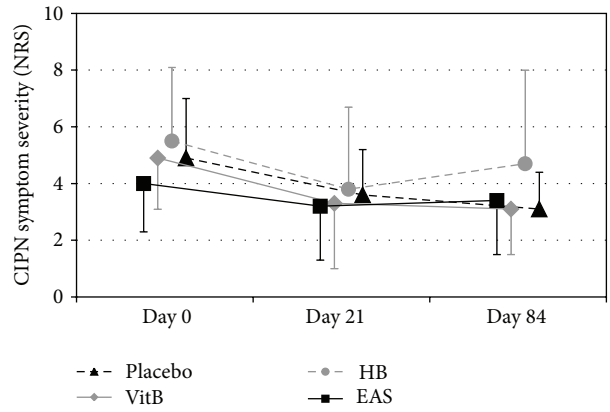

Figure 2: CIPN perceived symptom severity day 0, day 21, and day 84 (severity rated on a 10-point numerical rating scale (NRS); all values are mean ± SD).

Table 5: Neuropathy score (mean ± SD).

	Group	Day 0	Day 21	Change day 0 to 21
Neuropathy score (0–15)	EA	4.5 ± 1.5	3.5 ± 1.2	−1.0 ± 1.1
	HB	5.0 ± 2.2	4.4 ± 2.1	−0.6 ± 0.9
	VitB	3.9 ± 1.2	3.2 ± 1.3	−0.7 ± 0.8
	Placebo	4.1 ± 1.5	3.5 ± 1.7	−0.6 ± 1.0

B1/B6 were demonstrated. It should, however, been taken into consideration that the statistical power of the later tests was low because the study was powered to test the effectiveness of electroacupuncture.

When evaluating our results, several limitations must be considered. Unexpectedly, the intensity of CIPN complaints at baseline was relatively low, particularly in the electroacupuncture group. Our study design, including sample size calculations, was based on experiences with patients with more severe CIPN symptoms and higher pain scores showing larger clinical effects of electroacupuncture treatment. Consequently, there were no large margins for many patients to improve considerably, and our study results might have been influenced by a floor effect.

In spite of the limitation of this study, we are convinced that reporting negative results is of importance. Studies reporting positive results are more likely to be published [29], while negative results more often have to be published in journals with lower impact factors [30]. By publishing our negative results, we hope our data will have an impact on the critical discussion on study designs and acupuncture concepts for CIPN.

In 2006, a small case series ($n = 5$) reported encouragingly positive results on acupuncture without electrical stimulation for the treatment of CIPN [31]. All five patients showed improvements in pain, numbness, and tingling. In contrast to our study, all patients suffered from painful peripheral neuropathy with high symptom scores and were treated over a time period of 12 weeks. The authors pointed out that they are carrying out a larger trial, the results of

Table 6: Electroneurographical tests (amplitude and nerve conduction velocity) day 21–day 0 (mean ± SD).

Group	Day	Sural nerve		Median nerve	
		Amplitude (norm > 10 μV)	NCV (norm > 42 m/s)	Amplitude (norm > 7 μV)	NCV (norm > 45 m/s)
EA (N = 13)	0	5.4 ± 3.6	46.4 ± 4.7	19.0 ± 10.7	48.2 ± 4.7
	21	7.4 ± 6.0	46.0 ± 3.7	16.8 ± 7.0	48.4 ± 4.4
Diff. mean day 21–day 0		*+2.0*	*−0.4*	*−2.2*	*+0.2*
HB (N = 12)	0	4.3 ± 2.2	46.2 ± 3.4	15.5 ± 7.8	40.7 ± 9.7
	21	6.3 ± 2.6	46.8 ± 7.1	15.9 ± 9.8	46.3 ± 6.3
Diff. mean day 21–day 0		*+2.0*	*+0.6*	*+0.4*	*+5.6*
VitB (N = 13)	0	3.9 ± 2.7	45.0 ± 4.3	15.9 ± 9.8	46.9 ± 4.1
	21	5.1 ± 3.8	45.0 ± 3.8	15.3 ± 6.4	51.2 ± 3.1
Diff. mean day 21–day 0		*+1.2*	*±0*	*−0.6*	*+4.3*
Placebo (N = 14)	0	5.3 ± 3.1	45.3 ± 4.2	17.5 ± 9.5	47.1 ± 7.5
	21	6.3 ± 3.4	45.9 ± 5.8	16.8 ± 8.5	50.6 ± 6.0
Diff. mean day 21–day 0		*+1.0*	*+0.6*	*−0.7*	*+3.5*

Table 7: NCI common toxicity criteria (sensory item) day 21–day 0.

Group	Day	CTC sensory item			
		0	1	2	3
		N (%)	N (%)	N (%)	N (%)
EA (N = 13)	0	0 (0%)	10 (76.9%)	3 (23.1%)	0 (0%)
	21	2 (15.4%)	9 (69.2%)	2 (15.4%)	0 (0%)
HB (N = 12)	0	0 (0%)	4 (33.3%)	6 (50.0%)	2 (16.7%)
	21	2 (16.7%)	7 (58.3%)	2 (16.7%)	1 (8.3%)
VitB (N = 13)	0	0 (0%)	11 (84.6%)	2 (15.4%)	0 (0%)
	21	3 (23.1%)	9 (69.2%)	1 (7.7%)	0 (0%)
Placebo (N = 14)	0	0 (0%)	10 (71.4%)	3 (21.4%)	1 (7.1%)
	21	4 (28.6%)	7 (50.0%)	3 (21.4%)	0 (0%)

which are eagerly awaited. A randomized controlled trial on acupuncture for the treatment of CIPN without electrical stimulation was conducted in Beijing, China. The authors describe significant better treatment effects in the acupuncture group in relation to the control group treated with adenosylcobalamin [32]. A recently published small pilot study on acupuncture for the treatment of CIPN (n = 11) reports improvements in nerve conduction studies as well as in the subjective rating of the patients [33].

Several different acupuncture concepts have been used in the treatment of CIPN. Different to our protocol, other studies that reported positive effects of acupuncture treatments in CIPN used local points on the extremities like EX-LE 10 (Bafeng), EX-UE 9 (Baxie), and EX-LE 12 (Qiduan) [31, 33–35] or ear acupuncture for neuropathic pain [36]. In particular the local points on the affected limb/region might activate local, segmental, and spinal and central reflexes in accordance with results on animal models [20].

In traditional Chinese medicine an individualized approach with personalized acupuncture treatment is usually expected, while in acupuncture studies there is the necessity of standardization of the procedures, as done in this study. So our results only indicate that our particular standardized acupuncture protocol might not be effective in the treatment of CIPN, but the results cannot be generalized to other acupuncture concepts.

This study was embedded in a routine care rehabilitation program delivered at a specialized clinic in Southwest Germany. Participating patients came from many regions in Germany and thus were only available for a three-week treatment and observation period. Considering the frequent long-term chronic course of the CIPN symptoms it cannot be excluded that a longer treatment period might have yielded different results for electroacupuncture or one of the other therapies under study. Prior studies on acupuncture treatment in peripheral neuropathy have shown that measurable results can only be found after a longer period of treatment up to 10 weeks [17]. While peripheral neuropathy is a disease with structural damage of the nerves, any stable successful treatment has to induce neuroregeneration. The time of functional recovery varies, ranging usually from 3 to 6 months, depending on the level of the lesion and factors in regeneration [37]. So effects of treatment naturally take longer than in other indications of acupuncture treatment with functional states like in pain or vegetative imbalance.

All participating patients were not treated exclusively by the therapies under study but concomitantly received various medical and psychological interventions, depending on the individual need of each patient. This included regular participation in sport therapy sessions, psychoeducative groups, art therapies, ergotherapies, relaxation methods, physiotherapies, massages, and lymph drainages. Effects of these interventions may have contributed to a remarkable effect in the placebo group—as well as to all other groups—thus possibly diminishing the estimated group differences. Positive effects of this program—as delivered in the study center—have already been demonstrated [38].

Nevertheless further studies are necessary, to evaluate the role of acupuncture in the treatment of CIPN. The use of nerve conduction studies as an objective parameter for the evaluation of treatment effects [17] as well as recently introduced better outcome measures for the standardization of studies of CIPN will be helpful to improve the quality of future studies [39].

Table 8: Quality of life (EORTC QLQ-C30) day 0–day 21–day 84.

EORTC QLQ-C30 scale	Day	EA		HB		VitB		Placebo		Sum	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Physical functioning	0	72.4	22.2	61	22.4	68.4	15.8	75.3	15.6	69.7	19.4
	21	84.4	13.2	66.7	24.5	75.1	12.7	87.5	10.2	79.1	17.3
	84	82.3	12.2	67.3	26.5	80.5	15.7	85.6	11.2	79.5	17.9
Role functioning	0	58.3	34.4	38.5	30.7	37.8	27.8	52.9	28.4	47.2	30.8
	21	72.6	26.6	61.5	37.5	58.9	20.8	74.5	23.7	67.2	27.5
	84	64.5	30.3	56.4	31.6	53.3	29.7	71.4	16.2	61.9	27.4
Emotional functioning	0	63.1	21.6	44.2	25.3	54.4	27.1	62.3	26.4	56.5	25.7
	21	72.6	15.1	62.2	28.0	66.7	24.6	70.4	27.3	68.2	24.1
	84	70.6	18.6	52.3	35.5	62.2	32.7	72.0	19.1	64.8	27.5
Cognitive functioning	0	66.7	23.6	39.7	30.1	62.2	28.5	67.6	24.6	59.9	28.2
	21	70.2	18.7	55.1	30.0	71.1	24.0	79.4	18.2	69.8	23.9
	84	76.7	21.8	58.5	36.2	61.6	25.8	71.0	13.2	67.2	25.2
Social functioning	0	66.7	32.0	50.0	34.0	46.7	34.6	63.7	27.8	57.1	32.3
	21	83.3	17.3	62.8	36.1	60.0	32.0	77.5	25.6	71.2	29.3
	84	81.9	20.3	60.8	40.6	64.7	26.9	80.2	21.4	72.4	28.6
Global health	0	58.9	12.9	55.1	21.7	51.7	20.7	58.3	17.7	56.1	18.2
	21	67.9	13.4	62.8	26.0	61.7	17.2	67.6	17.4	65.1	18.5
	84	71.0	12.4	59.1	27.0	64.7	14.3	72.4	15.0	67.1	18.0

7. Conclusion

A specific standardized electroacupuncture concept, as well as vitamin B1/B6 and hydroelectric baths, showed similar treatment effects on CIPN and was not superior to placebo control. While contrary to our results other studies with different acupuncture concepts and longer treatment periods showed a positive effect on CIPN, the effect of acupuncture on CIPN remains unclear. Further randomized, placebo controlled studies seem necessary.

Conflict of Interests

All authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

Authors' Contribution

M. Rostock, K. Jaroslawski, C. Guethlin, H. H. Bartsch, and R. Ludtke designed the study, M. Rostock, K. Jaroslawski, and C. Guethlin collected the data, R. Ludtke carried out the statistical analyses, M. Rostock, K. Jaroslawski, S. Schröder, C. Guethlin, H. H. Bartsch, and R. Ludtke drafted the paper, and all authors participated in the interpretation of the findings, reviewed the paper, and approved the final paper.

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Review Article

Can Medical Herbs Stimulate Regeneration or Neuroprotection and Treat Neuropathic Pain in Chemotherapy-Induced Peripheral Neuropathy?

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Chemotherapy-induced neuropathy (CIPN) has a relevant impact on the quality of life of cancer patients. There are no curative conventional treatments, so further options have to be investigated. We conducted a systematic review in English and Chinese language databases to illuminate the role of medical herbs. 26 relevant studies on 5 single herbs, one extract, one receptor-agonist, and 8 combinations of herbs were identified focusing on the single herbs *Acorus calamus rhizoma*, *Cannabis sativa fructus*, *Chamomilla matricaria*, *Ginkgo biloba*, *Salvia officinalis*, *Sweet bee venom*, *Fritillaria cirrhosae bulbus,* and the herbal combinations Bu Yang Huan Wu, modified Bu Yang Huan Wu plus Liuwei Di Huang, modified Chai Hu Long Gu Mu Li Wan, *Geranii herba* plus *Aconiti lateralis praeparata radix* , Niu Che Sen Qi Wan (Goshajinkigan), Gui Zhi Jia Shu Fu Tang (Keishikajutsubuto), Huang Qi Wu Wu Tang (Ogikeishigomotsuto), and Shao Yao Gan Cao Tang *(Shakuyakukanzoto)*. The knowledge of mechanism of action is still limited, the quality of clinical trials needs further improvement, and studies have not yielded enough evidence to establish a standard practice, but a lot of promising substances have been identified. While CIPN has multiple mechanisms of neuronal degeneration, a combination of herbs or substances might deal with multiple targets for the aim of neuroprotection or neuroregeneration in CIPN.

1. Introduction

Several chemotherapeutic drugs are known to be neurotoxic and can lead to chemotherapy-induced peripheral neuropathy (CIPN). It is one of the main dose-limiting toxicities in oncologic treatments and a potential reason to terminate or suspend chemotherapy, in some cases leading to disease progression [1]. CIPN involves damage to the peripheral nervous system and can produce severe neuropathic pain [2, 3], sensory deficits, or gait impairment [4] and can severely decrease the patient's quality of life [5]. Sensory symptoms usually develop before motor symptoms, because motor neurons are more myelinated [6, 7]. Distal parts of the axons are the first affected, so sensory symptoms typically start symmetrically and bilaterally from the tips of the toes and fingers and progress proximally in a "stocking-glove" distribution [8]. The incidence of CIPN can reach levels of up to 92% [9]. The major groups of drugs that induce CIPN include the antitubulins (paclitaxel, docetaxel, ixabepilone, and vincristine), platinum analogs (cisplatin, carboplatin, and oxaliplatin), and the proteasome inhibitors bortezomib and thalidomide [1]. Patients with a preexisting other cause of

peripheral neuropathy develop more severe and persistent CIPN [10–12].

The development of CIPN is usually dependent on the cumulative dose and symptoms may progressively aggravate [10]. After sustaining therapy symptoms are usually reversible, but in some cases they may be irreversible [10, 13] and sometimes even progress after stopping medication, especially after treatment with vinca alkaloids (e.g., vincristine), platinum analogues (e.g., cisplatin and oxaliplatin), and taxanes (e.g., paclitaxel) [14, 15]. Mostly the sensory nerve cell bodies of the dorsal root ganglia are affected, or the afferent and efferent distal peripheral axons are damaged [16].

Some differences concerning the mechanisms of CIPN are described for different therapeutic drugs.

In platinum compounds the dorsal root ganglia are the main parts of the nervous systems that are injured [17] and apoptosis is induced via structural alterations of DNA and of cell-cycle kinetics [18], triggered by oxidative stress and mitochondrial dysfunction but possibly downregulated by a reduction of enzymes like p53 [19–22].

The taxanes paxlitaxel and docetaxel are antitubulins and mainly damage the soma of the sensory neurons and the nerve axons. This is induced by an interference with the axonal transport, which is caused by enhancement of microtubule polymerization [23]. Microglial activation in the spinal cord and high concentration in the dorsal root ganglia tissue induce CIPN [24]. Like platinum compounds, taxanes can damage dorsal root ganglia. This is induced by macrophage activation in the dorsal root ganglia but occurs also in the peripheral nerve [24]. Paclitaxel induces a massive polar reconfiguration of axonal microtubules and an impairment of organelle transport [25].

Vinca alkaloids (e.g., vincristine) prevent tubulin polymerization from soluble dimers into microtubules [26]. By affecting the tubulin dimers loss of axonal microtubules and alterations in their length, arrangement and orientation are produced [27, 28]. This alters the neuronal cytoskeleton, leading to abnormalities in axonal transport and axonal degeneration [28, 29]. Decreasing affinity for tubulin of the vinca alkaloids explains the different neurotoxicity profiles and the severity of CIPN [30].

Epothilones (e.g., ixabepilone) are like the taxanes antitubulins, so there might be similar mechanisms of peripheral neurotoxicity. They damage the ganglion soma cells and peripheral axons through disruption of microtubules of the mitotic spindle and interfere with the axonal transport in the neurons [31] and can also induce polymerization of tubulin dimers in microtubules. Additionally they stabilize preformed microtubules against conditions favouring depolymerization [32, 33].

Bortezomib might induce pathological changes in Schwann cells and myelin, axonal degeneration, and dorsal root ganglia neuron changes [34, 35], as well as chromatolysis of dorsal root ganglial neurons. It causes cytoplasmic accumulation of eosinophilic material [36] and interferes with the transcription, nuclear processing and transport, as well as with the cytoplasmic translation of messenger RNAs in dorsal root ganglions [37]. Mitochondrial and endoplasmic reticulum-mediated calcium dysregulation plays an important part [38, 39]. The activation of the mitochondria-based apoptotic pathway or inhibition of the transcription of the nerve growth factor by interference with the nuclear factor-κB pathway can lead to disarrangement of the neurotrophin network [39, 40]. Bortezomib can induce changes in all major primary afferent fibres [41].

The structure of thalidomide is characterized by a 3-substituted glutarimide ring and a ph-thalimide ring, which are sensitive to enzymatic or nonenzymatic hydrolysis [42], but despite several studies it has not been possible to identify the responsible enzymes for the production of neurotoxic metabolites, so its mechanism of peripheral neurotoxicity is still elusive [43].

Multiple drugs have been tested, mainly in animal studies, for their putative neuroprotective activity in CIPN. A few components which can protect different tissues from toxic agents were clinically tested, showing conflicting results. No conclusive reports confirming their effectiveness have been provided [44, 45] and a reduction of anticancer activity was suspected [46]. Several neurotrophins were tested but there was no evidence of neuroprotection [47–50]. Erythropoietin, a multifunctional trophic factor, showed promising results in preclinical studies [51, 52] but has relevant safety problems [53].

Antioxidants have been tested as neuroprotectants [54–59], but no conclusive evidence of neuroprotection has yet been found.

Neuropathic pain is conventionally treated by antiepileptic and tricyclic antidepressant drugs, but these drugs are ineffective for treatment of decreased sensation and cannot induce neuroprotection or neuroregeneration. Other compounds with different mechanisms (e.g., acetyl-L-carnitine, glutamate carboxypeptidase II inhibitors, calpain inhibitors, a solution of calcium and magnesium) have now been investigated in preclinical stages with unknown value for clinical routine [59–63].

Thus specific and effective curative treatments for CIPN are lacking, especially those meant for enhancing neuroregeneration or neuroprotection [64–67], and the evaluation of further treatment options is of great importance. While the effectiveness of herbal treatment is not well known yet, this review was done to illuminate the actual and potential future role of herbal treatment in CIPN.

2. Methods

To review the existing clinical and experimental studies of herbal treatment in CIPN, a systematic literature search was performed from the databases from inception up until January 2013 using MEDLINE, Google Scholar, Cochrane Database, CINHAL (Cumulative Index to Nursing and Allied Health Literature), CNKI (China National Knowledge Infrastructure), and Wanfang Med Online and ISI Proceedings for conference abstracts. The keywords searched were as follows:

(Chinese herbs or herbs or plants or Chinese medicine as MeSH term) AND (neuropathy or chemotherapy). The CINHAL, CNKI, and Wanfang Med Online Databases did

not allow logical searches with AND, so we used simple combinations of the search words. Historical searches of reference lists of relevant articles were also undertaken.

To be included in our review a study had to focus on the topic CIPN and neuropathy in human and animal models irrespective of design. Papers with at least an English abstract were included. Study selection was performed by two reviewers (SSch and KB) with disagreement resolved by discussion and adjudication.

Listed articles concerning diseases other than CIPN and herbal treatment were excluded, while animal products used in the tradition of herbal medicine were included.

3. Results

A total of 3474 (1477 in English and 1997 in Chinese databases) articles were retrieved by way of electronic searches and examination of reference lists of clinical and review articles. After screening titles and/or abstracts, 3376 articles were excluded because either the focus was on an intervention other than CIPN and herbal treatment or they were duplicated studies or not relevant. From a total of 98 articles which were retrieved for detailed evaluation, 15 were included in the review, focusing on 5 single herbs, one extract, one receptor agonist, and 8 combinations of herbs. For a summary of the experimental and clinical studies see Table 1.

3.1. Single Herbs or Single Herbal Compounds

3.1.1. Acorus calamus rhizoma . This herb (family: Araceae) is traditionally used in the treatment and management of pain and severe inflammatory in Ayurveda. It is commonly used to relieve the muscle, joint, vascular and nerve injury associated with severe inflammatory and neuropathic pain [68]. In a rat model a hydroalcoholic extract of *Acorus calamus rhizoma* has been shown to exert beneficial effect on neuropathic pain induced by tibial and sural nerve transection [69]. In a further study *Acorus calamus rhizoma* extract attenuated sciatic nerve chronic constriction injury and induced ameliorated behavioral (hyperalgesia and allodynia), biochemical (superoxide anion, myeloperoxidase, and total calcium), and histopathological (axonal degeneration) changes [68]. Another study investigated the protective effect of *Acorus calamus rhizoma* extract in vincristine-induced painful neuropathy. Hydroalcoholic extracts of *Acorus calamus rhizoma* attenuated vincristine-induced behavioral and biochemical changes to an extent comparable to pregabalin (positive control) and attenuated vincristine-induced painful neuropathy, which probably may be attributed to its multiple effects including antioxidative, anti-inflammatory, and calcium inhibitory activity [70].

3.1.2. Cannabis sativa. Two structurally distinct cannabinoid CB2 agonists—the aminoalkylindole (R,S)-*AM1241* ((R,S)-(2-iodo-5-nitrophenyl)-[1-((1-methyl-piperidin-2–yl)methyl)-1H-indol-3-yl]-methanone) and the cannabilactone *AM1714* (1,9-dihydroxy-3-(1′,1′-dimethylheptyl)-6H-benzo[c]chromene-6-one))—had been tested for their dose related suppression of established paclitaxel-evoked mechanical allodynia in a rat model. *(R)-AM1241*, but not *(S)-AM1241*, suppressed paclitaxel evoked mechanical allodynia, and *AM1714* induced a modest antinociceptive effect. So the authors suggested that cannabinoid CB2 receptors may be important therapeutic targets for the treatment of chemotherapy-evoked neuropathy [71]. *Cannabis sativa* or *Cannabis* extracts have not been clinically explicitly tested for CIPN but for other clinical conditions like neuropathic pain in HIV [72]. Multiple clinical trials examined the effect on neuropathic pain and found positive effects on central and peripheral neuropathic pain with different forms of application [73–82].

3.1.3. Matricaria chamomilla. This is a commonly used herb in western as well as in eastern phytopharmacological tradition. Flavonoids from *Matricaria chamomilla* seem to have an antispasmodic effect and main components such as α-bisabolol or chamazulene have anti-inflammatory effects [83]. En-In-Dicycloether has both antispasmodic and anti-inflammatory effects together [84]. In a mouse model it was shown that *Matricaria chamomilla extract*-treated mice had a significant reduction of cisplatin-induced peripheral pain [85]. *Matricaria chamomilla* hydroalcoholic extract was able to decrease cisplatin-induced pain and inflammation better than morphine [85].

3.1.4. Ginkgo biloba. The popular herb from the maidenhair tree that has shown some promising effects as an neuroprotectant. The most unique components of the extracts are the terpene trilactones, that is, ginkgolides and bilobalide [109]. In vitro and ex vivo studies indicate that bilobalide has multiple mechanisms of action that may be associated with neuroprotection, including its preservation of mitochondrial ATP synthesis, its inhibition of apoptotic damage induced by staurosporine or by serum-free medium, its suppression of hypoxia-induced membrane deterioration in the brain, and its action in increasing the expression of the mitochondrial DNA-encoded COX III subunit of cytochrome C oxidase and the ND1 subunit of NADH dehydrogenase [110]. Because multiple modes of action may apply to bilobalide, it could be useful in developing therapy for neurodegeneration [109–111]. Oztürk et al. investigated *Ginkgo biloba* alcoholic extract in cisplatin-induced peripheral neuropathy in mice [86]. Development of neuropathy was evaluated with changes in sensory nerve conduction velocity (NCV) and *Ginkgo biloba* extract prevented reduction in NCV. In another study a *Ginkgo biloba* extract prevented some functional and morphological deteriorations induced by cisplatin, antagonizing the decrease in the number of migrating cells and in the length of outgrowing axons [86]. Marshall et al. investigated retrospectively 17 patients with colorectal cancer who received oxaliplatin along with *Ginkgo biloba* extract, but no specification of the extraction method was provided in the published abstract. The researchers found that 11 of the 17 patients developed a grade 1 peripheral neuropathy (PN) after the first cycle of oxaliplatin. Five of six patients who received *Ginkgo biloba* after the second cycle of oxaliplatin

Table 1: Medical herbs tested for CIPN.

Section no.	Medical herb(s)	Study design	Chemotherapy	Outcome	Author/year
			Single herbs or single compounds		
3.1.1	*Acorus calamus rhizoma* (hydroalcoholic extract)	Rat model	Vincristine	Improvement of neuropathic pain	Muthuraman and Singh, 2011 [70]
3.1.2	*Cannabis*-Receptor agonists, R-AM1241 and Am-1714	Rat model	Paclitaxel	Allodynia, antinociceptive	Rahn et al., 2008 [71] multiple trials for neuropathic pain not due to chemotherapy, for example, Ellis et al. [72], Karst et al. [73], Ware et al. [74], Rog et al. [75], Blake et al. [76], Berman et al. [77], Wilsey et al. [78] with Cannabis sativa
3.1.3	*Matricariachamomilla* (hydroalcoholic extract)	Mouse model	Cisplatin	Improvement of neuropathic pain	Abad et al. 2011 [85]
3.1.4	*Ginkgo biloba* (alcoholic extract)	(1) Mouse model (2) Retrospective study ($n = 17$)	Oxaliplatin	(1) Neuroprotection (2) Improvement of neuropathic pain	(1) Oztürk et al., 2004 [86] (2) Marshall et al., 2004 [87]
3.1.5	*Salvia officinalis* (hydroalcoholic extract)	Mouse model	Vincristine	Improvement of neuropathic pain	Abad et al., 2011 [88]
3.1.6	*Sweet bee venom* (pharmacopuncture)	(1) Case series ($n = 5$) (2) Case series ($n = 11$)	Taxol, paclitaxel, or carboplatin	Injection into acupoint (Zusanli, St 36) (1) Effect on pain and neuropathy scales (2) Effect on VAS and HRQOL scores	(1) Park et al., 2011 [89] (2) Yoon et al., 2012 [90]
3.1.7	Verticinone, hydroalcoholic extracted from *Fritillaria bulbus*	Mouse model and rat model	Paclitaxel	Inflammatory and neuropathic pain, dose dependent, no tolerance	Xu et al., 2011 [91]
			Herbal combinations		
3.2.1	*Bu Yang Huan Wu* [*1] (*decoction*)	Randomized trial, $n = 44$, control = 40	Oxaliplatin	Improvement of clinical scales	Sun et al., 2008 [92]
3.2.2	modified *Bu Yang Huan Wu* plus *Liuwei Di Huang* [*2] (*decoction*)	Randomized trial, $n = 32$, control Vit. B1 = 32	Different chemotherapies	Improvement of clinical scales	Deng and Zou, 2007 [93]
3.2.3	modified *Chai Hu Long Gu Mu Li Wan* [*3] (*decoction*)	Randomized trial, $n = 26$, control = 22	Paclitaxel	neuroprotection	Pan et al., 2012 [94]
3.2.4	*Geranii herba* plus Aconiti radix (granule)	(1) Rat model (2) Randomized, prospective trial $n = 30$, control = 28	(1) Oxaliplatin (2) Oxaliplatin, taxol or capecitabine	(1) Allodynia (2) Neuropathic pain, paraesthesia, selling. external washing	Sima and Pan, 2009 [95]
3.2.5	Goshajinkigan = *Niu Che Sen Qi Wan* [*4] (granule)	(1) Rat model (2) Rat/mouse model	(1) Paclitaxel (2) Oxaliplatin	(1) No neurogeneration, improvement of allodynia (2) Improvement of neuropathic pain, no neuroregeneration	(1) Hashimoto et al., 2004 + 2006 [96, 97] (2) Ushio et al., 2012 [98]
		(1) Noncontrolled study $n = 14$ (2) Retrospective study GJG = 22, control = 23 (3) Retrospective study GJG = 45, control = 45 (4) Retrospective study $n = 82$ (5) Randomized prospective study Vit B12 = 14, VitB12/GJG = 15	(1) Oxaliplatin (2) Folfox[*8] (3) Folfox[*8] (4) Paclitaxel (5) Paclitaxel/ carboplatin	(1) Reduced acute neurotoxicity (2) Neuroprotection (3) Neuroprotection No change of anticancer activity (4) Neuroprotection, better when administered early (5) Less severe neurotoxicity, better CPT in GJG group	(1) Shindo et al., 2008 [99] (2) Nishioka et al., 2011 [100] (3) Kono et al., 2011 [101] (4)Yamamoto et al., 2009 [102] (5) Kaku et al., 2012 [103]

Table 1: Continued.

Section no.	Medical herb(s)	Study design	Chemotherapy	Outcome	Author/year
3.2.6	Keishikajutsubuto = Gui Zhi Jia Shu Fu Tang[*5] (granule)	Uncontrolled study $n = 15$ (3 dropouts)	Folfox[*8]	76.6% mean improvement on VAS	Yamada et al., 2012 [104]
3.2.7	Ogikeishigomotsuto = Huang Qi Wu Wu Tang[*6] (granule)	Case report $n = 1$	Oxaliplatin	neuropathic pain	Tatsumi et al., 2009 [105]
3.2.8	*Shakuyakukanzoto* = Shao Yao Gan Cao Tang[*7] (granule)	(1) Mouse model (2) Retrospective case analysis $n = 23$ (3) Retrospective clinical study, comparison GJG = 20, SYK = 24	(1) and (2) paclitaxel (3) Folfox[*8]	(1) Allodynia, hyperalgesia (2) Effect on neuropathic pain (3) 50% response in Shakuyu-kanzoto and 65% in Goshajinkigan on prevention of neurotoxicity	(1) Hidaka et al., 2009 [106] (2) Fujii et al., 2004 [107] (3) Hosokawa et al., 2012 [108]

[*1] *Astragalus membranaceus radix, Angelica sinensis radix, Prunus persicae semen, Paeoniae rubra radix, Ligustici chuanxiong rhizoma, Lumbricus terrestris, Spatholobi caulis, Curcuma radix, Chaenomeles lagenaria fructus*, and *Achyranthes bidentata*.

[*2] *Astragalus membranaceus radix, Ligustrum lucidum fructus, Paeoniae rubra radix, Lumbricus terrestris, Prunus persicae semen, Rehmanniae viridae radix, Corni officinalis fructus, Dioscorea opposita* radix; and *Alismatis rhizoma, Poria alba, Spatholobi caulis, Scolopendra, Mori fructus, Glycyrrhizae Radix, Dipsaci fructus, Lycii fructus, Coicis semen, Atractylodis rhizoma, Phellodendri cortex, Scorpio, Mori ramulus*, and *Cyathula officinalis*.

[*3] *Pseudostellaria heterophylla, Pinelliae rhizoma, Glycyrrhizae radix, Scutellaria baicalensis radix, Bupleuri radix*, *Fossilia ossis mastodia draconis*,
Ostrae concha,
Rubiae cordifoliae radix, Scutellariae barbatae herba, and *Fritillaria thunbergii bulbi*. The external washing was done with *Astragali radix, Angelica sinensis radix, Paeoniae rubra radix, Lumbricus terrestris, Ligustici chuanxiong rhizoma*, *Prunica persicae semen*, and *Carthami flos*.

[*4] *Rehmannia viride radix, Achyranthis bidentatae radix, Corni fructus, Dioscoreaopposita rhizome, Plantaginis semen, Alismatis rhizoma, Moutan cortex, Cinnamomi cortex, Aconiti lateralis praeparata tuber*, and *Poria alba*.

[*5] *Cinnamomi cortex, Aconiti lateralis praeparata tuber, Zingiberis rhizoma, Jujubae fructus, Glycyrrhizae radix*, and *Atracylodis macrocephalae rhizoma*.

[*6] *Astragali membranaceus radix, Cinnamomi cortex, Paeonia alba radix, Jujubae fructus*, and *Zingiberis rhizoma*.

[*7] *Paeonia alba radix* and *Glycyrrhizae radix*.

[*8] Chemotherapeutic regime with FOL: Folinic acid (leucovorin), F: Fluorouracil (5-FU), and OX: Oxaliplatin (Eloxatin).

reported decreased intensity and duration of sensory PN. No *Ginkgo biloba* related side effects have been observed. The data suggested that *Ginkgo biloba* extract appears to attenuate the intensity and duration of acute dysesthesias caused by oxaliplatin and may yield synergistic antitumor activity [87].

3.1.5. Salvia officinalis. *Salvia* species and their isolated constituents possess significant antioxidant activity in enzyme-dependent and enzyme-independent systems [112–115]. The flavonoid apigenin, for example, has been shown to protect neurons against Aβ-induced toxicity [116]. In addition to antioxidant activity, many *salvia* species and their isolated constituents showed anti-inflammatory properties [117, 118]. *Salvia officinalis* extract can have anti-inflammatory and also antinociceptive effects on chemical behavioral models of nociception in mice that involve an opioid mechanism [119]. An animal study showed the effects of the *Salvia officinalis* hydroalcoholic extract on vincristine-induced PN in mice in comparison with morphine with a decrease of pain response, suggesting that *Salvia officinalis* extract could be useful in the treatment of vincristine-induced peripheral neuropathic pain [88].

3.1.6. Sweet bee venom. The venom of honey bees with its active peptide Melittin has been tested for injection into the acupuncture point Zusanli (ST 36) for its effect on CIPN in animal models. It showed to alleviate thermal hyperalgesia and mechanical allodynia. The results indicated an association with the activation of the LC noradrenergic system and with a reduction in spinal pNR1 [120, 121].

In a first case series this was tested in 5 patients in a 1-week course of treatment, which showed no side effects and gave evidence of clinical improvement [89]. Another prospective case series of this procedure analyzed the clinical observations made on 11 CIPN patients treated with *Sweet bee venom*. A total of 11 eligible consecutive CIPN patients were treated for 3 weeks and observed for another 3 weeks. A significant intraindividual improvement was found for pain and neuropathy scales [90].

3.1.7. Verticinone from Fritillaria bulbus. Verticinone, an isosteroidal alkaloid isolated from *Fritillaria bulbus*, was evaluated in mice for its analgesic activities in murine models of inflammatory and neuropathic pain. It was shown that oral administration of hydroalcoholic extracted verticinone could significantly inhibit acetic acid-induced writhing response in a dose-dependent manner superior to acetylsalicyl acid. In the rat model of paclitaxel induced neuropathic pain, in contrast to the declined analgesic effect of morphine after repeated administration with the same dose, a relatively constant analgesic effect of verticinone was observed, so verticinone is expected to become a potentially novel sedative-analgesic agent without producing tolerance [91].

3.2. Herbal Combinations

3.2.1. Bu Yang Huan Wu (Chin.) = Tonify the Yang to Restore Five-Tenths Decoction (Engl.). *Bu Yang Huan Wu* is a classical combination of Chinese herbs, first mentioned in Wang Qing-Ren's *Yi Lin Gai Cuo* (Correcting the Errors in the Field of Medicine) published in 1830 [122]. This recipe contains *Astragalus membranaceus radix, Angelica sinensis radix, Prunus persicae semen, Paeoniae rubra radix, Ligustici chuanxiong rhizoma, Lumbricus terrestris, Spatholobi caulis, Curcuma radix, Chaenomeles lagenaria fructus*, and *Achyranthes bidentatae radix*.

The decoction has been used in a randomized Chinese study of 84 patients (treatment group $n = 44$, control group $n = 40$) after the treatment of oxaliplatin and showed reduced development of CIPN in the treatment group tested by standardized clinical tests [92].

3.2.2. Modified Bu Yang Huan Wu (Chin.) = Tonify the Yang to Restore Five-Tenths Decoction (Engl.) plus Liuwei Di Huang (Chin.) = Rokumijiogan (Jap.) = Pilula Rehmannia Sex Saporum (Lat.) = Six Ingredients Pill with Rehmannia (Engl.). In another randomized Chinese study a modified combination of two standard recipes *Bu Yang Huan Wu* and *Liuwei Di Huang* was tested as a decoction. *Liuwei Di Huang* was first described in the Yozheng Zhijue [123]. The mixture of both recipes contains *Astragalus membranaceus radix, Ligustrum lucidum fructus, Paeoniae rubra radix, Lumbricus terrestris, Prunus persicae semen, Rehmanniae viride radix, Corni officinalis fructus, Dioscorea opposita radix; and Alismatis rhizoma, Poria alba, Spatholobi caulis, Scolopendra, Mori fructus, Glycyrrhizae radix, Dipsaci fructus, Lycii fructus, Coicis semen, Atractylodis rhizoma, Phellodendri cortex, Scorpio, Mori ramulus*, and *Cyathula officinalis*.

The remaining dregs of decoction were additionally used for washing the lower extremities. The treatment was used on 32 patients with existing CIPN following different chemotherapies and compared with 32 patients who were treated orally with vitamin B1 2500 ug plus by intramuscular injection with vitamin B1 100 mg. Herbal treatment was found to be significantly more effective to vitamin treatment ($P < 0.05$) [93].

3.2.3. Modified Chai Hu Long Gu Mu Li Wan (Chin.) = Modified Saikokaryukotsuboreito (Jap.) = Modified Formula bupleurum cum ostrea et Fossilia ossis (Lat.) = Modified Bupleurum, Dragon Bone, and Oyster Shell Formula (Engl.). *Chai Hu Long Gu Mu Li Wan* is a traditional recipe derived from the *Shang Han Lun* [124]. A modification of this recipe was used in a Chinese randomized trial in which 48 patients with ovarian cancer were examined parallel to chemotherapy with placitaxel. They were divided into a treatment group with paclitaxel alone and a treatment group with paclitaxel plus a combination of oral Chinese herbal decoction treatment and external washing of the feet with Chinese herbs.

The oral combination of herbs consists of *Pseudostellaria heterophylla*, *Pinelliae rhizoma*, *Glycyrrhizae radix*, *Scutellaria baicalensis radix*, *Bupleuri radix*, *Fossilia ossis mastodi*, *Ostreae concha*, *Rubia cordifolia radix*, *Scutellariae barbatae herba,* and *Fritillariae thunbergii bulbi*. The external washing

was done with *Astragali membranaceus radix*, *Angelica sinensis radix*, *Paeoniae rubra radix*, *Lumbricus terrestris*, *Ligustici chuanxiong rhizoma*, *Prunus persicae semen,* and *Carthami flos*.

The incidence of CIPN was almost half as high in the patients treated additionally with Chinese herbs as evaluated by clinical testing ($P < 0.05$) [94].

3.2.4. Geranii herba plus Aconiti radix. External application of a combination of *Geranii herba* and *Aconiti radix* extract has been shown to be effective in a rat model of oxaliplatin evoked neuropathy. Mechanical allodynia and thermal hyperalgesia were alleviated. NGF was increased, substance P decreased in the group treated with *Geranii herba* and *Aconiti radix* extract additionally to oxaliplatin compared to oxaliplatin alone [95].

In the following randomized clinical study 58 patients with CIPN from oxaliplatin, taxol, or capecitabine were assigned prospectively in a controlled randomized trial: 30 patients were assigned to the study group and 28 were used as a control. The clinical study revealed that symptoms of pain, paraesthesia, and swelling were relieved after one week of therapy and it was concluded that *Geranii herba* plus *Aconiti radix* granule can relieve neuropathy and improve the quality of life. Unfortunately the authors did not provide data in the published abstract, which species of *Geranii herba* or *Aconiti radix* they used [95].

3.2.5. Goshajinkigan (Jap.) = Niu Che Sen Qi Wan (Chin.) = Pilula renales plantaginis et achyranthis (Lat.) = Life Preserving Kidney Qi Pill (Engl.). This formula derives from the *Jisheng Fang*, written by Yan Yonghe, a little known but highly regarded physician of the Song Dynasty, published in 1253 [125]. In Japanese Kampo medicine it is called Goshajinkigan (GJG) and is frequently used as a standardized granule. It contains 10 different herbs (*Rehmannia viride radix, Achyranthis bidentatae radix, Corni fructus, Dioscorea opposita rhizoma, Plantaginis semen, Alismatis rhizoma, Moutan cortex, Cinnamomi cortex, Aconiti lateralis praeparata tuber,* and *Poria alba*) [126]. GJG has antioxidant properties [127, 128].

GJG was tested for its effect on CIPN in animal studies. In a rat model of oxaliplatin-induced neuropathy repeated administration of GJG prevented the oxaliplatin-induced cold hyperalgesia but not mechanical allodynia and axonal degeneration of the rat sciatic nerve. A single administration of GJG reduced both cold hyperalgesia and mechanical allodynia after the development of neuropathy. GJG did not affect the antitumour effect of oxaliplatin on the tumour cells or mice implanted with tumour cells [98].

GJG was also tested in a rat model for paclitaxel-induced peripheral neuropathy, but as with oxaliplatin no regeneration was found in histological examination [96]. Nevertheless further rat animal studies showed a positive effect of GJG on cold allodynia [97].

GJG has been widely used to treat symptoms like numbness, vibration sensation, cold sensation, and limb pain associated with diabetic neuropathy [129].

It has been shown to prevent oxaliplatin-induced peripheral neuropathy in a FOLFOX-regimen (FOL: Folinic acid (leucovorin), F: Fluorouracil (5-FU), OX: Oxaliplatin (Elox-atin)) in clinical studies [99, 100]. In a noncontrolled study 14 patients received GJG every day after the first oxaliplatin infusion. GJG seemed to prevent acute oxaliplatin-induced neurotoxicity [99]. In a retrospective analysis of 45 patients, 22 received GJG during their FOLFOX regimen against nonresectable or recurrent colorectal cancer, while 23 did not get this additional therapy. The incidence of grade 3 PN in the GJG group was significantly lower than in the control group ($P < 0.01$, log-rank test). The incidence of grade 3 PN after 10 courses of chemotherapy was 0% in the GJG group and 12% in the control group, and after 20 courses was 33% in the GJG group and 75% in the control group [100]. A further retrospective analysis was performed in 90 patients who were given a FOLFOX regimen for metastatic colorectal cancer. Two treatment groups were compared: FOLFXOX plus GJG and FOLFOX plus GJG plus Ca^{2+}/Mg^{+}, and two control groups: FOLFOX without additional therapy and FOLFOX plus Ca^{2+}/Mg^{+}. When a cumulative dose of oxaliplatin exceeded 500 mg/m^2, the incidence of PN was 91% in the FOLFOX without additional therapy group, 100% in the FOLFOX group with additional Ca^{2+}/Mg^{+} therapy, and 79% in the FOLFOX plus GJG plus Ca^{2+}/Mg^{+} therapy group and 50% in the GJG plus FOLFOX group. The cumulative oxaliplatin dose when 50% of patients developed neurotoxicity was 765 mg/m^2 in the GJG plus FOLFOX and 340 mg/m^2 in the FOLFOX plus GJG plus Ca^{2+}/Mg^{+} group, respectively, and 255 mg/m^2 in both control groups. The authors concluded that concomitant administration of GJG reduced the neurotoxicity of oxaliplatin without having a negative influence on the response rate [101], so for further validation of these data a concept for a prospective, controlled, double blinded randomized study was developed [130].

GJG was also tested for paclitaxel-induced neuropathy in breast and gynecological cancer. A retrospective study on 82 patients found that GJG was possibly effective for the treatment and the prevention of peripheral neuropathy and seemed to be more effective when administered from the beginning of chemotherapy using paclitaxel [102].

In a prospective study in paclitaxel/carboplatin treated patients with ovarian or endometrial cancer, patients were randomly divided into two groups. 14 patients received vitamin B12 and 15 patients vitamin B12 plus GJG. The observation period was 6 weeks following treatment initiation. A NCI-CTCAE (*National Cancer Institute-Common Toxicity criteria)* grade 3 of neurotoxicity developed in 2 patients (14.3%) after 6 weeks in the vitamin B12, whereas no neurotoxicity was observed in the vitamin B12/GJG group. The change in the frequency of abnormal current perception threshold (CPT) ratio was significantly lower in the VB12/GJG group in comparison to VitB12 alone ($P < 0.05$), which suggests that GJG inhibits the progression of PN [103].

3.2.6. Keishikajutsubuto (Jap.) = Gui Zhi Jia Shu Fu Tang (Chin.) = Decoctum ramulorum cassiae cum atractylodis macrocephae et aconiti (Lat.) = Cinnamon Twig Decoction plus Atractylodes and Aconite (Engl.). This formula has its basis from the *Shang Han Lun* and was further developed as a Japanese experimental formula during the Edo period (1603 to 1868). It contains *Cinamomi cortex, Aconiti lateralis praeparata tuber, Zingiberis rhizoma, Jujubae fructus, Glycyrrhizae radix,* and *Atracylodis macrocephalae rhizoma*.

This herbal combination with an increased dose of *Aconiti lateralis praeparata tuber* was used as a granule for 11 patients with metastatic colorectal cancer receiving FOLFOX in a noncontrolled study. Reduction of neuropathy was observed in 5 cases after chemotherapy (45.5%) [104].

The same herbal granule was also used in a study on postherpetic neuralgia and was found to be effective. In three of 15 patients in this noncontrolled trial continuation of the treatment with *Keishikajutsubuto* was not possible due to hot flashes or gastric discomfort. The remaining 12 patients showed a VAS improvement rate of 76.5 ± 27.7% (mean ± standard deviation) [131].

3.2.7. Ogikeishigomotsuto (Jap.) = Huang Qi Wu Wu Tang (Chin.) = (Decotum quinque medicamentorum cum astragalo (Lat.) = Astragalus and Cinnamon Five Herb Combination (Engl.). This classical combination derives from *Jin Gui Yao Lue* (Essential Prescriptions from the Golden Chamber) [132]. In Kampo medicine it is called *Ogikeishigomotsuto*, containing *Astragali membranaceus radix, Cinnamomi cortex, Paeonia alba radix, Jujubae fructus,* and *Zingiberis rhizoma*. It has been used in individual cases for neuropathic pain due to ANCA-associated vasculitis [133].

In single case report the granule showed a positive effect on neuropathic pain and it allowed the continuation of the suspended chemotherapy with oxaliplatin [105].

3.2.8. Shakuyakukanzoto (Jap.) = Shao Yao Gan Cao Tang (Chin.), Formula glycyrrhizae et paeonia (Lat.), Peony and Licorice Decoction (Engl.). This classical formula derives from the *Shang Han Lun* [124]. In Kampo medicine it is called *Shakuyakukanzoto* and it is a herbal granule of *Paeonia alba radix* and *Glycyrrhizae radix*. It is used to relieve menstrual pain and muscle spasm as well as muscle pain due to the chemotherapeutic agents paclitaxel and carboplatin [134–136] and has also been tested for CIPN. A retrospective case analysis of 23 patients showed a positive effect on neuropathic pain in CIPN after paclitaxel for ovarian carcinoma [107].

This was supported by animal studies in a mouse model of paclitaxel-induced pain. *Shakuyakukanzoto* significantly relieved the allodynia and hyperalgesia induced by paclitaxel [106]. *Shakuyakukanzoto* has also been tested for preventing neurotoxic side effects of FOLFOX and the effect was retrospectively compared to the treatment with GJG (see Section 3.2.5). 44 patients with metastatic colorectal cancer received FOLFOX and concurrently received either GJG (n = 20) or *Shakuyakukanzoto* (n = 24). The response was 50.0% in the GJG and 65% in the *Shakuyakkanzoto* group. The authors concluded that both recipes are able to reduce the FOLFOX-induced neurotoxicity [108].

3.3. Herbs Tested or Herbal Ingredients for Neuropathic Pain, Not Specifically Tested to CIPN. Capsaicin is the active component of chili peppers, which are plants belonging to the genus *Capsicum*. Topical creams with capsaicin are used to treat peripheral neuropathic pain. Following application to the skin capsaicin causes enhanced sensitivity, followed by a period with reduced sensitivity and, after repeated applications, persistent desensitisation.

Topical capsaicin is used to treat postherpetic neuralgia and HIV-neuropathy and has been found to be effective in multiple trials. There are risks of epidermal innervation upon repeated application over long periods [137].

Aconiti lateralis praeparata radix is a herb used in many recipes for neuropathy like GJG (see Section 3.2.5) and *Keishikajutsubuto* (see Section 3.2.6) and is often used for several types of persistent pain. In a mouse model analgesic effects caused by inhibition of astrocytic activation by *Aconiti lateralis praeparata radix* were mimicked by the intrathecal injection of fluorocitrate. The study indicated that the activation of spinal astrocytes was responsible for the late maintenance phase of neuropathic pain, so *Aconiti lateralis praeparata radix* could be a therapeutic strategy for treating neuropathic pain [138]. In a rat model *Aconiti lateralis praeparata radix* was tested against a placebo for allodynia and thermal hyperalgesia. A dose-dependent effect was measured. The effects were inhibited by intraperitoneal and intrathecal nor-binaltorphimine, a selective kappa-opioid receptor antagonist, but not by intraperitoneal naloxone. The authors concluded that the effect against neuropathic pain is induced via spinal kappa-opioid receptor mechanisms [139].

Moutan cortex and *Coicis semen* have been tested positively in two different neuropathic pain mice models. In one model allodynia was induced by intrathecal administration of prostaglandin F2alpha (PGF2alpha) and in the second model by selective L5 spinal nerve transection. The extracts of *Moutan cortex* and *Coicis semen* dose dependently alleviated the PGF2alpha-induced allodynia. The increase in NADPH diaphorase activity in the spinal cord associated with neuropathic pain was also blocked by these extracts. These results suggest that *Moutan cortex* and *Coicis semen* are substances effective in the treatment of neuropathic pain [140].

Nigella sativa and one main compound thymoquinone were beneficial on histopathological changes of sciatic nerves in streptozotocin (STZ) induced diabetic rats. Evaluation of the tissues in the diabetic animals showed fewer morphologic alterations, and myelin breakdown decreased significantly after treatment with *Nigella sativa* and thymoquinone. The ultrastructural features of axons also showed improvement [141].

Ocimum sanctum was investigated in sciatic nerve transection induced peripheral neuropathy in rats. Axonal degeneration was assessed histopathologically. Paw pressure, Von Frey Hair, tail cold-hyperalgesia, and motor in-coordination tests were performed to assess the in vivo extent of neuropathy. Biochemical estimations of thiobarbituric acid reactive

species (TBARS), reduced glutathione (GSH), and total calcium levels were also performed. *Ocimum sanctum* attenuated axonal degeneration, rise in TBARS, total calcium, and decrease in GSH levels in a dose-dependent manner. In vivo reduction of nociceptive threshold and motor incoordination was found. The authors concluded that antioxidant and calcium attenuating actions may be responsible for the amelioration [142].

In an another animal study STZ-induced diabetic rats received intraperitoneal injection of this extract of *Teucrium polium*. The treated rats exhibited a lower nociceptive score as compared to untreated diabetic rats. The results may suggest a therapeutic potential of *Teucrium polium* for the treatment of hyperalgesia [143].

A hexanic extract from *Phyllanthus amarus* has been shown to be effective in a neuropathic model of nociception. The antiallodynic effects seemed not to be associated with the impairment of motor coordination or with the development of tolerance. Apart from its anti-inflammatory actions, which are probably linked to the presence of lignans, another as yet unidentified active principle(s) present in the hexanic extract of *Phyllanthus amarus* produces pronounced anti-allodynia [144].

An aqueous extract of *Sesbania sesban* was tested in STZ-induced diabetic rats. The treated group showed an increased tail flick latency significantly when compared with pregabalin and reduced superoxide anion and total calcium levels which gave evidence of neuroprotective effects [145].

4. Discussion

In spite of intense research in the last decades, no conventional pharmacological substance has been established as a sufficient and safe treatment of CIPN-induced neuroprotection and regeneration [31, 43–63, 65–67]. Herbal treatment is commonly used for different kind of therapies where western medicine does not offer a sufficient efficacy, but the evidence of the use of herbal treatment is not clear and has to be elucidated.

One main problem of doing research on herbs and understanding the mechanisms of their action is the fact that herbs contain a number of active compounds and by tradition, especially in Asian herbal therapy, combinations of multiple herbs are common.

Classical research mainly focusing on a single active compound has been done often without regard to historical knowledge of the therapeutic utility of the plant source [146]. This does not reflect the complexity of traditional Asian herbal recipes, while there is some evidence that single components extracted from plants are less potent than the complete extract [147] and a multitarget approach might be more effective than a single target approach [148]. Pharmacological mixtures can also have the advantage of potentiating the action of their multiple bioactive components and the option of an individualized therapy [149, 150]. For scientific understanding of the mechanisms of action there is still a need for basic research on single herbs and their active compounds, but research should not stop at this level but continue with research of multicompounds, their interactions, and increasing or decreasing activity in combinations.

The positive effect of a single herb on neuroprotection or regeneration was not found in any clinical study and rarely found in animal studies. Only the flavonoid apigenin from *Salvia officinalis* seems to protect neurons against toxicity [116] and *Ginkgo biloba* as a single herb shows some evidence of preventing neuronal degeneration and inducing neural regeneration in CIPN [109–111].

Mainly in animal studies only a few single herbs have been tested for treatment of CIPN improving neuropathic pain. This effect might be induced by *Acorus calamus rhizoma* due to its antioxidative, anti-inflammatory, and calcium inhibitory actions [70]. Flavonoids from *Matricaria chamomilla* have an antispasmodic and an anti-inflammatory effect [83, 84]. *Salvia officinalis* is probably working due to its antioxidant activity and anti-inflammatory properties [112–115] and is also involved in an opioid mechanism [119]. *Fritillaria bulbus* might be effective due to its isosteroidal alkaloid verticinone [91]. While *Cannabis sativa* is effective in multiple and central pain syndromes [72–78], the fact that two *Cannabis* agonists had been shown to be effective in CIPN in a rat model could be a hint that *Cannabis sativa* might be a promising substance for pain in CIPN in the future.

Only *Ginkgo biloba* extract has been positively tested in a small retrospective clinical study in humans [87] and Sweet bee venom had shown positive results by injection into acupuncture points in a small clinical case series [89, 90]. Topical capsaicin from chili peppers was found to be effective in multiple trials to treat pain from postherpetic neuralgia and HIV-neuropathy but has not been specifically tested against pain in CIPN [137].

There are a few more single herbs tested in animal models for the treatment of neuropathic pain in other conditions than CIPN. *Moutan cortex* and *Coicis semen* have been tested positively in two different neuropathic pain models. Both herbs dose dependently alleviated the PGF2alpha-induced allodynia and blocked NADPH diaphorase activity in the spinal cord associated with neuropathic pain [140].

Nigella sativa, *Ocimum sanctum*, *Teucrium polium*, *Phyllanthus amarus,* and *Sesbania sesban* also have been tested in single studies to ameliorate neuropathic pain [141–145].

Aconiti lateralis praeparata radix in a mouse model of CIPN had analgesic effects by inhibition of spinal astrocytic activation [138] and in a rat model *Aconiti lateralis praeparata radix* had a dose-dependent effect on allodynia and thermal hyperalgesia which was induced via spinal kappa-opioid receptor mechanisms [139], which confirmed similar animal studies on neuropathic pain in diabetic mice [148]. Active components are alkaloids, for example, aconitine and mesaconitine and have in addition pain-relieving effects, cardiotonic and vasodilator actions [151–155].

Aconiti lateralis praeparata radix is often used for several types of persistent pain while it is rarely used as a single herb for CIPN. But interestingly *Aconiti lateralis praeparata radix* is part of many recipes used for CIPN as GJG, *Shakuyakukanzoto, Keishikajutsubuto,* and *Geranii herba* plus *Aconiti radix* combination (see Sections 3.2.4, 3.2.5, 3.2.6, and 3.2.8).

Some of the pain-relieving effects of GJG are known to be induced by aconite [139] and it is considered that the analgesic effect is exerted by the suppression of pain-transmitting substances release by κ-opioid receptor stimulation mediated by dynorphin, an endogenous opioid substance released by processed aconite [148]. But on the other hand, these effects were stronger, when using of the full recipe in comparison to the use of *Aconiti lateralis praeparata radix* alone [155].

GJG is the best tested herbal recipe for CIPN. But not all details of its mechanism have been clearly identified and for some ingredients the effects are unknown. It is considered that the analgesic effect is exerted through the improvement of peripheral nocireceptor sensitivity, vasodilation, and peripheral circulation by the promotion of NO production due to the effects of *Alismatis rhizoma* and *Dioscorea opposita rhizoma* mediated by the bradykinin B2 receptor and the muscarinic acetylcholine receptors [148]. Another substance from GJG, *Rehmanniae praeparata radix,* could promote the function of learning and memory of MSG rats, and its mechanism may be related to the increase of the expression of hippocampal c-fos and nerve growth factor (NGF) [156], which can be relevant for regeneration in CIPN as well, while NGF exhibits potent biological activities such as preventing neuronal death, promoting neurite outgrowth, and supporting synapse formation [157]. One further mechanism of GJG might be its positive effect on improving the microcirculation, which might be helpful for the recovery of the nerves in CIPN [158].

Even though the full recipe was clinically positively tested in a couple of clinical trials, most of them were uncontrolled or retrospective analyses [99–102, 130] and the only prospective trial had a limited number of participants [103]. So the evidence of a positive effect is still low and needs further clinical and experimental confirmation.

GJG has also been compared to another herbal recipe, *Shakuyakukanzoto,* and both were found to be effective in the treatment of CIPN, but there was no negative control group [107]. *Shakuyakukanzoto* was analyzed in a mouse model [106] and in a retrospective analysis of 23 cases without controls [108]. Since *Shakuyakukanzoto* contains only two herbs, *Paeonia alba radix* and *Glycyrrhizae radix,* the future evaluation of its role for treating CIPN might be easier than for other herbal recipes. While neuronal apoptosis can be triggered by oxidative stress and mitochondrial dysfunction, substances with an antioxidative potency are possible candidates for treatment of CIPN [19–22], so the fact that paeoniflorin as one bioactive component of *Paeonia alba* radix has been positively tested as an antioxidant in a nonneuronal cell model might be relevant [159]. It has also neuroprotective effects which are closely correlated to activating the adenosine A1 receptor, ameliorating the function of the cholinergic nerve, regulating ion channel homeostasis, retarding oxidative stress, apoptosis of the neurocytes, and promoting nerve growth [160]. *Paeonia alba radix* was used in another recipe (see Section 3.2.7) and in two recipes *Paeonia rubra radix* (see Sections 3.2.1 and 3.2.2) has been used. *Paeonia alba radix* and *Paeonia rubra radix* have a lot of similarities in their components and paeoniflorin is a bioactive compound of both herbs [161].

While the reason for using herbal recipes derives from historical knowledge or transferral of historical concepts to modern diseases, interestingly four of eight herbal recipes tested for CIPN contain *Glycyrrhizae radix*. To find the scientific ratio behind this decision, analyses of its action in the context of neural cell damage might be fruitful.

Another herb that has been used in four of eight herbal recipes tested for CIPN is *Astragalus membranaceus radix*. This might be rational, while *Astragalus membranaceus radix* water extracts caused a marked enhancement of the NGF-mediated neurite outgrowth and the expression of growth-associated protein 43 from PC12 cells in vitro [162]. *Astragalus membranaceus radix* extracts can be a potential nerve growth-promoting factor, being salutary in aiding the growth of axons in the peripheral nerve [162]. Astragaloside IV (AGS-IV), one bioactive compound of *Astragalus membranaceus radix*, is an aldose-reductase inhibitor and a free-radical scavenger which suppressed a decrease in myelinated fibers, promoted an increase in myelinated fiber density and an increase in segmental demyelination in diabetic rats [163]. It also increased the activity of glutathione peroxidase in nerves, depressed the activation of aldose reductase in erythrocytes, decreased the accumulation of advanced glycation end products in both nerves and erythrocytes, and elevated Na+, K+-ATPase activity in both the nerves and erythrocytes of diabetic rats, so it is considered to be protective against the progression of peripheral neuropathy [163].

The herbal recipe *Bu Yang Huan* and a modified extension have been used in two clinical controlled randomized trials [92, 93] (3.2.1 and 3.2.2, see above) and was found to be effective in both. The formula used is very complex, and little is known about the mechanism of its compound, but herbs like *Astragalus membranaceus radix, Paeonia rubra radix,* and *Dioscorea opposita rhizoma* are part of the herbal combination where possible mechanisms are described (3.2.1, 3.2.2, 3.2.5, 3.2.7, see above), so at least something is known about its possible mechanisms of effect.

Another study used a modified classical prescription named *Chai Hu Long Gu Mu Li Wan* (see Section 3.2.3) and found positive results as well [94]. Even though it is a controlled randomized trial, the treatment protocol is very complicated, combining oral intake of medical herbs and external washing with other herbs. So also with this herbal combination little is known about the mechanism of its action.

Two other herbal recipes have been reported for successful clinical use, *Keishikajutsubuto* = *Gui Zhi Jia Shu Fu Tang* (see Section 3.2.6) and Ogikeishigomotsuto = *Huang Qi Wu Wu Tang* (see Section 3.2.7). But these studies are either uncontrolled or a case report, so the quality of the evidence is very low.

The trials on the single herbs and herbal combinations tested so far do not provide a clear recommendation for clinical use. But research on some single herbs as well as on combinations like GJG is promising, even though the level of knowledge is limited to basic research on the mechanisms of action and evidence from clinical trials. But it is necessary to continue research on this field, while treatment concepts for CIPN are lacking.

The most used herbs of herbal recipes in clinical trials on CIPN are *Aconiti lateralis praeparata radix*, *Rehmannia praeparata radix*, *Paeonia (alba* and *rubra) radix*, *Astragalus membranaceus radix,* and *Glycyrrhizae radix*.

In Chinese medicine experience *Astragalus membranaceus radix* is supporting the Qi, which means in western terms supporting the general energy level of the body, which is usually reduced in CIPN by activating the vegetative nervous system. *Rehmannia praeparata radix* is basically supporting the Yin, which in western terms reflects the structural damage of tissue, in the case of CIPN the peripheral nerves. *Paeonia alba radix* and *Paeonia rubra radix* promote the flow of the Xue, which in western terminology has its correlate in enhancing the microcirculation which might be reduced in CIPN; promotion of the perfusion might enhance the regeneration.

Aconiti lateralis praeparata radix supports in Chinese medicine theory the Yang. A typical symptom of Yang deficiency is ice-coldness, which is prominent in the extremities in some cases of CIPN. *Glycyrrhizae radix* supports the fluids and has a balancing effect on the whole recipe.

So in spite of using different terms, Chinese medicine theory describes physical reactions of the body that can be explained by western physiology and has found its proof by experimental studies [138, 139, 148, 151–156, 159–164].

So using these herbs has as rational foundation on the basis of Chinese medical theory as well as from experimental studies, but unfortunately still little is known about the complex physiological mechanisms of herbal combinations, the interactions of the substances, and the mechanisms of action of many other substances used in herbal medicine. The challenge for future research is bringing historical knowledge and modern scientific analysis together.

Due to this limited knowledge on the mechanisms of the action of herbs, we additionally collected data on herbs that are a putative treatment option for CIPN. While oxidative stress and mitochondrial dysfunction promote CIPN, substances with an antioxidative potency are possible candidates for the treatment of CIPN. So in the list below we list herbal antioxidants tested in neuronal cell or disease models. Additionally we added herbs for enhancement of nerve growth as putative treatment options for CIPN. NGF exhibits potent biological activities such as preventing neuronal death, promoting neurite outgrowth, and supporting synapse formation [157], which has a relevance for the development of CIPN [39].

Putative Herbs or Herbal Compounds for the Treatment of CIPN

(1) *Herbal Antioxidants Tested in Neuronal Cell or Disease Models.* Puerarin (from *Pueraria lobata radix*) [165, 166], Icariin (from Epimedii herba) [167], a fraction of polysaccharides (from *Lycium barbarum*) [168], Ginsenoside Rg1 (from Notoginseng panacis) [169], honokiol and magnolol (from *Magnolia officinalis*) [170], Ginkgolide A, B (from *Ginkgo biloba*) [171], huperzine A (from *Huperzia serrata*) [171], Ginseng radix [172], Notoginseng panacis radix [173], 3,5,4′-tetrahydroxystilbene-2-O-beta-D-glucoside (from *Polygonum multiflorum*) [174–176], celastrol (from *Tripterygium wilfordii* Hook) [177], Salvianolic acid B (from *Salvia miltiorrhiza*) [178, 179], tanshinone IIA (from *Salvia miltiorrhizae*) [180], *Gastrodia elata* rhizoma [181, 182], Astragaloside IV (from *Astragalus membranaceus radix*) [163], Tetramethylpyrazine (from *Ligusticum wallichi*) [183, 184], Ziziphus spinosus semen [185], Baicalein (from *Scutellaria baicalensis radix* Georgi) [186], *Uncaria rhynchophylla* [164], 6,7-dihydroxy-2-methoxy-1,4-phenanthrenedione, chrysoeriol 4′-O-beta-D-glucopyranoside, chrysoeriol 7-O-beta-D-glucopyranoside, and alternanthin (from *Dioscorea opposita radix*) [187].

(2) *Herbs or Herbal Compounds That Enhance Nerve Growth*. Cistanches herba [157, 188], Huperzine A (HupA) from *Huperzia serrata* [189, 190], a lipophilic fraction of panax ginseng [191]. *Astragalus membranaceus radix* [162], Gentiside C, a compound of *Gentiana rigescens radix* [191], *Rehmanniae praeparata radix* [192], *Paeoniflorin* of *Paeonia alba radix* [193].

Research with the aim of proving the benefits of promising substances or herbal combinations has to describe the mechanism of action for single herbs and single components, investigate the interactions of combinations of substances, and analyse the promoting effects of combinations.

From this viewpoint, research in this field has not very much progressed, but if research does not provide these data, herbal combinations will not find acceptance in mainstream treatments in non-Asian countries in Europe, North America, or Australia.

This challenge could be taken if more international cooperation of interested research groups would be organized.

5. Conclusion

Experimental and clinical studies have not yielded enough evidence to establish a standard practice for the treatment of CIPN, but from this literature review, a lot of promising substances, mainly Chinese medical herbs with possible effect in CIPN or a putative influence on mechanisms of CIPN, have been identified in the last years.

The knowledge of the mechanisms of action is still limited and the quality of the clinical trials needs further improvement. In the future not only the mechanisms of action for single herbs and single components have to be described, but interactions of combinations of substances as well as interactions with chemotherapy have to be investigated and analysed in depth. While CIPN has multiple possible mechanisms of neuronal degeneration, a combination of components might be a promising opportunity focusing on multiple targets of degeneration or activating regeneration.

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Review Article

A Systematic Review of Experimental and Clinical Acupuncture in Chemotherapy-Induced Peripheral Neuropathy

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Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect that can be very disabling and can limit or delay the dose of chemotherapy that can be administered. Acupuncture may be effective for treating peripheral neuropathy. The aim of this study was to review the available literature on the use of acupuncture for CIPN. The systematic literature search was performed using MEDLINE, Google Scholar, Cochrane Database, CINHAL, and ISI Proceedings. Hand searching was conducted, and consensus was reached on all extracted data. Only papers in the English language were included, irrespective of study design. From 3989 retrieved papers, 8 relevant papers were identified. One was an experimental study which showed that electroacupuncture suppressed CIPN pain in rats. In addition, there were 7 very heterogeneous clinical studies, 1 controlled randomised study using auricular acupuncture, 2 randomized controlled studies using somatic acupuncture, and 3 case series/case reports which suggested a positive effect of acupuncture in CIPN. *Conclusions*. Only one controlled randomised study demonstrated that acupuncture may be beneficial for CIPN. All the clinical studies reviewed had important methodological limitations. Further studies with robust methodology are needed to demonstrate the role of acupuncture for treating CIPN resulting from cancer treatment.

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a common side effect after patient exposure to chemotherapy agents such as vinca alkaloids, platinum derivatives, and taxanes and to newer agents such as bortezomib and thalidomide. CIPN is the second most important side effect in frequency after hematologic toxicity and can limit or delay the dose of chemotherapy that can be administered [1]. Sensory, motor, and/or autonomic neurotoxicity can be very disabling and/or painful, creating a major impact on patients' quality of life and adherence to treatment.

Conventionally, CIPN is prevented with the use of intravenous calcium and magnesium, without reducing treatment response. Pharmacological treatment of other CIPN symptoms like numbness or palsies is not usually effective, but there are some other options for treatment of neuropathic pain such as the use of antiepileptic and tricyclic antidepressant drugs (e.g., carbamazepine, lamotrigine, gabapentine or pregabalin, and venflaxine). There is no established pharmacological treatment for neuroregeneration [2, 3].

The mechanism of neurotoxicity of the antineoplastic agents is unclear. CIPN generally arises as a consequence of the disruption of axoplasmic microtubule mediated transport, distal axonal degeneration, and direct damage to the sensory nerve cell bodies of the dorsal root ganglia (DRG). Mitochondrial damage in the DRG neuron has been described as well [4]. The DRG neurons lack a vascular

barrier, in contrast to those in the central nervous system, and are more exposed to the neurotoxic effects of the antineoplastic agents. Both central and peripheral mechanisms seem to be involved.

Acupuncture stimulates areas of the skin using different methods including the insertion of thin needles that are then manipulated manually or electrically. In animal models acupuncture has been shown to reduce neuropathic pain in a variety of experimental conditions. Cold allodynia has been reduced in rats treated with electroacupuncture through the mediation of spinal alfa2-adrenergic receptors [5], while in mechanical allodynia electroacupuncture has been shown to act through mu and delta but not kappa opioid receptors [6]. Another possible effect of electroacupuncture in an experimental model of neuropathy may be the inhibition of COX2 expression [7]. It has been shown that electroacupuncture at the acupoint Zusanli (ST36) reduced the mechanical allodynia in a neuropathic model, normalising the expression profile of hypothalamic proteins, that have been mainly identified as being involved in inflammatory processes, metabolism and signal transduction [8]. Ko and colleagues [9] examined the mechanism of neuropathic pain and the analgesic effects of acupuncture at the molecular level by cDNA microarray analysis. They observed that the expression of 68 genes had more than doubled in a model of neuropathic pain in the rat but returned to normal after treatment with electroacupuncture. The genes were involved in biological processes such as signal translation, gene expression, and nociceptive pathways [9]. Also the expression of the sigma opioid receptor was decreased by 50% in the neuropathic pain model and was returned to normal after acupuncture. This could explain the poor response of neuropathic pain to the treatment with opioids, since the opioid receptor is downregulated in this condition, and could partially explain the analgesic action of acupuncture in neuropathic pain, because acupuncture normalises the opioid receptor expression and also increases the release of endogenous opioid peptides.

Cancer patients often seek additional help for their disease or for treatment-related side effects. A European survey in 13 countries [10] showed a prevalence of 35.9% of complementary therapy use (range among countries 14.8% to 73.1%). Acupuncture was used by 3.9% patients before cancer diagnosis and by 3% patients after cancer diagnosis. Acupuncture has also been shown to be effective for chemotherapy induced nausea and vomiting [11], xerostomia induced by radiation therapy [12, 13], fatigue [14], anxiety, depression, and insomnia [15]. Furthermore, acupuncture has been used encouragingly to treat peripheral neuropathy associated with diabetes and HIV [16–22].

In this paper the existing evidence of acupuncture effectiveness and/or efficacy for CIPN has been systematically reviewed.

2. Methodology

To review the existing clinical and experimental studies of acupuncture in CIPN, the systematic literature search was performed from the databases inception up until January 2012 using MEDLINE, Google Scholar, Cochrane Database, CINHAL (Cumulative Index to Nursing and Allied Health Literature), CNKI (China National Knowledge Infrastructure), Wanfang Med Online, and ISI Proceedings for conference abstracts. The keywords searched were (acupoint* OR acupuncture OR electro-acupuncture OR electroacupuncture OR moxibustion) AND "peripheral neuropathy." The CINHAL, CNKI, and Wanfang Med Online Databases did not allow logical searches with AND, so we used simple combinations of the search words. Historical searches of reference lists of relevant articles were also undertaken. To be included in the review, a study had to explore either the efficacy or the effectiveness of acupuncture needling for CIPN in either human or animal models irrespective of design. All papers with at least an abstract in English were included. Study selection was performed by two reviewers (Giovanna Franconi and Luigi Manni) with disagreement resolved by discussion and adjudication.

3. Results

A total of 3989 articles were retrieved from electronic searches and subsequent examination of reference lists of the clinical and review articles. After screening titles and/or abstracts, 3891 articles were excluded for the following reasons: the focus was on an intervention other than acupuncture, the neuropathy was not related to chemotherapy, the acupuncture treatment plan included additional interventions/modalities that were not acupuncture related, there were duplicated studies, or they were not relevant. From a total of 98 articles which were retrieved for detailed evaluation, 7 clinical studies and 1 experimental study were included in the review. For a summary of the clinical studies see Table 1.

Only one study was identified that addressed the topic of electroacupuncture (EA) and its effects on CIPN in an animal model. Meng and colleagues [30] demonstrated that EA at both low (10 Hz) and high (100 Hz) frequencies was able to improve neuropathic pain in paclitaxel-treated rats. The authors reported that low-frequency EA was more effective than high-frequency EA in relieving neuropathic symptoms and that opioid receptors antagonists (all types) abolished EA effects. It is known that low-frequency (2–15 Hz) EA engages centrally mediated endorphin, enkephalin, serotonergic, and noradrenergic analgesia, while high frequency EA (100 Hz) engages segmental-spinal opioids (dynorphin, enkephalin) and nonopioid (gamma aminobutyric acid, glycine) analgesia [31]. Thus it is conceivable that central-mediated effects of acupuncture are involved in suppression of neuropathic pain in CIPN.

For a summary of the clinical studies see Table 1.

The first published clinical study that explored the efficacy of acupuncture in cancer pain used auricular acupuncture in a population of 90 patients with neuropathic pain (despite stable medication) [23]. A small minority of patients also presented nociceptive pain. The patients were randomly divided into 3 arms: one arm with steel implants on auricular points eliciting an electrical response and 2 placebo arms with either steel implants or vaccaria seeds on auricular points

Table 1: Characteristics of the studies involving the use of acupuncture in CIPN.

Authors	Patients (n)	Design of the study	Intervention and control	Duration of intervention	Outcome(s)	Results
Alimi et al., 2003 [23]	90	Prospective randomized controlled trial	Auricular acupuncture versus placebo acupuncture and seeds	2 months	VAS pain score and medication consumption	True acupuncture better than placebo
Wong and Sagar, 2006 [24]	5	Prospective case series	Acupuncture (no control)	16 weeks (Two 6-week courses with a 4-week therapy free interval)	Pain score and WHO CIPN grade	Improvement
Xu et al., 2010 [25]	64	Controlled randomized trial	Acupuncture versus cobamamide	Not known	Questionnaire of peripheral neuropathy	Acupuncture better that cobamamide
Bao et al., 2011 [26]	1	Case report	Acupuncture (no control)	22 weeks	VAS pain score	No more symptoms
Donald et al., 2011 [27]	18	Retrospective case series	Acupuncture (no control)	6 weeks	Subjective symptoms	82% improved
Schroeder et al., 2012 [28]	11	Retrospective controlled nonrandomized trial	Acupuncture and best medical care versus best medical care	10 weeks	Nerve conduction studies	Acupuncture better than control
Tian et al., 2011 [29]	76	Controlled randomized trial	Warm acupuncture and moxibustion versus Neurotropin	Not known	Quality of life and neurotoxic symptoms	Acupuncture better than Neurotropin

Legend: VAS: visual analog scale; FACT-G: Functional Assessment of Cancer Therapy-General.

not eliciting an electrical response. Patients were treated for 2 one-month cycles. After 2 months the pain VAS score was significantly decreased in the true acupuncture group, while there was no effect of placebo.

In one prospective case series 5 patients were treated with manual acupuncture for their chemotherapy-induced neuropathy [24]. Acupuncture was performed once a week according to TCM diagnosis using acupoints CV6 (Qihai), ST36 (Zusanli), LI11 (Quchi), EX-LE10 (Bafeng), and EX-UE9 (Baxie) for two 6-week cycles, separated by 4 weeks. All patients showed an improvement in pain score and on the WHO grade of neuropathy after treatment with acupuncture. There were no observed side effects, and benefits persisted for 6 months of followup in 4/5 patients.

Xu et al. [25] studied 64 patients with CIPN induced by paclitaxel or oxaliplatin. The patients were randomized to an acupuncture group or a control group treated with cobamamide. The acupuncture treatment included points such as Hegu (LI4), Taichong (LR3), Zusanli (ST36), Qihai (CV6), and Quchi (LI11) and was performed for an unspecified length of time. The outcome was an evaluation of neurotoxicity assessed by a CIPN questionnaire. The twenty patients in the acupuncture group significantly improved, compared to the 12 patients in the control group.

A case report [26] described one patient with multiple myeloma and bortezomib-induced CIPN who was treated with 6 weekly sessions of acupuncture, followed by subsequent 8 sessions over the next 5.5 months. The treatment protocol included body acupuncture at LI4 (Hegu), TE5 (Waiguan), LI11 (Quchi), ST40 (Fenglong), EX-LE10 (Bafeng), and auricular acupuncture at shenmen, point zero, and 2 additional points were stimulated electrically. The VAS pain score decreased from 8/10 to 2/10 after 6 treatments, and the pain medication with morphine sulphate and oxycodone was stopped after 14 treatments. The patient remained pain-free for at least one year. There were no observed side effects.

A retrospective case series examined 18 patients affected by CIPN [27]. Patients were treated by 6 weekly acupuncture sessions, with acupoints selected on the basis of patient presentation at each session. No validated questionnaires were used, and side effects were not recorded. The most commonly used points were SP6 (Sanyinjiao) and ST36 (Zusanli), followed by LV3 (Taichong). After 6 weeks 82% ($n = 14$) patients reported an improvement of their neuropathy symptoms, 18% ($n = 3$) reported no change.

In a pilot controlled nonrandomised study [28], 6 patients with CIPN accepted acupuncture treatment, and 5 patients with CIPN who had refused acupuncture treatment served as controls. All patients received the best medical care, and the 6 patients in the acupuncture group were also treated with 10 weekly acupuncture treatments with a fixed protocol (ST34 Liangqiu, EX-LE12 Qiduan, and EX-LE10 Bafeng bilaterally). Nerve conduction studies (NCS) were done to confirm the presence of CIPN at baseline and 6 months later, that is, 3 months after the end of acupuncture treatment. Acupuncture significantly improved nerve conduction velocity and mean amplitude of NCS in treated patients, while there was no difference after the same time in the control group. There were no observed side effects.

The last study included in this review was a randomized controlled trial of 76 patients with gastrointestinal cancer and CIPN induced by oxaliplatin [29]. The intervention group received warm acupuncture and moxibustion, and the control group was treated with Neurotropin 4 mg given intramuscularly every day for 21 days. The intervention receiving acupuncture and moxibustion reported a significantly improved quality of life and reduction in neurotoxic symptoms.

4. Discussion

Complementary therapies in cancer care are used primarily to treat the symptoms associated with cancer and its treatments. This review suggests that although there are some indications that acupuncture may be effective in improving symptoms and neural damage associated with CIPN, the current evidence available is limited.

The positive effects of acupuncture in CIPN consist in a reduction in the pain score in most studies. Pain is the most common and the best studied indication for acupuncture, and acupuncture has been recommended as a complementary therapy for pain control or for reducing the amount of pain medicine in cancer patients. According to the evidence-based guidelines of the American College of Chest Physicians for lung cancer [32], acupuncture is recommended as a complementary therapy for lung cancer when pain is poorly controlled or when side effects such as neuropathy or xerostomia are clinically significant (grade 1A recommendation). The rationale is based on the analgesic action of acupuncture in acute and chronic pain and in cancer pain. Furthermore, studies on pain using functional magnetic resonance (fMRI) showed that acupuncture could modulate the cognitive-affective aspects of pain perception [33].

Improvement was also reported for other symptoms of CIPN in the paper by Wong and Sagar [24], where the effects of acupuncture were measured by the WHO CIPN score, which takes into account both the sensory and motor abnormalities of CIPN. One study [28] evaluated acupuncture effects with nerve conduction studies, which allowed a separate measurement of motor and sensory signals and showed a significant positive effect of acupuncture on motor and sensory parameters.

The studies included in this systematic review were very heterogeneous: 3 studies [23, 25, 29] were prospective randomized controlled trials, while another [28] was a retrospective analysis of a controlled study. These controlled studies showed a specific effect of acupuncture, unrelated to skin penetration. The remaining studies were uncontrolled case reports or case series. Such uncontrolled studies may present bias and lead to false positive results. The issue of choosing a control in acupuncture research is not a simple one, as placebo/sham acupuncture shares many pathways with true acupuncture (i.e., activation of opioid system as well as other pain-controlling neurotransmitters systems and activation of cerebral areas on fMRI), and the placebo/sham acupuncture used in acupuncture studies is not necessarily inert [34–37].

Different protocols were utilized to treat CIPN: auricular acupuncture only, and somatic acupuncture only, combined auricular and body acupuncture, each applied on different combinations of acupoints. Acupuncture protocols are usually standardized in acupuncture research, but this may not reflect what clinical acupuncturists do every day in their clinics, as acupuncture in TCM is a very individualized medicine [38]. Furthermore the choice of acupuncture points in a protocol depends on the reference system, which comprises many different schools and different approaches to acupuncture, such as acupuncture according to traditional Chinese medicine, medical acupuncture, Japanese acupuncture, French auricular acupuncture, trigger-point acupuncture, acupressure, electroacupuncture, and transcutaneous electrical nerve stimulation (TENS) of acupuncture points, among others [39], each one with a different approach to comparable problems. Future studies with sufficient number of patients should also address the issue of whether a pragmatic approach or a protocol approach should be employed.

Heterogeneity was also present when considering the outcome measurements, which ranged from subjective evaluation to pain VAS score to nerve conduction studies (NCS), which make it impossible to compare studies. More objective outcome measurements are advisable, and among them NCS which measures the number and conduction velocities of large myelinated fibers and relates to both the clinical subjective improvement and the histological nerve healing.

Neuronal damage by antineoplastic agents probably activates second messenger systems which cause hyperalgesia, allodynia, and pain, because it may be relieved by supplementation with trophic factors such as NGF, insulin growth factor 1 (IGF-1), and neurotrophin 3 (NT-3) [40]. There is a large experimental evidence base on the involvement of NGF in CIPN [41–44]. NGF promotes physiological maturation, survival, and expression of the specific phenotype in primary sensory neurons located in the DRG [45]. Acupuncture analgesia is an effect that has been amply demonstrated and occurs via the activation of different systems, involving nerves, hormones, cytokines, and other mediators [46]. At a neuroendocrine level acupuncture modulates various neurotrophins and growth factors including NGF [47], glial-derived neurotrophic factor (GDNF) [48–50], brain-derived neurotrophic factor (BDNF) [51, 52], and insulin growth factor (IGF) [53]. It is possible that the action of acupuncture on neuropathic pain be mediated by enhancement of spinal/central GABA-ergic, serotoninergic, and adrenergic neurotransmission [54–58] as well as by the action of acupuncture on the NGF system, driving NGF signalling toward its downregulation with parallel decrease in sensory neurons hypersensitization [59]. Thus, acupuncture can modify the expression of different genes and the expression of genes that control transcriptional factors that are crucial for cell homeostasis [60]. In Figure 1, we summarized the acupuncture mechanisms and mediators in CIPN based on what we know from animal studies of diabetic neuropathy [59, 61] and from human studies of brain imaging during acupuncture [62].

It is interesting to note that all the studies which used somatic acupuncture and described their protocol [24, 26–28] employed local points. EX-LE10 (Bafeng) is 4 points on the instep of each foot, proximal to the margin of

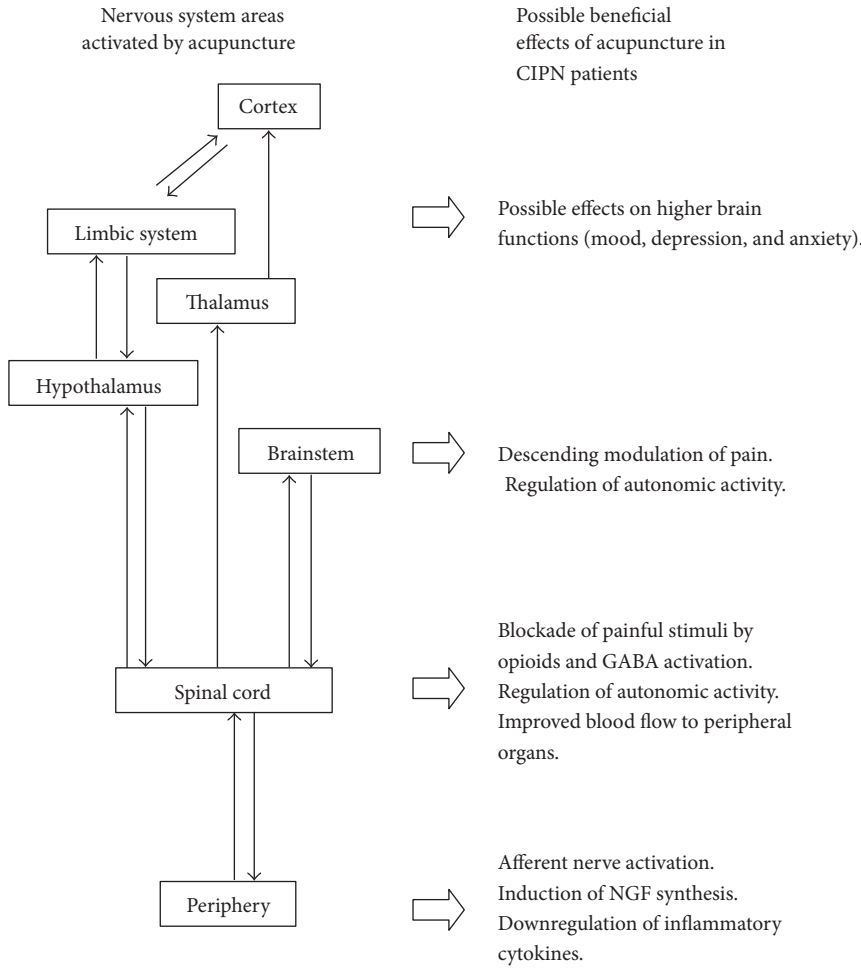

Figure 1: Possible targets of acupuncture treatment in CIPN. On the left side are the nervous system areas which are activated by acupuncture; on the right side are some possible beneficial effects of acupuncture in CIPN patients. CIPN = chemotherapy-induced peripheral neuropathy; GABA = gamma amino butyric acid; NGF = nerve growth factor.

the webs between each two neighbouring toes, while EX-UE9 (Baxie) is 4 points proximal to the margin of the webs between each two of the five fingers of a hand. The rationale behind the choice of points located nearby or in the same dermatome of the affected limb/region might lie in the activation of spinal response after acupuncture. Indeed the western neurophysiological hypothesis on the mechanism of acupuncture efficacy proposes that needle insertion and stimulation elicits a three-level response: local (at the site of needling) that could encompass the so called "flare reaction"; segmental, that includes all the acupuncture-induced reflex variations in spinal neurotransmission, that is, GABA-ergic one; central, that refers to the overall variation induced by needle stimulation in the activity and feedback response in the brain [35]. Thus, it is possible to link the positive outcome in such studies to the spinal/segmental activation of opioids and/or GABA signalling, in accordance with previous results on animal models [30].

The limitations of the studies reviewed include the small sample size of most studies, the presence of poor controls or no controls, poor randomization, and lack of blinding. However, the presence of some studies of good quality which suggest a positive effect of acupuncture in CIPN support the planning of more rigorous randomised controlled clinical studies evaluating the efficacy of acupuncture in CIPN. The advantages of acupuncture are its safety and low cost, and it would be very important to demonstrate its efficacy in such a disabling and potentially dangerous side effect of cancer treatment such as CIPN.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Antiangiogenic Activity and Pharmacogenomics of Medicinal Plants from Traditional Korean Medicine

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Aim. In the present study, we investigated the antiangiogenic properties of 59 plants used in traditional Korean medicine. Selected phytochemicals were investigated in more detail for their modes of action. *Methods*. A modified chicken-chorioallantoic-membrane (CAM) assay using quail eggs was applied to test for antiangiogenic effects of plant extracts. A molecular docking *in silico* approached the binding of plant constituents to the vascular endothelial growth factor receptors 1 and 2 (VEGFR1, VEGFR2). Microarray-based mRNA expression profiling was employed to correlate the 50% inhibition concentrations (IC_{50}) of a panel of 60 NCI cell lines to these phytochemicals. *Results*. Extracts from *Acer mono* leaves, *Reynoutria sachalniensis* fruits, *Cinnamomum japonicum* stems, *Eurya japonica* leaves, *Adenophora racemosa* whole plant, *Caryopteris incana* leaves-stems, and *Schisandra chinensis* stems inhibited angiogenesis more than 50% in quail eggs. Selected phytochemicals from Korean plants were analyzed in more detail using microarray-based mRNA expression profiles and molecular docking to VEGFR1 and VEGFR2. These results indicate multifactorial modes of action of these natural products. *Conclusion*. The antiangiogenic activity of plants used in traditional Korean medicine implicates their possible application for diseases where inhibition of blood vessel formation is desired, for example, cancer, macular degeneration, diabetic retinopathy and others.

1. Introduction

Traditional medicinal plants belong to the characteristics of most cultures on this earth. Medicinal plants helped to secure survival of our ancestors in a noncivilized world, which was not dominated by the technological achievements of the 20th and 21st centuries. Medicinal plants were not only indispensable for as basic health care of indigenous tribes in former ages as modern medicine was not in place. Even nowadays, phytotherapy is still used by a majority of the world's population. Over 50,000 plants would possess therapeutic virtues in the world and about 80% of human use herbal medicines at least once in their life [1, 2]. The pharmacological screening of plants is an important mean for the discovery of new, safe, and effective drugs in classical pharmacology [3]. Hence, it comes as no surprise that research on medicinal plants and natural products derived from them experiences a thriving revival in the past years.

Complementary and alternative medicine and traditional medicines are well acknowledged among the general population in industrialized countries [4, 5]. Many patients use complementary and alternative medicine, frequently without the knowledge of their doctors. Therefore, there is an urgent need for quality-controlled and safe but also effective products from complementary and alternative medicine. Clinical trials and cellular and molecular mechanistic studies on medicinal

herbs will help to improve their rational use and to better understand their modes of action. This was the motivation for many scientists from pharmacy and pharmacology, including our own group, to investigate the bioactivity of medicinal plants and phytochemicals isolated from them using techniques of molecular pharmacology and molecular biology [6–11]. With a strong commitment to traditional Chinese medicine [12–14] and traditional African medicine in the past years [15–17], we now focus on traditional Korean medicine.

Traditional Korean medicine is widely used in Korea and is the primary health care system for more than 20% of the population [18, 19]. Demographic studies confirm that traditional Korean medicine flourishes in modern Korea. One regional survey found that 36% of the Korean population had used complementary and alternative medicine in a 5-year period, although traditional Korean medicine use was not specified [20]. Forty percent of hypertensive patients used complementary and alternative medicine including traditional Korean medicine after discharge from hospitals [18]. A South Korean national survey was performed among 79% of people who are older than 18 years old and have health problems within one year. The result showed that 40% of those did not do anything, 23% of them visited western doctors' offices or western hospitals, and 19% used complementary and alternative medicine only (including traditional Korean medicine), with 18% using both western medical and TKM service [20].

It was reported that excessive angiogenesis is an important factor of the pathogenesis of many industrialized western countries [21]. Plants with anti-angiogenesis properties are therefore of considerable importance for diseases such as cancer, macular degeneration, diabetic retinopathy, and others [22–25].

Based on the antiangiogenic activity of the plant extracts in a modified *in vivo* chicken chorioallantoic membrane assay, selected phytochemicals were analyzed in more detail. A molecular docking approach was applied to investigate *in silico* the binding of selected phytochemicals to the vascular endothelial growth factor receptor (VEGFR1, FLT1) as an important angiogenic factor.

As previously shown for other antiangiogenic drugs [26], the microarray-based mRNA expression of VEGFR1/FLT1 and 89 other angiogenesis-regulating genes was correlated to the IC_{50} values of 60 cell lines of the National Cancer Institute, USA, for selected phytochemicals derived from traditional Korean medicine to identify possible cellular factors associated with their antiangiogenic activity.

2. Materials and Methods

2.1. Plant Material and Extraction. Medicinal plants used in the present work were collected at different localities of South Korea and provided by Professor Ik-Soo Lee (College of Pharmacy, Chonnam National University, Gwangju, South Korea). The plants were identified at the national herbarium, where voucher specimens were deposited under the references numbers (see Supplementary Table 1 in Supplementry Materials available online at http://dx.doi.org/10.1155/2013/131306). The extraction of the air-dried and powdered plant material was conducted using methanol (HPLC grade) with either ASE 300 (Dionex) or a sonicator (Branson Ultrasonics) at 50°C. The extracts were then conserved at 4°C until further use.

2.2. Angiogenesis Test

2.2.1. Cultivation of Quail Eggs. Quail eggs were cultured according to a described method [27]. Briefly, fertilized quail eggs were incubated for 70 h at 38°C and 80% relative humidity. After 70 h of incubation the eggs were opened. For this purpose, the eggs were placed in a vertical position to guarantee that the embryo floats in the upper part of the egg. Afterwards, a hole was cut into the top of the egg and the complete content of the egg, was transferred into a Petri dish. By using this method, it could be guaranteed that the albumin gets first into the Petri dish followed by the yolk with the embryo on top without exposing the embryo to shock forces which could damage the vitelline membrane.

2.2.2. Chicken Chorioallantoic Membrane Assay (CAM Assay). Plant extracts were tested for their antiangiogenic effects on quail eggs as previously described for chicken eggs with modifications [28, 29]. Briefly, the explanted embryo was placed in an incubator for 2 h at 38°C to acclimatize it to the new ambience. Subsequently, the test substances were placed on the CAM. Therefore, 2% agarose solution was prepared and mixed 1 : 10 with the plant extract prior diluted in DMSO 0.1% final concentration. The final concentration of the extract was 10 μg/mL. Pellets with 0.1% DMSO served as control. The agarose-pellets were then placed on the CAM after they cooled down to room temperature. The Petri dishes with the quail embryos were placed in the incubator again and incubated at 38°C and 80% relative humidity for 24 h before documenting the effect of the applied substances.

Imaging of the vascularized quail eggs was performed using a digital camera with 3x magnification objective (Canon eos 500 with a Canon mp-e 65 2.8 macro objective). For illumination, a mercury arc lamp was used which provided a high fraction of blue and UV light to obtain good contrast values between yolk and vessels. The pictured image section had a size of 5 × 5 mm. Following image acquisition, quantitative analysis was performed using a routine software which was written in the ImageJ-macro language, then the total small vessel number (or area) was then determined by the system, and the percentage inhibition of vascularization was calculated [30].

2.3. Correlation of Angiogenesis-Regulating Gene Expression with Cytotoxicity of Tumor Cell Lines. The mRNA microarray hybridization of the NCI cell line panel has been described [31, 32], and the data has been deposited at the NCI website (http://dtp.nci.nih.gov/). The performance of the COMPARE and hierarchical cluster analyses using mRNA-based microarray data of the database of the National Cancer Institute, USA, has been previously described by us [33]. A set of 89 genes were chosen because of their involvement in angiogenic processes [34]. The microarray data of this set of genes was exemplarily validated by real-time RT-PCR [26].

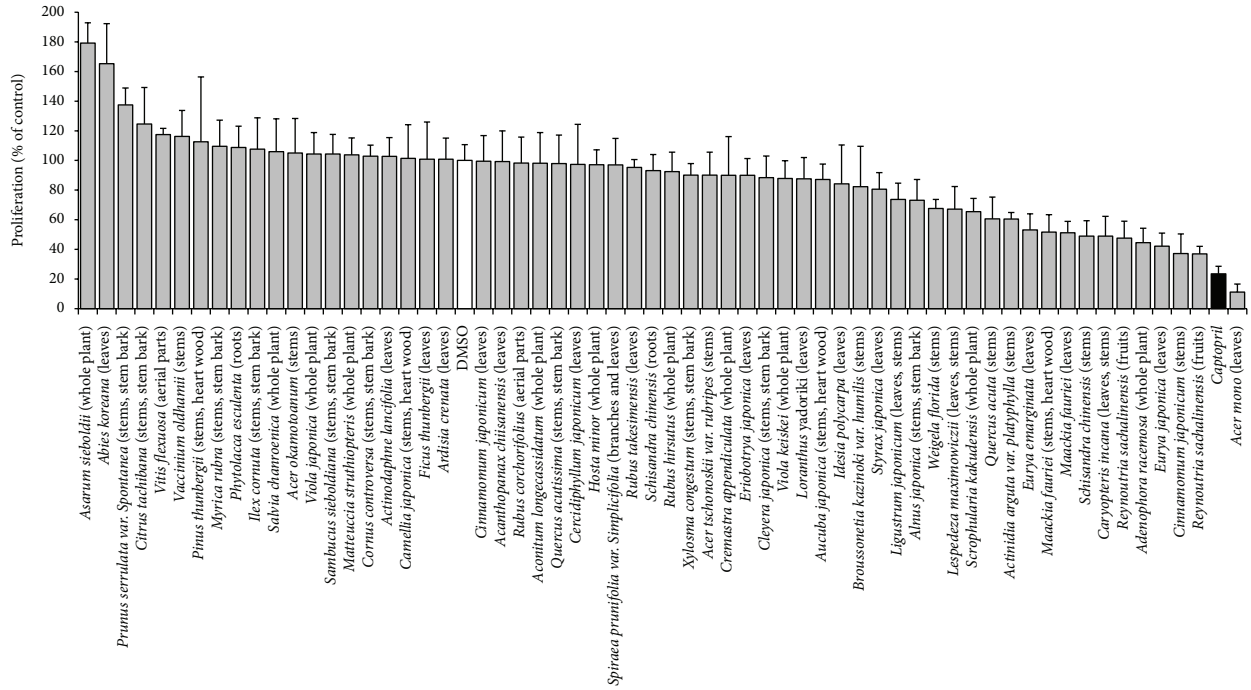

Figure 1: Antiangiogenic effects of the 59 Korean plant extracts (10 μg/mL) on the growth of blood capillaries on the CAM of quail eggs. Mean values ± SD of each five eggs are shown. DMSO: solvent control (white bar); captopril: positive control (10 μg/mL; black bar).

2.4. Statistics. Pearson's correlation test was used to calculate significance values and rank correlation coefficients as relative measure for the linear dependency of two variables. This test was implemented into the WinSTAT Program (Kalmia). The one-way ANOVA at 95% confidence level was used for statistical analysis.

2.5. Molecular Docking. Human vascular endothelial growth factor receptor 1 tyrosine kinase domain (VEGFR1-TK) structure was retrieved from PDB database (PDB code: 3HNG), which was submitted in complex with N-(4-chlo-rophenyl)-2-[(pyridin-4-ylmethyl)amino]benzamide). Chem-Spider and PubChem were referred for the 3D structures of control drugs and the Korean medicine compounds. Molecular docking calculations were performed with AutoDock4 [35]. Axitinib, which is an antiangiogenic compound and a known VEGFR1 and VEGFR2 inhibitor, was selected as the control drug to compare the binding energies and the docking sites of the candidate ligands. The residues of VEGFR1, which the N-(4-chlorophenyl)-2-[(pyridin-4-ylmethyl)amino]benzamide and control drugs in the literature make hydrogen bond with, were selected for the defined docking. Drug binding residues of VEGFR1 were identified as Val841, Ala859, Lys861, Glu878, Leu882, Val892, Val909, Cys912, Leu1029, and Asp1040. Furthermore, VEGFR2 PDB structure (PDB code: 3U6J), which was submitted in complex with a pyrazolone inhibitor (N-{4-[(6,7-dimethoxyquinolin-4-yl)oxy]-3-fluorophenyl}-1, 5-dim-ethyl-3-oxo-2-phenyl-2,3-dihydro-1H-pyrazole-4-carboxamide), was used. The drug interaction residues for VEGFR2 were Leu 840, Ala 866, Lys 868, Leu 889, Ile 892, Phe 918, Cys 919, Leu 1019, Leu 1035, Cys 1045, Asp 1046, and Phe 1047.

Grid maps were created covering those residues. For the docking calculations, the number of energy evaluations was set to 2,500,000 and the number of runs was set to 100. The lamarckian Genetic Algorithm was chosen for the docking calculations. For the visualization of the docking results, AutoDock Tools and Visual Molecular Dynamics were used. The surface representation image showing the binding pocket of human VEGFR1-TK was made with VMD software developed with NIH support by the theoretical and computational biophysics group at the Beckman Institute, University of Illinois at Urbana-Champaign.

3. Results

3.1. Antiangiogenic Activity In Vivo. Out of 59 plant extracts tested in the CAM assay, seven samples showed significant inhibition (>50%) of angiogenesis. They include extracts from *Acer mono* leaves, *Reynoutria sachalniensis* fruits, *Cinnamomum japonicum* stems, *Eurya japonica* leaves, *Adenophora racemosa* whole plant, *Caryopteris incana* leaves-stems, and *Schisandra chinensis* stems (Figure 1).

Representative images of the effect of antiangiogenic Korean plant extracts (10 μg/mL) on the growth of blood capillaries on the CAMs of quail eggs are shown in Figure 2.

3.2. Correlation of mRNA Expression of Angiogenic Genes with IC_{50} Values of NCI Cell Lines for Phytochemicals from Korean Plants. As a next step, we searched the literature on chemical constituents of antiangiogenic Korean plants (Table 1).

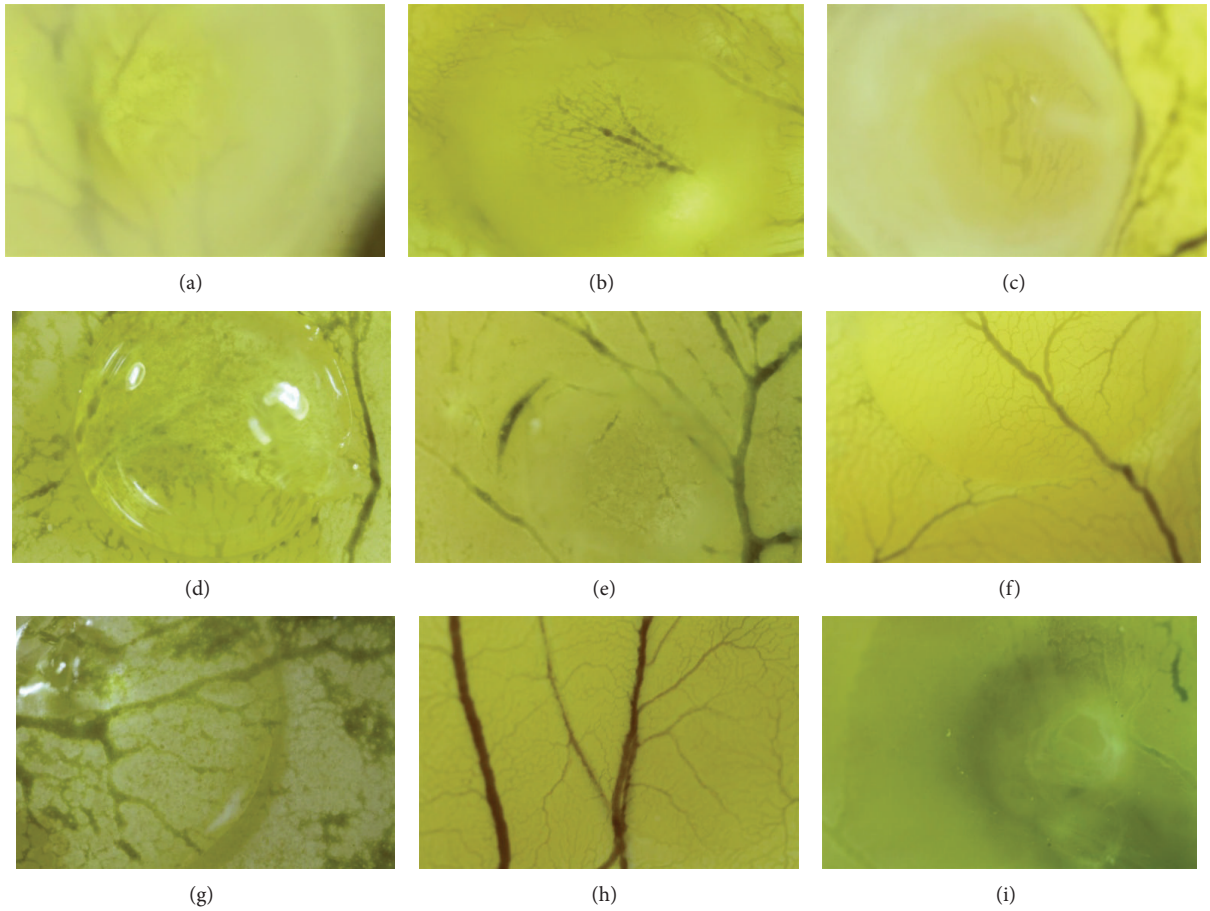

Figure 2: Representative images of the effect of antiangiogenic Korean plant extracts (10 μg/mL) on the growth of blood capillaries on the CAM of quail eggs. The tested extracts were from: *Acer mono* leaves (a); *Reynoutria sachalniensis* fruits (b); *Cinnamomum japonicum* stems (c); *Eurya japonica* leaves (d); *Adenophora racemosa* whole plant (e); *Caryopteris incana* leaves stems (f); *Schisandra chinensis* stems (g); DMSO or solvent control (h); captopril as positive control (i).

Then, we mined the NCI database for these compounds (http://dtp.nci.nih.gov/). Five compounds were found in the database and were exemplarily selected as possible antiangiogenic candidate compounds, that is, verbascoside, apigenin, emodin, resveratrol, and eriodictyol tetraacetate (Figure 3). Axitinib is a known VEGFR inhibitor and served as control drug. The average IC_{50} values over the entire range of NCI cell lines are shown in Figure 4.

The IC_{50} values of these phytochemicals were correlated with the baseline mRNA expression levels of 89 genes involved in angiogenic pathways for the NCI panel of tumor cell lines by the Pearson rank correlation test. Only those genes, whose expression correlated with $R > 0.3$ or $R < -0.3$ with the IC_{50} values of the five compounds were considered for further analyses (Table 2).

The gene expressions of these genes were then subjected to hierarchical cluster analysis. The dendrograms for verbascoside, apigenin, and emodin are shown in Figure 5. To investigate whether these gene expression profiles contain relevant information, we correlated them with the distribution of IC_{50} values for these three compounds of the cell lines. The IC_{50} values themselves were not used for generation of the cluster dendrograms. Therefore, we could address the question whether or not the gene expressions alone predicted the response of the cell line panel to these phytochemicals. As shown in Table 3, the distribution of the cell lines sensitive or resistant to the three natural products was significantly different, indicating that these angiogenesis-regulating genes indeed determined the response of tumor cells to verbascoside, apigenin, and emodin. These analyses were also performed for resveratrol and eriodictyol tetraacetate, but significant relationships were not found, indicating that these gene expression profiles were not predictive for response of tumor cell lines to these two compounds (Table 3).

3.3. Molecular Docking of Phytochemicals from Korean Plants to VEGFR1 and VEGFR2. Since antiangiogenic effects may not only be mediated by up- or downregulation of gene expressions but also by direct binding to angiogenic target molecules, we addressed the question whether the five selected phytochemicals may bind to VEGFR1 and VEGFR2. For this reason, we applied an *in silico* molecular docking approach. As a control drug, we used axitinib, a synthetic

Table 1: Korean plants with antiangiogenic potential.

Reference number	Plant species (and family)	Traditional uses	Part used	Previously reported activity	Reported chemical constituents
PB3699.1	*Acer mono* Maxim.	Leaves are an irritant and bark is astringent [28]	Leaves	The sap increases calcium ion absorption in mouse [29]	5-*O*-methyl-*(E)*-resveratrol 3-*O*-*β*-D-glucopyranoside; 5-O-methyl-*(E)*-resveratrol 3-*O*-*β*-D-apiofuranosyl-(1 → 6)-*β*-D-glucopyranoside; quercetin, quercitrin; eriodictyol; naringenin; eriodictyol-7-*O*-*α*-*D*-glucopyranoside; 5,7-dihydroxychromone 7-*O*-*α*-D-glucopyranoside; naringenin 7-*O*-*α*-D-glucopyranoside [30]
PB4737A.1	*Adenophora racemosa* J. Lee and S. Lee (Campanulaceae)	—	Whole plant	—	—
PB4338.1	*Caryopteris incana* (Thunb.) Miq. (Verbenaceae)	In China for the relief of colds, coughs, and rheumatic pains [36]	Leaves and stems	Antioxidant and cytotoxic activity reported for plant constituents [37, 38]	Incanone; suyiol [37]; incanoside; verbascoside, isoverbascoside, phlinoside A, 6-*O*-caffeoyl-beta-D-glucose; incanoside C, incanoside D and incanoside E; *β*-D-fructofuranosyl-*α*-D-(6-*O*-[*E*]-sinapoyl) glucopyranoside [38, 39]; 8-*O*-acetylharpagide; 6′-*O*-*p*-coumaroyl-8-*O*-acetylharpagide; (3*R*)-oct-1-en-3-ol *O*-*α*-*L*-arabinopyranosyl-(1″ → 6′)-*O*-*β*-D-glucopyranoside; apigenin 7-O-neohesperidinoside; 6′-*O*-caffeoylarbutin; leucosceptoside A; phlinoside A; 6′-*O*-Caffeoyl-8-*O*-acetylharpagide; (3*R*)-Oct-1-en-3-ol *O*-*β*-D-glucopyranosyl-(1″ → 2′)-*O*-*β* -D-glucopyranoside; (3*R*)-Oct-1-en-3-ol *O*-*α*-L-arabinopyranosyl- (1‴ → 6″)-*O*-*β*-D-glucopyranosyl-(1″ → 2′)-*O*-*β* -D-glucopyranoside [40]
PB2906.2	*Cinnamomum japonicum* Sieb. (Theaceae)	—	Stems	—	—
PB3828.1	*Eurya japonica* Thunb. (Theaceae)	As an ornamental [41]	Leaves	—	cyanidin 3-glucoside; Cyanidin 3-O-(6″-O-(4‴-*O*-acetyl-*α*-L-rhamnopyranosyl)-*B*-D-glucopyranoslde) [42]
PB2552.1	*Reynoutria sachaliensis* (F. Schmidt) Nakai.(Polygonaceae)	Crops protection against phytopathogenic fungi [43]	Fruits	Antioxidant activity [44]	Emodin; emodin-8-*O*-*β*-D-glucopyranoside; physcion-8-O-beta-D-glucopyranoside; quercetin-3-O-alpha-L-arabinofuranoside; quercetin-3-O-beta-D-galactopyranoside; quercetin-3-O-beta-D-glucuronopyranoside; anthraquinones, stilbenes [44]
PB2892.1	*Schisandra chinensis* (Turcz.) Bail. (Magnoliaceae)	Protective effect against deficits of the lung, liver, and gall bladder, alleviate cough and satisfy thirst [45]	Stems	Antihepatotoxic [46], enhance hepatic glutathione regeneration capacity [47], anti-inflammatory [45]	Lignans (schizandrin; gamma-schizandrin; gomisins A, B, C, D, E and F); nortriterpenoids (pre-schsanartanin and schindilactones A–C; schintrilactones A and B; wuweizidilactones A–F) [46]

(—): not reported. The complete list of the tested plants is available in supporting information.

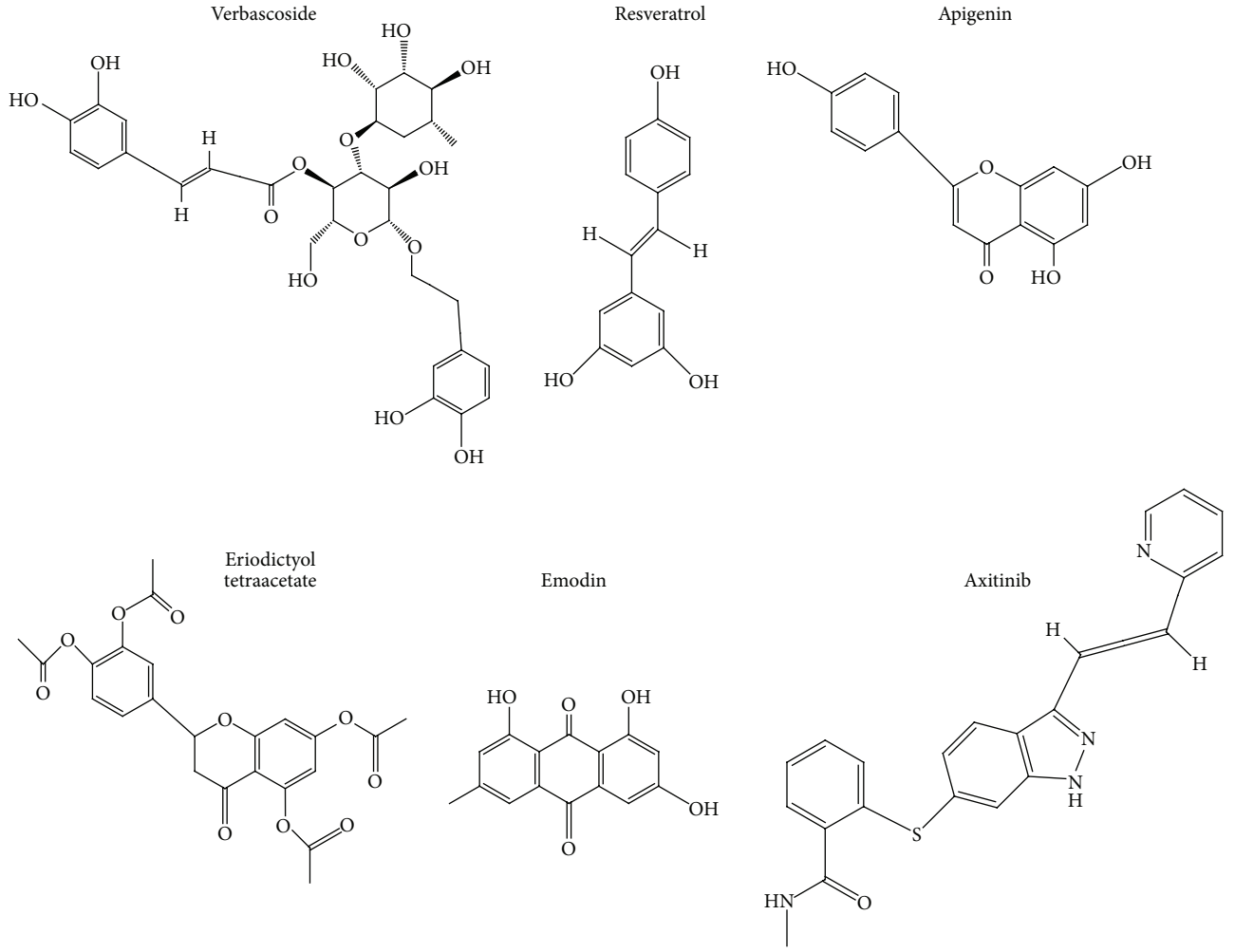

Figure 3: Chemicals structures of selected phytochemicals derived from Korean medicinal plants.

Table 2: Expression of angiogenesis-regulating genes correlating with IC_{50} values of selected phytochemicals in the NCI panel of tumor cell lines ($R > 0.3$; $R < -0.3$). The full names of the abbreviations given are listed in Supplementary Table 1.

Compound	Direct correlation with gene expression ($R > 0.3$)	Inverse correlation with gene expression ($R < -0.3$)
Verbascoside	*MPDZ, MMRN, F3, PECAM1, DDAH2,* and *NOTCH3, DVL3*	NOS2A, C1QR1, PML, and STC1
Emodin	*PECAM1, NID2*	TIMP3, SNX17, TFPI2, SPR, and ANG
Apigenin	*NRCAM*	FGR2
Eriodictyol tetraacetate	*FGFR2, SST, TEK, PML,* and *ANGPTL3*	EFEMP1, C1QR1, PECAM1, STC1, and TGFB1
Resveratrol	*TIMP3, EphA2, PLT, PIN,* and *COL4A2*	FN1, PECAM1, PML, ABCG1, and CXCR4
Axitinib (control drug)	*COL5A1, BPR2*	THBS4

small molecule inhibitor which binds to defined pharmacophores of VEGFR1 and VEGFR2. Remarkably, all five natural products bound to the same pharmacophores as axitinib, albeit at lower binding affinities (Table 4). Eriodictyol tetraacetate showed the lowest binding energy for both VEGFR1 and VEGFR2. Quercetin might be an efficient VEGFR1 inhibitor since it made hydrogen bond with drug binding residues (Glu878 and Cys912) with low binding energy. Apigenin was observed to make hydrogen bond with drug binding residues on VEGFR1 (Glu878, Cys912, and Asp1040) and VEGFR2 (Lys868, Cys919, Asp1046) like axitinib. Thus, apigenin seems to be a promising candidate as an antiangiogenic compound. Moreover, it was found that resveratrol interacted with VEGFR1 and VEGFR2 with high affinity and made hydrogen bond with drug binding residues (Glu878 and Cys912 on VEGFR1, Lys868, Cys919, and Asp1046 on VEGFR2). Besides, verbascoside bound to VEGFR2 (−9.99 kcal/mol) with higher affinity than VEGFR1 (6.93 kcal/mol). Emodin showed moderately low binding energies compared to other compounds.

Table 3: Separation of clusters of the NCI cancer cell lines obtained by hierarchical cluster analysis for selected phytochemicals from antiangiogenic plants derived from traditional Korean medicine. The $\log_{10}$ IC_{50} median values (M) of each compound were used as cutoff values to define cell lines as being sensitive or resistant. $P > 0.05$ was considered as not significant (χ^2 test).

Compounds	Clusters	Sensitive	Resistant	P-value (χ^2 test)
Verbascoside	Partition*	≤−4.278	>−4.278	$P = 3.35497 \times 10^{-4}$
	Cluster 1	3	14	
	Cluster 2	4	9	
	Cluster 3	9	1	
	Cluster 4	8	1	
Emodin	Partition*	≤−4.607	>−4.607	$P = 0.0142$
	Cluster 1	7	2	
	Cluster 2	5	1	
	Cluster 3	8	15	
	Cluster 4	8	4	
	Cluster 5	1	6	
Apigenin	Partition*	≤−4.543	>−4.543	$P = 0.02628$
	Cluster 1	2	9	
	Cluster 2	5	4	
	Cluster 3	9	1	
	Cluster 4	6	7	
Eriodictyol tetraacetate	Partition	≤−4.358	>−4.358	n.s.**
	Cluster 1, 2, 3	6	2	
	Cluster 4	2	5	
Resveratrol	Partition*	≤−4.223	>−4.223	n.s.**
	Cluster 1	3	2	
	Cluster 2	9	5	
	Cluster 3	5	4	
	Cluster 4	3	10	
	Cluster 5	4	3	
Axitinib (control drug)	Partition*	<−5.015	>−5.015	n.s.**
	Cluster 1	10	15	
	Cluster 2	4	6	
	Cluster 3	15	8	

*$\log_{10}$ IC_{50} (M).
**n.s.: not significant ($P > 0.05$).

The binding of the five phytochemicals and axitinib to VEGFR1 is shown in Figure 6. Similar binding modes were found for VEGFR2 (data not shown).

4. Discussion

4.1. Antiangiogenic Activity In Vivo. Antiangiogenic compounds are gaining more and more interest as a new approach in the prevention and treatment of cancer and inflammatory diseases [48]. The CAM assay is a sensitive, easily feasible, and cheap *in vivo* test for investigations of the antiangiogenic potential of individual compounds and plant extracts [49]. The assay does not only provide information on the efficacy of test samples *in vivo* but also on their toxicity *in vivo*.

To the best of our knowledge, their antiangiogenic property is being reported here for the first time. The best antiangiogenic effect was recorded with the extract from *Acer mono* (11.14% proliferation), this activity being better than that of captopril (23.54% proliferation), highlighting its possible importance in cancer therapy. Captopril served as control drug, since its antiangiogenic activity is well known and the drug also inhibited angiogenesis in the CAM-assay [50, 51]. *Schisandra chinensis* exhibited a good but different extent of angiogenesis inhibition with both leaves and stems extracts, strengthening the hypothesis that it is necessary to screen various plant organs when evaluating their pharmacological activities. A comparison of our Korean plant extracts showed that there was no correlation between cytotoxicity and antiangiogenic activity [52]. Therefore, these extracts might not only be used to inhibit angiogenesis in tumors but also for treatment of noncancerous diseases such as diabetic retinopathy or macular degeneration. It has been shown during the past years that therapeutic antibodies which target VEGF are not only active in cancer but are also a considerable

Table 4: *In silico* molecular docking to VEGFR1 and VEGFR2 of selected phytochemicals from antiangiogenic plants derived from traditional Korean medicine. (Residues marked bold are the drug binding residues).

Receptors	Compounds	Lowest energy of docking (kcal/mol)	Mean binding energy (kcal/mol)	Residues involved hydrogen bond interaction with the ligand	Number of residues involved in hydrophobic interaction with ligand
VEGFR1	Axitinib (control drug)	−12.71	−12.38	**Glu 878, Cys 912**, Glu 910, **Asp 1040**	12
	Eriodictyol tetraacetate	−9.92	−9.27	**Asp 1040**	14
	Quercetin	−9.01	−8.51	**Glu 878**, Glu 910, **Cys 912**	9
	Apigenin	−8.85	−8.56	**Glu 878,Cys 912, Asp 1040**	11
	Resveratrol	−7.89	−7.72	Lys 861, **Glu 878,Cys 912**	10
	Emodin	−7.30	−7.30	Leu 833, Glu 910, **Cys 912**	10
	Verbascoside	−6.93	−4.95	Arg 1021, Asp 1022, **Asp 1040**	11
VEGFR2	Axitinib (control drug)	−12.39	−12.20	Glu 917, **Asp 1046**	14
	Eriodictyol tetraacetate	−10.56	−9.85	Ala 1050	18
	Verbascoside	−9.99	−5.33	His 816, Thr 916, **Asp 1046**, Ala 1050	19
	Apigenin	−9.04	−9.01	**Lys 868, Cys 919, Asp 1046**	11
	Quercetin	−8.29	−8.18	Ala 881, Glu 885, Ile 1025, Ile 1044	11
	Resveratrol	−8.15	−8.05	**Lys 868, Cys 919, Asp 1046**	9
	Emodin	−7.63	−7.35	Ile 1025, **Asp 1046**	9

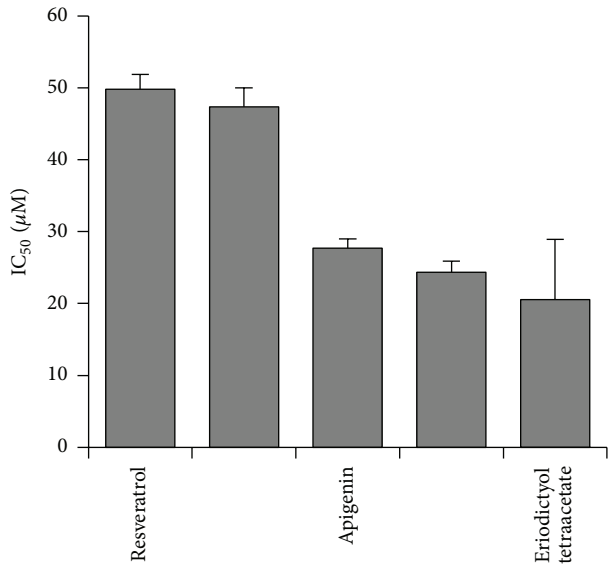

Figure 4: Cytotoxic activity of selected phytochemicals derived from Korean medicinal plants for tumor cell lines from the NCI cell line panel.

potential for ophthalmologic applications [23]. It is reasonable to speculate that plant extracts with antiangiogenic properties may not only be candidate for cancer therapy but also for therapeutic applications in ophthalmology.

4.2. Microarray-Based Gene Expression Profiling. In the present investigation, the IC_{50} profiles of five phytochemicals for the panel of 60 cell lines of the National Cancer Institute (NCI), USA, were correlated with the microarray-based expression profiles of the cell lines. The intention was to identify molecular determinants which predict sensitivity or resistance of tumor cells to these compounds. This concept was developed in the 1990s at the Developmental Therapeutics Program of the NCI to extract meaningful information of large-scale drug screenings [53]. During the past years, this concept provided a fertile ground to unravel mechanisms of action of new drugs and to use gene expression profiles for the prediction of chemosensitivity of tumor cells [54–56]. We applied this approach to gain insight of determinants of activity of natural products derived from traditional Chinese medicine, for example, homoharringtonine, artemisinin, cantharidin, arsenic trioxide, and others [36–40, 57].

In the present investigation, we focused on compounds derived from traditional Korean medicine. It was a striking feature that genes with diverse functions correlated with the response of the NCI cell lines to phytochemicals (verbascoside, apigenin, emodin, quercetin, eriodictyol, and resveratrol). This result may be taken as a hint that these natural products affect several targets and intracellular signaling pathways. This hypothesis is supported by similar observations of other authors.

Emodin inhibits tumor growth *in vitro* and *in vivo* [41, 42]. Several proteins involved in angiogenesis have been associated with this effect, including matrix metalloproteinases 2 and 9, basic fibroblast growth factor, urokinase plasminogen activator, plasminogen inhibitor 1, the extracellular signal-regulated kinases 1 and 2 (ERK1/2), and the chemokine CXCR4 receptor [41, 42, 58, 59]. Furthermore, emodin inhibits the phosphorylation of the VEGF receptors 1, 2, and 3 [60].

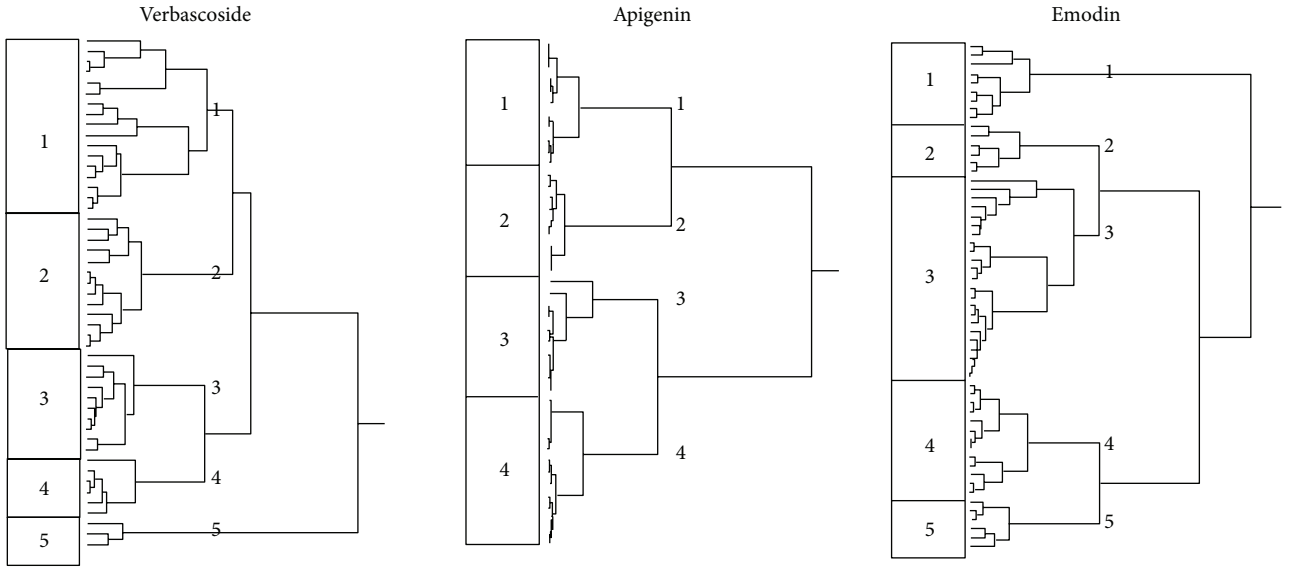

Figure 5: Dendrograms obtained by hierarchical cluster analysis of microarray-based expressions of angiogenesis-regulating genes for selected phytochemicals derived from Korean medicinal plants for tumor cell lines from the NCI cell line panel. The dendrograms were obtained by clustering using the WARD method. Extended versions of these dendrograms showing the exact positions of each cell line are included as Supplementary Material.

Apigenin inhibits angiogenesis by inhibiting VEGF and HIF-1 expression via the PI3 K/AKT/P70S6 K1 and HDM2/p53 pathways [61, 62]. Further antiangiogenic mechanisms are downregulation of type I collagen, vimentin, matrix metalloproteinase 8, and of the cytokine IL6/STAT3 pathway [63, 64].

Angiogenesis is inhibited by resveratrol both *in vitro* and *in vivo* [65–67]. Several mechanisms have been unraveled, for example, downregulation and/or inhibition of VEGF, HIF-1α, Flk-1, and Src [68, 69]. Various signaling pathways contribute to inhibition of angiogenesis such as the eukaryotic elongation factor-2 kinase-regulated pathway, the GSK3β/β-catenin/TCF-dependent pathway, NF-κB-related signaling, and cytokine signaling (IL8/CXCL8) [43, 44, 70, 71]. The antiangiogenic activity of verbascoside and eriodictyol tetraacetate has not been described yet.

Microarray analyses have been previously performed for emodin, verbascoside, and resveratrol and a huge number of genes have been found to be regulated by treatment with these compounds [45–47, 72–74]. Together with the microarray data of the present investigation, these results further emphasize the multifactorial activity of natural products. While some scientists from conventional academic medicine have called natural products as "dirty drugs" for their multiple modes of action, the past years of intense research on synthetic and monospecific drugs showed that synthetic drugs are not superior. Tumor cells readily develop resistance to monospecific drugs, for example, by point mutations in the corresponding target proteins preventing drug binding, by downregulation of target gene expression, or by activation of alternative signaling routes and bypassing of inhibited pathways [75]. The probability is much less that tumor cells escape treatment with multifactorial drugs, since resistance to one mode of drug action does not affect the drug's activity on other cellular signaling pathways. The fact that organisms developed rather multi- than monotarget compounds during evolution of life on earth may be taken as a clue that the concept of multitargeted therapy is superior [76, 77].

4.3. Molecular Docking of VEGFR1 and VEGFR2. Antiangiogenic compounds may not only exert their blood vessel inhibiting effects by up- or downregulation of angiogenesis-regulating genes, but also by targeting and binding to key regulators of angiogenesis. The specific targeting of growth factor receptors by therapeutic antibodies and small molecules is currently one of the most thriving fields in drug development with a plethora of new drugs on the market. This is also true for antiangiogenic therapies [22]. VEGFRs are exquisite targets to inhibit angiogenesis. Inhibition of the tyrosine kinase activity of VEGFR by small molecules leads to blockage of VEGFR-related downstream-signaling pathways, hence, inhibition of blood vessel sprouting.

The number of VEGFR inhibitors was steadily increasing over the past few years [78]. One of them is axitinib, which specifically binds to all three VEGF receptors, VEGFR1, VEGFR2, and VEGFR3 [79, 80]. Therefore, this drug served as control for our bioinformatical docking studies. The idea was to investigate whether or not the five selected phytochemicals from Korean plants might bind to the same pharmacophore as axitinib. As the crystal structure of VEGFR3 was not available in the PDB database, we only analyzed VEGFR1 and VEGFR2. As expected, axitinib was predicted to bind with high affinity to both receptors, which is indicated by low free binding energies (<−12 kcal/mol). Molecular docking of verbascoside, apigenin, emodin, quercetin, eriodictyol

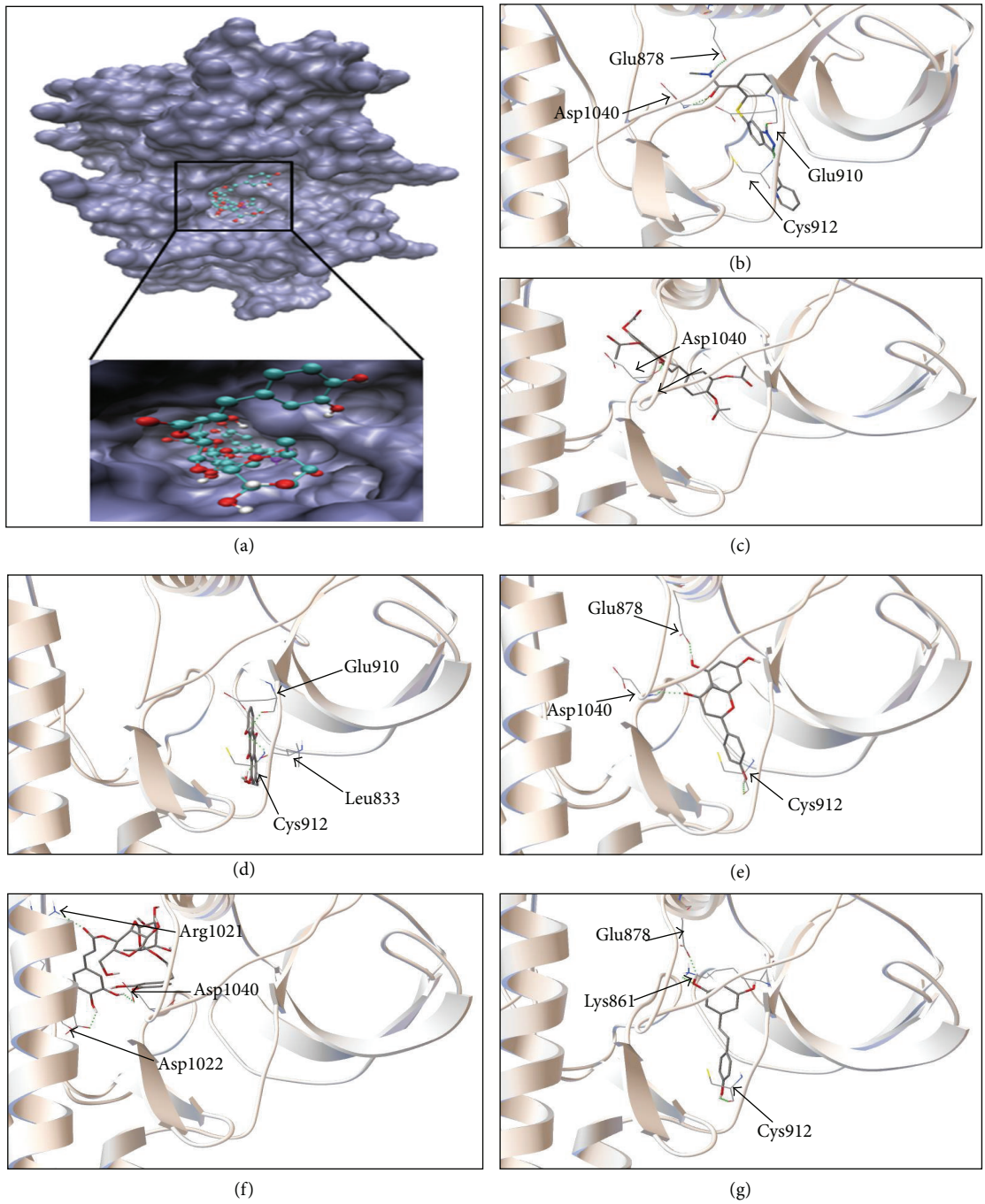

Figure 6: Docking studies of candidate antiangiogenic compounds. (a) Docking of 6 compounds into the binding site of VEGFR1-TK (PDB code: 3HNG in blue surface representation). The compounds occupy the same binding site as axitinib, a known antiangiogenic compound (in violet). Docked structure of axitinib (b), eriodictyol tetraacetate (c), emodin (d), apigenin (e), verbascoside (f), and resveratrol (g) in VEGFR1-TK binding pocket. The residues involved in hydrogen bond interaction are labeled, and hydrogen bonds are shown as green dots. Axitinib is a known VEGFR-TK inhibitor and was used as control drug.

tetraacetate, and resveratrol yielded free binding energies in a range from −6 to −9 kcal/mol. This indicates that these phytochemicals may bind to the receptors at lower affinity than axitinib. It can be speculated that these compounds efficiently inhibit angiogenesis by combining different mechanisms, such as VEGFR binding as well as up/downregulation of angiogenic genes and proteins. Therefore, these natural products may be efficient angiogenesis inhibitors, even if they bind with lower affinities to VEGF receptors than axitinib. For drug development, these phytochemicals may serve as lead compounds to synthesize novel derivatives with improved binding properties to VEGF receptors.

5. Conclusion

We estimate medicinal plants in general and especially plants from traditional Korean medicine as valuable and indispensable resources for the development of new drugs and the rational use of phytotherapy. This point of view is supported by a comprehensive survey of the NCI, USA, showing that the vast majority of clinically established cancer drugs during the past three decades were based on natural products [81]. It can be expected that natural products and evidence-based complementary and alternative therapies such as inhibition of angiogenesis by Korean medicinal plants will lead to considerably improve the treatment of patients in the future.

Conflict of Interests

No potential conflict of interests was disclosed.

Authors' Contribution

Ean-Jeong Seo and Victor Kuete contributed equally.

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Review Article

Chinese Medicines as an Adjuvant Therapy for Unresectable Hepatocellular Carcinoma during Transarterial Chemoembolization: A Meta-Analysis of Randomized Controlled Trials

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Objective. To conduct a comprehensive PRISMA-compliant systematic review and meta-analysis to evaluate the efficacy and safety of Chinese medicines (CMs) as an adjuvant therapy for unresectable HCC during transarterial chemoembolization (TACE). *Methods*. Main databases were searched up to October 2012 for randomized controlled trials (RCTs) evaluating the effects of CMs plus TACE on unresectable HCC compared with TACE alone. References of relevant reviews and eligible studies were also assessed. Risk ratios with 95% confidence intervals and mean difference were calculated. Heterogeneity and publication bias were examined. *Results*. Sixty-seven trials ($N = 5{,}211$) were included in the meta-analysis. Sensitivity analysis and random-effects model were performed for assessing significant heterogeneity. CMs plus TACE showed beneficial effects on tumor response, survival at 6, 12, 18, 24, and 36 months, quality of life, and TACE toxicity reduction compared with TACE alone. *Conclusion*. The results show that the use of CMs may increase the efficacy and reduce the toxicity of TACE in treating patients with unresectable HCC. These findings suggest that CMs could be considered as an adjuvant therapy for unresectable HCC patients during TACE. Larger-scale RCTs using standard methods and long-term follow-up are warranted to confirm these findings.

1. Introduction

Liver cancer, mainly hepatocellular carcinoma (HCC), ranks the sixth most common cancer and the third leading cause of cancer-related death worldwide [1, 2]. Annually, more than 748,000 new cases are diagnosed and 695,000 died with liver cancer. HCC is mostly unresectable as many were detected at advanced stage with poor liver function, high tumor recurrence rate, and metastasis [3]. As most HCC patients are not suitable candidates for curative resection, transarterial chemoembolization (TACE) is the most commonly used for unresectable HCC patients as a primary and palliative therapy because of improvement in survival [4–6]. However, severe side effects including liver and renal failure, bone marrow depression, postembolization syndrome, and liver abscess were observed with the use of TACE [4, 7].

Chinese medicines (CMs) were commonly used in treating HCC with side effects seldom reported. Increasing number of studies was conducted in assessing the effects of CMs on HCC. Experimental studies found the chemopreventive effects and anti-HCC properties of CMs mainly through the induction of apoptosis and autophagy and cytotoxicity on cancer cells [75–78]. Although three systematic reviews evaluating the efficacy of CMs on HCC had been published [79–81], the effect of CMs combined with TACE in treating HCC remains uncertain. No systematic review was conducted

according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) [82]. Moreover, two of these reviews included nonrandomized controlled trials which probably overestimated the beneficial effects of CMs [79, 81]. Another review had not focused on specific stage of HCC [80]. In addition, a significant proportion of related randomized controlled trials (RCTs), especially those published recently (2007 afterward, 27 studies), were not included in these reviews. Therefore, we conducted a comprehensive and PRISMA-compliant systematic review and meta-analysis to investigate the efficacy of CMs on unresectable HCC including updated trials published after 2007. Specifically, we aim to critically appraise the efficacy and safety of CMs as an adjuvant therapy for unresectable HCC patients during TACE treatment focusing on outcomes of survival, tumor response, quality of life (QoL), and TACE toxicity.

2. Methods

This systematic review was conducted according to the PRISMA statement [82].

2.1. Search Strategies. Main electronic databases including MEDLINE (1946–2012), EMBASE (1947–2012), AMED (1985–2012), CINAHL Plus (1937–2012), PubMed (January 1966–2012), the Cochrane Library (1996–2012), Chinese Biomedical CD Database (CBM, January 1980–2012), China Network Knowledge Infrastructure (CNKI, 1911–2012), TCMOnline (1949–2012), Chinese Medical Current Contents (CMCC, 1994–2012), and WanFang Data (1989–2012) were searched for eligible studies. The latest search was performed on October 2012. References of relevant reviews and eligible studies were also checked.

The search terms used were "liver cancer," "hepatocellular carcinoma," "primary liver carcinoma," "Chinese medicine," "herbal medicine," "traditional medicine," and "complementary medicine" without restriction on publication language and publication type. Free-text and MeSH terms were used when allowed. The search strategies in Chinese and English were slightly adjusted to suit the instructions of different databases.

2.2. Study Selection Criteria. Eligible RCTs examining the efficacy of CMs plus TACE in treating unresectable HCC were assessed. Inclusion criteria were as follows: (a) RCTs; (b) participants in treatment group received combination therapy consisting of CMs and TACE and TACE alone in control group; (c) participants had unresectable or stage II or above primary HCC which were confirmed by cytological or pathological results, or met the criteria of the European association for the study of the liver guideline; (d) reported data on at least one of the outcomes including survival, tumor response, QoL using the Karnofsky performance scale (KPS), or TACE-related toxicity.

Primary outcomes were 6-month, 12-month, 18-month, 24-month, and 36-month survival and tumor response. Secondary outcomes included KPS (QoL) and TACE toxicity. Survival was defined as the number of patients in each intervention group who were alive at 6, 12, 18, 24, or 36 months. Tumor response has to be assessed using the World Health Organization (WHO) criteria, which were commonly used to evaluate therapeutic efficacy on solid tumors [83, 84]. According to the results of CT and/or MRI, the efficacy of anticancer agents was classified as follows: complete response (CR) refers to the disappearance of all visible tumor lesions; partial response (PR) refers to 50% or more decrease in the lesions; no change (NC) refers to either less than 50% decrease in total tumor size or at least 25% increase in the lesions; and progressive disease (PD) refers to at least 25% increase in the size of the lesions. Tumor response was defined as CR plus PR and compared before and after treatment. TACE-related toxicity including gastrointestinal and bone marrow toxicities was evaluated using the 5-point WHO scale (grade 0–4) on reporting acute and subacute toxic effects [85].

Exclusion criteria included the following: (a) using other complementary medicines in treatment or control group; (b) metastatic HCC; (c) inconsistency of reporting on methods, results, or both; and (d) duplicated or redundant publications.

2.3. Study Selection. All searched titles and abstracts were screened independently by two authors (Fan Cheung and XuanbinWang) according to the predefined eligibility criteria. Disagreements were resolved by consensus or consulting a third author (Yibin Feng). Full texts of the potentially eligible studies were retrieved and further assessed by these two authors (Fan Cheung and XuanbinWang) using the same method.

2.4. Data Extraction. Data of the included studies were extracted independently and cross-checked by two authors (FC and XBW) using a standardized extraction form which was generated at the protocol stage. The extracted items comprised (1) authors and year of publication; (2) study design; (3) participant characteristics; (4) intervention details, and (5) outcome measures.

2.5. Study Quality Assessment. Study quality was independently evaluated by two authors (Fan Cheung and Xuanbin-Wang) using the six dimensions of Cochrane "risk of bias" assessment [114]. The assessment criteria included sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome data, and other bias. Each dimension was rated as "yes" (low risk of bias), "unclear" (unclear risk of bias), or "no" (high risk of bias). Studies with 3 or more "yes" were classified as high quality with low risk of bias and 0–2 poor quality with high risk of bias. As bias of blinding may be more severe for subjective outcomes (e.g., QoL) than for objective outcomes (e.g., survival and tumour response), separate analyses for different outcomes were conducted as recommended by Cochrane collaboration [114].

2.6. Statistical Analysis. Review Manager 5.1 (The Nordic Cochrane Centre, Copenhagen, Denmark) was used for data analysis. Risk ratios (RRs) with 95% confidence intervals (CIs) and mean difference (MD) were calculated for dichotomous and continuous data, respectively. Heterogeneity was

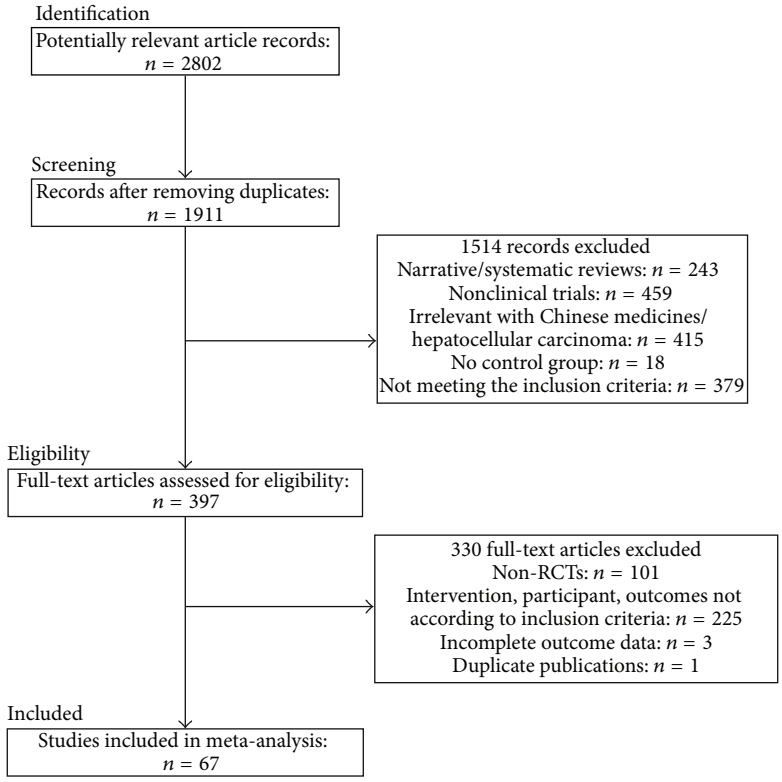

Figure 1: Flow diagram of the study selection for this systematic review.

assessed using X^2 test and I^2 statistic with $P < 0.1$ or $I^2 > 50\%$ was treated as substantial heterogeneity [114]. Significant statistical heterogeneity was further assessed using sensitivity analyses and results were estimated using random-effects model. In contrast, a fixed-effect model was used for homogeneous studies. Publication bias was examined using funnel plots [115] and Egger's test [116] (STATA 10.0, StataCorp LP, College Station, TX, USA). P values lower than 0.05 were considered statistically significant.

3. Results

A total of 2802 potential trials were identified for this review, of which 891 were duplicate records and 1514 were excluded because of narrative/systematic review, nonclinical trials, irrelevance, no comparison group, or not meeting the inclusion criteria of this study (Figure 1). The full text of 397 articles was retrieved for further evaluation, of which 330 were excluded for the reasons of not RCTs ($n = 101$), not according to the inclusion criteria ($n = 255$), incomplete outcome data ($n = 3$), or duplicate publication ($n = 1$). Finally, 67 RCTs with a total of 5211 patients (study sample size ranged from 25 to 236) [8–74] were included in this study. Two of the included studies were retrieved from the relevant reviews and studies [20, 21].

3.1. Study Descriptions. All studies were conducted in hospital settings in China, of which 6 were multicentre studies [18, 36, 39, 57, 58, 67] and the remaining were single-centre studies (Table 1). All studies adapted parallel-arm group design. Nearly, all studies, except one [18], were published in Chinese from 1999 to 2011. Participants aged from 18 to 78 years old. Near half ($n = 32$) described the enrollment criteria (diagnosis, inclusion and exclusion criteria).

Three studies used individualized prescriptions according to traditional CM syndrome patterns [17, 63, 73], while 46 standardized CM formulae including 4 single herbs and 42 composite formulae were tested in the remaining 64 studies. Ai Di injection ($n = 8$) was the most popularly used standardized CM formula. The duration of CMs treatment ranged from 14 days to 3 years.

3.2. Methodological Quality. Of the 67 included studies, only 15 studies reported the methods of allocation sequence generation, which included using a random number table [11, 26, 43, 60, 65], drawing of lots [17], shuffling envelops [28, 56], stratified randomization [30, 63], and referring to the sequence of admission [12, 49, 64, 69, 70]. The remaining 52 studies described that the participants were "randomly allocated," but the allocation procedures were not reported. None of the studies mentioned the method of allocation concealment. Twenty studies reporting objective outcomes were rated as at low risk of blinding bias. Most studies (82%) reported no significant difference of baseline characteristics. No study described intention-to-treat analysis. Only 6 studies [21, 38, 47, 59, 60, 65] reported the information of dropouts, in which 3 studies [21, 38, 59] provided reasons of withdrawal. Forty studies were rated as at low risk of bias for incomplete

Table 1: Characteristics of the included studies.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Ayi and Liu 2011 [8]	108 (54/54)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 56 (28–77) Disease stage: NA Child-Pugh score: C KPS: >60	5-FU, HCPT, LP	Ai Di injection (60 L/d)	56 ds	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) KPS (QoL increase) (4) AE
Bao 2007 [9]	54 (28/26)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 51 (25–68) Disease stage: II, III Child-Pugh score: A, B KPS: NA	5-FU, DDP, MMC, HCPT, EPI, LP, GSP	Kang Ai injection (40–60 mL/d)	1 mon	(1) TR (short-term effectiveness) (2) AE
Cao et al. 2005 [10]	100 (50/50)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: NA Child-Pugh score: A KPS: NA	5-FU, MMC, LP	Gan Fu Kang capsule (1 capsule, t.i.d.)	60–80 ds	(1) Survival at 6/12/24/36 mons
Chen and Ding 2007 [11]	60 (32/28)	Single centre, parallel group, unblinded RCT (random number table)	Age range: 36–70 Disease stage: NA Child-Pugh score: NA KPS: >60	5-FU, MMC, OX, LP, GSP	Ai Di injection (60 mL/d)	42 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Dan et al. 2007 [12]	70 (35/35)	Single centre, parallel group, unblinded RCT (sequence of admission)	Age range: 29–70 Disease stage: II, III, IV Child-Pugh score: NA KPS: NA	5-FU, DDP, THP, LP	Fu Zheng Ping Gan Xiao Liu Tang (1 dose/d)	1–6 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/18 mons (3) KPS (QoL increase)
Deng et al. 2009 [13]	40 (20/20)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 52 (26–66) Disease stage: III, IV Child-Pugh score: NA KPS: ≥70	THP, LP	Fu Fang Ku Shen injection (20 mL/d)	2 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Dong et al. 2007 [14]	65 (33/32)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 56.5 Disease stage: II, III, IV Child-Pugh score: NA KPS: ≥60	5-FU, DDP, THP, LP	Ai Di injection (80–100 mL/d)	56 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Dong et al. 2008 [15]	133 (67/66)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 56 (28–77) Disease stage: NA Child-Pugh score: NA KPS: >60	5-FU, THP, LP	Jing Long capsule (1 g, t.i.d.)	56 ds	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) KPS (QoL increase) (4) AE
Han 2009 [16]	48 (30/18)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 49.9 (32–70) Disease stage: II, III Child-Pugh score: NA KPS: NA	ADM, MMC, CBDCA, LP, GSP	Blood-activating and stasis-resolving herbs (NA)	NA	(1) TR (short-term effectiveness) (2) KPS (QoL increase)

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Hou and Lu 2009 [17]	72 (36/36)	Single centre, parallel group, unblinded RCT (drawing of lots)	Age range: 34–72 Disease stage: NA Child-Pugh score: NA KPS: >70	DDP, BLM-A5, GC, LP, GSP	CMs given according to CM syndrome differentiation (1 dose/d)	4 wks	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Huang et al. 2002 [18]	57 (30/27)	Multicentre, parallel group, unblinded RCT (unreported)	Mean age (range): 59.5 (35–70) Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, MMC, HCPT, LP	Kang Lai Te injection (200 mL/d) plus Bai Hua She She Cao injection (30 mL/d)	2–4 mons	(1) TR (short-term effectiveness) (2) Survival at 12 mons (3) AE
Huang 2008 [19]	50 (30/20)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP, MMC, HCPT, LP	Ci Dan capsule (5 capsules, t.i.d.)	4 mons	(1) TR (short-term effectiveness) (2) Survival at 12 mons (3) AE
Jia et al. 2003 [20]	66 (34/32)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: T3-4N0M0 Child-Pugh score: NA KPS: >60	5-FU, DDP, ADM, MMC, LP, GSP	*Brucea javanica* oil Injection (30 mL/d)	2–4 mons	(1) TR (short-term effectiveness) (2) AE
Li et al. 2009 [21]	64 (32/32)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: II, III Child-Pugh score: NA KPS: ≥60	5-FU, DDP, ADM, LP	Kang Lai Te capsule (6 capsules, q.i.d.)	42–63 ds	(1) TR (short-term effectiveness) (2) AE
Li 2007 [22]	36 (20/16)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 50.9 (32–70) Disease stage: II, III Child-Pugh score: NA KPS: NA	MMC, THP, CBDCA, LP, GSP	CMs for fortifying the spleen and activating the blood (1 dose/d)	NA	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Li and Fan 2008 [23]	128 (64/64)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: NA Child-Pugh score: NA KPS: >70	5-FU, EPI, MMC, LP	Fu Zheng Kang Ai Tang (1 dose/d)	3 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) AE
Liang et al. 2005 [24]	68 (35/33)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 29–70 Disease stage: II, III Child-Pugh score: NA KPS: NA	DDP, MMC, EPI, LP	Matrine injection (150 mL/d)	28 ds	(1) TR (short-term effectiveness) (2) AE

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Liang et al. 2008 [25]	121 (64/57)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 44.8 (30–70) Disease stage: II, III Child-Pugh score: NA KPS: ≥60	5-FU, ADM, MMC, CBDCA, LP	Ci Dan capsule (5 capsules, q.i.d.)	2 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12 mons (3) KPS (QoL increase)
Liang et al. 2005 [26]	146 (75/71)	Single centre, parallel group, unblinded RCT (random number table)	Mean age (range): 50.7 (20–74) Disease stage: III, IV Child-Pugh score: A, B, C KPS: NA	MMC, EPI, CBDCA, LP	Bu Zhong Yi Qi Tang (1st–3rd month: 1 dose/d; 4th–6th month: 2 doses/w)	6 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24/36 mons
Ling 2010 [27]	128 (64/64)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 39–62 Disease stage: II, III, IV Child-Pugh score: NA KPS: NA	5-FU, DDP, EPI, LP	Xiao Liu Tang (1 dose/d)	2-3 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Liu et al. 2007 [28]	70 (34/36)	Single centre, parallel group, unblinded RCT (shuffling envelops)	Mean age (range): 50.7 (28–67) Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP, ADM, MMC, HCPT, LP, GSP	Kang Ai injection (40 mL/d)	20 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Lu and He 2009 [29]	48 (24/24)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 28–68 Disease stage: II, III Child-Pugh score: NA KPS: NA	DDP, MMC, EPI, LP	Experience CMs formula (NA)	3–12 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Lu et al. 2010 [30]	60 (30/30)	Single centre, parallel group, unblinded RCT (stratified randomization)	Mean age: 49.4 Disease stage: II, III, IV Child-Pugh score: NA KPS: NA	DDP, ADM, MMC, LP	Yang Gan Kang Ai Wan (9 g, t.i.d.)	135–270 ds	(1) Survival at 6/12/18 mons
Lu et al. 2007 [31]	63 (33/30)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 18–71 Disease stage: II, III Child-Pugh score: A, B, C KPS: 50–90	DDP, GC, LP	Kang Ai injection (40 mL/d)	40 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Meng 2008 [32]	148 (75/73)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 56 Disease stage: II, III	5-FU, THP, LP	Ai Di injection (50 mL/d)	28 ds	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) KPS (QoL increase) (4) AE

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Shi and Sun 2005 [33]	50 (30/20)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 52.3 (37–65) Disease stage: NA Child-Pugh score: NA KPS: NA	5-FU, MMC, LP, GSP	Tan Re Qing injection (40 mL/d)	≥14 ds	(1) KPS (QoL increase)
Qiao 2010 [34]	40 (20/20)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 18–65 Disease stage: II, III Child-Pugh score: NA KPS: >50	5-FU, DDP, ADM, LP	Ai Tong Xiao granule (1 pack/d)	56 ds	(1) KPS (QoL increase) (2) AE
Sun et al. 2002 [35]	236 (118/118)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 51.4 (26–74) Disease stage: NA Child-Pugh score: NA KPS: NA	MMC, EPI, CBDCA, LP	Hua Chan Su injection (20 mL/d)	1–24 Ks	(1) Survival at 12/24/36 mons (2) AE
Tang et al. 2010 [36]	50 (30/20)	Multicentre, parallel group, unblinded RCT (unreported)	Mean age: 54.1 Disease stage: II, III Child-Pugh score: NA KPS: >60	ADM, MMC, LP, GSP	Fu Gan injection (20 mL/d)	2 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) KPS (QoL increase)
Tian et al. 2001 [37]	43 (23/20)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 52.2 (23–73) Disease stage: II, III Child-Pugh score: NA KPS: 60–80	5-FU, DDP, ADM, LP	Fu Zheng Jie Du Tang (1 dose/d)	18–88 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Tian 2006 [38]	72 (36/36)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 53 (33–75) Disease stage: NA Child-Pugh score: NA KPS: ≥70	5-FU, DDP, ADM, MMC, LP	Ai Yi Shu injection (0.5 mg/d)	NA	(1) TR (short-term effectiveness) (2) Survival at 6/12/18/24 mons (3) KPS (QoL increase) (4) AE
Wang et al. 2002 [39]	95 (47/48)	Multicentre, parallel group, unblinded RCT (unreported)	Mean age (range): 50.5 (28–73) Disease stage: NA Child-Pugh score: NA KPS: ≥60	5-FU, ADM, MMC, THP, HCPT, CBDCA, LP, GSP	960 mixture (NA)	42–210 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Wang and Cheng 2009 [40]	57 (27/30)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 48 (30–65) Disease stage: II, III Child-Pugh score: NA KPS: ≥70	5-FU, DDP, ADM, LP, GSP	Fu Fang Ku Shen injection (20 mL/d)	≥20 ds	(1) TR (short-term effectiveness) (2) Survival at 12/24/36 mons (3) KPS (QoL increase)
Wang and Yang 2002 [41]	60 (30/30)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 55.7 Disease stage: II, III Child-Pugh score: NA KPS: >60	ADM, DDP, MMC, LP	Gan Ji granule (1 pack, b.i.d.)	3-4 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12 mons
Wang et al. 2008 [42]	86 (43/43)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 55.56 Disease stage: II, III Child-Pugh score: NA KPS: >60	DDP, ADM, MMC, LP	Jian Pi Qing Gan He Ji (200 mL, b.i.d.)	2 mons	(1) TR (short-term effectiveness)

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Wang et al. 2007 [43]	43 (22/21)	Single centre, parallel group, unblinded RCT (random number table)	Mean age (range): 64 (35–78) Disease stage: II, III Child-Pugh score: NA KPS: NA	ADM, MMC, CBDCA	Qi Shu Fang (1 dose/d)	56 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Wang 2008 [44]	59 (30/29)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 61 (38–78) Disease stage: NA Child-Pugh score: NA KPS: NA	5-FU, ADM/EPI, MMC, LP, GSP	Experience CMs formula (1 dose/d)	>2 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Weng et al. 2008 [45]	96 (55/41)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 28–76 Disease stage: NA Child-Pugh score: A, B KPS: NA	5-FU, DDP, ADM, MMC, LP, GSP	Experience CMs formula plus CM patch (NA)	NA	(1) TR (short-term effectiveness) (2) Survival at 6/12/24/36/48/60 mons (3) KPS (QoL increase) (4) AE
Wu et al. 2000 [46]	80 (36/44)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 51.4 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP, MMC, LP, GSP	Hua Chan Su injection (20 mL/d)	20 ds	(1) TR (short-term effectiveness) (2) Survival at 6/12 mons (3) AE
Wu 1999 [47]	25 (13/12)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 55.4 (38–69) Disease stage: II Child-Pugh score: NA KPS: NA	5-FU, DDP, MMC, LP	Yi Guan Jian Jia Wei (NA)	24 wks	(1) Survival at 36 mons
Wu et al. 2003 [48]	60 (30/30)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 51.9 (28–72) Disease stage: II, III	5-FU, ADM (or DDP), MMC, LP, GSP	Hu Gan Ruan Jian Fang (NA)	NA	(1) TR (short-term effectiveness) (2) Survival at 6/12 mons
Xu et al. 2006 [49]	57 (30/27)	Single centre, parallel group, unblinded RCT (sequence of admission)	Mean age (range): 52.3 (39–72) Disease stage: NA Child-Pugh score: NA KPS: NA	5-FU, MMC, HCPT, LP	Fu Zheng Jie Du Tang (1 dose/d)	2–4 mons	(1) TR (short-term effectiveness) (2) Survival at 12 mons
Xu et al. 2007 [50]	52 (32/20)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 38–75 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP, THP, LP	Ai Di Injection (50 mL/d)	4 wks	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Xu et al. 2007 [51]	60 (40/20)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 35–72 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP/OX, HCPT, LP	CMs for fortifying the spleen and resolving dampness and activating the blood and detoxifying (1 dose/d)	2 mons	(1) TR (short-term effectiveness) (2) AE
Xue et al. 2002 [52]	70 (34/36)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 51.3 (26–72) Disease stage: III, IV Child-Pugh score: NA KPS: NA	5-FU, DDP, ADM, MMC, LP	Si Jun Zi Tang (1 dose/d)	NA	(1) TR (short-term effectiveness) (2) Survival at 12/24/36 mons (3) AE

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Yang 2010 [53]	50 (25/25)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 54 (31–74) Disease stage: NA Child-Pugh score: A, B KPS: NA	5-FU, DDP, ADM, EPI, LP	Lian Hua Qing Gan Yin (1 dose/d)	NA	(1) Survival at 12/24 mons
Yang 2006 [54]	62 (31/31)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 27–68 Disease stage: NA Child-Pugh score: NA KPS: NA	5-FU, DDP, EPI, LP, GSP	Ai Di injection (50 mL/d)	>1 mon	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Yang 2006 [55]	50 (28/22)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 50.6 (26–75) Disease stage: II, III, IV Child-Pugh score: NA KPS: >60	5-FU, DDP, MMC, THP, LP	Ai Di injection (50 mL/d)	32 ds	(1) TR (short-term effectiveness) (2) AE
Yi et al. 2008 [56]	67 (36/31)	Single centre, parallel group, unblinded RCT (shuffling envelops)	Mean age (range): 53.9 (25–69) Disease stage: II, III Child-Pugh score: NA KPS: >60	5-FU, ADM, HCPT, LP, GSP	Kang Ai injection (40 mL/d)	12 wks	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Yu and Kang 2010 [57]	96 (48/48)	Multicentre, parallel group, unblinded RCT (unreported)	Mean age (range): 53.1 (30–69) Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, ADM, HCPT, LP, GSP	Fu Fang Ku Shen injection (20 mL/d)	45 ds	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Yuan et al. 2010 [58]	62 (31/31)	Multicentre, parallel group, unblinded RCT (unreported)	Mean age (range): NA Disease stage: NA Child-Pugh score: NA KPS: NA	5-FU, DDP, MMC, THP, LP, GSP	CMs for soothing the liver, fortifying the spleen, and tonifying the kidney (NA)	3 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Yuan and Yu 2005 [59]	73 (35/38)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 34–69 Disease stage: NA Child-Pugh score: NA KPS: >50	5-FU, DDP, MMC, HCPT	Ai Di injection (50 L/d)	>20 ds	(1) TR (short-term effectiveness)
R. Q. Zhai and H. Y. Zhai 2010 [60]	62 (32/30)	Single centre, parallel group, unblinded RCT (random number table)	Mean age (range): 55.3 (28–72) Disease stage: III, IV Child-Pugh score: NA KPS: ≥50	MMC, EPI, CBDCA, LP	Hu Gan Xiao Zheng Tang (1st–3rd month: 1 dose/d; 4th month: 1 dose/2 d) plus San Jie Xiao Tong Gao (plaster therapy; 1 dose/2 ds)	4 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24/36 mons (3) KPS (QoL increase) (4) AE
Zhang et al. 2005 [61]	224 (116/108)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 51.3 (29–76) Disease stage: NA Child-Pugh score: NA KPS: NA	MMC, EPI, CBDCA, LP	Jing Long capsule (4 capsules, t.i.d.)	≥3 yrs	(1) TR (short-term effectiveness) (2) Survival at 6/12/24/36 mons

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Zhang et al. 2007 [62]	60 (30/30)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 50 (24–75) Disease stage: II, III Child-Pugh score: NA KPS: ≥60	5-FU, DDP, ADM, MMC, LP, GSP	Chai Shao Liu Jun Zi Tang (1 dose/d)	>1 mon	(1) TR (short-term effectiveness) (2) KPS (QoL increase) (3) AE
Zhang et al. 2008 [63]	61 (31/30)	Single centre, parallel group, unblinded RCT (stratified randomization)	Mean age (range): 50.2 (24–67) Disease stage: II, III Child-Pugh score: A, B, C KPS: NA	5-FU, MMC, THP, LP	CMs given according to CM syndrome differentiation (1 dose/d)	≥2 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/18/24 mons (3) KPS (QoL increase)
Zhang et al. 2008 [64]	64 (31/33)	Single centre, parallel group, unblinded RCT (sequence of admission)	Age range: 39–73 Disease stage: II, III, IV Child-Pugh score: NA KPS: >60	5-FU, DDP, HCPT, OX, LP	Jian Pi Fu Gan Tang (1 dose/d)	>2 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/18 mons (3) KPS (QoL increase)
Zhang et al. 2007 [65]	112 (58/54)	Single centre, parallel group, unblinded RCT (random number table)	Mean age (range): 57.2 (18–70) Disease stage: II Child-Pugh score: NA KPS: ≥50	5-FU, DDP, EPI, VDS	Gu Ben Yi Liu II (NA)	≥2 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons (3) KPS (QoL increase) (4) AE
Zhang 2011 [66]	49 (25/24)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 49.9 (33–71) Disease stage: II, III Child-Pugh score: NA KPS: >60	NA	Blood-activating and stasis-resolving herbs (NA)	2-3 mons	(1) TR (short-term effectiveness) (2) KPS (QoL increase)
Zhang et al. 2000 [67]	95 (50/45)	Multicentre, parallel group, unblinded RCT (unreported)	Age range: 29–60 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP/ADM, MMC, LP	CMs for soothing the liver and regulating Qi, fortifying the spleen and harmonizing the stomach, tonifying the liver and kidney, and softening hardness and dissipating binds (1 dose/d)	≥2-3 mons	(1) TR (short-term effectiveness) (2) Survival at 6/12/24 mons
Zhao and Huang 2005 [68]	60 (30/30)	Single centre, parallel group, unblinded RCT (unreported)	Age range: 38–72 Disease stage: II Child-Pugh score: NA KPS: NA	5-FU, EPI, HCPT, LP	Can Qi capsule (NA)	NA	(1) TR (short-term effectiveness) (2) AE

Table 1: Continued.

Study	Sample size (T/C)	Design (sequence generation)	Baseline characteristics	Intervention		Duration	Outcome measures
				TACE	Experimental CMs		
Zhao et al. 2006 [69]	94 (48/46)	Single centre, parallel group, unblinded RCT (sequence of admission)	Mean age (range): 52.8 (40–64) Disease stage: II, III Child-Pugh score: NA KPS: >70	5-FU, DDP, ADM, HCPT, LP, GSP	Liu Jun Zi Tang (1 dose/d)	126–168 ds	(1) TR (short-term effectiveness) (2) Survival at 12/24/36 mons
Zhou et al. 2002 [70]	228 (118/110)	Single centre, parallel group, unblinded RCT (sequence of admission)	Age range: 28–72 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, DDP, ADM, MMC, LP, GSP	Liu Jun Zi Tang (1 dose/d)	NA	(1) Survival at 6/12/24/36 mons
Zhou et al. 2010 [71]	64 (32/32)	Single centre, parallel group, unblinded RCT (unreported)	Mean age (range): 49.7 (22–70) Disease stage: NA Child-Pugh score: A, B KPS: ≥60	MMC, BLM-A5, LP, GSP	Kang Ai Fang (1 dose/d)	3 mons	(1) TR (short-term effectiveness) (2) Survival at 12 mons (3) KPS (QoL increase)
Zou 2004 [72]	50 (25/25)	Single centre, parallel group, unblinded RCT (unreported)	Mean age: 41 Disease stage: II, III Child-Pugh score: NA KPS: NA	5-FU, MMC, HCPT, LP	Fu Fang Ku Shen injection (16 mL/d)	40 ds	(1) TR (short-term effectiveness) (2) AE

5-Fu: fluorouracil; AE: TACE toxicity; b.i.d.: two times a day; BLM-A5: bleomycin A5; C: control group; CBDCA: carboplatin; CM: Chinese medicine; d: day; DDP: cisplatin; EPI: epirubicin; GC: gemcitabine; HCPT: hydroxycamptothecin; KPS: Karnofsky performance scale; MMC: mitomycin; mon: month; NA: not available; OX: oxaliplatin; q.i.d.: four times a day; QoL: quality of life; T: treatment group; TACE: transarterial chemoembolization; THP: pirarubicin; t.i.d.: three times a day; TR: tumor response; VDS: vindesine; wk: week; yr: year.

Table 2: Risk of bias assessment.

Study	Sequence generation	Allocation concealment	Blinding: outcomes				Blinding	Incomplete outcome data	Selective outcome data	Other bias	Risk of bias score
			Survival	Tumor response	KPS	AE					
Ayi and Liu 2011 [8]	U	U	Y	Y	/	Y	Y	Y	U	Y	3
Bao 2007 [9]	U	U	/	Y	/	N	N	U	U	Y	1
Cao et al. 2005 [10]	U	U	Y	/	/	/	Y	Y	Y	Y	4
Chen and Ding 2007 [11]	Y	U	/	Y	N	N	N	Y	U	Y	3
Dan et al. 2007 [12]	N	U	Y	Y	N	/	N	Y	Y	U	2
Deng et al. 2009 [13]	U	U	/	Y	N	/	N	Y	Y	U	2
Dong et al. 2007 [14]	U	U	/	Y	N	/	N	Y	U	U	1
Dong et al. 2008 [15]	U	U	Y	Y	N	Y	N	N	U	Y	1
Feng 2002 [73]	U	U	/	Y	/	/	Y	Y	U	Y	3
Guo et al. 2005 [74]	U	U	/	Y	/	N	N	U	Y	U	1
Han 2009 [16]	U	U	/	/	N	/	N	N	Y	Y	2
Hou and Lu 2009 [17]	Y	U	/	Y	N	N	N	U	Y	Y	3
Huang et al. 2002 [18]	U	U	Y	Y	/	N	N	Y	U	U	1
Huang 2008 [19]	U	U	Y	Y	/	N	N	Y	U	U	1
Jia et al. 2003 [20]	U	U	/	Y	/	N	N	Y	Y	Y	3
Li et al. 2009 [21]	U	U	/	Y	/	N	N	N	Y	Y	2
Li 2007 [22]	U	U	/	Y	N	/	N	U	Y	U	1
Li and Fan 2008 [23]	U	U	Y	Y	/	N	N	Y	U	Y	2
Liang et al. 2005 [24]	U	U	/	Y	/	Y	Y	U	Y	Y	3
Liang et al. 2008 [25]	U	U	Y	Y	N	/	N	U	Y	Y	2
Liang et al. 2005 [26]	Y	U	Y	Y	/	/	Y	U	Y	Y	4
Ling 2010 [27]	U	U	/	Y	N	/	N	Y	Y	U	2
Liu et al. 2007 [28]	Y	U	/	Y	N	N	N	Y	Y	Y	4
Lu and He 2009 [29]	U	U	/	Y	N	/	N	Y	Y	Y	3
Lu et al. 2010 [30]	Y	U	Y	/	/	/	Y	Y	Y	Y	5
Lu et al. 2007 [31]	U	U	/	Y	N	N	N	Y	Y	Y	3
Meng 2008 [32]	U	U	Y	Y	N	Y	N	Y	U	Y	2
Qiao 2010 [34]	U	U	/	/	N	N	N	U	Y	Y	2
Shi and Sun 2005 [33]	U	U	/	/	N	/	N	Y	U	Y	2
Sun et al. 2002 [35]	U	U	Y	/	/	N	N	U	U	Y	1
Tang et al. 2010 [36]	U	U	Y	Y	N	/	N	U	Y	Y	2
Tian et al. 2001 [37]	U	U	/	Y	N	/	N	U	Y	Y	2
Tian 2006 [38]	U	U	Y	Y	N	U	N	Y	Y	Y	3
Wang et al. 2002 [39]	U	U	/	Y	N	N	N	U	Y	Y	2
Wang and Cheng 2009 [40]	U	U	Y	Y	N	/	N	Y	Y	Y	3
Wang and Yang 2002 [41]	U	U	Y	Y	/	/	Y	Y	U	Y	3
Wang et al. 2008 [42]	U	U	/	Y	/	/	Y	Y	U	Y	3
Wang et al. 2007 [43]	Y	U	/	Y	N	Y	N	Y	Y	Y	4
Wang 2008 [44]	U	U	/	Y	N	/	N	Y	Y	U	2
Weng et al. 2008 [45]	U	U	Y	Y	N	U	N	Y	Y	Y	3
Wu et al. 2000 [46]	U	U	Y	Y	/	N	N	Y	Y	Y	3
Wu 1999 [47]	U	U	Y	/	/	/	Y	U	U	U	1
Wu et al. 2003 [48]	U	U	Y	Y	/	/	Y	U	U	Y	2
Xu et al. 2006 [49]	N	U	Y	Y	/	/	Y	U	U	U	1
Xu et al. 2007 [50]	U	U	/	Y	N	N	N	Y	Y	Y	3
Xu et al. 2007 [51]	U	U	/	Y	/	N	N	U	U	Y	1

Table 2: Continued.

Study	Sequence generation	Allocation concealment	Blinding: outcomes				Blinding	Incomplete outcome data	Selective outcome data	Other bias	Risk of bias score
			Survival	Tumor response	KPS	AE					
Xue et al. 2002 [52]	U	U	Y	Y	/	Y	Y	Y	Y	Y	4
Yang 2010 [53]	U	U	Y	/	/	/	Y	U	N	Y	2
Yang 2006 [54]	U	U	/	Y	N	N	N	Y	Y	U	2
Yang 2006 [55]	U	U	/	Y	/	Y	Y	U	U	U	1
Yi et al. 2008 [56]	Y	U	/	Y	N	N	N	U	N	Y	2
Yu and Kang 2010 [57]	U	U	/	Y	N	Y	N	U	N	Y	1
Yuan et al. 2010 [58]	U	U	/	Y	N	/	N	Y	Y	Y	3
Yuan and Yu 2005 [59]	U	U	/	Y	/	/	Y	Y	U	U	2
R. Q. Zhai and H. Y. Zhai 2010 [60]	Y	U	Y	Y	N	N	N	Y	Y	Y	4
Zhang et al. 2005 [61]	U	U	Y	Y	/	/	Y	N	U	Y	2
Zhang et al. 2007 [62]	U	U	/	Y	N	N	N	Y	Y	Y	3
Zhang et al. 2008 [63]	Y	U	Y	Y	N	/	N	Y	Y	Y	4
Zhang et al. 2008 [64]	N	U	Y	Y	N	/	N	Y	U	Y	2
Zhang et al. 2007 [65]	Y	U	Y	Y	N	Y	N	Y	Y	Y	4
Zhang 2011 [66]	U	U	/	Y	N	/	N	U	Y	Y	2
Zhang et al. 2000 [67]	U	U	Y	Y	/	/	Y	Y	U	Y	3
Zhao and Huang 2005 [68]	U	U	/	Y	/	/	Y	Y	Y	Y	4
Zhao et al. 2006 [69]	N	U	Y	Y	/	/	Y	U	U	U	1
Zhou et al. 2002 [70]	N	U	Y	/	/	/	Y	Y	Y	U	3
Zhou et al. 2010 [71]	U	U	Y	Y	N	/	N	N	U	Y	1
Zou 2004 [72]	U	U	/	Y	/	N	N	Y	Y	U	2

AE: transarterial chemoembolization toxicity; KPS: Karnofsky performance scale. Y: yes; N: no; U: unclear.

outcome reporting, 5 (7%) at high risk, and 22 could not be rated due to insufficient information. Thirty-nine studies (58%) were rated as at low risk of bias for selective outcome data, 3 (4%) at high risk, and 25 (37%) did not provide sufficient information to permit judgment. Consequently, 29 studies were assigned as high quality with a low risk of bias (Table 2).

3.3. Meta-Analysis of Primary Outcomes

3.3.1. Tumor Response (Short-Term Effectiveness). Fifty-eight RCTs involving 4482 participants reported tumor response as an outcome for testing the short-term effect of CMs plus TACE (combination therapy). The combination therapy was found to be superior to TACE alone in increasing the short term effectiveness (RR = 1.33; 95% CI = 1.25 to 1.41; $P < 0.00001$) (Figure 2). The fixed-effect model was used to combine the data, whereas both X^2 and I^2 test suggested a low risk of heterogeneity ($P = 1.00$; $I^2 = 0\%$).

3.3.2. Survival (Long-Term Effectiveness). Thirty-two trials presenting 3038 participants reported the number of patients surviving for 6 to 60 months. Survival at 48 and 60 months were not evaluated as only 1 study [45] reported the results on this. Significant increases of survival at 6, 12, 18, 24, and 36 months for combination therapy were found with corresponding RRs (95% CI) of 1.12 (1.07 to 1.16), 1.39 (1.31 to 1.48), 1.89 (1.44 to 2.49), 1.75 (1.55 to 1.97), and 2.51 (1.97 to 3.19), all $P < 0.00001$ (Figure 3). The results were homogenous although significant heterogeneity was observed for survival at 18 months ($P = 0.03$; $I^2 = 63\%$). However, similar estimates (RR = 2.52; 95% CI = 1.67 to 3.82; $P < 0.0001$) and homogeneity ($P = 0.66$; $I^2 = 0\%$) were observed in sensitivity analysis by excluding a study with outlier.

3.4. Meta-Analysis of Secondary Outcomes

3.4.1. KPS. KPS was measured in 36 studies for assessing the effect on QoL, in which continuous data was reported in 9 studies ($n = 477$ participants) and dichotomous data (KPS > 10) reported in 27 studies ($n = 2041$ participants). Significant differences in favor of combination therapy were found for continuous outcome of KPS (MD = 9.12; 95% CI = 4.17 to 14.07) (Figure 4). Random-effects model was used as heterogeneity was observed ($P < 0.00001$, $I^2 = 95\%$). Heterogeneity was reduced ($P = 0.03$, $I^2 = 18\%$) after excluding studies with outliers and the significant difference between treatment and control groups was robust (RR = 3.94; 95% CI = 2.30 to 5.59; $P < 0.00001$).

Study or subgroup	Treatment Events	Total	Control Events	Total	Weight	Risk ratio M-H, fixed, 95% CI
Ayi 2011	43	54	34	54	3.9%	1.26 [0.99, 1.62]
Bao 2007	17	28	8	26	0.9%	1.97 [1.03, 3.78]
Chen 2007	18	32	10	28	1.2%	1.57 [0.88, 2.82]
Dan 2007	10	35	13	35	1.5%	0.77 [0.39, 1.52]
Deng 2009	2	20	1	20	0.1%	2.00 [0.20, 20.33]
Dong 2007	19	33	13	32	1.5%	1.42 [0.85, 2.36]
Dong 2008	42	67	34	66	3.9%	1.22 [0.90, 1.64]
Feng 2002	5	35	4	33	0.5%	1.18 [0.35, 4.02]
Guo 2005	10	33	8	33	0.9%	1.25 [0.56, 2.77]
Hou 2009	18	36	12	36	1.4%	1.50 [0.85, 2.64]
Huang 2002	17	30	9	27	1.1%	1.70 [0.92, 3.16]
Huang 2008	21	30	7	20	1.0%	2.00 [1.05, 3.80]
Jia 2003	19	34	10	32	1.2%	1.79 [0.99, 3.24]
Li 2009	12	32	8	32	0.9%	1.50 [0.71, 3.17]
Li 2007	8	20	6	16	0.8%	1.07 [0.47, 2.45]
Li 2008	27	64	19	64	2.2%	1.42 [0.88, 2.28]
Liang J. X. 2005	13	35	6	33	0.7%	2.04 [0.88, 4.74]
Liang 2008	49	64	35	57	4.2%	1.25 [0.97, 1.60]
Liang T. J. 2005	54	75	38	71	4.4%	1.35 [1.04, 1.74]
Ling 2010	28	64	24	64	2.7%	1.17 [0.77, 1.78]
Liu 2007	16	34	16	36	1.8%	1.06 [0.64, 1.76]
Lu 2009	17	24	14	24	1.6%	1.21 [0.79, 1.86]
Lu 2007	18	33	11	30	1.3%	1.49 [0.85, 2.61]
Meng 2008	47	75	37	73	4.3%	1.24 [0.93, 1.65]
Tang 2010	16	30	4	20	0.5%	2.67 [1.04, 6.81]
Tian 2001	8	23	6	20	0.7%	1.16 [0.48, 2.77]
Tian 2006	16	36	12	36	1.4%	1.33 [0.74, 2.40]
Wang C. J. 2002	25	47	21	48	2.4%	1.22 [0.80, 1.85]
Wang 2009	21	27	19	30	2.0%	1.23 [0.88, 1.72]
Wang R. P. 2002	6	30	5	30	0.6%	1.20 [0.41, 3.51]
Wang R. P. 2008	5	43	3	43	0.3%	1.67 [0.42, 6.54]
Wang 2007	10	21	11	22	1.2%	0.95 [0.52, 1.76]
Wang Z. X. 2008	18	30	8	29	0.9%	2.17 [1.13, 4.20]
Weng 2008	28	55	16	41	2.1%	1.30 [0.82, 2.07]
Wu 2000	17	36	9	44	0.9%	2.31 [1.17, 4.54]
Wu 2003	19	30	15	30	1.7%	1.27 [0.81, 1.99]
Xu 2006	17	30	9	27	1.1%	1.70 [0.92, 3.16]
Xu 2007a	20	32	10	20	1.4%	1.25 [0.75, 2.09]
Xu 2007b	22	40	10	20	1.5%	1.10 [0.65, 1.85]
Xue 2002	27	34	19	36	2.1%	1.50 [1.06, 2.14]
Yang J. M. 2006	16	31	14	31	1.6%	1.14 [0.68, 1.92]
Yang X. L. 2006	12	28	5	22	0.6%	1.89 [0.78, 4.55]
Yi 2008	16	36	13	31	1.6%	1.06 [0.61, 1.84]
Yu 2010	37	48	28	48	3.2%	1.32 [0.99, 1.76]
Yuan 2010	11	31	9	31	1.0%	1.22 [0.59, 2.53]
Yuan 2005	18	35	13	38	1.4%	1.50 [0.87, 2.60]
Zhai 2010	18	32	10	30	1.2%	1.69 [0.93, 3.05]
Zhang 2005	73	116	53	108	6.2%	1.28 [1.01, 1.63]
Zhang H. 2007	11	30	11	30	1.2%	1.00 [0.51, 1.94]
Zhang H. T. 2008	22	31	16	30	1.8%	1.33 [0.89, 1.99]
Zhang J. M. 2008	11	31	11	33	1.2%	1.06 [0.54, 2.09]
Zhang Q. 2007	32	58	27	54	3.2%	1.10 [0.77, 1.57]
Zhang X. Y. 2011	8	25	6	24	0.7%	1.28 [0.52, 3.14]
Zhang 2000	30	50	25	45	3.0%	1.08 [0.76, 1.53]
Zhao 2005	24	30	14	30	1.6%	1.71 [1.12, 2.62]
Zhao 2006	26	48	21	46	2.4%	1.19 [0.79, 1.79]
Zhou 2010	29	32	21	32	2.4%	1.38 [1.05, 1.82]
Zou 004	13	25	10	25	1.1%	1.30 [0.71, 2.39]
Total (95% CI)		**2248**		**2126**	**100.0%**	**1.33 [1.25, 1.41]**
Total events	1212		861			

Heterogeneity: $\chi^2 = 29.83$, df = 57 ($P = 1.00$); $I^2 = 0\%$

Test for overall effect: $Z = 9.01$ ($P < 0.00001$)

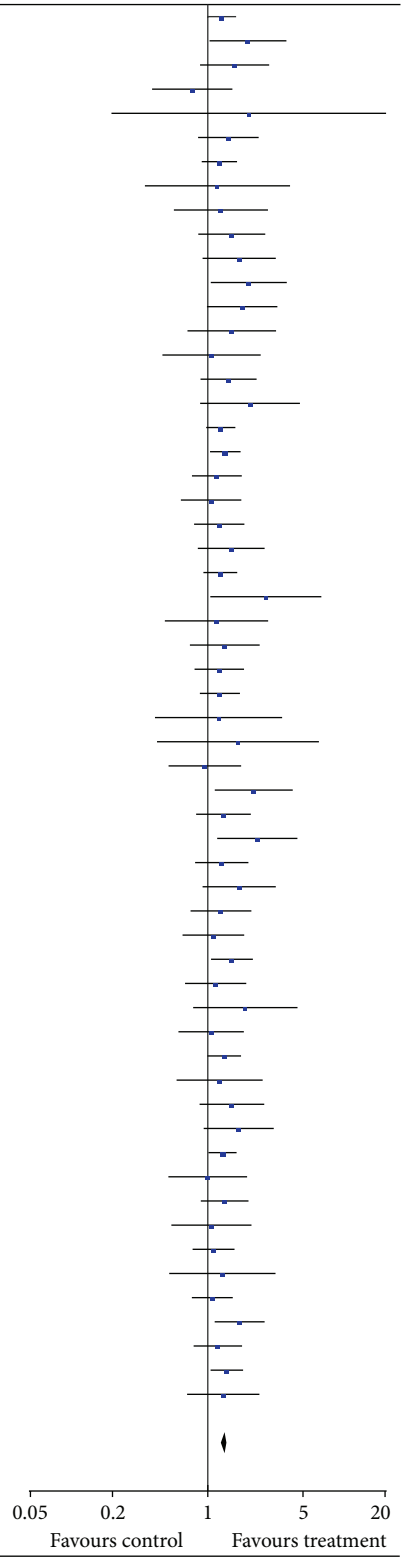

Figure 2: Results of Forest plots of comparison of CMs plus TACE versus TACE alone on tumor response (complete response + partial response) for HCC patients at middle and late stages. M-H: Mantel-Haenszel estimates; CI: confidence interval; CMs: Chinese medicines; TACE: transcatheter arterial chemoembolization.

Study or subgroup	Treatment Events	Treatment Total	Control Events	Control Total	Weight	Risk ratio M-H, fixed, 95% CI
3.6.1 6 months						
Ayi 2011	48	54	45	54	2.4%	1.07 [0.92, 1.24]
Cao 2005	50	50	42	50	2.3%	1.19 [1.05, 1.35]
Dan 2007	29	35	24	35	1.3%	1.21 [0.92, 1.58]
Dong 2008	41	67	35	66	1.9%	1.15 [0.86, 1.55]
Li 2008	48	64	38	64	2.0%	1.26 [0.99, 1.62]
Liang 2008	44	64	37	57	2.1%	1.06 [0.82, 1.36]
Liang T. J. 2005	67	75	59	71	3.2%	1.08 [0.94, 1.23]
Lu 2010	27	30	22	30	1.2%	1.23 [0.96, 1.57]
Meng 2008	46	75	37	73	2.0%	1.21 [0.91, 1.62]
Tang 2010	28	30	15	20	1.0%	1.24 [0.95, 1.63]
Tian 2006	33	36	29	36	1.6%	1.14 [0.94, 1.37]
Wang R. P. 2002	22	30	19	30	1.0%	1.16 [0.82, 1.64]
Weng 2008	51	55	37	41	2.3%	1.03 [0.91, 1.16]
Wu 2000	30	36	32	44	1.5%	1.15 [0.91, 1.45]
Wu 2003	22	30	19	30	1.0%	1.16 [0.82, 1.64]
Zhai 2010	26	32	22	30	1.2%	1.11 [0.84, 1.46]
Zhang 2005	104	116	90	108	5.0%	1.08 [0.97, 1.19]
Zhang H. T. 2008	30	31	29	30	1.6%	1.00 [0.91, 1.10]
Zhang J. M. 2008	26	31	21	33	1.1%	1.32 [0.98, 1.78]
Zhang Q. 2007	48	58	40	54	2.2%	1.12 [0.92, 1.36]
Zhang 2000	42	50	35	45	2.0%	1.08 [0.89, 1.32]
Zhou 2002	94	118	86	110	4.8%	1.02 [0.89, 1.17]
Subtotal (95% CI)		**1167**		**1111**	**44.6%**	1.12 [1.07, 1.16]
Total events	956		813			
Heterogeneity: $\chi^2 = 15.27$, df = 21 ($P = 0.81$); $I^2 = 0\%$						
Test for overall effect: $Z = 4.97$ ($P < 0.00001$)						
3.6.2 12 months						
Ayi 2011	34	54	25	54	1.3%	1.36 [0.96, 1.94]
Cao 2005	42	50	30	50	1.6%	1.40 [1.08, 1.81]
Dan 2007	18	35	8	35	0.4%	2.25 [1.13, 4.48]
Dong 2008	11	67	10	66	0.5%	1.08 [0.49, 2.38]
Huang 2002	24	30	14	27	0.8%	1.54 [1.03, 2.31]
Huang 2008	27	30	11	20	0.7%	1.64 [1.08, 2.48]
Li 2008	38	64	29	64	1.6%	1.31 [0.94, 1.84]
Liang 2008	36	64	20	57	1.1%	1.60 [1.06, 2.43]
Liang T. J. 2005	57	75	43	71	2.4%	1.25 [1.00, 1.57]
Lu 2010	25	30	17	30	0.9%	1.47 [1.03, 2.09]
Meng 2008	12	75	11	73	0.6%	1.06 [0.50, 2.25]
Sun 2002	94	118	56	118	3.0%	1.68 [1.36, 2.07]
Tang 2010	26	30	9	20	0.6%	1.93 [1.16, 3.19]
Tian 2006	24	36	18	36	1.0%	1.33 [0.89, 1.99]
Wang 2009	18	27	16	30	0.8%	1.25 [0.81, 1.92]
Wang R. P. 2002	11	30	4	30	0.2%	2.75 [0.99, 7.68]
Weng 2008	33	55	22	41	1.3%	1.12 [0.78, 1.60]
Wu 2000	24	36	18	44	0.9%	1.63 [1.07, 2.49]
Wu 2003	19	30	16	30	0.9%	1.19 [0.77, 1.83]
Xu 2006	24	30	14	27	0.8%	1.54 [1.03, 2.31]
Xue 2002	20	34	16	36	0.8%	1.32 [0.83, 2.10]
Zhai 2010	22	32	13	30	0.7%	1.59 [0.99, 2.54]
Zhang 2005	90	116	70	108	3.9%	1.20 [1.01, 1.42]
Zhang H. T. 2008	28	31	22	30	1.2%	1.23 [0.96, 1.57]
Zhang J. M. 2008	17	31	7	33	0.4%	2.59 [1.24, 5.37]
Zhang Q. 2007	40	58	26	54	1.4%	1.43 [1.03, 1.98]
Zhang 2000	31	50	18	45	1.0%	1.55 [1.02, 2.36]
Zhao 2006	25	48	22	46	1.2%	1.09 [0.73, 1.63]
Zhou 2002	63	118	42	110	2.3%	1.40 [1.04, 1.87]
Zhou 2010	31	32	25	32	1.3%	1.24 [1.02, 1.50]
Subtotal (95% CI)		**1516**		**1447**	**35.7%**	1.40 [1.32, 1.50]
Total events	964		652			
Heterogeneity: $\chi^2 = 24.97$, df = 29 ($P = 0.68$); $I^2 = 0\%$						
Test for overall effect: $Z = 10.24$ ($P < 0.00001$)						
3.6.3 18 months						
Dan 2007	12	35	4	35	0.2%	3.00 [1.07, 8.40]
Lu 2010	21	30	11	30	0.6%	1.91 [1.13, 3.23]
Tian 2006	12	36	4	36	0.2%	3.00 [1.07, 8.43]
Zhang H. T. 2008	25	31	20	30	1.1%	1.21 [0.89, 1.64]
Zhang J. M. 2008	10	31	3	33	0.2%	3.55 [1.08, 11.70]
Subtotal (95% CI)		**163**		**164**	**2.3%**	1.89 [1.44, 2.49]
Total events	80		42			
Heterogeneity: $\chi^2 = 10.80$, df = 4 ($P = 0.03$); $I^2 = 63\%$						
Test for overall effect: $Z = 4.54$ ($P < 0.00001$)						
3.6.4 24 months						
Ayi 2011	15	54	9	54	0.5%	1.67 [0.80, 3.48]
Cao 2005	35	50	24	50	1.3%	1.46 [1.04, 2.05]
Dong 2008	8	67	8	66	0.4%	0.99 [0.39, 2.47]
Li 2008	23	64	18	64	1.0%	1.28 [0.77, 2.13]
Liang T. J. 2005	38	75	24	71	1.3%	1.50 [1.01, 2.22]
Meng 2008	9	75	8	73	0.4%	1.09 [0.45, 2.68]
Sun 2002	80	118	38	118	2.0%	2.11 [1.58, 2.81]
Tang 2010	17	30	3	20	0.2%	3.78 [1.27, 11.23]
Tian 2006	5	36	0	36	0.0%	11.00 [0.63, 191.88]
Wang 2009	13	27	11	30	0.6%	1.31 [0.71, 2.42]
Weng 2008	21	55	5	41	0.3%	3.13 [1.29, 7.61]
Xue 2002	13	34	6	36	0.3%	2.29 [0.98, 5.35]
Zhai 2010	14	32	6	30	0.3%	2.19 [0.97, 4.95]
Zhang 2005	55	116	37	108	2.0%	1.38 [1.00, 1.91]
Zhang H. T. 2008	21	31	17	30	0.9%	1.20 [0.80, 1.78]
Zhang Q. 2007	30	58	17	54	0.9%	1.64 [1.03, 2.62]
Zhang 2000	4	50	0	45	0.0%	8.12 [0.45, 146.71]
Zhao 2006	11	48	4	46	0.2%	2.64 [0.90, 7.69]
Zhou 2002	42	118	12	110	0.7%	3.26 [1.81, 5.87]
Subtotal (95% CI)		**1138**		**1082**	**13.5%**	1.75 [1.55, 1.99]
Total events	454		247			
Heterogeneity: $\chi^2 = 25.65$, df = 18 ($P = 0.11$); $I^2 = 30\%$						
Test for overall effect: $Z = 8.83$ ($P < 0.00001$)						

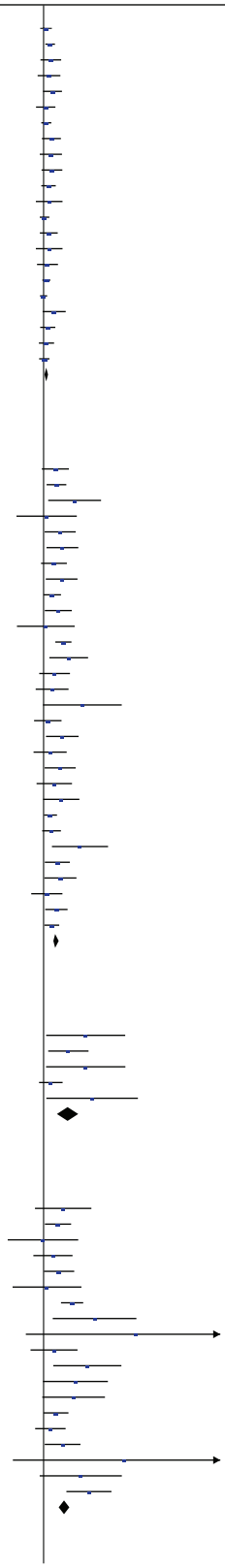

Figure 3: Continued.

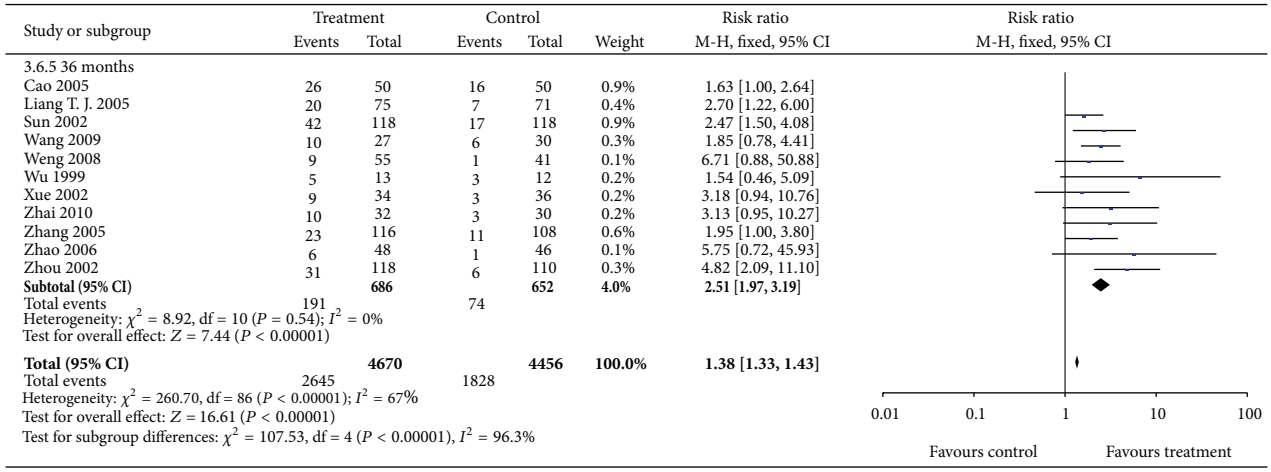

Figure 3: Results of Forest plots of comparison of CMs plus TACE versus TACE alone on 6-month, 18-month, 24-month, and 36-month survival for HCC patients at middle and late stages. M-H: Mantel-Haenszel estimates; CI: confidence interval; CMs: Chinese medicines; TACE: transcatheter arterial chemoembolization.

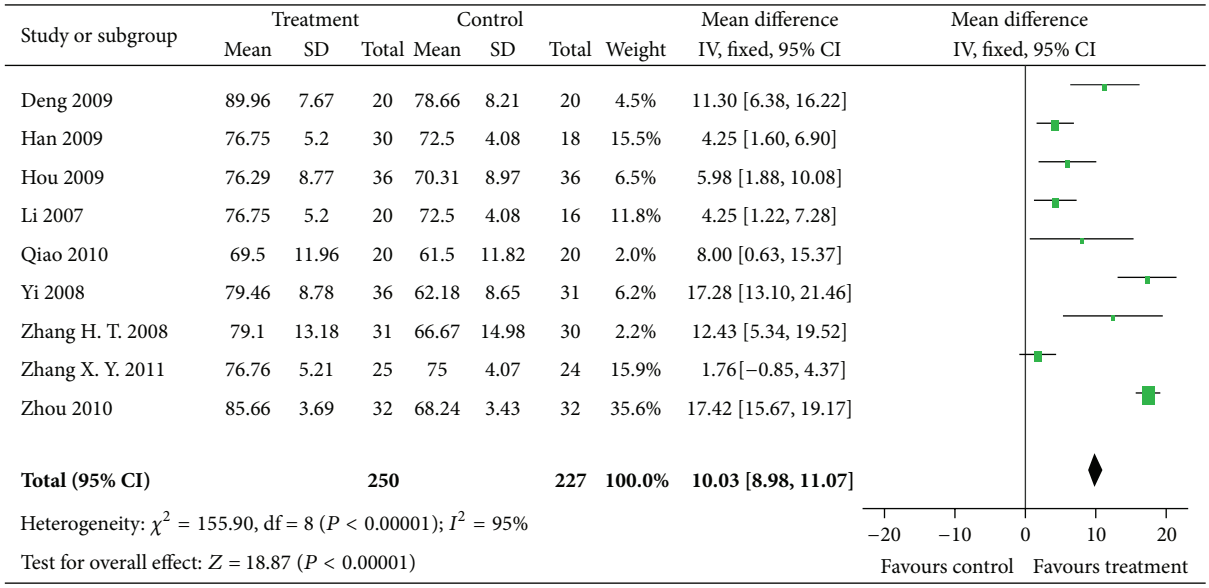

Figure 4: Results of Forest plots of comparison of CMs plus TACE versus TACE alone on Karnofsky score (continuous data) for HCC patients at middle and late stages. M-H: Mantel-Haenszel estimates; CI: confidence interval; CMs: Chinese medicines; TACE: transcatheter arterial chemoembolization.

KPS >10 indicated that the results of KPS increased more than 10 points after treatment. A superior effect on the improvement of QoL in combination therapy compared with TACE alone was observed (RR = 1.74; 95% CI = 1.57 to 1.93; P < 0.00001) (Figure 5). As the result was homogenous (P = 0.83; I^2 = 0%), fixed-effect model was used.

3.4.2. Reduction in TACE Toxicity (Short-Term Effectiveness). Results of fixed-effect model in 12 studies showed that TACE toxicity including nausea and vomiting, alanine transaminase (ALT) elevation, and bone marrow depression were significantly reduced in treatment groups compared with TACE alone with corresponding RRs (95% CI) of 0.86 (0.76 to 0.96), 0.61 (0.04 to 0.93), and 0.71 (0.58 to 0.86) (Figure 6). Heterogeneity was not observed in the analysis (P = 0.1, 0.47, 0.85; I^2 = 40%, 0%, 0%; resp.). No chronic adverse reaction was reported in the studies.

3.4.3. CMs-Related Side Effects. CMs-related side effects were rarely reported. Only 3 studies (4%) [20, 60, 74] reported low-grade fever (2 cases), dizziness (1 case), gastrointestinal discomfort (28 cases), and mild skin itch and rashes (3 cases). These symptoms were generally alleviated or recovered after symptomatic treatment. No severe side effects associated with CMs were reported in the included trials. The long-term side effects of the treatment were uncertain as only short-term effects were measured.

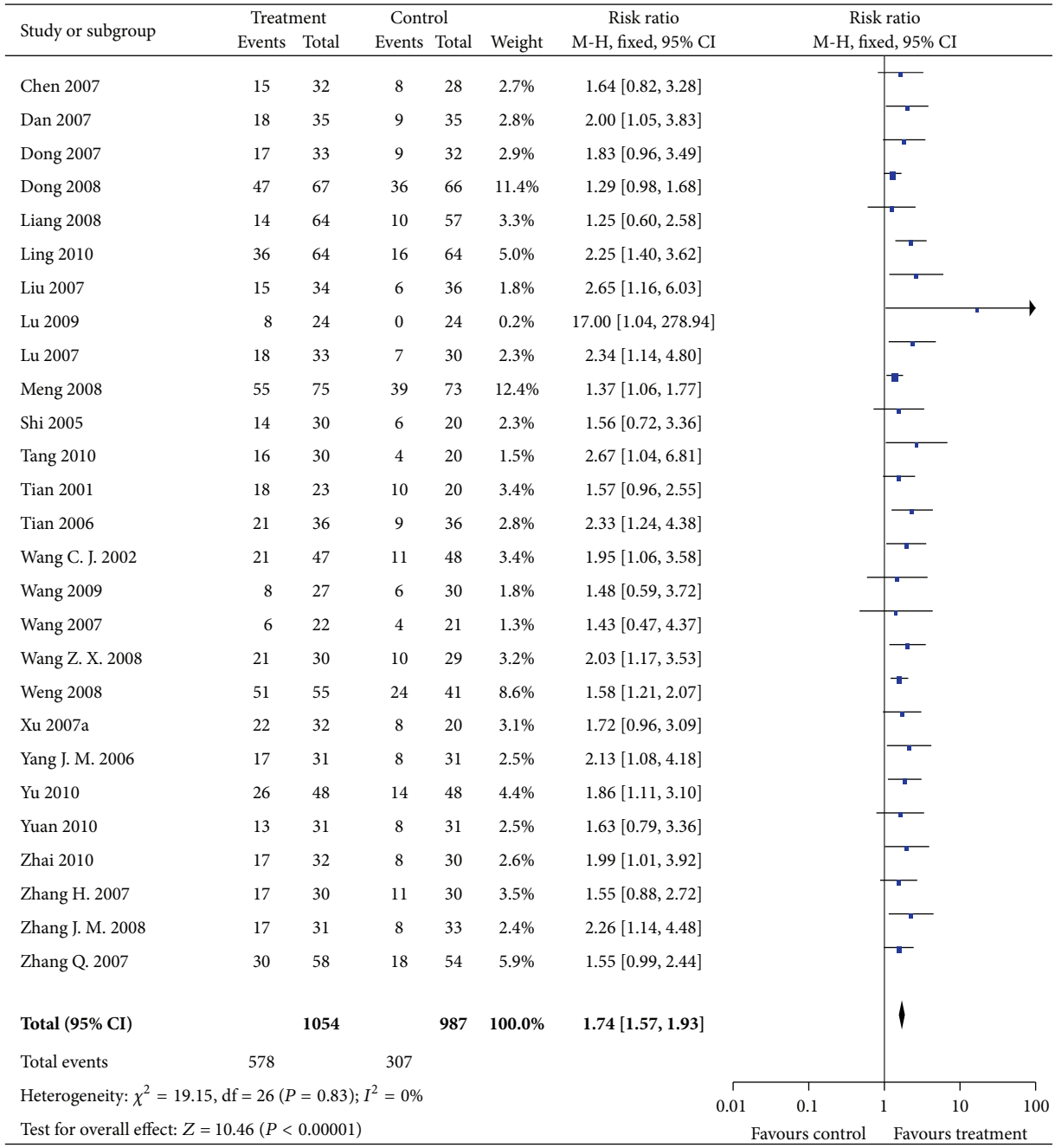

Figure 5: Results of Forest plots of comparison of CMs plus TACE alone versus TACE on KPS increased >10 points for HCC patients at middle and late stages. M-H: Mantel-Haenszel estimates; CI: confidence interval; CMs: Chinese medicines; TACE: transcatheter arterial chemoembolization; KPS: karnofsky performance score.

3.5. Risk of Bias across Studies. Risk of publication bias was assessed using funnel plot to compare symmetry for all studies except for one with outliers (Figure 7). Results of Egger's test suggested no significant publication bias of the included studies (t = 1.99, P = 0.051).

3.6. Common Herbs. The top 10 most frequently used herbs in the included trials were listed in Table 3 together with the potential pharmacological properties. Although the constituents of the formulae were varied across the trials, there was a general consensus in diagnosis based on unique Chinese medicine theory. Reinforcing healthy Qi and blood, clearing fire toxin, and resolving dampness were the most concerned therapeutic principles which were associated with improvement in short-term and long-term effectiveness. Radix Astragali (Huang Qi) (n = 35) was the most frequently used herb in the trials, followed by Poria Cocos, Rhizoma Atractylodis Macrocephalae, Radix Ginseng, Radix Bupleuri, Radix Codonopsis, Semen Coicis, Herba Oldenlandia Diffusa, Radix Paeoniae Alba, and Rhizoma Curcumae. These 10

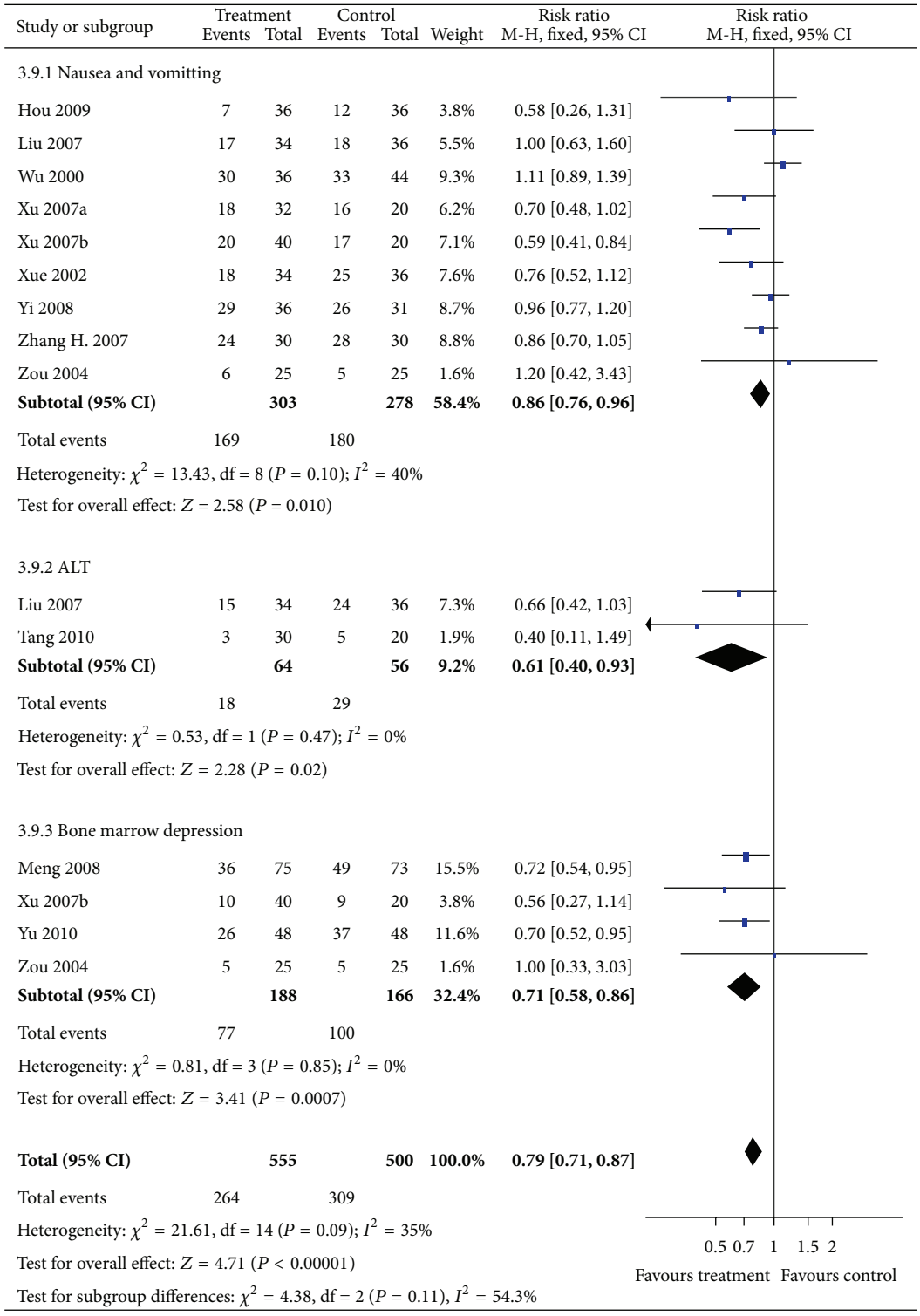

Figure 6: Results of Forest plots of comparison of CMs plus TACE versus TACE alone on TACE toxicity (grade 1–4) for HCC patients at middle and late stages. M-H: Mantel-Haenszel estimates; CI: confidence interval; CMs: Chinese medicines; TACE: transcatheter arterial chemoembolization.

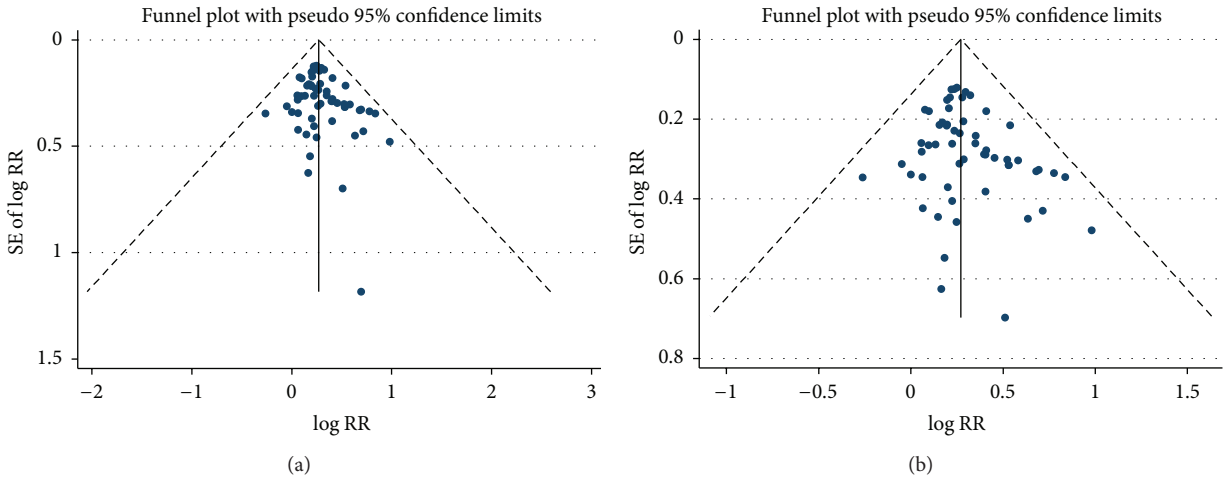

Figure 7: Funnel plots of (a) tumor response of the included studies and (b) tumor response of the included studies excluding a study with outlier results.

herbs are worthy of additional investigation to examine the possible active components for the use in HCC treatment.

4. Discussion

TACE is one of the few therapeutic treatments for unresectable HCC patients. Although CMs are increasingly used to enhance the treatment effects of TACE and reduce the side effects, the effectiveness is uncertain as the updated evidence has not been systematically summarized.

This is the first PRISMA-compliant systematic review and meta-analysis for examining the efficacy of CMs plus TACE (combination therapy) in treating unresectable HCC patients. Sixty-seven studies involving 5,211 participants met the study selection criteria. The meta-analysis showed that the combination therapy was significantly better than TACE alone in increasing tumor response, prolonging survival, improving QoL, and reducing TACE toxicity. CM-related side effects were mild and rarely reported in the studies. The above findings suggested that CMs might play a potentially beneficial role in assisting TACE therapy to improve tumor response, survival, and QoL, as well as reduction in TACE toxicity.

The results are robust as the analyses were based on 67 RCTs with a large sample size of more than 5000 subjects, and both short-term (tumor response, QoL, and TACE toxicity reduction) and long-term (survival) effectiveness were assessed. Although most of the included studies (n = 66) were published in Chinese, the trials in this review represent the best available evidence on the efficacy of CMs as an adjuvant therapy for unresectable HCC patients during TACE treatment. Moreover, this review was conducted using comprehensive, rigorous, and PRISMA-compliant methods. An extensive search was conducted for RCTs published before October 2012. Between-study heterogeneity was further assessed by sensitivity analysis and random-effects model. Publication bias was investigated by both visual funnel plots and Egger's test.

Considerable variety in the ingredients of the CM formulae was found in this review which might be due to different TCM diagnosis and the CM practitioners' personal experience. However, a common consensus in TCM diagnosis and treatment principle was observed among the included trials. According to our review, CM herbs might enhance the tumor response by inhibiting tumor angiogenesis and cancer cell proliferation, inducing apoptosis, and increasing immune response (Table 3). The enhancement of tumor response may contribute to the improvement of survival. Moreover, CMs may reduce the acute and subacute adverse reactions induced by TACE, thus improve the QoL. Further investigation on the therapeutic mechanism, pharmacokinetics, pharmacodynamics and their possible active components of the frequently used herbs could bring new insight into the treatment of HCC.

4.1. Limitations. Although extensive searches and strict methods were used to select studies and estimate the effects, there are several potential limitations. First, only studies published in English or Chinese were included, and studies published in other languages cannot be assessed. Second, as most studies were conducted among Chinese, the generalizability to other population needed to be further assessed. Third, clinical heterogeneity may be detected as CM preparations, dose, and treatment duration are varied across the included studies. Further studies are warranted to investigate the effects of different CM preparations in treating middle or late stage HCC. Fourth, sample size, selection criteria of subjects, and TACE drugs varied across the included studies, and the heterogeneity was not reflected in the data analyses. Fifth, most of the studies did not report the method of randomization, and all studies failed to report the method of allocation concealment and blinding (subjective outcomes), which might lead to the potential selection bias. Moreover, reasons for dropouts and withdrawals were mostly not described. Overall, these items were mostly at unclear or high risk of bias which could bias the findings of this review resulting in overestimation of the CMs beneficial effects.

Table 3: The top 10 most frequently used CMs of the included studies.

CM herb Latin name (Chinese Pinyin)	No.	TCM diagnosis	Pharmacological properties
Radix Astragali (Huang Qi)	35	Qi deficiency	(1) Suppresses the oncogenic transformation of cancer cells [86] (2) Induces apoptosis [87] (3) Induces macrophage, LAK and NK cell activity [88, 89] (4) Inhibits T-helper cell type 2 cytokines [89]
Poria Cocos (Fu Ling)	25	Dampness accumulation	(1) Induces apoptosis [90, 91] (2) Cytotoxicity against cancer cell lines [90] (3) Inhibits tumor angiogenesis [92]
Rhizoma Atractylodis Macrocephalae (Bai Zhu)	23	Qi deficiency	(1) Induces apoptosis [93, 94]
Radix Ginseng (Ren Shen)	19	Qi deficiency	(1) Induces apoptosis [95, 96] (2) Inhibits tumor cell proliferation [96] (3) Cytotoxicity against cancer cell lines [97, 98] (4) Inhibits tumor angiogenesis [99]
Radix Bupleuri (Chai Hu)	19	Qi stagnation	(1) Induces apoptosis [100] (2) Activates macrophages, NK and LAK cells [101] (3) Downregulates TNF-α, IL-6, and NF-κB p65 expression [102]
Radix Codonopsis (Dang Shen)	18	Qi deficiency	(1) Inhibits cancer cells invasion and migration [103] (2) Enhances T cell, B cell, and macrophage production, and activates macrophages [104]
Semen Coicis (Yi Yi Ren)	15	Dampness accumulation	(1) Induces apoptosis [105] (2) Inhibits NF-κB signaling and protein kinase C activity [106] (3) Stimulates T cell proliferation [107]
Herba Oldenlandia Diffusa (Bai Hua She She Cao)	14	Fire toxin	(1) Inhibits cancer cell proliferation and induces apoptosis [108, 109]
Radix Paeoniae Alba (Bai Shao)	13	Blood deficiency	(1) Inhibits angiogenesis and induces apoptosis [110, 111]
Rhizoma Curcumae (E Zhu)	12	Blood stagnation	(1) Inhibits cancer cell proliferation and angiogenesis, induces cell cycle arrest and apoptosis [112] (2) Inhibits platelet aggregation [113]

CM: Chinese medicine; LAK: lymphokine activated killer; NF-κB: nuclear factor kappa-light-chain enhancer of activated B cells; NK: natural killer; No.: number of studies; TCM: traditional Chinese medicine; TNF-α: tumor necrosis factor-alpha; IL: interleukin.

4.2. Implications for Practice and Research. As most included studies have poor quality, future trials should be rigorously implemented using standard procedures following a standardized trial protocol (e.g., consolidated standards of reporting trials statement) [117, 118]. Another crucial issue is the quality control of CM preparations which consist of various CMs from different batches. As different properties of CMs might exist in different batches in the same CM formula, the quality control of further CM preparations should be established based on scientific practice including chemical and bioresponse fingerprint to ensure the quality and consistency of CM preparations and the validity of study results [119, 120]. In addition, CMs should be provided by a consistent and reliable supply to maintain the effective treatment effect of CM preparations. Given that oral administration and intravenous injection of CMs were used by all included studies, further reviews should compare the effects between these routes. Moreover, as no data on the possible interaction between TACE and CMs preparations was reported, the interaction should be assessed further. Only a small number of studies (33%) showed that the results of at least 12-month survival (the long-term effectiveness) of CMs treatment need to be determined in more RCTs with long-term follow-up. Acute and subacute CMs-related side effects in the studies were slight and alleviated spontaneously after symptomatic treatment. However, these were only reported in few studies (4%). Only short-term CMs-related side effects were reported. And all the side effects were not measured by standard criteria. Additional researches should evaluate both acute and chronic CMs-related side effects according to standard criteria to confirm the safety of CMs treatment in treating patients with HCC.

5. Conclusion

The positive results in this meta-analysis show that CMs treatment appears to increase the efficacy of TACE by prolonging survival, increasing tumor response, improving QoL, and reducing TACE toxicity for unresectable HCC. Although making a definitive recommendation is currently premature with low quality of the most studies, these findings suggest that CMs could be considered as an adjuvant therapy for unresectable HCC patients during TACE treatment. RCTs with rigorous methods, long-term follow-up, and standard

reporting (consolidated standards of reporting trials statement) are recommended to further evaluate the clinical effects of combining CMs and TACE use for HCC patients [117, 118].

Abbreviations

ALT: Alanine transaminase
CBM: Chinese biomedical CD database
CIs: Confidence intervals
CMs: Chinese medicines
CMCC: Chinese medical current contents
CNKI: China network knowledge infrastructure
CR: Complete response
HCC: Hepatocellular carcinoma
KPS: Karnofsky performance scale
MD: Mean difference
PR: Partial response
PRISMA: Preferred reporting items for systematic reviews and meta-analyses
QoL: Quality of life
RCT: Randomized controlled trial
RRs: Risk ratios
TACE: Transarterial chemoembolization
CM: Chinese medicine
CMs: Chinese medicines
WHO: World health organization.

Conflict of Interests

All the authors declare no conflict of interests.

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Research Article

Distributions of Usage and the Costs of Conventional Medicine and Traditional Chinese Medicine for Lung Cancer Patients in Taiwan

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Background. This study aims to analyze the utilization patterns of patients with lung cancer stratified by surgery status. *Methods*. A retrospective cohort study was conducted from 1996 to 2010 by using the Longitudinal Health Insurance Database 2005. *Results*. Among the 7,677 lung cancer patients, 230 (31.17%) and 1,826 (26.32%) who have and have not undergone surgery have used TCM outpatient services, respectively. For lung cancer patients who have not undergone surgery, patients who are aged 70 years and older, males, occupational members, and farmers and fishermen are less likely to avail of TCM services. For lung cancer patients who have undergone surgery, the likelihood of TCM users is higher in residents who used TCM one year prior to lung cancer diagnosis and in patients with insurance amounts ranging from ≥NT$60,000. The total amount paid per visit for WM is higher than that for one year of TCM outpatient care before and after lung cancer diagnosis. *Conclusion*. The factors associated with TCM use varied according to surgery status. The costs of insurance covering TCM were consistently lower than those covering WM for lung cancer patients. These findings would be useful for health policy makers who are considering TCM and WM integration.

1. Introduction

1.1. Incidence and Mortality of Lung Cancer. Lung cancer is the leading cause of cancer-related mortality worldwide. Northern America, Eastern Asia, and Western Europe have the highest incidence of lung cancer, whereas the Eastern, Western, and Middle Africa have the lowest incidence of lung cancer. In particular, Hungary, French Polynesia, United States of America, Poland, and Serbia have the highest incidence rates of lung cancer in the world; their age-standardized incidence rates in 2008 were 52.0, 43.6, 42.1, 40.9, and 40.7 per 100,000 persons, respectively [1]. About 55% of lung cancer cases occur in less developed countries. Lung cancer accounts for more than 28% of the total deaths in Taiwan [2]. Lung cancer also ranks first as the most common cancer in both men and women, accounting for about 20% of all cancer deaths. In addition, the annual standardized mortality incidence increased from 22.7/100,000 in 1991 to 25.9/100,000 in 2009 [2]. Lung cancer accounts for more than 20% of all cancer deaths in Hong Kong and ranks first as the most common cancer in the country [3].

1.2. Use of Complementary and Alternative Medicine (CAM) Worldwide. The use of CAM has gained worldwide popularity. According to a survey across a number of European countries, the top three motives for using CAM in cancer patients include directly fighting the disease with alternative therapy for decreasing the tumor, increasing the ability of

the body to fight the cancer, and improving the physical well-being of the patient [4]. CAM, which is commonly used with conventional medicine for cancer care, is covered by the National Health Insurance (NHI) Program of Taiwan [5–8]. According to the 2007 National Health Interview Survey, the prevalence of CAM use was 42% in Asian Americans and 38% in American adults [9]. The percentage of CAM use was 23.6% for lung cancer patients, which is slightly higher than that for head and neck cancer (22.7%), but lower than that for pancreatic cancer patients (56.3%) [4].

1.3. Use of Traditional Chinese Medicine (TCM) in Asian Countries. TCM is one of the most popular forms of CAM worldwide. Cancer patients use TCM because they believe that TCM is a self-help cultural process and because they believe that conventional therapy has adverse effects and individualized and tailored prescriptions are important [10]. TCM is commonly used with conventional medicine and has entered the mainstream society and culture. TCM is even covered by the NHI program of Taiwan. In addition, TCM plays an active role in the modern health care system of Chinese and East Asian societies. One important feature of the NHI program is the coverage of both biomedicine (WM) and TCM. By 2003, more than 99% of the 23 million residents of Taiwan had been covered by the NHI program after the implementation of this universal health insurance plan. Thus, TCM has a higher level of accessibility because of less financial barrier. A study on the determinants of TCM and acupuncture utilization of patients with cervical, breast, lung, liver, or colorectal cancers in Taiwan has shown that the prevalence of TCM and acupuncture use for lung cancer patients is 16.03% and 2.53%, respectively [11].

1.4. Therapeutic Effect of TCM against Non-Small-Cell Lung Cancer (NSCLC) in Previous Studies. TCM is commonly used in combination with chemotherapy or radiotherapy in treating patients with unresectable NSCLC [12–16]. The effect of treatment with TCM has been focused on the stimulation of the host immune response, thus activating a cytotoxic response against NSCLC. A systematic review including publications in 11 electronic databases and 24 trials has evaluated the efficacy of Chinese herbal medicine (CHM) combined with conventional chemotherapy (CT) in treatment of advance NSCLC [17]. Their findings indicated that the most five commonly used herbs were *Radix Adenophorae, Radix Ophiopogonis, Radix Glycyrrhizae,* and *Poria*. In addition, CHM as an adjuvant therapy can reduce CT toxicity in terms of reduction of nausea and vomiting, decrease of hemoglobin, inhibition of white blood cells, and decrease of platelet, prolong one-year survival rate, enhance immediate tumor response, and improve Karnofsky performance score in advanced NSCLC patients [17]. Another systematic review on oral CHM trials involving 862 patients with NSCLC showed that TCM plus chemotherapy improves the quality of life, tumor response, and survival rate of patients, as well as alleviate the symptoms experienced by patients [12]. The systematic review also demonstrated that aidi injection, a type of TCM, combined with cobalt-60 or navelbine and platinol has adjuvant therapeutic effects in improving the response rate, bone marrow hematopoietic function, and quality of life but not the survival rate of patients [13]. In a randomized control, TCM by stages plus chemotherapy significantly increases the survival and quality of life of patients with advanced NSCLC [14]. *Astragalus mongholicus, Gynostemma pentaphyllum, Ganoderma lucidum, Ligustrum lucidum, Atractylodes, Coptis chinensis,* and *Coptis chinensis* are the most commonly used ingredients that have demonstrated oncologic and immunologic pharmacology for lung cancer [14, 15]. These ingredients either inhibit lung adenocarcinoma cell migration and invasion or have an anticancer effect by inhibiting human cancer cell growth and inducing apoptosis [14–16]. The possible anticancer mechanisms of these ingredients include cell cycle arrest following apoptotic death by numerous and competing degenerative pathways [15].

1.5. How the Current Study Helps Resolve Uncertainties regarding TCM Use. Several studies have explored the prevalence of TCM use. However, these studies have been conducted either in general populations [18–23], in a single clinical setting [24], or for acupuncture use only [25]. Two studies have explored TCM use in Taiwan in 1996–2001 [26] and in 1997–2003 [27] in a general population by using complete NHI datasets for TCM. Two recent studies have explored TCM use among patients with prostate [28] and liver cancers [5]. However, TCM use among patients with lung cancer has not yet been reported and the impact of surgery on TCM use has not yet been explored. This study aims to compare the differences between the characteristics, types of care provider, existing diseases, and expenditures for outpatient services of TCM and non-TCM users with lung cancer enrolled in the NHI program. This study, which is stratified by the surgery status of lung cancer patients in Taiwan from 1996 to 2010, utilized a population-based random sample of one million insured patients.

2. Methods

2.1. Data Sources. This study used NHI Research Database (NHIRD) claim datasets from the NHI program of Taiwan. The NHI program was initiated in Taiwan in March 1995 and covers approximately 99% of the 23.74 million Taiwanese residents [29]. The national government-run Bureau of National Health Insurance (BNHI) had contracts with 97% of the hospitals and 92% of the clinics all over the nation. The NHIRD provides registration and claim datasets from a random sample of one million beneficiaries for research use. The Longitudinal Health Insurance Database 2005 (LHID2005) contains all the ambulatory and inpatient claim data of one million beneficiaries who were randomly sampled from the entire enrollees in the NHI and was released in 2007. Similar distributions of beneficiary age and gender in the LHID2005 and the original NHI database were observed. The registration and claim datasets from the LHID2005 for the years 1996 to 2010 were used in the current study. The LHID2005 database contains comprehensive information, such as the demographic data, dates of

clinical visits, diagnostic codes, details of prescriptions, and expenditure amounts of beneficiaries. The data for registered beneficiaries, expenditures of ambulatory care visits, and inpatient admission were obtained for analysis. Every individual has a unique personal identification number (PIN). The data on patient identities and institutions were scrambled cryptographically by the NHIRD to protect the privacy of the beneficiaries. All datasets can be interlinked by using an individual's PIN. The BNHI conducts an expert review of random samples of every 50 to 100 ambulatory and inpatient claims in each hospital and clinic quarterly; several penalties are given to hospitals and clinics that generate false reports of diagnosis [30]. The International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM) was used in disease diagnosis.

2.2. Study Subjects. A retrospective cohort study for patients with lung cancer was conducted. An individual with lung cancer had to have at least three ambulatory claims or at least one inpatient claim with diagnosis of ICD-9-CM code 162 from 1997 to 2009, in which the first diagnosis date is the index date. A total of 7,677 lung cancer patients utilized ambulatory or inpatient care during the period. The surgery status of the patients was identified by using procedures including wedge resection (32.29), thoracoscopic wedge resection (32.20), segmental resection (32.39), thoracoscopic segmental section (32.30), lobectomy (32.49), thoracoscopic lobectomy (32.41), pneumonectomy (32.59), or thoracoscopic pneumonectomy (32.50).

2.3. Variables for Expenditures and Coexisting Diseases. The NHI covered TCM outpatient care, but not TCM inpatient care. Therefore, only TCM and WM ambulatory care were analyzed in this study. The outpatient datasets contained encounter forms based on dates of visit, patient gender and date of birth, medical facility visited, department visited, prescribing physician, dispensing pharmacist, three items from the ICD-9-CM codes, primary procedure (e.g., drug or diagnostic procedure), type of copayment, and billed and paid amounts. All outpatient visits within one year, particularly before and after the index date, were analyzed for expenditure and coexisting diseases.

2.4. Sociodemographic Factors and Urbanization Levels of Residential Area. The sociodemographic factors studied included age, gender, insurance premium, and insured unit. Age was categorized into four levels: <50, 50–59, 60–69, and ≥70 (in years). Amount of insurance premium was categorized into four levels: <20,000, 20,000–39,999, 40,000–59,999, and ≥60,000. The insurance premium amount of an individual was determined by his/her work salary. The residential areas of study subjects consisted of 6 areas: Northern, Taipei, Central, Southern, Eastern, and Kao-Ping areas. The Kao-Ping area is located at southwestern Taiwan, consisting of Kaohsiung City, Kaohsiung County, and Ping-Tung County. The urbanization level of 365 Taiwan townships was classified into seven levels according to the method developed by Lin et al. [31]. The seven levels of urbanization were high-density urban area, medium-density urban area, newly developed area, general area, aging society area, rural area, and nondeveloped area (seclusion area). The indicators used in developing the township stratification for urbanization level included the population density (people/km^2), population ratio of people with an educational level of college or above, population ratio of elder people over 65 years old, population ratio of agricultural workers, and the number of physicians per 100,000 people, among others. The insured unit consisted of governmental department and public school, private enterprise, occupational member, farmers and fishermen, and low-income households and veterans.

2.5. Statistical Analysis. The mean and 95% confidence interval (CI) were reported for the continuous variables, whereas the number, percentage, and 95% CI were reported for the categorical variables. The chi-square test or the two-sample t-test was used to compare the differences in proportions and means of the variables. The adjusted odd ratios (ORs) of TCM use for each factor were estimated by multivariate logistic regression analysis. Cox proportional hazard models were used to evaluate the association between TCM use and 5-year mortality. We calculated hazard ratios and their 95% confidence intervals (CIs) by adjusting age, gender, and multiple variables. All analyses were stratified by surgery status to examine the status of one of the major lung cancer treatments on TCM use and pattern. All P values were reported from two-sided tests, in which the level of statistical significance was set at 0.05. All analyses were performed by using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Factors Associated with TCM Use. Among the 7,677 lung cancer patients, 1,826 (26.32%) lung cancer patients who have not undergone surgery have availed of TCM outpatient services, whereas 230 (31.17%) lung cancer patients who have undergone surgery have availed of TCM outpatient services. Among the lung cancer patients who have not undergone surgery, participants aged 70 years and older (OR = 0.60, 95% confidence interval, CI: 0.49–0.73), males (0.86, 0.75–0.98), occupational members (0.69, 0.51–0.93), and farmers and fishermen (0.72, 0.53–0.97) are less likely to avail of TCM services. By contrast, patients who are TCM users one year prior to lung cancer diagnosis (4.55, 4.00–5.17), who have insurance amounts ranging from NT$20,000 to NT$39,999 (1.34, 1.12–1.60), who are residents of urban levels 2 (1.35, 1.03–1.77) and 4 (1.47, 1.14–1.91) and Central (1.55, 1.21–1.97) and Kao-Ping areas (1.32, 1.02–1.71) are more likely to avail of TCM services. Among the lung cancer patients who have undergone surgery, the likelihood of TCM users is higher in residents who used TCM one year prior to lung cancer diagnosis (4.07, 2.74–6.04) and in patients with insurance amount ranging from ≥NT$60,000 (3.18, 1.18–8.56) (Table 1).

3.2. Medical Institutes. For patients who have not undergone surgery, most WM and TCM outpatient services one

Table 1: Sociodemographic factors of patients with lung cancer.

Characteristic	Nonsurgery (N = 6,939)			Surgery (N = 738)			P value for interaction
	TCM nonusers	TCM users	Adjusted[a] OR (95% CI)	TCM nonusers	TCM users	Adjusted[a] OR (95% CI)	
Patient no.	5113	1826		508	230		
Age	66.95 ± 14.95	61.59 ± 14.66		61.02 ± 12.82	59.85 ± 12.56		0.45
<50	789 (15.43)	412 (22.56)	1.00	88 (17.32)	47 (20.43)	1.00	
50s	784 (15.33)	339 (18.57)	0.85 (0.69–1.05)	132 (25.98)	72 (31.30)	1.24 (0.72–2.13)	
60s	1186 (23.20)	469 (25.68)	0.85 (0.70–1.04)	148 (29.13)	59 (25.65)	0.88 (0.48–1.61)	
≥70s	2354 (46.04)	606 (33.19)	0.60 (0.49–0.73)$^{+}$	140 (27.56)	52 (22.61)	0.68 (0.35–1.31)	
Gender							0.43
Female	1855 (36.28)	796 (43.59)	1.00	195 (38.39)	115 (50.00)	1.00	
Male	3258 (63.72)	1030 (56.41)	0.86 (0.75–0.98)*	313 (61.61)	115 (50.00)	0.69 (0.46–1.04)	
TCM use one year prior to lung cancer diagnosis							0.45
TCM nonusers	4024 (78.70)	799 (43.76)	1.00	397 (78.15)	111 (48.26)	1.00	
TCM users	1089 (21.30)	1027 (56.24)	4.55 (4.00–5.17)$^{+}$	111 (21.85)	119 (51.74)	4.07 (2.74–6.04)$^{+}$	
Insured amount (NT$/month)							0.53
<20,000	3292 (64.38)	1079 (59.09)	—	297 (58.46)	127 (55.22)	1.00	
20,000–39,999	1476 (28.87)	527 (31.33)	1.34 (1.12–1.60)$^{\#}$	159 (31.30)	67 (29.13)	1.46 (0.83–2.58)	
40,000–59,999	218 (4.26)	113 (6.19)	1.23 (0.92–1.66)	41 (8.07)	22 (9.57)	1.93 (0.95–3.91)	
≥60,000	127 (2.48)	62 (3.40)	1.10 (0.75–1.61)	11 (2.17)	14 (6.09)	3.18 (1.18–8.56)*	
Urban level							0.67
1	1217 (25.80)	478 (28.901)	1.00	141 (29.87)	74 (36.45)	1.00	
2	1298 (27.52)	407 (24.61)	1.35 (1.03–1.77)*	128 (27.12)	51 (25.12)	2.05 (0.83–5.04)	
3	704 (14.92)	293 (17.71)	1.08 (0.84–1.40)	76 (16.10)	31 (15.27)	1.70 (0.71–4.08)	
4	818 (17.34)	300 (18.14)	1.47 (1.14–1.91)$^{\#}$	77 (16.31)	28 (13.79)	1.28 (0.54–3.05)	
≥5	680 (14.42)	176 (10.64)	1.22 (0.96–1.55)	50 (10.59)	19 (9.36)	1.49 (0.66–3.32)	
Residential area							0.79
Northern	592 (12.27)	177 (10.45)	1.00	50 (10.44)	19 (9.18)	1.00	
Taipei	1655 (34.31)	543 (32.05)	1.09 (0.85–1.39)	197 (41.13)	80 (38.65)	0.82 (0.38–1.78)	
Central	886 (18.37)	434 (25.62)	1.55 (1.21–1.97)$^{+}$	71 (14.82)	44 (21.26)	1.44 (0.64–3.21)	
Southern	799 (16.57)	246 (14.46)	1.12 (0.86–1.46)	86 (17.95)	32 (15.46)	1.02 (0.45–2.33)	
Eastern	143 (2.96)	39 (2.30)	1.10 (0.70–1.73)	6 (1.25)	2 (0.97)	1.49 (0.21–10.54)	
Kao-Ping	748 (15.51)	256 (15.11)	1.32 (1.02–1.71)*	69 (14.41)	30 (14.49)	0.96 (0.41–2.23)	
Insured unit							0.96
Government, school employees	310 (6.81)	134 (8.10)	1.00	44 (9.57)	17 (8.67)	1.00	
Private enterprise employees	914 (20.09)	436 (26.34)	1.00 (0.77–1.29)	123 (26.74)	61 (31.12)	1.13 (0.55–2.32)	
Occupational member	600 (13.19)	235 (14.20)	0.69 (0.51–0.93)*	88 (19.13)	33 (16.84)	0.75 (0.33–1.74)	
Farmers, fishermen	1351 (29.69)	404 (24.41)	0.72 (0.53–0.97)*	101 (21.96)	40 (20.41)	1.45 (0.60–3.52)	
Low-income households and veterans, other regional	1375 (30.22)	446 (26.95)	0.98 (0.75–1.28)	104 (22.61)	45 (22.96)	1.88 (0.84–4.24)	

TCM: traditional Chinese medicine; OR: odds ratio; CI: confidence interval. *<0.05; $^{\#}$<0.01; $^{+}$<0.001; [a]adjusted ORs were from the model considering age, gender, visit one year ago, insured amount, residential area, and insured unit.

year after lung cancer diagnosis were provided by private hospitals (43.76% for WM; 36.07% for TCM) and private clinics (26.75% for WM; 40.46% for TCM) (Table 2). For patients who have undergone surgery, most WM outpatient services one year after lung cancer diagnosis were provided by private hospitals (45.65%), followed by public hospitals (32.14%). Most TCM outpatient services one year after lung cancer diagnosis were provided by private hospitals (45.22%), followed by private clinics (29.91%). The outpatient services one year before lung cancer diagnosis of patients who have and have not undergone surgery had similar patterns: most WM and TCM outpatient services were provided by private clinics, followed by private hospitals.

3.3. Coexisting Diseases. The diagnoses in all ambulatory claim data were recorded in the ICD-9-CM format. One year after lung cancer diagnosis, lung cancer was the top disease code for WM and TCM patients who have and have not undergone surgery. Except in TCM users who have undergone surgery, the three most frequently recorded coexisting diseases in patients were essential hypertension, diabetes mellitus, and acute upper respiratory infection. For TCM users who have undergone surgery, the three most frequently recorded coexisting diseases were essential hypertension, diabetes mellitus, and general symptoms. The most frequently recorded coexisting diseases of patients one year before lung cancer diagnosis were acute upper respiratory infection, followed by essential hypertension, diabetes mellitus, and general symptoms (see Table 3).

3.4. Expenditures. Table 4 shows the expenditure details of the lung cancer patients. For the patients who have not undergone surgery, the WM outpatient services accounted for 64.40% of all outpatient visits and 71.15% of the total expenditures for one-year outpatient care after cancer diagnosis, whereas the WM outpatient services accounted for 70.12% of the visits and 66.67% of the expenditures for one-year outpatient care before cancer diagnosis. For the patients who have undergone surgery, the WM outpatient services accounted for 61.64% of all outpatient visits and 43.21% of the total expenditures for one-year outpatient care after cancer diagnosis, whereas the WM outpatient services accounted for 63.05% of the visits and 48.25% of the expenditures for one-year outpatient care before cancer diagnosis. The cost of consultation, treatment, medical supplies, drugs, and visitations of TCM nonusers who have not undergone surgery were much higher than those of TCM users who undergone one-year outpatient care before and after cancer diagnosis. The average total expenditure of TCM nonusers was NT$2990.92 (US$98.39) per visit after cancer diagnosis and NT$1257.98 (US$41.38) per visit before cancer diagnosis. The average total expenditure of TCM users was NT$2193.82 (US$72.17) per visit after cancer diagnosis and NT$982.16 (US$32.31) per visit after cancer diagnosis (US$ 1 = NT$30.4 in 2010). For patients who have undergone surgery, the fee for consultation, treatment, and medical supplies, as well as the cost for the total amount per visit of WM, was also much higher than those of TCM for one-year outpatient care before and after cancer diagnosis. The average total expenditure of TCM nonusers was NT$3104.64 (US$102.13) per visit after cancer diagnosis and NT$1270.36 (US$41.79) per visit before cancer diagnosis. The average total expenditure of TCM users was NT$2783.77 (US$91.57) per visit after cancer diagnosis and NT$1121.71 (US$36.90) per visit before cancer diagnosis.

3.5. Overall Mortality. Table 5 shows the univariate and multivariate Cox's analyses of TCM use for overall mortality stratified by surgery status. We found there was no statistical difference in overall mortality between TCM and non-TCM users with surgery. On the contrary, TCM users were associated with lower mortality than non-TCM users in patients without surgery (adjusted hazard ratio for mortality: 0.64; 95% CI: 0.55–0.76).

4. Discussion

This study is the first large-scale survey in the literature that focuses on TCM use for lung cancer patients who undergo one-year outpatient care before and after lung cancer diagnosis. In this study, for patients who have not undergone surgery, the proportions of TCM outpatient visits increased from 29.88% before lung cancer diagnosis to 35.60% after lung cancer diagnosis. On the contrary, the proportions of TCM outpatient expenditures decreased from 33.33% to 28.85%. For patients who have undergone surgery, the proportions of TCM outpatient visits and expenditures increased from 36.95% and 51.75% before lung cancer diagnosis to 38.36% and 56.79% after lung cancer diagnosis, respectively. The possible explanation for this finding is that TCM use is associated with longer survival in lung cancer patients with surgery as found in our study.

Among the lung cancer patients who have not undergone surgery, TCM use was lower among ≥70 year old patients, males, occupational members, farmers, and fishermen. On the contrary, the percent of TCM use was higher among those who reside in the regions of central Taiwan. These findings are similar to those of a previous study on patients with mild diseases or prostate cancer [5, 26–28]. The overall prevalence of TCM utilization in the current study is higher than that reported by Lin et al. for patients with prostate cancer (26.78% versus 2.6%) [28] and that by our previous results for patients with liver cancer (26.78% versus 19.50%) [5]. Lin et al. considered only the outpatient visits specific for prostate cancer. By contrast, the current study considered all outpatient visits. Therefore, the current study reports a higher prevalence of TCM use compared with that of Lin et al. On the basis of our previous work on liver cancer, a slightly higher prevalence of TCM utilization was shown in patients with lung cancer compared with patients with liver cancer. The possible explanation for this higher prevalence is that patients with lung cancer were associated with higher prevalence of respiratory disease, and the Chinese are more likely to seek TCM to relieve symptoms caused by respiratory diseases. In this study, we further consider the effects of the surgery status of TCM users prior to cancer diagnosis on their TCM use after cancer diagnosis. Results show that surgery status

Table 2: Lung cancer outpatient service providers during the period 1996–2010.

Outpatient service providers	Nonsurgery (N = 6,939)					Surgery (N = 738)				
	TCM nonusers (N = 5,113)		TCM users (N = 1,826)		P value for χ^2	TCM nonusers (N = 508)		TCM users (N = 230)		P value for χ^2
	Visits	Percentage (95% CI)	Visits	Percentage (95% CI)		Visits	Percentage (95% CI)	Visits	Percentage (95% CI)	
Visits one year after lung cancer diagnosis										
Type of providers					<0.001					<0.001
Public hospitals	37,079	26.15 (25.92, 26.38)	15,825	20.19 (19.91, 20.47)		5,832	32.14 (31.46, 32.82)	2,616	23.17 (22.39, 23.95)	
Public Chinese medicine hospitals	0	—	129	0.16 (0.14, 0.19)		0	—	6	0.05 (0.01, 0.1)	
Private hospitals	62,051	43.76 (43.5, 44.02)	28,271	36.07 (35.74, 36.41)		8,284	45.65 (44.92, 46.37)	5,106	45.22 (44.3, 46.14)	
Private Chinese medicine hospitals	0	—	577	0.74 (0.68, 0.8)		0	—	22	0.19 (0.11, 0.28)	
Public clinics	4,325	3.05 (2.96, 3.14)	1,712	2.18 (2.08, 2.29)		419	2.31 (2.09, 2.53)	141	1.25 (1.04, 1.45)	
Private clinics	37,930	26.75 (26.52, 26.98)	31,712	40.46 (40.12, 40.81)		3,594	19.8 (19.22, 20.38)	3,377	29.91 (29.06, 30.75)	
Other medicine service providers	407	0.29 (0.26, 0.31)	146	0.19 (0.16, 0.22)		19	0.10 (0.06, 0.15)	24	0.21 (0.13, 0.30)	
Total	141,792	100	78,372	100		18,148	100	11,292	100	
Visits one year prior to lung cancer diagnosis										
Type of providers					<0.001					<0.001
Public hospitals	22,613	17.02 (16.81, 17.22)	8,176	14.44 (14.15, 14.73)		2,456	19.16 (18.48, 19.84)	1,138	15.15 (14.34, 15.96)	
Public Chinese medicine hospitals	38	0.03 (0.02, 0.04)	85	0.15 (0.12, 0.18)		6	0.05 (0.01, 0.08)	0	—	
Private hospitals	42,914	32.29 (32.04, 32.55)	15,163	26.78 (26.41, 27.14)		4,227	32.98 (32.17, 33.79)	2,334	31.07 (30.02, 32.12)	
Private Chinese medicine hospitals	206	0.16 (0.13, 0.18)	491	0.87 (0.79, 0.94)		39	0.30 (0.21, 0.4)	14	0.19 (0.09, 0.28)	
Public clinics	5,826	4.38 (4.27, 4.49)	1,929	3.41 (3.26, 3.56)		579	4.52 (4.16, 4.88)	202	2.69 (2.32, 3.05)	
Private clinics	60,971	45.88 (45.61, 46.15)	30,716	54.24 (53.83, 54.65)		5,493	42.86 (42, 43.71)	3,804	50.64 (49.51, 51.77)	
Other medicine service providers	318	0.24 (0.21, 0.27)	71	0.13 (0.10, 0.15)		17	0.13 (0.07, 0.2)	20	0.27 (0.15, 0.38)	
Total	132,886	100	56,631	100		12,817	100	7,512	100	

TCM: traditional Chinese medicine; CI: confidence interval.

Table 3: Top 5 disease codes among lung cancer patients during the years 1996–2010 for all outpatient visits one year before and after lung cancer diagnosis.

Ranking	Nonsurgery (N = 6,939)						Surgery (N = 738)					
	TCM nonusers (N = 5,113)			TCM users (N = 1,826)			TCM nonusers (N = 508)			TCM users (N = 230)		
	Disease (code)	No.	Percentage (95% CI)	Disease (code)	No.	Percentage (95% CI)	Disease (code)	No.	Percentage (95% CI)	Disease (code)	No.	Percentage (95% CI)
Outpatient visits one year after lung cancer diagnosis												
	Total visits = 141,792			Total visits = 78,372			Total visits = 18,148			Total visits = 11,292		
1	Lung cancer (162)	41,431	29.22 (28.98, 29.46)	Lung cancer (162)	16,308	20.81 (20.52, 21.09)	Lung cancer (162)	8,117	44.73 (44.00, 45.45)	Lung cancer (162)	4,674	41.39 (40.48, 42.3)
2	Essential hypertension (401)	11,917	8.40 (8.26, 8.55)	Acute upper respiratory infections (465)	4,989	6.37 (6.19, 6.54)	Diabetes mellitus (250)	1,150	6.34 (5.98, 6.69)	Essential hypertension (401)	661	5.85 (5.42, 6.29)
3	Diabetes mellitus (250)	11,480	8.10 (7.95, 8.24)	Essential hypertension (401)	4,692	5.99 (5.82, 6.15)	Essential hypertension (401)	1,135	6.25 (5.9, 6.61)	Diabetes mellitus (250)	658	5.83 (5.40, 6.26)
4	Acute upper respiratory infections (465)	7,968	5.62 (5.50, 5.74)	Diabetes mellitus (250)	4,450	5.68 (5.52, 5.84)	Acute upper respiratory infections (465)	798	4.40 (4.10, 4.70)	General symptoms (780)	560	4.96 (4.56, 5.36)
5	General symptoms (780)	6,652	4.69 (4.58, 4.80)	General symptoms (780)	3,526	4.50 (4.35, 4.64)	Symptoms involving respiratory system and other chest symptoms (786)	736	4.06 (3.77, 4.34)	Acute upper respiratory infections (465)	487	4.31 (3.94, 4.69)
Outpatient visits one years before lung cancer diagnosis												
	Total visits = 132,886			Total visits = 56,631			Total visits = 12,817			Total visits = 7,512		
1	Acute upper respiratory infections (465)	10,986	8.27 (8.12, 8.42)	Acute upper respiratory infections (465)	7,093	12.52 (12.25, 12.8)	Acute upper respiratory infections (465)	1,182	9.22 (8.72, 9.72)	Acute upper respiratory infections (465)	643	8.56 (7.93, 9.19)
2	Essential hypertension (401)	10,845	8.16 (8.01, 8.31)	Essential hypertension (401)	4,478	7.91 (7.69, 8.13)	Essential hypertension (401)	903	7.05 (6.60, 7.49)	Diabetes mellitus (250)	624	8.31 (7.68, 8.93)
3	Diabetes mellitus (250)	9,095	6.84 (6.71, 6.98)	Diabetes mellitus (250)	3,958	6.99 (6.78, 7.20)	Diabetes mellitus (250)	811	6.33 (5.91, 6.75)	Essential hypertension (401)	407	5.42 (4.91, 5.93)
4	General symptoms (780)	5,586	4.20 (4.10, 4.31)	General symptoms (780)	3,909	6.90 (6.69, 7.11)	General symptoms (780)	516	4.03 (3.69, 4.37)	General symptoms (780)	367	4.89 (4.40, 5.37)
5	Acute bronchitis and bronchiolitis (466)	4,747	3.57 (3.47, 3.67)	Symptoms involving respiratory system and other chest symptoms (786)	3,029	5.35 (5.16, 5.53)	Hypertensive heart disease (402)	432	3.37 (3.06, 3.68)	Acute bronchitis and bronchiolitis (466)	351	4.67 (4.20, 5.15)

Table 4: Expenditures for outpatient services for lung cancer patients (NT$) during the period 1996–2010 one year before and after lung cancer diagnosis.

Characteristic	Non-surgery (N = 6,939)							Surgery (N = 738)						
	TCM nonusers (N = 5,113)			TCM users (N = 1,826)			t value	TCM nonusers (N = 508)			TCM users (N = 230)			t value
	Total	Percentage (95% CI)	Average (95% CI)	Total	Percentage (95% CI)	Average (95% CI)		Total	Percentage (95% CI)	Average (95% CI)	Total	Percentage (95% CI)	Average (95% CI)	
Outpatient visits one year after lung cancer diagnosis	141,792			78,372				18,148			11,292			
Fees for consultation, treatment, and medical supply	181,842,461	42.88 (42.87, 42.88)	1282.46 (1249.09, 1315.83)	71,784,259	41.75 (41.74, 41.76)	915.94 (878.61, 953.28)	14.35***	29,957,288	53.17 (53.16, 53.18)	1650.72 (1538.08, 1763.36)	14,166,877	45.07 (45.05, 45.09)	1254.59 (1137.20, 1371.98)	4.77***
Diagnosis fee	30,666,528	7.23 (7.23, 7.23)	216.28 (215.63, 216.93)	17,116,958	9.96 (9.95, 9.96)	218.41 (217.67, 219.14)	−4.26***	3,764,169	6.68 (6.67, 6.69)	207.42 (205.82, 209.01)	2,437,653	7.75 (7.75, 7.76)	215.87 (213.89, 217.86)	−6.51***
Drug fee	211,579,372	49.89 (49.89, 49.90)	1492.18 (1462.74, 1521.62)	83,032,934	48.29 (48.29, 48.30)	1059.47 (1026.91, 1092.03)	19.32***	22,621,500	40.15 (40.14, 40.16)	1246.50 (1180.61, 1312.39)	14,829,824	47.18 (47.16, 47.19)	1313.30 (1219.56, 1407.04)	−1.14
Total amount	424,088,361	100	2990.92 (2945.98, 3035.86)	171,934,151	100	2193.82 (2143.54, 2244.10)	23.17***	56,342,957	100	3104.64 (2973.91, 3235.37)	31,434,354	100	2783.77 (2631.91, 2935.63)	3.14**
Outpatient visits one year before lung cancer diagnosis	132,886			56,631				12,817			7,512			
Fees for consultation, treatment, and medical supply	82,399,038	49.29 (49.28, 49.3)	620.07 (600.21, 639.94)	23,736,072	42.67 (42.66, 42.69)	419.14 (399.76, 438.51)	14.19***	8,969,651	55.09 (55.06, 55.11)	699.82 (633.15, 766.49)	3,851,397	45.71 (45.67, 45.74)	512.7 (474.3, 551.1)	4.77***
Diagnosis fee	29,313,743	17.54 (17.53, 17.54)	220.59 (220.01, 221.18)	12,468,308	22.42 (22.41, 22.43)	220.17 (219.39, 220.94)	0.86	2,752,677	16.91 (16.89, 16.92)	214.77 (212.98, 216.55)	1,661,546	19.72 (19.69, 19.75)	221.19 (218.79, 223.59)	−4.20***
Drug fee	55,455,774	33.17 (33.17, 33.18)	417.32 (409.28, 425.36)	19,416,544	34.91 (34.90, 34.92)	342.86 (329.2, 356.52)	9.20***	4,559,885	28.01 (27.98, 28.03)	355.77 (339.48, 372.06)	2,913,321	34.57 (34.54, 34.61)	387.82 (354.65, 421)	−1.70
Total amount	167,168,555	100	1257.98 (1236.48, 1279.48)	55,620,684	100	982.16 (958.35, 1005.97)	16.85***	16,282,213	100	1270.36 (1202.13, 1338.59)	8,426,264	100	1121.71 (1069.67, 1173.75)	3.40***

TCM: traditional Chinese medicine; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; CI: confidence interval.

Table 5: Univariate and multivariate Cox's analyses of TCM use for overall mortality stratified by surgery status. (n = 7,677).

	N	Person-years	Cases	IR	Mortality	
					Age & gender adjusted HR (95% CI)	Adjusted[a] HR (95% CI)
Patients with surgery						
TCM users						
No	508	2810.47	68	24.19	1.00	1.00
Yes	230	1090.53	29	26.59	1.06 (0.68–1.64)	0.95 (0.56–1.60)
Patients without surgery						
TCM users						
No	5113	25551.53	1184	46.34	1.00	1.00
Yes	1826	10908.33	272	24.94	0.67*** (0.58–0.76)	0.64*** (0.55–0.76)

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[a]Multivariate adjustment for age, sex, visit one year ago, insured amount, urban level, residential area and insured unit.
IR: Incidence density rate = Number of incidence cases/Person-years ∗ 1000.

did not exert a significant effect on TCM utilization. Prior TCM use before cancer diagnosis is shown to be the most significant factor for TCM users who have not undergone and have undergone surgery. The significant factor of prior TCM use before cancer diagnosis, a socio-cultural characteristics of individuals that exist prior to their illness, corresponds to the predisposing factor that proposed by Andersen's health behavior model [32].

On the basis of the ICD-9-CM codes, and with the exception of the malignant lung neoplasm, we found that acute upper respiratory infections, diabetes mellitus, and essential hypertension were the primary indications in TCM users and nonusers. In TCM users who have and have not undergone surgery, the other primary indications were symptoms involving the respiratory system and other chest symptoms. Pulmonary tuberculosis was shown to be another indication for TCM use. These findings on disease pattern of health care use may be explained by the fact that patients seek TCM to relieve respiratory symptoms.

Using the prevalence of surgery and difference in total costs for each TCM and WM user per year from our study, and number of lung cancer incidence cases in 2008 reported by Department of Health, Taiwan, we estimated that TCM use would save at least 7.1 millions per year. Given that costs for TCM and WM use per session in other developed countries are much higher than those in Taiwan, we believe that TCM use would potentially save much more costs in other developed countries.

This study is the first large-scale study of TCM use in Taiwanese society, in which a million residents were randomly selected from the 23 million population of Taiwan. The NHI database has high comprehensiveness because the NHI covers more than 99% of the population of Taiwan and 93% of the medical institutes. Previous studies of health care utilization in cancer patients did not consider the status of TCM use prior to cancer diagnosis. Several previous studies on TCM/CAM use have been conducted via telephone interviews, self-administered interviews, household interviews, and hospital- and clinical-based survey. These studies usually had limited sample sizes and were conducted in countries in which TCM/CAM was not covered by insurance. Thus, the pattern and characteristics of TCM/CAM use may be affected by the socioeconomic status of individuals.

This study has several limitations. First, several herbal medicines were not covered by NHI, and visits at clinics not in contract with the BNHI were not considered in this study. About 10% of the TCM clinics do not have contracts with NHI because of the low insurance amount given by NHI. In addition, cancer patients need rare and expensive Chinese herbal medicine , which is not covered by NHI. The NHIRD data may lead to an underestimation of TCM costs. Second, the data for the clinical stages and types of lung cancer were not available for the study; thus, TCM use and costs stratified by these factors cannot be explored. We also observed that the pattern of TCM use varied according to surgery status. However, we did not observe a significant correlation of surgery and other covariates because of limited sample size of TCM users who have undergone surgery. Thus, we described the utilization pattern of TCM according to surgery status.

5. Conclusions

Our study reports the prevalence and pattern of TCM use under NHI, a comprehensive and universal health insurance program in Taiwan. The NHI program covers both conventional WM and TCM services. The prevalence of TCM use among lung cancer patients who have and have not undergone surgery is similar. The factors associated with TCM use varied according to surgery status. The total costs of insurance-covered TCM were lower than those of WM for both patients who have and have not undergone surgery. This study provides information about the frequency of TCM use and the coexisting diseases treated by WM and TCM in lung cancer patients according to surgery status. The results of this study would be useful for health policy makers and for researchers considering TCM and WM integration.

Abbreviations

TCM: Traditional Chinese medicine
CAM: Complementary and alternative medicine
LHID2005: Longitudinal Health Insurance Database 2005
LC: Lung cancer
NSCLC: Non-small-cell lung cancer
NHI: National Health Insurance
BNHI: Bureau of National Health Insurance.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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Research Article

Cytotoxicity and Pharmacogenomics of Medicinal Plants from Traditional Korean Medicine

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Aim. The present study was designed to investigate the cytotoxicity of a panel of 280 Korean medicinal plants belonging to 73 families and 198 species against human CCRF-CEM leukemia cells. Selected phytochemicals were investigated in more detail for their mode of action. *Methods*. The resazurin assay was used to determine cytotoxicity of the plant extracts. Microarray-based mRNA expression profiling, COMPARE, and hierarchical cluster analyses were applied to identify which genes correlate with sensitivity or resistance to selected phytochemicals of the Korean plants. *Results*. The results of the resazurin assay showed that cytotoxicity extracts tested at 10 μg/mL from 13 samples inhibited proliferation more than 50% ($IC_{50} < 10\,\mu g/mL$) and the most active plants are *Sedum middendorffianum* (15.33%) *and Lycoris radiata* (17.61%). Out of 13 selected phytochemicals from these plants, hopeaphenol and deoxynarciclasine were the most cytotoxic ones. Genes from various functional groups (transcriptional or translational regulation, signal transduction, cellular proliferation, intracellular trafficking, RNA metabolism, endoplasmic/sarcoplasmic reticulum function, etc.) were significantly correlated with response of tumor cell lines to these two compounds. *Conclusion*. The results provide evidence on the possible use of selected Korean medicinal plants and chemical constituents derived from them for the treatment of tumors.

1. Introduction

Since ancient times, humans have derived many benefits from medicinal plants. A variety of different medicinal plants have traditionally been used in Asian cultures as medicinal plants to treat cancers [1]. The pharmacological screening of plants is an important mean for the discovery of new, safe, and effective drugs. Over 50,000 plants, possess therapeutic virtues in the world, and about 80% of human use herbal medicines at least once in their life [2, 3]. Medicinal plants through the multiplicities of their chemical constituents are important for the discovery of new substances active against tumors and other cancers. Screenings of medicinal plants used as anticancer drugs have provided modern medicine with effective cytotoxic pharmaceuticals. More than 60% of the approved anticancer drugs in the United State of America (from 1983 to 1994) were from natural origin [4, 5].

Traditional Korean medicine is widely used in Korea and is the primary health care system for more than 20% of the population [6, 7]. As previously emphasized in a regional demographic survey, about 30–40% of the Korean population had used complementary and alternative medicine (including traditional Korean medicine) within a 5-year period [6, 8].

In the pharmacopoeia of many countries worldwide including Korea, there is still a serious lack of information on

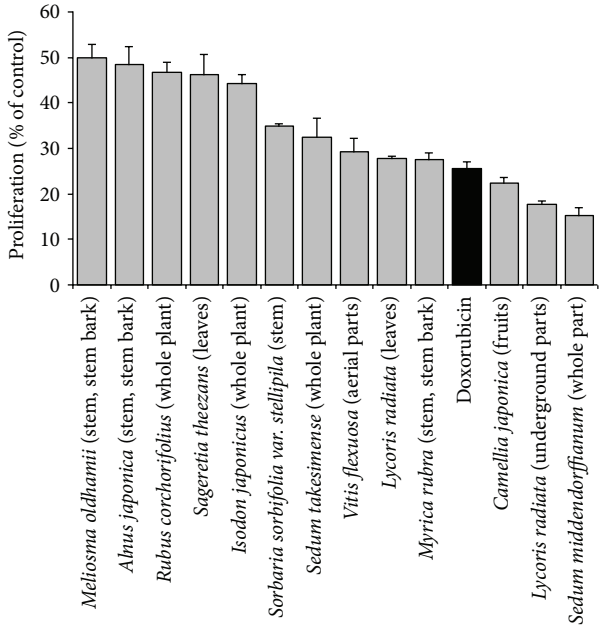

Figure 1: Cytotoxicity of the 13 most active samples from selected Korean medicinal plants extract (at 10 μg/mL) and doxorubicin (1 μM) on CCRF-CEM leukemia cells. Data with different superscript letters are significantly different ($P < 0.05$). (See Supplementary Data for the overview of the cytotoxicity of all 280 tested extracts. Are shown mean values ± SD of each five measurements.) The established anticancer drug, doxorubicin was used as positive control (black bar).

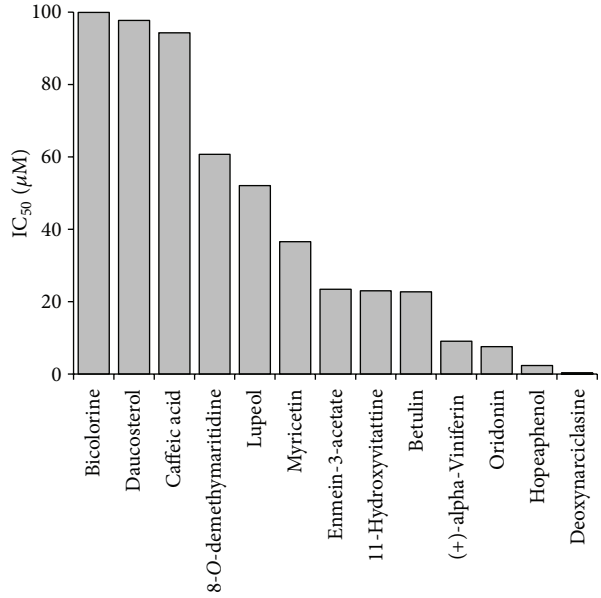

Figure 2: Mean $\log_{10}IC_{50}$ values for selected phytochemicals derived from Korean medicinal plants for tumor cell lines from the NCI cell line panel.

the use of medicinal plants in the treatment of cancer. However, it has been recommended that ethnopharmacological usages such as for immune and skin disorders, inflammatory, infectious, parasitic, and viral diseases should be taken into account when selecting plants used to treat cancer, since these reflect disease states bearing relevance to cancer or cancer-like symptoms [9, 10]. In general, leukemia cells are often more sensitive to cytotoxic drugs than adherent cancer cells, making them a suitable tool for primary bioactivity screenings. Hence, the present work was designed to investigate the cytotoxicity of a panel of 280 Korean medicinal plants against human CCRF-CEM leukemia cells. Based on the bioactivity of the extracts, selected phytochemicals of active plants were analyzed in more detail. Genes determining sensitivity or resistance to selected compounds were identified by microarray-based mRNA expression profiles and hierarchical cluster analyses in a panel of tumor cell lines of the National Cancer Institute, USA.

2. Material and Methods

2.1. Plant Material and Extraction. Medicinal plants used in the present work were collected at different localities of the Republic of Korea and provided by Prof. Ik-Soo Lee (College of Pharmacy, Chonnam National University, Kwangju, South Korea). The plants were identified at the National herbarium, where voucher specimens were deposited under the references numbers (see Supplementary material available online at http://dx.doi.org/10.1155/2013/341724). The extraction of the air-dried and powdered plant material was conducted using methanol (HPLC grade) with either ASE 300 (Dionex) or a sonicator (Branson Ultrasonics) at 50°C. The extracts were then conserved at 4°C until further use.

2.2. Cell Culture. Human CCRF-CEM leukemia cells were obtained from Professor Axel Sauerbrey (University of Jena, Jena, Germany). Cells were maintained in RPMI 1640 containing 100 units/mL penicillin and 100 mg/mL streptomycin and supplemented with heat-inactivated 10% fetal bovine serum (FBS), in a humidified environment at 37°C with 5% CO_2. Doxorubicin ≥ 98.0% (Sigma-Aldrich, Schnelldorf, Germany) was used as a positive (cytotoxic) control.

2.3. Resazurin Cell Growth Inhibition Assay. Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay [11] was used to assess the cytotoxicity of the studied samples. The assay tests cellular viability and mitochondrial function. Briefly, aliquots of 5×10^4 cells/mL were seeded in 96-well plates, and extracts were added immediately. After 24 h incubation, 20 μL resazurin 0.01% w/v solution was added to each well and the plates were incubated at 37°C for 1-2 h. Fluorescence was measured on an automated 96-well Infinite M2000 Pro plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Doxorubicin was used as positive control. The concentration of DMSO was kept at or below 0.1% in all experiments. Each assay was done at least three times, with three replicates each. All samples were tested at a single concentration of 10 μg/mL.

2.4. COMPARE and Hierarchical Cluster Analysis of mRNA Microarray Data. The mRNA microarray hybridization of

Table 1: Korean plants with cytotoxic activity.

Plants (and family)	Traditional uses	Part used	Previously reported activity of the plant	Reported chemical constituents
Alnus japonica Steudel (Betulaceae)	In oriental traditional medicine as remedies for fever, hemorrhage, diarrhea, and alcoholism [43]	Stems-stem bark	Hepatoprotective and antioxidant activities [44], antiviral activity against the influenza virus [45]	1,7-Bis-(3,4-dihydroxyphenyl)-3-hydroxyheptane-5-*O*-*β*-D-xylopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-*O*-*β*-D-glucopyranosyl(1 → 3) -*β*-D-xylopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-*O*-*β*-D-apiofuranosyl(1 → 6)-*β*-D-glucopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-*β*-D-glucopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxylheptane; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-*β*-D-glucopyranoside; oregonin; hirsutanonol; hirsutenone; platyphylloside [44]; tannins (alnusjaponins A and B); 5-*O*-galloyl-(−)-shikimic acid, 2,3-(*S*)-hexahydroxydiphenoyl-D-glucose, 4,6-di-*O*-galloyl-D-glucose, 1,4-di-*O*-galloyl-*β*-D-glucose, 4,6-(*S*)-valoneoyl-D-glucose; strictinin; gemin D; pedunculagin; praecoxin A; flosin A; stachyurin; casuarinin [46], lupeol; betulin; betulinic aldehyde; 3-acetoxybetulinic aldehyde, *β*-sitosterol [45]
Camellia japonica L. (Theaceae)	Cosmetic protectant to keep the skin and hair healthy and as a soothing agent [47]	Fruits	Antibacterial activity [48], inhibitor of human immunodeficiency virus type 1 protease [49], Epstein-Barr virus inhibitor [50], antimetastasis activity [51], antioxidant activity [52, 53], inhibitor of human type I procollagen production [54], and antiallergic responses [55], anti-inflammatory [47]	3*β*,18*β*-dihydroxy-28-norolean-12-en-16-one; 18*β*-hydroxy-28-norolean-12-ene-3,16-dione; camelliagenin A, B, and C [56], camellenodiol 3-*O*-*β*-D-galactopyranosyl(1 → 2)[*β*-D-xylopyranosyl(1 → 2)-*β*-D-galactopyranosyl(1 → 3)]-*β*-D-glucuronopyranoside; camellenodiol 3-*O*-4″-*O*-acetyl-*β*-D-galactopyranosyl(1 → 2)[*β*-D-xylopyranosyl(1 → 2)-*β*-D-galactopyranosyl(1 → 3)]-*β*-D-glucuronopyranoside; camellenodiol 3-*O*-(*β*-D-galactopyranosyl(1 → 2)[*β*-D-xylopyranosyl(1 → 2)-*β*-D-galactopyranosyl(1 → 3)]-6′-methoxy-*β*-D-glucuronopyranoside; maragenin II 3-*O*-(*β*-D-galactopyranosyl(1 → 2)[*β*-D-xylopyranosyl(1 → 2)-*β*-D-galactopyranosyl(1 → 3)]-6′-methoxy-*β*-D-glucuronopyranoside, camellioside A; camellioside B [57]
Isodon japonicus (Burman f.) H. Hara (Labiatae)	Antibacterial, anti-inflammation, and anthelmintic [58]	Whole plant	Cytotoxicity on K562 human leukemia cells and immunomodulatory activity [16], antibacterial activity for plant constituents [59]	Isadonol; epinodosin; sodoponin; epinodosinol [60, 61]; epinodosin; oridonin; taihangjaponicain A; lushanrubescensin J; bisjaponins A and B [58]; isodonal, trichodonin; nodosin; enmein; oridonin; enmein-3-acetate [59]
Lycoris radiata (L'Her.) Herbert (Amaryllidaceae)	Laryngeal trouble, furuncle, carbuncle, suppurative wounds [62]	Leaves, underground parts	Cytotoxicity against B16F10 melanoma cells [17]	Different types of alkaloids (crinine-type; galanthamine-type; lycorine-type homolycorine-type; tazettine-type; narciclasine-type; and lycorine-type alkaloids); trisphaeridine; galanthine; bicolorine; 11-hydroxyvittatine; 8-*O*-demethymaritidine; *O*-demethylgalanthamine; *O*-demethyllycoramine [63]

Table 1: Continued.

Plants (and family)	Traditional uses	Part used	Previously reported activity of the plant	Reported chemical constituents
Meliosma oldhamii Miq. ex. Maxim. (Sabiaceae)	Liver ailments [64]	Stems-stem bark	Low Cholinesterase inhibition (12–19% at 5 mg/mL) [65], moderate alpha glucosidase activity [64]	—
Myrica rubra Sieb. and Zucc. (Myricaceae)	Diarrhea; gastroenteritis in China [66]	Stems-stem bark	Antioxidant activity [67]; anti-influenza virus activity [68]	Taraxerone; taraxerol; myricadiol; sitosterol; 28-hydroxy-D-friedoolean-14-en-3-one (); myricanol 5-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranoside; myricanol 5-*O*-α-L-arabinofuranosyl-(1 → 6)-β-D-glucopyranoside; isomyricanone [69]; cyanidin-3-*O*-glucoside; myricetin; quercetin-3-*O*-rutinoside [67]
Rubus corchorifolius L. f. (Rosaceae)	Stomachache, diarrhea, and dysentery [18]	Whole plant	Antioxidant activity of essential oil [70]	*Ent*-kauran-3β, 16β, 17, 19-tetrol; *ent*-2-carbonyl-16β-hydroxy-kauran-17β-D-glucoside [18]; rubusin A; quercetin; kaempferol [25]
Sageretia theezans (L.) Brongn (Rhamnaceae)	Tea materials [71]	Leaves, Stems	Antioxidant activity [72]	7-*O*-methylmearnsitrin; myricetrin, kaempferol 3-*O*-α-L-rhamnopyranoside, europetin 3-*O*-α-L-rhamnoside, and 7-*O*-methyl quercetin 3-*O*-alpha-L-rhamnopyranoside; 7-*O*-methylmearnsetin 3-*O*-rhamnoside [71, 72]
Sedum middendorffianum Maxim. (Crassulaceae)	—	Whole plant	—	kaempferol; quercetin; myricetin; arbutin [24]
Sedum takesimense Nakai (Crassulaceae)	—	Whole plant	Antioxidant activities [26]	Ferulic acid; caffeic acid; gallic acid; methyl gallate; myricetin; quercetin; luteolin; rhodalin; rhodalidin; luteolin-7-*O*-β-D-glucoside; arbutin; 1-(4-hydroxyphenyl)-2-(3,5-dihydroxyphenyl)-2-hydroxyethanone; gossypetin-8-*O*-β-D-xylopyranoside; 2,6-di-*O*-galloylarbutin [26]
Sorbaria sorbifolia (L.) A. Br. var. *stellipila* MAX. (Rosaceae)	—	Stems	Antioxidant activities, cytotoxicity [73, 74]	Sutherlandin-5-trans-*p*-coumarate; cardiosdiospermin-5-(4-hydroxy) benzoate [75]; noreugenin; wogonin; 5,7,3′,4′-tetrahydroxy-3-methoxyflavone; protocatechuic acid; benzoic acid; emodin; daucosterol [76]; 5,2′,4′-trihydroxy-6,7,5′-trimethoxyflavone; succinic acid; *p*-hydroxybenzoic acid [77]
Vitis flexuosa Thunb. (Vitaceae)	—	Aerial parts	—	Flexuosol A; gnetin A; (+)-epsilon-viniferin; vitisin A; hopeaphenol [78]

(—): not reported; the complete list of the tested plants is available in supplementary material.

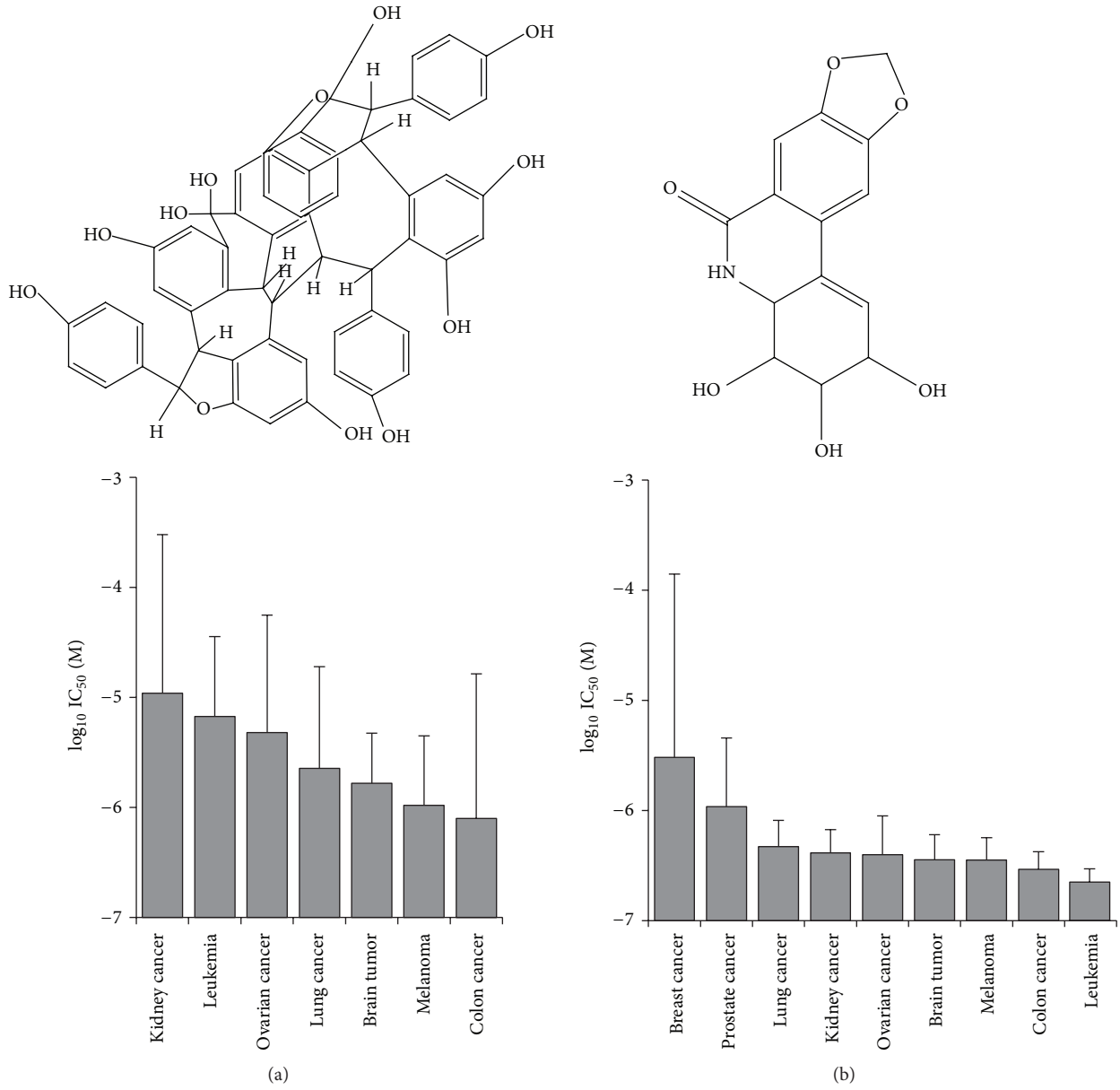

Figure 3: Cytotoxic activity of hopeaphenol (a) and deoxynarciclasine (b) towards cell lines of different tumor types (mean ± SD).

the NCI cell line panel has been described [12, 13] and the date has been deposited at the NCI website (http://dtp.nci.nih.gov/). For hierarchical cluster analysis, objects were classified by calculation of distances according to the closeness of between-individual distances by means of hierarchical cluster analysis. All objects were assembled into a cluster tree (dendrogram). The merging of objects with similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relation. The distance of a subordinate cluster to a superior cluster represents a criterion for the closeness of clusters as well as for the affiliation of single objects to clusters. Thus, objects with tightly related features appear together, while the separation in the cluster tree increases with progressive dissimilarity. Previously, cluster models have been validated for gene expression profiling and for approaching molecular pharmacology of cancer [12–14]. Hierarchical cluster analyses applying the WARD method were done with the WinSTAT program (Kalmia, Cambridge, MA, USA). Missing values were automatically omitted by the program, and the closeness of two joined objects was calculated by the number of data points they contained. In order to calculate distances between all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean = 0 and a variance = 1. For COMPARE analysis, the mRNA expression values of genes of interest and IC_{50} values for selected phytochemicals of the NCI cell lines were selected from the NCI database (http://dtp.nci.nih.gov/). The mRNA expression has

Table 2: Cross-resistance profile of a panel of cell lines towards hopeaphenol and deoxynarciclasine determined by correlating the IC_{50} values by Pearson's correlation test.

	Hopeaphenol	Deoxynarciclasine
Doxorubicin		
R-value	<0.30	0.340
P-value	>0.05	0.010
Daunorubicin		
R-value	<0.30	0.430
P-value	>0.05	0.001
Vincristine		
R-value	<0.30	0.331
P-value	>0.05	0.012
Paclitaxel		
R-value	<0.30	0.330
P-value	>0.05	0.012
Cisplatin		
R-value	<0.30	0.300
P-value	>0.05	0.020
Melphalan		
R-value	<0.30	0.388
P-value	>0.05	0.004
Carmustin		
R-value	<0.30	0.394
P-value	>0.05	0.003
Erlotinib		
R-value	−0.353	>−0.30
P-value	0.004	>0.05

Pearson's rank correlation test.

been determined by microarray analyses as reported [12]. COMPARE analyses were performed by a web-based algorithm (http://dtp.nci.nih.gov/) to produce rank-ordered lists of genes expressed in the NCI cell lines. The methodology has been described previously in detail [15]. Briefly, every gene of the NCI microarray database was ranked for similarity of its mRNA expression to the IC_{50} values for the corresponding compound. Scale indices of correlations coefficients (*R*-values) were created. Pearson's correlation coefficients with positive algebraic signs indicate that greater mRNA expression in cell lines correlate with enhanced drug resistance, whereas coefficients with negative algebraic signs indicate that greater mRNA expression in cell lines was associated with drug sensitivity. Pearson's correlation test and χ^2 test were implemented into the WinSTAT Program (Kalmia). The one-way ANOVA at 95% confidence level was used for statistical analysis.

3. Results

3.1. Cytotoxic Activity. In the present work, we screened 280 plant extracts derived from traditional Korean medicine belonging to 73 plant families and 198 species (see Supplementary Table) for their cytotoxicity against human CCRF-CEM leukemia cells.

The results of the cytotoxicity assay (Figure 1; Supplementary Table) indicated that is among the 280 studied plant extracts, 58 promoted the growth (% growth > 100%) of CCRF-CEM cells, while the remaining samples showed various extents of growth inhibition. Out of these extracts, 207 extracts showed no or a weak inhibition of cancer cell proliferation (<50%) at the tested concentration of 10 μg/mL. However, 13 samples induced the proliferation of less 50% cells (IC_{50} < 10 μg/mL). These samples included *Sedum middendorffianum* whole plant (15.33%), *Lycoris radiata* underground parts (17.61%), *Camellia japonica* fruits (22.28%), *Myrica rubra* stem-stem bark (27.61%), *Lycoris radiata* leaves (27.73%), *Vitis flexuosa aerial parts* (29.32%), *Sedum takesimense* whole plant (32.39%), *Sorbaria sorbifolia var. stellipila* stems (34.98%), *Isodon japonicus* whole plant (44.15%), *Sageretia theezans* leaves and stems (46.35%), *Rubus corchorifolius* aerial part (46.81%), *Alnus japonica* stem-stem bark (48.43%), and *Meliosma oldhamii* stem-stem bark (49.94%) (Figure 1). The traditional used and reported bioactivities are compiled in Table 1.

3.2. Cytotoxicity of Phytochemicals Derived from Koran Medicinal Plants. We searched the literature on the chemical constituents of the cytotoxic Korean plants (Table 1). Subsequently, we mined the NCI database for these compounds. Thirteen compounds with average $\log_{10}IC_{50}$ values over the entire NCI cell line panel below −4.0 M are depicted in Figure 2. Hopeaphenol and deoxynarciclasine were the most cytotoxic compounds with $\log_{10}IC_{50}$ values of 0.346×10^{-6} M (=0.346 μM) and 0.233×10^{-5} M (=2.33 μM), respectively.

If the average IC_{50} values over the entire range of cell lines were diversified regarding their tumor types, colon cancer and melanoma cell lines were most sensitive towards hopeaphenol, whereas leukemia and kidney cancer cell lines were most resistant (Figure 3(a)). The cell lines reacted in a different manner towards deoxynarciclasine. Leukemia and colon cancer were most sensitive towards this compound, whereas breast cancer and prostate cancer cell lines were most resistant (Figure 3(b)).

3.3. Cross-Resistance of the NCI Cell Line Panel towards Hopeaphenol and Deoxynarciclasine. The $\log_{10}IC_{50}$ values of hopeaphenol and deoxynarciclasine were correlated with clinically well-established anticancer drugs. The cell line panel showed statistically significant correlations between deoxynarciclasine and doxorubicin, daunorubicin, vincristine, paclitaxel, cisplatin, melphalan and carmustin. By contrast, cross-resistance was not found between hopeaphenol and these standard drugs, indicating that hopeaphenol might be useful for the treatment of otherwise drug-resistant tumors (Table 2). Interestingly, an inverse correlation was found between the $\log_{10}IC_{50}$ values for hopeaphenol and the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. Such a relationship was not found between deoxynarciclasine and erlotinib (Table 2).

Table 3: Genes identified by standard or reverse COMPARE analyses, whose mRNA expression in a panel of 60 cell lines correlated with IC_{50} values for hopeaphenol.

Pearson's correlation coefficient	Experimental ID	Gene symbol	Name	Function
0.552	GC63503	HMGN4	High mobility group nucleosomal binding domain 4	Binds nucleosomal DNA
0.527	GC190712	UAP1	UDP-N-acetylglucosamine pyrophosphorylase 1	Nucleotidyltransferase
0.521	GC45602	TBC1D2	TBC1 domain family, member 2	GTPase activator
0.521	GC188142	ERBB2IP	Erbb2 interacting protein	Receptor adapter, structural constituent of cytoskeleton
0.515	GC26884	PRPS1	Phosphoribosyl pyrophosphate synthetase 1	Involved in nucleotide synthesis
0.513	GC38343	CNNTAL1		Unknown
−0.591	GC97260	MANBA	Mannosidase, beta A, lysosomal	Exoglycosidase
−0.574	GC73531	FGF9	Fibroblast growth factor 9 (glia-activating factor)	Cell growth and differentiation during development
−0.534	GC55495	CYP7B1	Cytochrome P450, family 7, subfamily B, polypeptide 1	Monooxygenase
−0.516	GC94617	GABRA3	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	Neurotransmission
−0.515	GC184017	HES1	Hairy and enhancer of split 1, (Drosophila)	Transcriptional repressor

Information on gene functions was taken from the OMIM database, NCI, USA, (http://www.ncbi.nlm.nih.gov/Omim/) and from the GeneCard database of the Weizmann Institute of Science, Rehovot, Israel (http://bioinfo.weizmann.ac.il/cards/index.html).

3.4. COMPARE and Hierarchical Cluster Analyses of mRNA Microarray Data. We further investigated the microarray-based transcriptomic mRNA expression by COMPARE analyses to test whether the responses of the tumor cells lines to hopeaphenol and deoxynarciclasine were associated with specific gene expression profiles. For this reason, we mined the transcriptome-wide mRNA expression database of the NCI and correlated the expression data with the IC_{50} values for both compounds. This represents a hypothesis-generating bioinformatical approach, which allows the identification of novel putative molecular determinants of cellular response for hopeaphenol and deoxynarciclasine. The scale rankings of genes obtained by COMPARE computation were subjected to Pearson's rank correlation tests. The thresholds for correlation coefficients were $R > 0.50$ for direct correlations and $R < -0.50$ for inverse correlations. The genes fulfilling these criteria are shown in Table 3 (for hopeaphenol) and Table 4 (for deoxynarciclasine). These genes were from diverse functional groups for hopeaphenol (Table 3). For deoxynarciclasine, genes were found involved in transcription (*ILF2, BCLAF1, MATR3, PSPC1, ZBTB11, CNOT8, IKZF5, SF1, EBP*, and *MYBL1*), RNA metabolism (*HNRNPA1, LARS, SFRS1, FARSA, NUDT21, DDX39,* and *SERBP1*), translation (*GOT1, NGDN*), cellular proliferation (*CWF19L1, MKI67, HSPA9, MLF1IP*, and *DCBLD2*), intracellular trafficking (*OPTN, SNX6*, and *RAB11FIP5*), endoplasmic/sarcoplasmic reticulum function (*SLN, DNAJC10, LMAN2L, SEC24D*, and *KDELR2*), signal transduction (*FRAT2, CHUK, GNA12*, and *LPP*), and others.

As a next step, the genes identified by COMPARE analyses and Pearson's rank correlation tests were subjected to hierarchical cluster analyses. Only the mRNA expression levels but not the IC_{50} values of the compounds for the cell line panel were used for cluster analyses. Four cluster branches were found in the hopeaphenol-related dendrogram (Figure 4) and three clusters were obtained in the deoxynarcilasine-related cluster analysis (Figure 5). Remarkably, the distribution of cell lines sensitive or resistance to both drugs considerably varied between the different clusters of the dendrograms. Since the IC_{50} values of the compounds were not included into the cluster analysis, we could address the question whether the gene expression profiles alone are sufficient to predict sensitivity or resistance of cell lines to the compounds.

As shown in Table 5, the distributions of cell lines sensitive or resistant to hopeaphenol and deoxynarciclasine, respectively, were significantly different between the clusters of the dendrograms, indicating that the expression of these genes was not only responsible for the branching of the dendrograms, but also predicted sensitivity or resistance of these cell lines towards these compounds.

4. Discussion

4.1. Cytotoxic Activity. The cytotoxicity observed in *Isodon. japonicus* in this work is in concordance with previous reports. In effect, the cytotoxic effect of *I. japonicus* extract was reported against five human cancer cell lines (IC_{50} values below 10 μg/mL on stomach MKN-45, breast MCF-7, leukemia K562, colon HT29, and lung A549 cell lines) with leukemia K562 (IC_{50} : 2.70 μg/mL) being the most sensitive [16]. Also, the ethanol extract of *Lycors radiata* exhibited significant antiproliferative effect against B16F10 melanoma cells and induced apoptosis through the activation of p38 and AP-1 pathway [17]. The present report, therefore, provides evidence on the activity of *L. radiata* not only against cell lines derived from solid tumors but also derived from the

Table 4: Genes identified by standard or reverse COMPARE analyses, whose mRNA expression in a panel of 60 cell lines correlated with IC_{50} values for deoxynarciclasine.

Pearson's correlation coefficient	Experimental ID	Gene symbol	Name	Function
0.572	GC74997	SNAP25	Synaptosomal-associated protein, 25 kDa	Regulation of neurotransmitter release
0.56	GC32186	OPTN	Optineurin	Maintenance of membrane trafficking
0.557	GC52658	FAM116B	Family with sequence similarity 116, member B	Unknown
0.531	GC187393	ZDHHC7	Zinc finger, DHHCtype containing 7	Palmitoyltransferase
0.529	GC12575	CLEC9A	C-type lectin domain family 9, member A	Endocytic receptor for necrotic cells
0.528	GC188718	SNX6	Sorting nexin 6	Involved in intracellular trafficking
0.528	GC154565	RAB11FIP5	RAB11 family interacting protein 5 (class I)	Involved in protein trafficking
0.527	GC18484	NTAN 1	N-terminal asparagine amidase	Ubiquitin-dependent turnover of intracellular proteins
0.526	GC16433	DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	Endoplasmic reticulum cochaperone
0.524	GC10009	PCOLCE2	Procollagen C-endopeptidase enhancer 2	Binds to procollagens
0.524	GC45803	LMAN2L	Lectin, mannose-binding 2-like	Regulation of export from the endoplasmic reticulum
0.524	GC82947	NGDN	Neuroguidin, EIF4E binding protein	Involved in the translational repression
0.519	GC75800	SEC24D	SEC24 family, member D (*S. cerevisiae*)	Transport of ER-derived vesicles
0.517	GC90440	KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	Retention of luminal endoplasmic reticulum proteins
0.514	GC173264	GNA12	Guanine nucleotide binding protein (G protein) α12	Transducer in transmembrane signaling
0.512	GC170473	MYBL1	V-Myb myeloblastosis viral oncogene homolog (avian)-like 1	Transcriptional activator
0.512	GC175225	LPP	LIM domain containing preferred translocation partner in lipoma	Signal transduction from cell adhesion sites to the nucleus
0.511	GC14006	ITGB5	Integrin, β5	Receptor for fibronectin
0.509	GC150035	DCBLD2	Discoidin, CUB, and LCCL domain containing 2	Involved in tumor progression
0.508	GC73833	NCEH1	Neutral cholesterol ester hydrolase 1	Promotes tumor cell migration
0.507	GC177466	AHNAK	*AHNAK* nucleoprotein	Involved in neuronal cell differentiation
0.502	GC40315	ADAL	Adenosine deaminaselike	Putative nucleoside deaminase
0.502	GC28174	MPG	N-methylpurine-DNA glycosylase	Hydrolysis of alkylated DNA
0.501	GC18079	CR1	Complement component (3b/4b) receptor 1 (Knops blood group)	Mediates cellular binding of particles and immune complexes
−0.658	GC18354	ILF2	Interleukin enhancer binding factor 2, 45 kDa	Transcription
−0.608	GC44240	NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2, 8 kDa	Part of mitochondrial membrane respiratory chain
−0.605	GC65788	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	Involved in RNA replication
−0.589	GC43237	CWF19L1	CWF19-like 1, cell cycle control (*S. pombe*)	Cell cycle control
−0.572	GC174320	BCLAF1	BCL2-associated transcription factor 1	Death-promoting transcriptional repressor
−0.572	GC40779	LARS	Leucyl-tRNA synthetase	Editing of tRNA
−0.567	GC151509	FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	Wnt signaling regulator
−0.561	GC175610	IK	*IK* cytokine, downregulator of HLA II	May bind to chromatin
−0.555	GC30213	SLN	Sarcolipin	Sarcoplasmic reticulum proteolipid
−0.553	GC162737	ANKHD2		Unknown
−0.552	GC17532	SFRS28		Unknown
−0.55	GC185046	SFRS1	Serine/arginine-rich splicing factor 1	Splicing regulator
−0.55	GC37292	FARSA	Phenylalanyl-tRNA synthetase, α subunit	Phenylalanine-tRNA ligase
−0.548	GC32318	MKI67	Antigen identified by monoclonal antibody Ki-67	Cell proliferation
−0.548	GC149101	MATR3	Matrin 3	Regulator of transcription

Table 4: Continued.

Pearson's correlation coefficient	Experimental ID	Gene symbol	Name	Function
−0.547	GC150688	PSPC1	Paraspeckle component 1	Regulator of androgen receptor-mediated gene transcription
−0.546	GC80581	ZBTB11	Zinc finger and BTB domain containing 11	Regulator of transcription
−0.546	GC52470	MLF1IP	MLF1 interacting protein	Involved in mitotic progression
−0.545	GC81490	NUDT21	Nudix (nucleoside diphosphate linked moiety X)-type motif 21	Involved in pre-mRNA 3′-processing
−0.543	GC33504	DDX39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39A	Involved in pre-mRNA splicing
−0.543	GC35760	NDUGB8		Unknown
−0.539	GC36766	CHUK	Conserved helix-loop-helix ubiquitous kinase	Involved in NF-kappa-B signaling
−0.538	GC33463	POLD1	Polymerase (DNA directed), δ1, catalytic subunit	DNA synthesis
−0.537	GC83208	SERBP1	SERPINE1 mRNA binding protein 1	Regulation of mRNA stability
−0.536	GC153558	CNOT8	CCR4-NOT transcription complex, subunit 8	Transcription factor
−0.535	GC17771	GOT1	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)	Involved in amino acid metabolism
−0.535	GC43091	NUDCD2	NudC domain containing 2	Unknown
−0.534	GC31949	HSPA9	Heat shock 70 kDa protein 9 (mortalin)	Control of cell proliferation and cellular aging
−0.533	GC91403	IKZF5	IKAROS family zinc finger 5 (Pegasus)	Transcriptional repressor
−0.533	GC31641	SF1	Splicing factor 1	Transcriptional repressor
−0.532	GC161453	KDM2B	Lysine (K)-specific demethylase 2B	Histone demethylase
−0.53	GC35520	EBP	CCAAT/enhancer binding protein (C/*EBP*), gamma	Transcription factor
−0.53	GC10226	ABCB7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	Heme transport

Information on gene functions was taken from the OMIM database, NCI, USA, (http://www.ncbi.nlm.nih.gov/Omim/) and from the GeneCard database of the Weizmann Institute of Science, Rehovot, Israel (http://bioinfo.weizmann.ac.il/cards/index.html).

hematopoietic system, that is, CCRF-CEM leukemia cells. *Sorbaria sorbifolia* was cytotoxic towards HepG-2 cells via induction of apoptosis and cell cycle arrest [18], and evidence of this activity towards CCRF-CEM leukemia cells is provided herein.

In the US NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity, if the IC_{50} value following incubation between 48 and 72 h is less than 20 μg/mL [19]. In this study, we reduced the cutoff point to 10 μg/mL. All extracts were tested at this concentration and only samples with an inhibitory effect >50% were considered to have highly promising activities against leukemia cells. Under this condition, 13 samples from 12 medicinal plants (Figure 1) were identified as promising anticancer products and should be screened for more cancer cell lines. It is noteworthy that only *Lycoris radiata* exhibited a significant (<50% growth proliferation) activity with both aerial and subterraneal parts, suggesting that different parts of the plant should be considered when screening the cytotoxicity of medicinal plants.

To the best of our knowledge, the cytotoxic effect of several active plants (Table 1) against leukemia cells was reported here for the first time. Nevertheless, some of the analyzed plants contain compounds with known cytotoxicity against cancer cells. In fact, the diarylheptanoids [1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-*O*-β- D-glucopyranosyl (1 → 3)-β-D-xylopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-heptane- 3-*O*-β- D-apiofuranosyl(1 → 6)-β-D-glucopyranoside; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-5-*O*-β- D-glucopyranoside, 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane; 1,7-bis-(3,4-dihydroxyphenyl)-heptane-3-one-5-*O*-β-D-glucopyranoside; oregonin; hirsutanonol; hirsutenone; 1,7-bis-(3,4-dihydroxyphenyl)-5-hydroxyheptane-3-*O*-β- D-xylopyranoside and platyphylloside], isolated from the bark of *A. japonica*, showed cytotoxic activities on human, B16 mouse melanoma cells and SNU-1 gastric cancer cell lines with IC_{50} values varying from 17.02 to 55.47 μM [20]. Interestingly, the extract of the stem bark of this plant exhibited significant antiproliferative activity on leukemia CCRF-CEM cells (Figure 1), inducing less than 25% growth at 10 μg/mL. In addition, Kim et al. [21] reported the presence of two well-known cytotoxic compounds, betulin and lupeol [22] in the extract of this plant. Apoptosis induction *via* Fas-mediated pathway was also reported in human MCF-7 breast adenocarcinoma cells by prodelphinidin B-2 3,3′-di-*O*-gallate, a constituent of *M. rubra* [23]. The presence of such cytotoxic compounds could explain the good activity of the crude extract of *A. japonica* and *M. rubra*. Similarly, the presence of cytotoxic compounds such as quercetin or ferulic acid [22], in *R. corchorifolius* and *S. middendorffianum* extracts [24, 25]

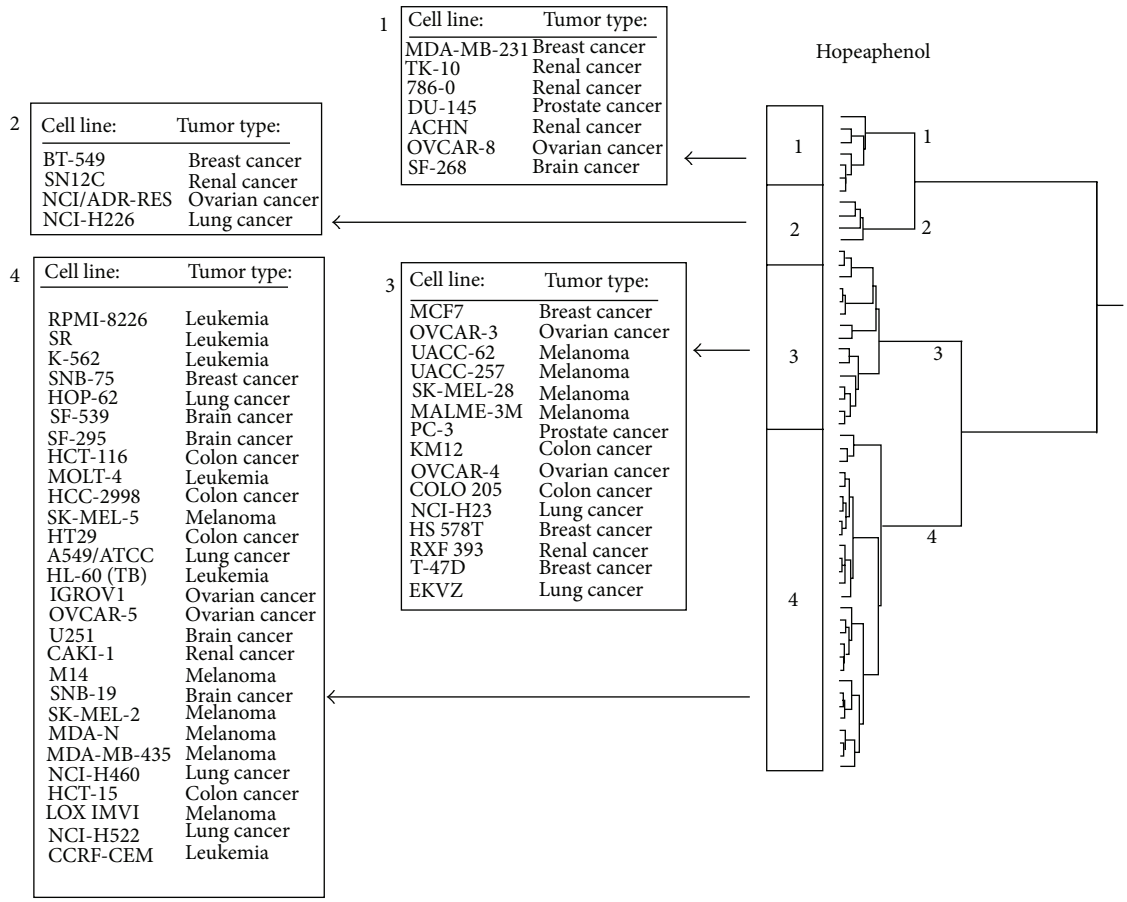

Figure 4: Dendrograms obtained by hierarchical cluster analysis of microarray-based gene expressions for hopeaphenol of the panel of NCI cell lines. The dendrograms were obtained by clustering using the WARD method.

and *Sedum takesimense* [26] could also provide some explanation on their antiproliferative potentials. Nonetheless, it is interesting to know that the activity does not only depend on the presence of a cytotoxic substance in a plant, but also their quantities and possible interaction with other plant constituents. For example, cytotoxic compounds such as incanone {known to be active on HL60 leukemia cells (IC_{50} value of 6 μM) [25]} or verbascoside [27] {active on human HEP-2 larynx epidermoid carcinoma, human RD rhabdomyosarcoma and human MCF-7 breast adenocarcinoma cell lines [28]} were reported in *Caryopteris incana*. But extract from *Caryopteris incana* did not show significant activities against CCRF-CEM leukemia cells in the present study.

We screened the NCI database of the Developmental Therapeutics Program of the NCI for the constituents of our panel of Korean medicinal plants. The two most cytotoxic compounds were hopeaphenol and deoxynarciclasine. Both compounds have been previously reported to be cytotoxic [29–32]. While hopeaphenol's activity has been demonstrated in mouse tumors *in vitro* and *in vivo* [31, 32], the present investigation shows that this compounds also active against human tumor cells. To the best of our knowledge, the mechanisms of action of these two compounds have not been investigated in the past and are addressed in our analysis for the first time.

4.2. COMPARE and Hierarchical Cluster Analyses of mRNA Microarray Data. To gain insight into possible modes of action of both phytochemicals, we investigated gene expression profiles of the NCI cell line panel. By microarray-based gene expression and COMPARE analyses, we correlated the IC_{50} values for both compounds of 60 tumor cell lines with transcriptomic mRNA expression levels of this cell line panel [12]. This approach has been successfully used to unravel the mode of action of novel compounds [33]. Cluster and COMPARE analyses are also useful for comparing gene expression profiles with IC_{50} values for investigational drugs to identify candidate genes for drug resistance [34, 35] and to identify prognostic expression profiles in clinical oncology [36, 37].

Eleven genes passing the correlation thresholds of $R > 0.5$ and $R < -0.5$ were identified to significantly correlate with sensitivity or resistance to hopeaphenol. Except for fibroblast growth factor 9 [38], none of them have been associated with response of tumor cells to cytostatic drugs. This point of view is supported by the fact that the NCI cell lines did not exert cross-resistance between hopeaphenol and anticancer drugs, such as doxorubicin, vincristine, cisplatin, and others.

The genes correlating with the response of tumor cells to deoxynarciclasine were also not known to confer drug resistance as of yet, but many of them belong to functional

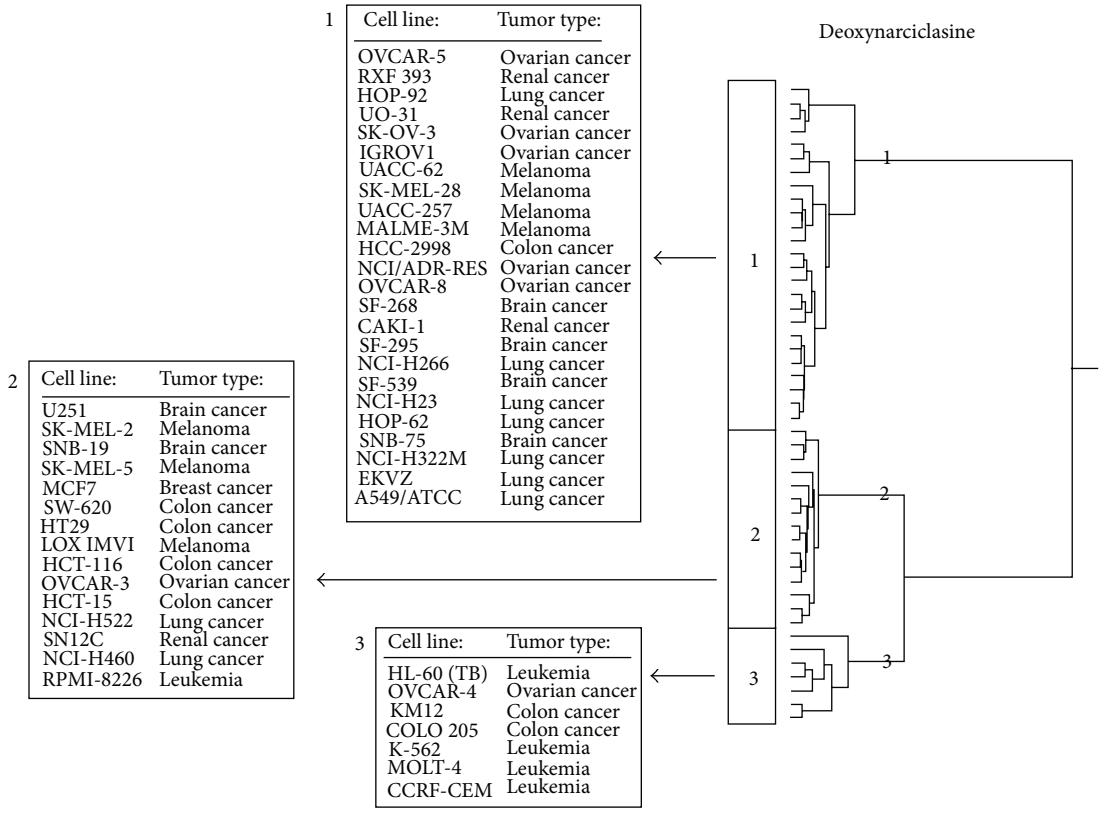

Figure 5: Dendrograms obtained by hierarchical cluster analysis of microarray-based gene expressions for deoxynarciclasine of the panel of NCI cell lines. The dendrograms were obtained by clustering using the WARD method.

groups, which are associated with response to chemotherapy. For example, transcriptional regulation, signal transduction, and cell proliferation are well-known processes influencing the success of cancer chemotherapy [39–42]. This might also explain why the NCI cell line panel exhibited cross-resistance between deoxynarciclasine and standard chemotherapy. On the other hand, genes involved in cellular trafficking or endoplasmic/sarcoplasmic reticulum functions have not been recognized as possible mechanisms of drug resistance of tumors. This finding merits more detailed investigations in the future.

4.3. Conclusion. The present work provides evidence that some plants derived from Korean medicine could be useful in the treatment of leukemia and supports the advanced investigation of the most active plants extracts. It also provides first pharmacological data on the cytotoxicity of some plants, such as *Adenophora racemosa, Cinnamomum japonicum, Eurya japonica, Sedum middendorffianum,* and *Vitis flexuosa*. The identification of cytotoxic phytochemicals from these plants, for example, hopeaphenol, and deoxynarciclasine, and the investigation of their possible molecular modes of action may foster the development of novel treatment strategies against otherwise drug-resistant and refractory tumors.

Conflict of Interests

No potential conflict of interests was disclosed.

Table 5: Separation of clusters of 60 cancer cell lines obtained by hierarchical cluster analysis for hopeaphenol and deoxynarciclasine. The $\log_{10}$ IC_{50} median values (M) of each compound were used as cut-off values to define cell lines as being sensitive or resistant. $P > 0.05$ was considered as not significant (χ^2 test).

	Sensitive	Resistant	χ^2 test
	Hopeaphenol		
Partition*	<−5.80	>−5.80	
Cluster 1	0	7	
Cluster 2	0	4	
Cluster 3	15	0	
Cluster 4	12	16	$P = 4.49 \times 10^{-6}$
	Deoxynarciclasine		
Partition*	<−6.40	>−6.40	
Cluster 1	3	22	
Cluster 2	13	2	
Cluster 3	7	0	$P = 1.76 \times 10^{-6}$

*$\log_{10}$ IC_{50} (M).

Authors' Contribution

Victor Kuete and Ean-Jeong Seo contributed equally to this paper.

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Review Article

Therapeutic Applications of Herbal Medicines for Cancer Patients

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Medicinal herbs and their derivative phytocompounds are being increasingly recognized as useful complementary treatments for cancer. A large volume of clinical studies have reported the beneficial effects of herbal medicines on the survival, immune modulation, and quality of life (QOL) of cancer patients, when these herbal medicines are used in combination with conventional therapeutics. Here, we briefly review some examples of clinical studies that investigated the use of herbal medicines for various cancers and the development of randomized controlled trials (RCTs) in this emerging research area. In addition, we also report recent studies on the biochemical and cellular mechanisms of herbal medicines in specific tumor microenvironments and the potential application of specific phytochemicals in cell-based cancer vaccine systems. This review should provide useful technological support for evidence-based application of herbal medicines in cancer therapy.

1. Clinical Uses of Herbal Medicine with Anticancer Effects

A range of clinical studies have indicated that a spectrum of anticancer activities from various herbal medicines can be detected. In this section, we have organized and classified the clinical use of a number of herbal medicines according to their suppressive effect on specific cancer types (Table 1).

1.1. For Breast Cancer. Although a specific role for vitamins and selenium in the prevention of breast cancer has not been established, some anticancer activities have been shown *in vitro* [1–3]. In a randomized controlled trial, 2972 patients with invasive or noninvasive breast carcinoma received either 200 mg of vitamin A preparation (Fenretinide) per day or no therapy. At 97 months posttreatment there was a significant reduction in recurrence of local breast cancer in premenopausal women (HR: 0.65; 95% CI: 0.46–0.92). However, no significant difference in metastasis or overall survival time could be demonstrated [4]. Interestingly, other studies have shown that long-term uptake of vitamin E may in fact have a negative effect on breast cancer patients [5, 6]. Currently, their rule seems to cause malabsorption or maldigestion in cancer patients suffering from a concomitant illness, moreover, providing patients adopt a balanced and healthy diet [4, 7].

Phytoestrogens are classified into water-soluble isoflavones and lipophilic lignans. Isoflavones are found in high abundance in soy beans, and lignans are found in linseeds wheat, fruit, flaxseeds, and vegetables [8, 9]. Among six related clinical trials conducted so far, only one concluded that isoflavone was associated with a reduced risk of breast cancer [10]. Soy-derived phytoestrogens are popularly recommended for treating postmenopausal symptoms in women with breast cancer undergoing tamoxifen therapy. The principal constituents of soy bean plant extracts, including isoflavones genistein and daidzein, are structurally similar to 17β-estradiol and can confer weak estrogenic effects [11]. However, there is no evidence to support the recommendation of use of phytoestrogens either in treating breast cancer or for easing climacteric symptoms [12].

Investigations of traditional Chinese medicines (TCM) have uncovered a number of antibreast cancer agents, although most of their mechanisms of action have not yet been elucidated. These TCM herbs with antibreast cancer activities can be classified into six categories: alkaloids [13, 14], coumarins [15, 16], flavonoids and polyphenols [17, 18], terpenoids [19], quinone [20], and artesunate [21] (Table 1).

Table 1: Clinical use of herbal medicines exhibiting anticancer activities.

Herbal medicine	Suppressive effects on carcinogenesis and cancer metastasis	References
	For breast cancer	
Vitamin A (fenretinide)	200 mg/day significantly reduces the recurrence of local breast cancer in premenopausal women	[4]
Vitamin E	Leads to malabsorption or maldigestion in cancer patients; balanced and healthy diet	[5, 6]
Isoflavone	To reduce risk of breast cancer	[10]
Isoflavones genistein and daidzein	To confer weak estrogenic effects	[11]
Alkaloids	Inhibition of cancer cell growth	[13, 14]
Coumarins	Inhibition of cancer cell growth	[15, 16]
Flavonoids and polyphenols	Antiproliferation	[17, 18]
Terpenoids	MCF-7 cell apoptosis	[19]
Quinone	To induce G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells	[20]
Artemisunate	Decrease the proliferation of human breast cancer cells from expressing a high ERα : ERβ ratio	[21]
	For prostate cancer	
Vitamins A-D and retinoid	Maintain homeostasis and prevent various metabolic disorders	[23]
Vitamin E	Reduce the risk of lethal or advanced prostate cancer relative to nonusers	[30]
Epigallocatechin-3-gallate (EGCG)	Arrest LNCaP and DU145 prostate cancer cells at the G0-G1 phase of the cell cycle	[34]
	Inhibit metalloproteinase *in vitro*	[35]
Soy isoflavones	Inhibit 5α-reductase activity	[37]
	Chemopreventive activities	[22]
Scutellaria baicalensis (baicalin)	Inhibit enzymatic synthesis of eicosanoids	[38]
Baicalein	Impair the proliferation of androgen-independent PC-3 and DU145 prostate cancer cells in culture	[39]
	Induces cell-cycle arrest at the G0-G1 phase	[39]
	Induces apoptosis of prostate cancer cells at concentrations achievable in humans	[40]
	Suppresses the expression of specific androgen receptor in prostate cancer	[40]
Lycopenes	Decreases prostate cancer risk	[41]
	Diminishes oxidative damage in lymphocytes	[42]
	Significantly decreases levels of PSA and less oxidative damage	[42]
PC-SPES	Decreases serum testosterone concentrations ($P < 0.05$); decreases serum concentrations of prostate-specific antigen	[43]
	Antitumor efficacy against cancer cell lines	[44]
Wedelia chinensis (Asteraceae)	Inhibits the androgen receptor (AR) signaling pathway	[47]
	For lung cancer	
Platycodon grandiflorum (Campanulaceae)	Anticancer effect in lung cancer patients	[49–51]
Morus alba (Moraceae)	Anticancer effect in lung cancer patients	
Prunus armeniaca (Rosaceae)	Anticancer effect in lung cancer patients	
Rhus verniciflua (Anacardiaceae)	Anticancer effect in lung cancer patients	
Perilla frutescens (Labiatae)	Anticancer effect in lung cancer patients	
Stemona japonica (Stemonaceae)	Anticancer effect in lung cancer patients	
Tussilago farfara (Compositae)	Anticancer effect in lung cancer patients	
Draba nemorosa (Brassicaceae)	Anticancer effect in lung cancer patients	

Table 1: Continued.

Herbal medicine	Suppressive effects on carcinogenesis and cancer metastasis	References
	For liver fibrosis and cancer	
Inchin-ko-to (TJ-135)	Preventive effect on liver fibrosis	[53]
Yi Guan Jian (YGJ)		[53]
Yi Guan Jian (YGJ)		[54]
Fufang-Liu-Yue-Qing		[55]
Danggui Buxue Tang (DBT)		[56]
Salvianolic acid B		
Curcumin	Suppressive effect on hepatic fibrogenesis and carcinogenesis	[57]
Oxymatrine		
Compound 861	Suppressive effect on hepatic fibrogenesis	[58, 59]
Sho saiko-to (TJ-9)	Reduces/limits the progression of hepatocellular carcinoma	[60]
	For pancreatic cancer	
GDC-0449, IPI-926, XL-139 and PF-04449913	SMO antagonists; deregulation of sonic hedgehog homology (SHH)	[61]
Cyclopamine	Inhibit SHH signaling by directly binding to the 7-helix bundle of the SMO protein; arrest the growth of pancreatic tumors	[62]
	Weakens the recruitment of BMPCs into cancer cells and reduces the formation of tumor vasculature	[63]
	The cancerous vascular system becomes unstable after treatment with cyclopamine due to the expression of angiopoietin-1	[63]

Some of these phytochemicals, such as curcumin and artemisinin, have well-known chemical structures. Compounds in these categories have been taken as health foods or dietary supplement for decades. However, evidence-based *in vivo* studies and clinical trials are still recommended for routine public use or specific clinical applications.

1.2. For Prostate Cancer. Prostate cancer is characterized by a long latency period, a strong dietary influence, and limited treatment strategies for the advanced disease; therefore, many patients turn to complementary and alternative medicine (CAM) with the belief that these medicines represent a viable therapeutic option that may be free of adverse side effects [22]. This folkloric belief, strongly upheld in many Asian cultures, needs to be substantiated with systematic, evidence-based research. Vitamins, including vitamins A–D and retinoids, are organic compounds that cannot be synthesized by humans and must be ingested to maintain homeostasis and prevent various metabolic disorders [23]. Emerging evidence indicates that inflammation might have a crucial role in the genesis of prostate carcinoma [24–26]. A number of clinical trials have sought to evaluate the anti-inflammatory activities of vitamins on prostate carcinogenesis [27]. Despite a lack of convincing evidence, vitamin and mineral supplements are used extensively by patients that are diagnosed with prostate cancers [28, 29]. The belief is that such supplements might actually prevent or treat inflammation-associated disease and at the very least not cause harm [22]. Among smokers, daily ingestion of >100 IU of vitamin E was reported to produce a 56% reduction in risk in lethal or advanced prostate cancer relative to nonusers [30]. On the other hand, a selenium and vitamin E chemoprevention trial (SELECT) that aimed to determine whether vitamin E and/or selenium supplementation could reduce prostate carcinogenesis showed that dietary supplementation with vitamin E in fact statistically increased the risk of prostate cancer among healthy men [31, 32]. Together these clinical data suggest that the application of vitamin E may be specific for only treating inflammation-associated features in prostate cancer patients, rather than affecting anticancer activity. Future studies are needed to address such an apparent contradiction between effects in healthy and cancer populations.

The use of medicinal herbs and their derivative herbal extracts that contain numerous polyphenolic compounds may contribute to the lower incidence of prostate cancer in Asian populations relative to Caucasians and African Americans [33]. Many polyphenols (e.g., isoflavones) are phytoestrogens that can bind to estrogen receptors and elicit estrogenic effects in target tissues or organs. Some specific compounds, in particular the four active polyphenolic compounds in green tea, epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG), the soy isoflavones, as well as *Scutellaria baicalensis*, β-carotene, and the lycopenes have all been studied for their effect on prostate carcinoma [22]. Using specific bioassays, EGCG in green tea was demonstrated to arrest LNCaP and DU145 prostate cancer cells at the G0-G1 phase of the cell cycle [34] and inhibit metalloproteinase *in vitro*, although the effect was achieved at a much higher concentration than the serum levels detected in humans who consumed moderate amounts of green tea [35]. In a Phase II study of green tea in the treatment of patients with androgen-independent metastatic prostate carcinoma, one patient achieved a prostate-specific antigen (PSA) response of >50% that lasted

for approximately one month. Patients, however, suffered marked symptoms of toxicity in this study, most notably diarrhea, nausea, and fatigue. Investigators concluded that green tea has limited antineoplastic activity, as defined by a decline in PSA levels, against androgen-independent prostate cancers [36]. For soy isoflavones, they have been shown to inhibit 5α-reductase activity, the enzyme that is functionally responsible for the conversion of testosterone to the more potent androgen dihydrotestosterone [37]. Several preclinical randomized studies have also evaluated the potential therapeutic effects of soy isoflavones as chemopreventive agents [22]. For *Scutellaria baicalensis*, it contains very high levels of *baicalin*, a flavone glycoside that inhibits enzymatic synthesis of eicosanoids, which are important mediators of inflammation and prostate tumor cell proliferation [38]. Baicalein (a flavone) impairs the proliferation of androgen-independent PC-3 and DU145 prostate cancer cells in culture and induces cell-cycle arrest at the G0-G1 phase [39] and apoptosis at concentrations achievable in humans [40]. Baicalein drastically suppressed the expression of specific androgen receptor in prostate cancer at clinically achievable concentrations [40]. For lycopenes, although most studies have used mixtures of tomato products, the association of decreased prostate cancer risk and tomato product intake has led to the specific focus on the use and effect of lycopene [41]. Chen and colleagues studied the effect of lycopene levels and measures of oxidative damage in patients undergoing prostatectomy for localized prostate cancer [42]. They found that oxidative damage in lymphocytes from these patients was diminished after dietary intervention compared with pretreatment levels and that the prostate tissues in treated patients revealed significantly decreased levels of PSA and less oxidative damage. Since it still remains uncertain whether lycopene itself causes the effect, or whether a more complex food extract is responsible, additional randomized trials are needed to assess the efficacy of lycopene in chemoprevention activities.

Sales of the herbal extract PC-SPES as a dietary supplement for "prostate health" began in the mid-1990s. The name PC-SPES is derived from PC for "prostate cancer" and "spes," the Latin word for hope. This botanical mixture is used primarily for treatment of prostate carcinoma [43]. The formulation contains extracts of eight herbs, *Ganoderma lucidum*, *Scutellaria baicalensis*, *Rabdosia rubescens*, *Isatis indigotica*, *Dendranthema morifolium*, *Serenoa repens*, *Panax notoginseng*, and *Glycyrrhiza uralensis*, that were selected based on either their use in Chinese medicinal therapy for urinary problems or their antitumor efficacy against cancer cell lines [44]. In addition, a series of clinical studies have described the effects and mechanisms of PC-SPES activity [45]. Although the therapeutic application of PC-SPES seemed to be promising, unfortunately, PC-SPES was recalled and withdrawn from the market because certain batches of testing PC-SPES samples were found to be contaminated with US Food and Drug Administration-(FDA-)controlled prescription drugs. To our knowledge, the FDA has not approved so far the use of PC-SPES in cancer treatment. More evidence and correlative information to demonstrate the *in vitro* and clinical efficacy of this herbal mixture are needed.

Lin et al. [46] showed that *Wedelia chinensis* (Asteraceae), an oriental medicinal herb containing various compounds such as indole-3-carboxylaldehyde, wedelolactone, luteolin, and apigenin, is capable of suppressing androgen activity. Moreover, oral administration of *W. chinensis* extract impeded prostate cancer tumorigenesis. This anticancer action of *W. chinensis* extract was subsequently demonstrated to be due to three active compounds that can inhibit the androgen receptor (AR) signaling pathway [47]. Recently, our own study showed that a different set of phytochemicals extracted from *W. chinensis* plants can confer potent and specific anti-inflammatory bioactivities *in vitro* and *in vivo*. These activities resulted in strong anticolitis activities in test mice [48]. Future studies of *W. chinensis* for chemoprevention or as a complementary medicine against prostate cancer in humans are warranted.

1.3. For Lung Cancer. Lung cancer is one of the most deadly cancers, and the lung is a common site of metastasis of tumors from other tissues in the body. Standard chemotherapy regimes often have limited survival benefits due to the severe toxicity [49, 50] of the various anticancer agents, such as gemcitabine, paclitaxel, docetaxel, etoposide, and vinorelbine. Recent reports have suggested that herbal medicines and their phytochemicals which seem to have lower or little toxicity may provide an attractive strategy for lung cancer therapy. Traditionally, herbal plants such as *Platycodon grandiflorum* (Campanulaceae), *Morus alba* (Moraceae), *Prunus armeniaca* (Rosaceae), *Rhus verniciflua* (Anacardiaceae), *Perilla frutescens* (Labiatae), *Stemona japonica* (Stemonaceae), *Tussilago farfara* (Compositae), and *Draba nemorosa* (Brassicaceae) have been used to treat lung cancer [51]. Clinically, the proportion of patients that use herbal medicines as adjuvants alongside conventional (e.g., chemotherapy) treatment for lung cancer is as high as 77% [52]. Herbs are mainly used in lung cancer to reduce therapy-associated toxicity and cancer-related symptoms and sometimes to directly increase anticancer effects [4]. However, it is important to note that some CAM methods or remedies may have adverse effects or reduce the efficacy of conventional treatment, and the primary justification for use of traditional herbal medicines remains empirical evidence, case studies, and hypothetical physiological effects [4].

1.4. For Liver Fibrosis and Cancer. Liver fibrogenesis is a gradual process of increased secretion and decreased degradation of extracellular materials, which can be initiated by activation of hepatic stellate cells (HSCs) [64, 65]. The number of deaths due to hepatocellular carcinoma (HCC) has steadily increased over the last decade. Unfortunately, there are no successful, clinically satisfactory therapies for patients suffering HCC. Herbal medicines are being considered as one possible strategy against liver fibrosis and HCC. Three medicinal herbs are already used as official drugs in China, Japan, and other parts of Asia. Different chemically induced fibrosis models were designed using the rat liver system to assess the preventive effects of specific herbal extracts on liver fibrosis. Formulations assessed include Inchin-ko-to (TJ-135) [53], Yi

Guan Jian (YGJ) [53], Yi Guan Jian (YGJ) [54], Fufang-Liu-Yue-Qing [55], and Danggui Buxue Tang (DBT) [56]. In 2007, Luk et al. [57] provided a systematic review of the mechanisms of action of medicinal herbal compounds, such as salvianolic acid B (SAB), oxymatrine, and curcumin in the treatment of hepatic fibrogenesis and carcinogenesis. Although some of these herbal medicines, such as YGJ, are traditionally used to treat human liver fibrosis, the therapeutic or clinical anticancer effect of these herbal mixtures in liver tissue has not been fully elucidated. The further identification of as yet unknown effective components in these herbal extract is critical for their pharmacological use and improvement.

A combination of 10 herbs (named compound 861), including *Salvia miltiorrhiza* (sage), *Astragalus membranaceus*, and *Spatholobus suberectus* known in TCM as the "king herb" components of the formula, that is, the herbs that are pharmacologically active, and seven others (modifiers of toxicity that act synergistically with the king herbs to improve immune function), has been tested in a number of experimental studies for antifibrotic properties. Two uncontrolled open trials of 60 and 22 patients with chronic hepatitis B who were treated with compound 861 reported a beneficial effects on liver fibrosis, with the majority of treated patients showing both clinical and histological improvement [58, 59]. Since these clinical studies of compound 861 did not satisfy quality control criteria, clinicians consider that additional well-designed trials are needed for routine and authorized clinical use of compound 861 for the treatment of hepatitis B-induced liver fibrosis.

A large number of clinical reports have indicated the therapeutic effects of one Japanese traditional medicine (*kampo yaku*) Sho saiko-to (TJ-9). This medicine is a combination of seven herbs traditionally used for treating liver diseases [66, 67]. However, little clinical data on the efficacy of TJ-9 in preventing human liver cancer has been reported. In a long-term (5 years) randomized controlled study, patients that were positive for hepatitis B surface antigen (HBsAg) received a dose of 7.5 g/day aqueous TJ-9 extract together with the standard treatment using interferon. Upon followup, the cumulative development of hepatocellular carcinoma (HCC) was found to be significantly lower than that of the controls (i.e., patients without TJ-9 treatment) [60]. Unfortunately, TJ-9 is contraindicated for patients with hepatic cirrhosis or acute respiratory failure in Japan, because some of these patients were found to contract interstitial pneumonia after drug administration [68]. Therefore, well-designed future trials that can address the specificity of TJ-9 or its major active components in inhibition or suppression of the progression of viral hepatitis-induced hepatocellular carcinoma or metabolic liver cancers are needed [69].

1.5. For Pancreatic Cancer. Smoothened (SMO), a component of the sonic hedgehog homology (SHH) signaling pathway, has been shown to play a key role in the cellular behavior of cancer stem cells [70]. The deregulation of SHH was considered as an important factor that can drive or maintain the progression of pancreatic cancer [71]. There are some SMO antagonists that, such as GDC-0449, IPI-926, XL-139, and PF-04449913, are being evaluated with high hope for treatment of pancreatic cancers [61]. Cyclopamine, a steroidal alkaloid extracted from *Veratrum californicum*, can efficiently inhibit SHH signaling by directly binding to the 7-helix bundle of the SMO protein. This complex can further impact upon the function of 12-transmembrane receptor patched-1 (PTCH-1) and thereby influence the structure of SMO [62]. It needs to be noted here that cyclopamine not only can weaken the recruitment of bone marrow precursor cells (BMPCs) into cancer cells, but also can reduce the formation of tumor vasculature [63]. The cancerous vascular system becomes unstable after treatment with cyclopamine due to the expression of angiopoietin-1, an angiogenic factor found in the tumor microenvironment, which is under the regulation of SHH. Cyclopamine has been explored as an SMO activity suppression agent and to arrest the growth of pancreatic tumors [63]. Encouraging findings suggest that this phytochemical obtained from a traditionally used TCM herb should be systematically explored in the future for efficacious SMO-targeting anticancer drugs.

2. Use of Herbal Supplements as Adjuvants in Conventional AntiCancer Therapies

Numerous Chinese herbal medicines are being used in combination with chemotherapy or radiotherapy to improve the efficacy of cancer therapy and reduce side effects and complications (Figure 1), although this practice is highly frowned upon by many western physicians. Understanding of the use of specific herbal medicines as adjuvants to conventional therapy, therefore, needs to be increased in consultation and coordination with physicians and other health care providers. This section outlines evidence for use of herbal medicines as adjuvants to conventional drug-based, chemo- or radiotherapy regimes in cancer treatment. On the other hand, this section also summarizes the challenges or limitations for clinical use of these herbal medicines.

2.1. Common Use of Herbal Medicines as Adjuvant Treatment in Chemo- or Radio-Cancer Therapy. For the above adjuvant anticancer therapy studies, herbal medication in general was applied as a combination therapy with the conventional chemotherapy to hopefully increase the therapeutic benefit and quality of life (QoL) as well as to decrease the side effects or complications. Between 28% and 98% of ethnic Chinese cancer patients in Asia [72–74] and 25% to 47% of those living in North America are reported to have used herbal medicines as part of their cancer care [75, 76]. Although a number of herbal medicines have been found to be adjunctive in chemo- and radiotherapy, most clinical trials or studies have been reported mainly, if not only, in China or other Asian countries and they are virtually not cited on PubMed. In 2010, Qi and colleagues [77] provided a systematic review of Chinese herbal medicines in clinical trials, mainly as adjuvant treatments to reduce complications and side effects of chemo- or radiotherapy. Several traditionally used Chinese herbal medicines, including astragalus [78, 79], Turmeric (curcumin)

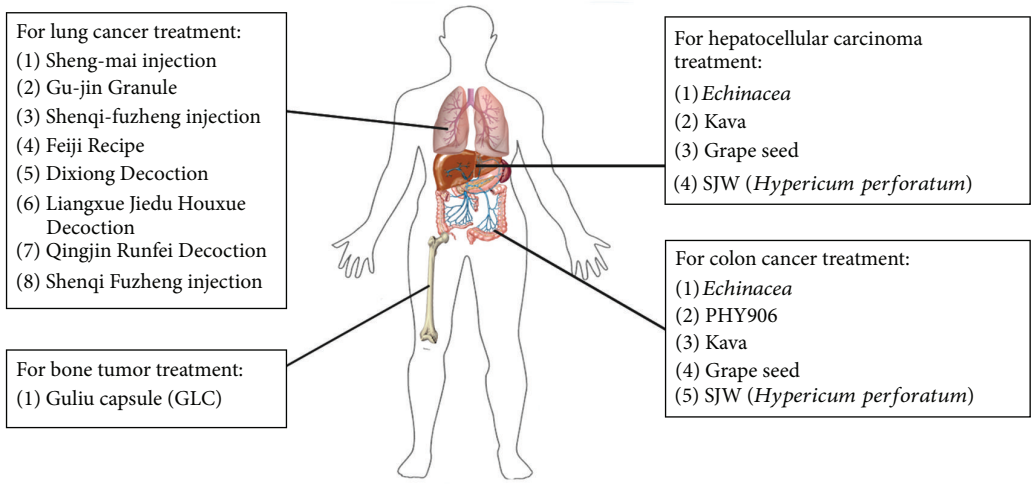

Figure 1: Medicinal herb "extracts" or "formulations" being tested as adjuvant treatments for chemo- or radiotherapies against various cancers.

[80–82], Ginseng [83–85], TJ-41 (Bu-Zhong-Yi-Qi-Tang) [86, 87], PHY906 [74, 88–90], Huachansu [91, 92], and Kanglaite [93, 94], are commonly used by cancer patients to either "treat" cancer and/or "reduce the toxicity" induced by chemotherapy or radiotherapy. Preclinical and clinical studies have indicated that these herbal medicines may possess a number of advantages in terms of suppression of tumor progression, by increasing the sensitivity of chemo- and radiotherapeutics, improving immune system function, and easing the tissue/physiology damage caused by chemo- and radiotherapeutics. However, most studies to date are empirical (i.e., not well controlled) clinical studies or observations that mainly report reduction in side effects and complications during or after chemotherapy and radiotherapy. Some traditional herbal formulations, including Bojungikki-tang [87], Kang-Fu-Zhi-Tong [95], PHY906 [88], Xiao-Chai-Hu-Tang, Huang-Lian-Jie-Du-Tang, and Yin-Chen-Wu-Ling-San [96], have been observed, "detected," or "claimed" to protect liver function, reduce cancer-related fatigue and pain, improve respiratory tract infections and gastrointestinal side effects, and even ameliorate the symptoms of cachexia. Often, these clinical results do not meet the standard US FDA requirements for clinical trials, but they may still offer some insight into traditionally used herbal medicines as adjuvant treatments for cancers. They may also provide useful pointers for the development of future botanical drugs as cancer primary or adjuvant therapies [74, 77, 97].

2.2. For Lung Cancer. In a randomized controlled trial (RCT) with 63 patients with non-small-cell lung cancer (NSCLC), Sheng-mai Injection (Ya'an Sanjiu Pharmaceutical Co., China) and Gu-jin Granule (Jiangyin Tianjiang Pharmaceutical Co., China) were observed to enhance median survival time ($P = 0.014$) and response rate increase to 48.5% (16/33), compared to the untreated control (32.2% = 9/28) in the control group ($P = 0.0373$), while all test groups were treated with a combination of navelbine and cisplatin (NP) chemotherapy [98]. In another clinical trial with Shenqi-fuzheng injection (Lizhu Co., China) among 232 NSCLC patients enrolled, herbal injection significantly improved the response rate and QoL of lung cancer patients, evaluated by using the QoL scale of European Organization for Research on Treatment of Cancer (QLQ-C30) [99]. Furthermore, the randomized controlled trial for Feiji Recipe treatment was also observed to enhance the clinical therapeutic efficacy and alleviate side effects of chemotherapy [100], as shown with an increase on scores in role, social, and economic status ($P < 0.05$ or $P < 0.01$), again based on QLQ-C30 questionnaire [101]. Recently, Xu et al. [102] applied a high quality of clinical trial methodology to examine the effect of TCM on improving QoL of postoperative non-small-cell lung cancer (NSCLC) patients. They clearly presented the design and protocol for a placebo-controlled, double-blinded RCT and were able to systematically provide evidence for the effectiveness of chemotherapy combined with TCM in improving QOL of postoperative NSCLC patients. The result is expected to provide support for integrative optimization of "combined" treatment of lung cancer patients.

One of the major risks of conventional treatment in lung cancer patients is radiation pneumonitis, caused by radiotherapeutic intervention [103]. Increasing evidence has been reported on the beneficial efficacy of certain herbal medicines such as Dixiong Decoction [104], Liangxue Jiedu Huoxue Decoction [105], Qingjin Runfei Decoction [106], and Shenqi Fuzheng injection [107] (Figure 1). These herbal formulations were reported to significantly lower the incidence of radiation pneumonitis and improve clinical radiographic physiologic (CRP) dyspnea score and the Radiation Therapy Oncology Group (RTOG) grading score, in groups of NSCLC patients undergoing radiotherapy treatment. These findings also revealed some of the possible adverse effects and potential uses of specific herbal medicines in combinational therapy alongside conventional chemotherapy. The broad range and heterogeneity of herbal medicine intervention and the resultant effects still pose a challenge to high-powered analysis of specific herbal medicines and their applications for evidence-based use in cancer therapies. Therefore, high level quality control to ensure consistent batch preparation and

systematic pharmacokinetic studies are required for all test herbal medicines and their activity against lung cancer [108], not only in human studies, but also in experimental animal systems to support evidence-based application.

2.3. For Colon Cancer. In oncology, drug interactions are important because of the narrow therapeutic index and inherent toxicity of many anticancer agents [109]. Previous studies indicated that the activity of cytochrome P450 enzymes (CYP enzymes) in the gastrointestinal wall is one of the most important factors that can alter the bioavailability of orally administered anticancer agents that are substrates of CYP3A [110]. A number of herbal supplements including *Echinacea*, kava, grape seed, and St John's wort (*Hypericum perforatum*) are also considered to be inducers of CYP [111] (Figure 1). Because of the increased use of herbal products by cancer patients, more consideration needs to be given to their combined use with anticancer agents [109, 112]. The administration of St. John's wort was shown to induce intestinal and hepatic expression of CYP3A [113] and be beneficial for the metabolism of irinotecan [114], a camptothecin derivative that can result in DNA damage on interaction with topoisomerase I. St. John's wort is hence used empirically in the treatment of metastatic carcinoma of the colon or rectum.

Recent studies based on epidemiological modeling have demonstrated interesting patterns suggesting that herbal treatment may improve prognosis in advanced colon cancer patients when used as an adjuvant therapy [115, 116]. The therapeutic mechanisms of traditional Chinese medicine in metastatic cancer have been discussed in terms of a hypothetical, dualistic antiproliferation and immune-stimulation model of tumor progression and regression [117]. Clinically, between 30% and 75% of patients with colon cancer are estimated to use CAM, but systematic or statistical evidence of survival efficacy is still limited. In one study with a 10-year followup of colon cancer patients ($n = 193$) who presented to a San Francisco Bay Area Center for Chinese medicine, authors compared the survival rate in patients choosing a short-term treatment regime lasting for the duration of their chemotherapy/radiotherapy period with those choosing a continuing long-term treatment. They also compared the survival of patients treated with Pan-Asian medicine plus vitamins (PAM+V) with that of concurrent external controls from the Kaiser Permanente Northern California and California Cancer Registries [118]. In this study, some modern methods, including Kaplan-Meier and traditional Cox regression, were used for analyses of causal inference, namely, propensity score and marginal structural models (MSMs), which have not been previously used in studies of cancer survival in response to treatment with Chinese herbal medicine. Results indicated that PAM+V combined with conventional therapy, as compared with conventional therapy alone, reduced the risk of patient death at stage I by 95%, stage II by 64%, stage III by 29%, and stage IV by 75%. No significant difference was observed between short-term *versus* long-term PAM+V administration [118]. This was apparently a sound clinical investigation and suggests that prospective trials combining PAM+V with conventional chemotherapy/radiotherapy may be clinically justifiable in future systematic studies.

Accumulating clinical studies show that some TCM preparations, including Pi-Sheng Decoction and Yi-Qi-Zhu-Yu Decoction, may be useful in reducing side effects and enhancing the drug effect of chemotherapy for colorectal cancer [119]. For preventing recurrence and metastasis, Jian-Pi-Xiao-Liu Decoction, Fu-Zheng Capsule, and Qu-Xie Capsule were used to decrease the recurrence and metastasis of colorectal cancer in a subsequent consolidation therapy after radical resection of patient's tumor. For improving the quality of life, Jian-Qi-Jie-Du Decoction, Jian-Pi-Yi-Qi Decoction, Fu-Pi-Yi-Wei Decoction, and Ai-Di injection were reported to enhance the antitumor "curative" effect of chemotherapy, reduce the side effects of chemotherapy, improve the immune function, and extend survival time in colorectal cancer patients. However, with the advancement of colorectal cancer treatment model, TCM theories and clinical studies on the typing of syndrome differentiation apparently are still lagging behind. In addition, current studies often have not addressed the issues on the anticancer properties or the observed beneficial health maintain/survival effects of treated TCMs. It is not only desirable, but also in fact necessary to further study the action model and the associated biochemical and physiological mechanisms of these anticancer mode herbs, as a milestone for future TCM research [119].

2.4. For Hepatocellular Carcinoma. The traditional Chinese medicine term or pathological classification of unresectable hepatocellular carcinoma (UHCC) is "liver stasis" [120]. Many clinical studies from China have indicated that TCM, such as Shentao Ruangan pills and hydroxycamptothecin, plus chemotherapy can significantly alleviate the symptoms, enhance therapy tolerance, stabilize tumor size and augment immunological function, reduce the incidence rate of adverse events, and prolong survival time of UHCC patients [121–123]. Although these studies may be criticized individually for lacking quality at the international level, together they do seem to suggest that the administration of TCM may warrant additional trials for patients with UHCC. Future clinical trials with TCM for UHCC need to have sufficient methodological quality and should be pursued in accordance with the Consolidated Standards of Reporting Trials (CONSORT) statement (see Section 3). In particular, rigorously designed, multicenter, large, randomized, double-blind, controlled trials are necessary [124].

2.5. For Other Cancers. Over the past two decades, a number of Chinese herbal medicines have been noted for their radiosensitization and radioprotection effects during radiotherapy of cancers, including bone cancer as well as head and neck tumors [125]. Cho and Chen [126] reported that a combination of TCM with radiotherapy not only enhanced therapeutic outcomes, but also improved the performance status of patients with nasopharyngeal cancer. Su and colleagues [127] consistently found that Guliu capsules (GLC) combined with Sr-89 conferred therapeutic effects in the treatment of metastatic bone tumors. They found that combined GLC and Sr-89 treatment was effective against metastatic bone tumor and improved patients' QOF enhancing ostalgia relief rate

and decreasing hemotoxicity. In brain tumor treatment, Quan and colleagues [128] also reported that TCM, in combination with radio- or chemotherapy, had an effect on tumor growth inhibition, survival time, and QOL enhancement in brain tumor metastases. These findings further indicate the potential application of TCM in the therapy of different cancers (Figure 1).

2.6. Challenges for the Use of Herbal Medicine in Cancer Therapy. Although traditional herbal medicines, phytomedicines, medicinal foods, and complementary or alternative medicines have been increasingly used over the past decade in European and North American countries, they seem to have not generated interest or been accepted by mainstream medicine practitioners in western countries, especially in standard care for cancer patients. The key issue considered by many biomedical scientists has been the lack of evidence-based information/guidelines for routine and regulatory application of herbal medicines as "drugs" for use in public health. The sticking points hindering the use of phytomedicines can be attributed to six major issues: (1) lack of consistent and reliable sources of authentic medicinal plant materials, with respect to species verification and authentication, cultivation using good agricultural practice protocols, and standardized/normalized methods and technology for plant extraction/mixture preparation; (2) lack of definitions and routine preparation of the biochemical/biological ingredients and compositions of herbal medicines or the phytochemicals/phytocompounds derived from medicinal plants, with respect to identification of metabolite profiles, index compounds, and putative active compounds or metabolites; (3) general and specific safety considerations, including tolerable high dosage, minimal effective dosage, and specific usage; (4) proof of efficacy in treating or assisting specific cancer patients, including lack of results/data from preclinical animal studies, execution of bona fide, and double-blind, placebo-included, statistician-assisted clinical trial studies; (5) highly complex "personalized" prescriptions or formulations for the use of some traditional medicines (e.g., in TCM) that may be mystified by a "secret ingredient" in specific formulations; and (6) the criminal act of supplementation/"spiking" highly potent western chemical drugs into herbal medicines in counterfeit activities. Without addressing all of the above issues, we cannot meet the challenges of modernizing herbal medicines. Although we have reviewed a spectrum of laboratory, preclinical and clinical studies on potential applications of herbal medicines for cancer patients' care in an inclusive fashion, a great many of these studies did not follow the stringent requirements, procedures, and protocol needed for developing western style drug or medicinal foods. Systematic and correlated efforts among researchers of our scientific communities are therefore urgently needed.

It is also important to note that the central tenet in recent western medicine is that a drug should be composed of well-known chemical components or a pure single compound that selectively interacts with known and specific molecular target(s) in our body system. However, the search for single molecules that can modify single or highly specific key factors in a disease process is now recognized as a difficult and sometimes inappropriate strategy, because a large volume of studies on genomics, proteomics, and metabolomics studies have shown that many clinically used commercial drugs (e.g., aspirin, doxorubicin, etc.) may in fact bind and work on multiple molecular targets. Multiple cell types, target molecules, and/or multiple signaling pathways are known to contribute to various diseases. Herbal extracts/mixtures prepared as traditional phytomedicines represent a combinational chemistry and "thus claimed" to encompass a vast and useful repertoire of chemical entities that can confer a complex and yet integrated effect on a spectrum of molecular and cellular components and functions, resulting in a profound and balanced medicinal activity. Unfortunately, according to the current FDA and NIH cancer clinical trial regulations in USA, such "claims" often directly conflict with the present guidelines or guidance. One major drawback in the integration of herbal medicines into mainstream western medicines is, therefore, the lack of defined molecular targets. With regard to this concern, recent research findings revealed from a spectrum of omics studies strongly suggest that a multifactorial mode of action and multitarget pharmaceutical activity may in fact already be the "norm" for a spectrum of currently used clinical drugs. As a result, there may be much less difference in terms of the complexity of molecular targets aimed by single compound drugs versus complex herbal medicine extracts than was originally assumed, as we previously demonstrated in a cancer cell line study [129]. We may then further project that the "multi-target" approach or activity believed for various herbal medicines may in fact be "reasonable and understandable" and therefore be positively considered and prepared in botanical drug development. Pooling data from individual trials by using a meta-analysis approach may be a useful strategy to interpret at the results of a group of inconclusive trials [130].

The uncertain or not well-defined composition of herbal products also raises questions about their safety, such as the evidence indicating that some herbal extracts may have harmful interactions with specific prescription drugs [131]. To address this issue, the establishment of optimized CMC (chemistry, manufacture, and control) conditions for each herbal preparation will need to be considered as important technology for confirming and standardizing the composition of specific medicinal herb components. Toward this aim, we believe that the pattern-oriented approach (fingerprint analysis) is a good strategy, because it can evaluate the integrative and holistic properties of test herbal medicines by comparing the similarities, differences, and correlation of the results from analyses of the whole production process, including manufacture, processing and storage of raw materials for preparation, intermediate products, finished products, and the distribution products [132, 133]. Yongyu and colleagues [134] have systematically reviewed fingerprint methods for analyses of herbal medicines. The fingerprint profiling of therapeutically used herbal medicines can be employed as a reference or index for quality control of phytochemical composition, and the results can be used in future clinical applications. Furthermore, the fingerprinting profiles can also be coordinated with and employed for therapeutic

efficiency. This study approach was recently evaluated by an investigation of randomized controlled clinical trials (see Section 3).

In order to treat specific diseases, it is desirable that "modern drugs" can be generally applied to most patients with the same disease, although personalized medicine is becoming more popular. In traditional herbal medicines, mixtures of herbal extracts, comprising multiple phytocompounds, presumably regulating multiple targets for two or three medical indications are often used in a prescription. A major challenge for clinical use of such herbal remedies in cancer therapeutics is the evaluation of "true" active components and their targets for such multiple indications. Although modern chemical drugs and conventional herbal medicines may seem to be very different, they may, in fact, share some pharmacological foundations. As some herbal medicine classes have a common structural scaffold, this similarity may account for their potency in similar target groups [135]. It is believed that these structures and the activity/function information will become one of the most important indices for medicinal chemists to efficiently classify and seek specific pharmaceutical activities and for effectively optimizing herbal chemicals.

3. Evidence of the Effect of Herbal Medicines in Randomized Controlled Trials

Randomized controlled trials (RCTs) (or randomized comparative trials) currently serve as the gold standard for most clinical trials and provide the best evidence of the efficacy of healthcare interventions [136]. Carefully planned and well-executed RCTs often obtain the best estimates of treatment effect and thus help guide clinical decision making; therefore, considerable effort is put into improving the design and reporting of RCTs [137, 138]. Linde et al. [139] commented that reporting quality may vary across different types of complementary therapies, with herbal medicine trials being apparently superior to homeopathy and acupuncture trials. Also, reporting quality differed among different individual botanical medicines and improved continuously for decades from the 1980s to the 2000s [140]. With these controversies, in June 2004 an international group of pharmacologists, methodologists, pharmacognosists, and trialists met for a consensus-making meeting, which then led to the development of recommendations for the reporting of herbal medicine trials in Toronto, Canada [141]. An elaboration of CONSORT statement was put forward that aimed to aid researchers to more accurately assess the internal/external validity and reproducibility of herbal medicine trials, to allow a more accurate assessment of safety and efficacy of herbal medicines [141, 142]. Among the 22 CONSORT checklist items, 9 of them were elaborated to enhance their relevance to the trials of herbal interventions, including the detailed recommendations for 1 item (item 4 (interventions)) and minor recommendations for 8 items (item 1 (title and abstract), item 2 (background), item 3 (participants), item 6 (outcomes), item 15 (baseline data), item 20 (interpretation), item 21 (generalizability), and item 22 (overall evidence)) [141]. Specifically, the detailed recommendation in item 4 addressed the concerns of the herbal medicine intervention, which is still need extensive elaboration. These recommendations have been developed to improve the reporting of RCTs.

Although TCM and other herbal medicines are being used worldwide, their efficacies have only been studied in a sporadic way, with very few properly randomized and controlled studies. Trials of note that have employed a high standard of clinical trial methodology include Mok and colleagues [143] and Chan and colleagues [144]. Mok and colleagues examined the possible role of Chinese herbal medicine in reducing chemotherapy-induced toxicity. They reported that traditional Chinese herbal medicine seemed to have a significant effect on the control of nausea in patients with early-stage breast or colon cancers, but these herbal medicines did not reduce the hematologic toxicity associated with chemotherapy. In addition, Chan et al. [144] conducted a randomized, placebo-controlled trial to evaluate the efficacy of test TCMs in improving QOL and reducing chemotoxicity and possible decrease in the side effects of systemic chemotherapy and the immune system status of patients undergoing a standard treatment for ovarian cancers. In this study, ovarian cancer patients were randomized to receive either the test TCM formulation or a placebo in addition to standard chemotherapy. The primary outcome was recorded by the global health status (GHS) score and assessed by the European Organization for Research and Treatment of Cancer questionnaire, and the secondary outcomes were examined using other QOL items, chemotoxicity levels defined according to the World Health Organization (WHO) criteria, and alterations in specific immune functions. The results suggest that TCM exerted effects in maintaining immune function (e.g., lymphocyte count and cytokine activities) rather than improving QOL. However, as these randomized trials failed to recruit sufficient study numbers, we may need to conclude that, in order to fully evaluate and demonstrate specific bioactivities and the merits of various TCM formulations or plant extracts in cancer patients, continued, systematic efforts in conducting scientifically sound studies with RCTs are required [144].

4. Other Anticancer Applications of Specific Herbal Medicines

Tumor microenvironments are now recognized to play a critical role in cancer growth, progression, and metastasis [145, 146]. Intensive interactions between tumor or cancerous cells and their stromal microenvironments that involve a spectrum of immune cell types have received considerable research attention over the past few years [146–148]. There has been particular interest in the strong link between various immune activities at or surrounding tumor tissues and the progression of tumor growth. Enhancement of tumor surveillance by the host immune system has also been considered to be a key strategy to facilitate anticancer effect. In this section, we address the specific effects of herbal medicines on the enhancement of host immunity and review their molecular targets in anticancer activities.

4.1. Herbal Medicines as Adjuvant for Dendritic Cell-(DC-)Based Vaccines. By definition, the function of an adjuvant used with a vaccine is to aid or promote antigen delivery and presentation. An adjuvant can also assist in the induction of cytokines and stimulation/activation of antigen-presenting cells in the tumor or tissue microenvironment [149]. Specific herbal medicines such as *Ganoderma lucidum* or Dioscorea tuber have been reported to confer immunomodulatory activities [150, 151]. Bioactive polysaccharides from *Ganoderma lucidum* (Reishi) were investigated for their immunostimulatory and anticancer properties [152]. A specific polysaccharide fraction from Reishi stimulated immune cell activation including dendritic cell maturation and cytokine expression and displayed potent adjuvant activity in mice [153, 154]. Polysaccharides from *Dioscorea batatas* were found to induce TNF-α secretion via Toll-like receptor 4-mediated protein kinase signaling pathways [155]. A number of phytochemicals have also been demonstrated to effectively enhance the anti-tumor potency of gene-based cancer vaccines. For example, shikonin enhanced the anti-tumor potency of a cancer vaccine via the induction of RANTES expression at the skin immunization tissue site [156]. And a phytocompound mixture extracted from the butanol fraction of a stem and leaf extract of *Echinacea Purpurea* conferred immunomodulatory effects suggesting that it can effectively modulate DC mobility and related cellular physiology *in vivo* in the mouse immune system [157]. These studies suggest the potential application of herbal medicines in a cell-based vaccine system.

4.2. Induction of Immunogenic Cell Death by Herbal Medicines. Immunogenic cell death mediated by damage-associated molecular pattern (DAMP) signals was found to trigger an immunogenic response including maturation and antigen uptake of dendritic cells [158]. Recently, Chen and colleagues [159] demonstrated that shikonin can induce immunogenic cell death in treated tumor cells. Shikonin-treated, tumor cell lysate-loaded, mature DCs were shown to exhibit strong anticancer activities against test mouse melanoma, including the induction of cytotoxic activities of splenocytes against target tumor cells, inhibition of tumor growth, and improvement in mouse survival. The use of shikonin-treated tumor cells from patients to pulse their own DCs in culture should be evaluated in future clinical studies as a new approach for developing DC-based anticancer vaccines.

5. Conclusion and Future Development

For centuries if not millennia, various plants (many systematized in traditional Chinese medicine) have been used as medicines and disease therapeutics in most human cultures. As exemplified in this review, over the last two decades renewed public interest and research efforts from scientific and medical communities worldwide have generated a large volume of information including clinical studies and trials on the pharmacological effects, usage, and the development into future medicines of herbs and derivative medicinal phytochemicals as anti-tumor and chemoprevention agents. Although considerable effort has been put into the verification and upgrade of many traditional remedies or multiple-herb formulations, systematic, standardized research and the use of FDA regulatory protocols and defined clinical trials are still quite limited and need to be actively pursued. At the same time, it is necessary for scientists, clinicians, and regulatory agencies to actively consider how to create novel, improved, or modified clinical surveys, studies, and trial mechanisms that employ the stringent trial standards of the 21st century but also incorporate, at the international level, the wealth of old empirical but incomplete data from various records and documents accumulated by traditional medicine practices worldwide, to expedite the discovery and development of new phytomedicines and botanical drugs.

While continuous and systematic effort is needed, a number of notable "breakthroughs" have occurred in the field of medicinal plant research and botanical drugs in the last few years. In April 2008, the FDA approved the very first botanical drug, Veregen, a partially purified fraction of the water extract of green tea leaves from *Camellia sinensis*, for topical treatment of external genital and perianal warts [160]. Very recently (January 2013), the FDA approved, for the first time, an oral botanical drug, Crofelemer (a purified oligomeric proanthocyanidin from the latex of the South American *Croton lechleri* tree), for treatment of diarrhea in HIV/AIDS patients. Although these two pioneer FDA-approved botanical drugs are not therapies for cancer, they certainly pave way for such future developments. One possible example is the ongoing (2013) FDA clinical trial on "PHY906." This four-herbal-plant-composed TCM formulation has been shown to confer with good evidence [74]. It is our hope that the phase III clinical trial of this formula will lead the way in the development of CAM for cancer patients. With the various other new clinical trials ongoing, CAM may start playing critical roles in future health care of aging populations.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Traditional Herbal Medicine: A Review of Potential of Inhibitory Hepatocellular Carcinoma in Basic Research and Clinical Trial

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Although significantly develops in hepatocellular carcinoma (HCC), features of HCC remain an aggressive cancer with a dismal outcome. Traditional Chinese medicine (TCM), specifically Chinese herbal medicine (CHM), is one of the most popular complementary and alternative medicine modalities worldwide. The use of heat-clearing and detoxicating (Chinese named *qingre jiedu*) CHM has attracted great attention as an alternative antitumor including HCC considering its low toxicity and high activity. Together these reports indicate that CHM is a promising anti-HCC herbal remedy in basic research. For patients with advanced HCC, CHM including formula and single combined with transcatheter arterial chemoembolization or chemotherapy is able to decrease tumor growth and the side effect of toxicity and improve overall survival, quality of life, and immune function. Due to its abundance, low cost, and safety in consumption, CHM remains a species with tremendous potential for further investigation in HCC.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most common malignancies in China, ranks as the second leading cause of cancer mortality [1] and approximately accounts for half in the world [2]. Globally, there are approximately 750,000 new cases of HCC reported per year [3]. The incidence of HCC is increasing rapidly in the United States and other developed countries [3]. Moreover, features of HCC are an aggressive cancer with a dismal outcome largely due to metastasis and postsurgical recurrence [4].

As known, surgical resection, embolization, ablation, and chemotherapy play important roles in the treatment of HCC, but it is limited to a significant extent by its toxicities, significant resistance to available chemotherapeutic agents, side effects and complexities. Given the poor prognosis associated with HCC and limited treatment options outside of surgery, patients seek additional therapies to improve quality of life (QOL) or survival. One possible way to increase the efficacy of anticancer drugs and to decrease toxicities or side effects is to develop complementary and alternative medicine (CAM). Traditional Chinese medicine (TCM), one of the most popular CAM forms worldwide, has been widely used in cancer, especially from Chinese herbal medicine (CHM) [5].

In China, there has been a long history of using TCM in the treatment of liver cancer and other malignancies. The clinical diagnosis and treatment in TCM are mainly based on the *yin-yang* and five elements theories wood, fire, earth, metal, and water, an ancient philosophical concept used to explain the composition and phenomena of the physical universe. These theories apply the phenomena and laws of nature to the study of the physiological activities and pathological changes of the human body and its interrelationships.

The typical TCM therapies include acupuncture, CHM, and *qigong* exercises. The CHM contains hundreds of medicinal substances, mainly plants, but also some minerals and animal products classified by their performed function in the body. Different parts of CHM such as the leaves, roots, stems, flowers, and seeds are widely used. Usually, CHM is combined in formulas and single.

As the theory of TCM including CHM in the management of tumors concentrates on integrity and functional regulation of the organism, which coincides with oncology of modern medicine, its position in combination therapy for HCC has attracted more attention. Recently, accumulating evidences demonstrated that CHM attenuates HCC proliferation, invasion, and metastasis in basic research and improves patients with HCC survival and overall response rate as well in clinical study. This review will provide the reader with a new understanding of CHM's properties with an emphasis on regulation of multiple molecular targets of importance for HCC in basic research and representative CHM combined with classic treatment for HCC.

2. The Representative CHM Formula in Basic Research

2.1. Songyou Yin Formula. Nontoxic and supporting the healthy energy (*Fuzheng Guben*) herbal formulas play active roles in the anticancer. *Songyou Yin*, one of the important representative *Fuzheng Guben* herbal formulas, consists of five herbs: *Radix Salviae Miltiorrhiae, Radix Astragali, Fructus Lycii*, *Fructus Crataegi,* and *Trionyx sinensis Wiegmann*. With respect to HCC invasion and metastasis, *Songyou Yin* inhibited the growth and invasion of HCC (MHCC97H) cells with high metastatic potential both *in vitro* and *in vivo* through inducing apoptosis and downregulation of MMP2 and VEGF expression [6]. Moreover, the formula improved living life, minimized cancer-related weight loss, and prolonged the survival of nude mice bearing tumors [6]. In experiments conducted for opposite effects of chemotherapy enhancing the malignancy of treated HCC, Xiong et al. found that *Songyou Yin* attenuated epithelial-mesenchymal transition (EMT) and inhibited the enhanced metastatic potential of residual HCC in nude mice [7]. For another, the formula can render HCC sensitive to oxaliplatin via the inhibition of stemness by induction of cancer stem cells (CSCs) differentiation or direct elimination of CSCs to decrease expression of CSC-related markers in tumor tissues and cell lines [8]. As known, palliative resection increased markedly HCC metastasis in patients with HCC. *Songyou Yin* reinforced the ability of IFN-α to inhibit palliative resection that induced the metastasis-enhancing potential by downregulation of VEGF and MMP2/TIMP2 [9].

2.2. Jiedu Xiaozheng Yin Formula. The use of heat clearing and detoxicating (Chinese named *qingre jiedu*) CHM including *Jiedu Xiaozheng Yin* has attracted great attention as an alternative antitumor including HCC. *Jiedu Xiaozheng Yin* (JXY), a polyherbal formula of TCM, consists of the following herbs *Hedyotis diffusa, Pseudobulbus Cremastrae seu Pleiones, Spica Prunellae* and *Radix Sophorae Flavescentis*. With regard to the study performed in HCC proliferation and growth, Cao et al. found that JXY inhibited the growth of HCC and the formation of cell colonies and blocked the cell cycle to G1 phase in a dose-dependent manner *in vitro*. Meanwhile, JXY showed an obvious antitumor effect *in vivo* through increasing the expression of G1-related proteins (cyclin D and cyclin E) [10]. In HCC angiogenesis, using human umbilical vein endothelial cells (HUVECs), chick chorioallantoic membrane (CCM), and an HCC mouse xenograft model, JXY treatment significantly reduced tube formation of HUVECs, and angiogenesis in the CCM, and microvessel density of tumor *in vivo* [11]. Further studies showed that JXY suppressed the vascular endothelial growth factor A (VEGF-A) expression and its receptor 2 (VEGFR-2) among the HCC HepG2 cells, HUVECs, and tumor [11]. About research played in hepatocarcinogenesis, Li et al. found that activation of c-fos proto-oncogene, c-jun, and c-myc oncogenes plays a crucial role in the pathogenesis of HCC [12].

2.3. Huqi San Formula. Huqi San, one of the experiences of famous Chinese anticancer drugs, consists of the following herbs *Ramulus Visci*, *Radix Astragali seu Hedysari*, *Radix Curcumaez* and *Radix Salviae Miltiorrhiae*. It demonstrated the inhibitory effect of it on rat befor-hepatocarcinogenesis induced by diethylnitrosamine (DEN) by analyzing the mutational activation of c-fos and overexpression of c-jun and c-myc. And the formula can inhibit the overexpression of it and liver preneoplastic lesions induced by DEN [12]. Another, Wen et al. demonstrated the modulation effect of energy metabolic enzyme expression on DEN-mediated hepatocarcinogenesis by *Huqi San*. They found that the formula treated rats showed significant decrease in areas of gamma-GT positive foci and activity of AFP [13]. On the other hand, the formula obviously increased these activities of G-6-Pase, SDH, and ATPase. *Huqi San* is a potential anticarcinogenic agent that may induce apoptosis by reducing the inhibitory effects of X-linked inhibitor of apoptosis protein on caspase-3 [14].

2.4. Fuzheng Yiliu Formula. Fuzheng Yiliu granule, one of the famous *Fuzheng Guben* herbal formulas in China, consists of four herbs: *Radix Hedysari, Radix Angelicae Sinensis, Radix Patriniae Scabrae*, and *Rhizoma Curcumae Phaeocaulis.* With respect to HCC immune function, the formula could inhibit HCC growth by regulating immune function and inducing apoptosis of tumor cells *in vivo* and *in vitro*. Cao et al. found that mice treated with *Fuzheng Yiliu* granule had higher percentages of CD3(+) and CD4(+), and more NK cells, and highest serum levels of IL-2 and TNF-α in the peripheral blood than those in the animals treated with normal saline. The results indicated that high-dose *Fuzheng Yiliu* granule-containing serum significantly decreased HepG2 cell viability, inhibited cell proliferation, and induced apoptosis [15]. The present study suggests that representative CHM formula properties with an emphasis on regulation of HCC multiple molecular targets are of importance *in vitro* and *in vivo*.

3. The Representative CHM Formula in Clinical Research

HCC is a multifactor and multistage process, and it is relatively difficultly to find a specific medication. Therefore,

a wide consensus that combination therapy is most likely the way resulting in developing new therapy strategies for HCC in the future has been reached in academic institution. Transcatheter arterial chemoembolization (TACE) is the most widely used primary treatment for unresectable HCC because of its survival benefit, although its clinical effect is still far from satisfactory [16]. In China, there has been a long history of using CHM such as JDF granules, *Jiedu*, granules and *ganji* Decoction in the treatment of HCC and other malignancies. JDF granule preparation is composed of four CHM, root of *Actinidia valvata* and *Salvia chinensis*, bulb of *Cremastra appendiculata*, and *gizzard* membrane of *Gallus gallus domesticu* [17]. To evaluate the effect of combined therapy with TACE and JDF granules preparation in the treatment of patients with unresectable HCC on survival, current study data demonstrated that TACE combined with JDF granule preparation could improve the prognosis of patients and prolong the survival of patients with unresectable HCC [17]. Furthermore, the trial found that the median overall survival was 9.2 months (95% confidence interval [95% CI], 6.94–1.46) in the TACE combination with JDF formula versus 5.87 months (95% CI, 4.21–7.52) in the control group. In the TACE combination with JDF formula, 1-, 2-, and 3-year survival rates were 41.2%, 18.4%, and 9.6%, respectively. Additional, the Cox regression analysis revealed the therapy model to be an independent predictor of patient prognosis. Importantly, the current study provides preliminary and powerful data to support future evaluation of CHM in combination therapy for HCC in a large-cohort, randomized clinical trial (RCT).

To effetely prevent the recurrence of HCC after surgical resection, a case-control trial, *Jiedu* granules, a CHM compound, plus cinobufacini injection, extracted from skin of *Bufo bufo gargarizans Cantor* after operation versus TACE after operation, was performed. *Jiedu* Granules is composed of CHM, *Herba Salviae Chinensis, Radix Actinidiae valvatae, Semen Coicis, Fructus Crataegi, Massa Medicata Fermentata,* and *Fructus Hordei Germinatus*. The trials demonstrated that *Jiedu* granules plus cinobufacini injection, a combination that is commonly used for postoperation management of HCC, can postpone tumor recurrence and metastasis, prolong the survival time, and increase the survival rate of postsurgical patients with HCC [18].

To evaluate whether CHM improves immune response for 1,008 unresectable HCC after TACE by using meta-analysis of data from the literature involving available 12 RCT of CHM in combination with TACE compared with that of TACE alone, literature retrieval was conducted. Meng et al. showed that the differences of pooled weighted mean difference before and after treatment and 95% CI were 13.63 (8.96–18.69; $P = 0.0001$) for the proportion of CD3(+) T cells, 10.56 (6.91–14.21; $P = 0.0001$) for the proportion of CD4(+) T cells, 3.40 (6.83 to 0.03; $P = 0.052$) for the proportion of CD8(+) T cells, 0.54 (0.42–0.66; $P = 0.0001$) for the ratio of $CD4^+/CD8^+$, and 12.34 (7.26–17.41; $P = 0.0001$) for the proportion of NK cells. No serious adverse events were reported. Thus, TCM in combination with TACE improves the immune response of patients with unresectable HCC [19]. To observe the efficacy of TCM comprehensive therapeutic project in treating the middle/late stage patients with HCC, with prospective RCT, 97 patients with HCC were assigned to the test group (49 cases) treated with TCM comprehensive therapy using *Oleum fructus bruceas* intervention combining oral intake of *ganji* Decoction and external application of *Ailitong,* and the control group (48 cases) was treated with chemotherapeutic agents combining iodized oil chemoembolization and analgesics [20]. To compare the efficacy and safety of CHM plus TACE with that of TACE alone (therapy I versus therapy II) in treating unresectable HCC via a meta-analysis of all available 37 RCTs involving 2653 patients, an important research was performed [21]. The results showed that therapy I compared with therapy II improved the patient survival, QOF, alleviation of symptoms, and tumor response and was thus more therapeutically beneficial. Moreover, no serious adverse events were reported [21]. This trial study displayed that TCM comprehensive therapy is an effective treatment for the middle/late stage patients with HCC, and it could extend the pain-relieving sustained time and improve the patients' QOL and long-term survival with less adverse reaction. Evidence from patients with HCC trials suggests that representative CHM formula combined with TACE improve overall survival, QOL, and immune function.

4. The Representative CHM Single Herb in Basic Research

CHM, including animal and plant herbs, plays important roles in the treatment of malignancy including HCC. Based on TCM theory, heat-clearing and detoxicating (Chinese named *qingre jiedu*) CHM such as toad's skin (*Chan chu pi*), *Flos Chrysanthemi Indici*, *Herba Scutellariae Darbatae Radix, Radix Sophorae Flavescentis,* and *Radix Scutellariae* has increasingly become important for treatment of HCC. Bufalin, one of the representative animal herbs, is the major component of *Chan-Chu* extracted from toad's skins. With respect to autophagy in HCC, Tsai et al. investigated the pharmacological mechanisms of cell cycle arrest and autophagic cell death induced by bufalin in SK-HEP-1 human HCC cells *in vitro* [22]. Data showed that bufalin triggered autophagic cell death and G2/M phase arrest through the AKT/mTOR signaling pathway in SK-HEP-1 cells. For another, bufalin triggered autophagy and enhanced Beclin-1 expression, LC3-I to LC3-II conversion, as well as decreased p62 expression and mTOR signaling activation in HCC HepG2 cells [23]. Blockage of autophagy by selective inhibitor 3-MA decreased apoptotic ratio in bufalin-treated HepG2 cells, suggesting that bufalin induces HepG2 cells for autophagy at least in part via AMPK-mTOR dependent pathway. Similarly, arenobufagin, a bufadienolide from toad venom, had potent antineoplastic activity against HCC HepG2 cells as well as corresponding multidrug-resistant HepG2/ADM cells. Arenobufagin induced mitochondria-mediated apoptosis in HCC cells as well as increasing Bax/Bcl-2 expression ratio. They observed the inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway by arenobufagin. Interestingly, inhibition of mTOR by rapamycin or siRNA duplexes augmented the arenobufagin-induced apoptosis and autophagy. Based on

the evidenced above, underlying antineoplastic mechanisms of arenobufagin involve cross talk between apoptosis and autophagy via inhibition of the PI3K/Akt/mTOR pathway [24]. Regarding HCC angiogenesis, arenobufagin inhibited VEGF-induced viability, migration, invasion, and tube formation in HUVECs *in vitro* and suppressed sprouting formation from VEGF-treated aortic rings in an *ex vivo* model [25]. Furthermore, arenobufagin blocked angiogenesis in a matrigel plugs assay in HCC cells. Additionally, arenobufagin inhibited VEGF-induced VEGFR-2 autophosphorylation and suppressed the activity of VEGFR-2-mediated signaling cascades. With regard to HCC cell apoptosis and proliferation, our teams previous study showed that *Flos Chrysanthemi Indici* exerted a significant apoptotic effect via a mitochondrial pathway and arrested the cell cycle by regulation of cell cycle-related proteins in human MHCC97H cells, a HCC cell line with high metastatic potential [26]. Following, we investigated the effect of this herbon isoproterenol induced growth of human HCC cells in correlation with the intracellular activity of MAPK/ERK1/2 and found that the herb was effective in attenuating the mitogenic effect of isoproterenol on both HepG2 and MHCC97H cells. The inhibitory effect of the plant was mediated by inhibiting the isoproterenol-induced activation of MAPK/ERK1/2 via beta2-AR in tumor cells [27]. In addition, our study indicated that *Flos Chrysanthemi Indici* inhibited the invasion and migration ability of MHCC97H cells, as assesses through a matrigel-coated membrane invasion. Moreover, the herb could reduce the cancer cell metastatic ability, in part at least, through decrease of the MMP expression, simultaneous increase of the TIMP expression, and further restoring their balance as a therapeutic target in HCC [28]. Dai et al. found that *Herba Scutellariae Darbatae Radix* (HSDR) effectively inhibits the proliferation and induces apoptosis of H22 cells involving loss of mitochondrial transmembrane potential, release of cytochrome C, and activation of caspase-3 in a dose-dependent manner [29].The effect of HSDR on immune function was determined using hepatoma H22 tumor bearing mice. The results demonstrate that the plant increased weight, thymus, and spleen index of H22 bearing mice and phagocytotic function of macrophages by observing peritoneal macrophages phagocytize chicken red blood cells. It is suggested that HSDR improves the immune function of hepatoma H22 tumor bearing mice [30]. Regarding the detoxification and enhancement for chemotherapy, high-dose HSDR combined with 5-fluorouracil (5-FU) could significantly enhance the tumor inhibition rate, thymus, and spleen index in immunological organs. The data displayed that HSDR can significantly enhance the tumor inhibition rate of 5-FU, reduce the toxic effects, prolong the survival time, and improve immune function in the H22 tumor-bearing mice [31]. Matrine, an alkaloid purified from the Chinese herb *Radix Sophorae Flavescentis,* significantly inhibited the proliferation and induced G1-phase cell cycle arrest and apoptosis of HCC HepG2 cells in a dose-dependent manner. Fewer autophagic vacuoles were observed in the combined 3-MA and matrine treatment group when 3-MA was added before matrine treatment, indicating that both autophagy and apoptosis are activated when matrine-induced death of HepG2 cells occurs [32]. Baicalein, a purified flavonoid extracted from the roots of *Radix Scutellariae*, exhibited significant cytotoxicity to three HCC cell lines but marginal cytotoxicity to a normal liver cell line. Treatment with baicalein dramatically reduced mitochondrial transmembrane potential, activated caspase-9 and caspase-3, and significantly inhibited tumor growth of HCC xenografts in mice. Furthermore, Baicalein treatment dramatically decreased the levels of phosphorylation of MEK1, ERK1/2, and Bad *in vitro* and *in vivo*. Consequently, these findings suggest that Baicalein preferentially inhibits HCC tumor growth through inhibition of MEK-ERK signaling and by inducing intrinsic apoptosis [33]. These results suggest the representative CHM single herb properties with an emphasis on regulation of HCC multiple molecular targets of importance *in vitro* and *in vivo*.

5. The Representative CHM Single Herb in Clinical Research

Kanglaite injection, one of Chinese herb preparations, extracted from CHM coix seed oil, had been confirmed with antitumor activity including HCC [34]. Zhu et al. found that *Kanglaite* injection combined with TACE was significantly superior to TACE group in improving symptoms and Karnofsky scores, decreasing tumor growth and side-effect of marrow toxicity for patients with advanced stage HCC [35]. Regarding the single CHM use in combination with TACE, occasionally, single CHM was used to be combined with some first-line anticancer agents. *Kanglaite* injection reverses the multidrug resistance and has obvious chemotherapy sensitization [36]. Thus, to observe the therapeutic effect and safety, several researchers focused on studying the combination form of CHM and some chemotherapeutic agents. For instance, compared with Capecitabine group, *Kanglaite* injection combined with Capecitabine for patients with advanced stage HCC displayed better clinical benefit response and tolerance, longer time-to-progression, and median survival time [36]. *Fructus Bruceae* oil and *ganji* recipe combination of TACE showed that the improvements in the treatment group, including patients' physical energy enhancing, symptoms alleviating, and overall QOL improving, were superior to those in the TACE group [37]. Yang et al. observed that patients with advanced HCC were treated with TACE using B. javanica oil/lipiodol mixture perfusion remobilization (treatment group) or using conventional chemotherapy/lipiodol chemoembolization infusion (control group) [38]. The data displayed that the incidence of toxic side effects in the treatment group was less than that in the control group after the intervention. Compare with the 0.5 and 1-year survival rate, the treatment group was 96. 1% (25/26) and 50% (13/26) and the control group was 83.3% (20/24) and 33.3% (8/24). Additionally, two group cases with a median survival time were 8.9 and 6.7 months, respectively. It is suggests that representative single CHM combined with TACE improved the overall survivals, QOL and reduces the toxic side effects.

6. The Effects of Detoxification and Enhancement for Chemotherapy on CHM

Previous studies have demonstrated that, one of the representative CHM, HSDR increased the weight, thymus, and spleen index of H22 bearing mice and phagocytotic function of macrophages by observing peritoneal macrophages phagocytize chicken red blood cells [31]. Regarding the detoxification and enhancement for chemotherapy, high-dose HSDR combined with 5-FU could significantly enhance the tumor inhibition rate, thymus, and spleen index in immunological organs. The data displayed that CHM can significantly reduce the toxic effects and improve immune function. CHM plus TACE significantly increases nondeterioration performance, T cells, natural killer cells, and white blood cell count, significantly decreases the level of blood alpha-fetoprotein concentration, and significantly lowers the risk for nausea and vomiting [39]. Furthermore, meta-analysis has also demonstrated that CHM plus TACE increases the proportions of cluster differentiation CD3(+) T cells, CD4(+) T cells, and NK cells, as well as the ratio of $CD4^+/CD8^+$ before and after treatment [19]. A meta-analysis of RCT for CHM combined with chemotherapy has reported promising evidence that CHM plus chemotherapy may have benefits on patients with HCC [40]. *Astragalus* might also boost host immune function by stimulating macrophage and NK cell activity [31]. Moreover, *Astragalus* appears to restore *in vitro* T-cell function, which is suppressed in cancer patients [31]. The main ingredient of *Kanglaite* injection is coix seed oil. *Kanglaite* injection exerted a significant inducing apoptotic effect and arrested the cell cycle by inhibition of M stage in cancer cells. Another, *Kanglaite* injection actives NK cells, promotes phagocytosis, and improves immune function as well [34, 35]. And it can induce tumor cell apoptosis, block tumor cell mitosis, improve immune function, reduce the toxicity of chemotherapy, and relieve cancer pain [34]. *Fructus Bruceae* oil emulsion (*Yadanzi* oil), one of Chinese herb preparations, can significantly inhibit topoisomerase, reverses the multidrug resistance, and has obvious chemotherapy sensitization in HCC cells [37].

7. Side Effects of CHM

No significant drug-related side effects were observed in the study group. The majority of patients reported mild to moderate side effects. The main side effects associated with CHM were lower fever, gastrointestinal symptoms, bone marrow suppression, neurotoxicity, hand-foot syndrome, and so forth [17, 34–38].

8. Conclusions

The rising of the use of TCM, particularly CHM has worldwide sparked an extreme interest in more and more people and researchers. Recently, the use of heat-clearing and detoxicating (Chinese named *qingre jiedu*) CHM has attracted great attention as an alternative antitumor including HCC considering its low toxicity and high activity. For HCC in basic research, CHM has been found to be active against hepatocarcinogenesis, proliferation, invasion, metastasis, and angiogenesis. Additionally, CHM combined with chemotherapy could significantly enhance the HCC inhibition rate and immune function in immunological organs and reduce the toxic effects. All these beneficial effects will ameliorate the radiation, chemotherapy, and TACE induced adverse effects. These outcomes of such studies may be useful for the clinical applications of HCC in human beings against cancers and may open up a new therapeutic avenue. Due to its abundance, low cost, and safety in consumption, CHM remains a species with tremendous potential for further investigation in HCC.

For patients with advanced HCC, CHM including formula and single was used to be combined with TACE or chemotherapy usually, decreasing tumor growth and side effect of toxicity, and to prolong overall survival, QOF, and immune function. Together these reports indicate that CHM is a promising anti-HCC herbal remedy. However, due to various factors—including inconsistencies in treatment schemes, the limited sampling sizes, and lack of quality assurance of the herbal products well-designed RCTs to prove the effectiveness of TCM as adjuvant therapy for cancer are scarce. In general, most of the published clinical studies were trials without rigorous randomization or they involved single group pre-post, cohort, time series, or matched case-control studies. Fore another, studies should be performed on a large number of HCC patients receiving radiotherapy, chemotherapeutic, postoperative combination with CHM. Moreover, the clinical trials are insufficient to draw firm conclusions for the single use of CHM in patients with HCC.

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Review Article

Role of Kampo Medicine in Integrative Cancer Therapy

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Clinical trials to date demonstrate that standard cancer treatments are currently the most efficient treatments for large numbers of cancer patients. Cancer treatments will increasingly require approaches that allow patients to live with cancer, by increasing their natural healing power and tumor immunity, as well as attenuating the progression of their cancers, instead of only attacking the cancer cells directly. Complementary and alternative medicine, including Kampo medicine, compensates for the drawbacks of western medicine by increasing patients' self-defense mechanisms. In Japan, clinicians who have studied both western medicine and Kampo treat cancer patients by fusing the two medical systems into a unitary one. The goal of the system is to assist the functional maintenance and recovery of the living body complex with the physical, mental, social, and spiritual balance, rather than addressing direct antitumor effects. In this review, we describe the usefulness of Kampo medicine, especially juzentaihoto, and outline the reports on evidence, in addition to the report on an attitudinal survey about the use of Kampo medicine in cancer treatment in Japan.

1. Limitations of Standard Cancer Treatment

Western medicine should be used in preference to Kampo medicine in the diagnosis and treatment of cancer. Medical diagnosis based on western medicine is essential to determine the exact degree of progression and malignancy of cancer [1–4]. Western medicine successfully treats many types of cancer, when the appropriate treatment is used. Clinical trials to date demonstrate that standard cancer treatments are currently the most efficient treatments for large numbers of cancer patients [5–8]. The current standard cancer treatments include surgery, chemotherapy, and radiation therapy. Reliable therapy has not been established yet for refractory cancer, including advanced or recurrent cancer.

Advanced experimental therapies using special anticancer agents, radiation, immunotherapy, and gene therapy have been attempted. Radiation therapy, chemotherapy, and surgery are called "invasive treatments" [9–12]. The term "invasive" means "harmful to the body" as well as "attacking the cancer." Aggressive treatment has the disadvantage of damaging normal tissue and reducing tumor immunity and physical strength. This is especially true in bone marrow cells or intestinal mucosal epithelia, in which the cell cycle is vigorous [13–18]. The tissues and organs of the whole body maintain order in their structure and function by incorporating nutrients and oxygen from the blood into cells, excreting waste, and repairing old and scar tissues. This is called "natural healing power" or "self-healing power." Abnormal cells such as cancer cells are constantly produced in our body, but as long as the immune system is maintained properly, they are eliminated prior to growth or progression. This means that as long as the immune system is working

properly, cancer is constantly being "cured," without any treatment.

Cancer treatments will increasingly require approaches that allow patients to live with cancer, by increasing their natural healing power and tumor immunity, as well as attenuating the progression of their cancers, instead of attacking the cancer cells [19–21]. Complementary and alternative medicine, including Kampo medicine [22–25], compensates for the drawbacks of western medicine by increasing the body's healing power and resistance [26–29]. The term "multidisciplinary treatment of cancer" refers to treatment that appropriately utilizes a combination of treatments such as standard treatment, advanced experimental treatments, medical care to enhance the anticancer and healing power of the body, and palliative care, depending on the state of the cancer patient. The goal of holistic treatment, or integrated care, is not only to kill cancer cells but also to address the healing power and resistance of the body. Kampo treatment can provide very useful therapeutic options to achieve holistic therapy and multidisciplinary treatment for cancer [30–36].

2. Surveys of the Use of Kampo in Cancer Treatment in Japan

Three papers investigating the use of Kampo in cancer treatment in Japan were published in 2012. Takeda et al. [37] conducted a self-reported questionnaire on Kampo medicine involving a total of 476 patients with gynecologic cancers. Anxiety was assessed using the State-Trait Anxiety Inventory. It was confirmed that 22.9% of the women had used Kampo medicine. Kampo users were more likely to have had chemotherapy and were more likely to have experienced uncomfortable adverse effects of cancer treatment. Kampo users were more likely to believe that Kampo offers relief of symptoms with less adverse effects and that Kampo is more effective than western medicine. Kampo users expressed stronger attitudes in regard to taking Kampo medicine. Multiple regression analysis revealed that chemotherapy (RR, 1.82; 95% CI, 1.14–2.91), lower state anxiety (RR, 0.76; 95% CI, 0.58–1.00), and higher trait anxiety (RR, 1.46; 95% CI, 1.11–1.92) were independently associated with Kampo use. The study showed that approximately one-fourth of Japanese gynecologic cancer patients take Kampo medicine. Kampo users made more favorable comments on Kampo medicine than nonusers. The authors concluded that the psychological characteristics of individual patients are one of the factors that can influence the usage of Kampo.

Iwase et al. [38] conducted a cross-sectional self-administered anonymous questionnaire among 549 physicians working in palliative care teams at 388 core cancer treatment hospitals and 161 certified medical institutions that have palliative care units (PCUs). Valid responses were obtained from 311 physicians (56.7%) who were evenly distributed throughout the country without significant geographical biases. Kampo medicines were prescribed for controlling cancer-related symptoms by 64.3% of the physicians. The symptoms treated with Kampo medicines were numbness/hypoesthesia (n = 99, 49.5%), constipation (n = 76, 38.0%), anorexia/weight loss (n = 72, 36%), muscle cramps (n = 71, 35.5%), and languor/fatigue (n = 64, 32.0%). Regarding open issues about prescription, 60.7% (n = 173) of the physicians raised the issue that the dosage forms need to be better devised. The authors concluded that more evidence from clinical studies is needed to increase the clinical use of Kampo medicines and that the action mechanisms of Kampo should be clarified through laboratory research.

Ito et al. [39] conducted a nationwide survey to investigate the use of Kampo medicine by Japanese physicians in the core cancer treatment hospitals designated by the national Ministry of Health, Labour and Welfare. Among the 900 physicians surveyed, 92.4% reported having prescribed Kampo medications, and 73.5% of those physicians reported having prescribed Kampo medications for cancer patients. Despite this high percentage of usage and the finding that 9.7% of the physicians considered Kampo medications to be harmful, only 23.1% of the physicians expressed high expectations of the efficacy of Kampo medicine in tumor suppression and exertion of an immunostimulatory action. In contrast, many of cancer patients expressed the belief that Kampo can suppress tumor growth. The authors concluded that further research on the efficacy and safety of Kampo medicine in cancer treatment is warranted to resolve this discrepancy between patients' and physicians' expectations.

3. The Role of Kampo Medicine in Multidisciplinary Treatment of Cancer

The Kampo medicine approach is to prevent cancer by emphasizing the body's defense mechanism and natural healing power [22–25]. The existence of even early stage cancer indicates that the healing system is already in functional decline. The fundamental approach of Kampo treatment is to remove obstacles to healing, compensate for deficiencies, and consider the required combination of crude drugs. In western medicine, ideas aimed at activation and nourishing of tissue function are scant, whereas Kampo medicine considers these ideas as the most important treatment strategy. Kampo medicine recognizes that diseases are processes of struggling between individual-specific resistance power and external force of robing those power (e.g., virus). And the former directly determines the occurrence, progression, and outcome of disease. Sanity is a Kampo concept including all of the antidisease substances in the living body, which in modern medicine equates to natural healing power, comprising the mechanisms of self-defense, homeostasis, immune surveillance, and tissue repair. The immune system, antioxidant actions to prevent harm by active oxygen, and the tissue repair system also basically correspond to the concept of sanity in Kampo medicine. In Kampo cancer treatment, we try to treat dysfunction of the body that leads to cancer progression and also try to enhance body function to regain sanity. This is a core characteristic of Kampo treatment of cancer.

The present position of Kampo medicine in the medical treatment of cancer in Japan allows patients to access western and Kampo medical treatments simultaneously. Kampo

medicine [22–25] is a unique medical system that originated from ancient China, was gradually imported to Japan, and has been improved and refined by many excellent physicians since the Edo period. Most Kampo preparations (Japanese traditional herbal medicines) are available as extract formulations, which are greatly different from the herbal medicine practiced in China, Taiwan, and Korea. Four ethical Kampo extract formulations were approved in 1967 in Japan. Since then, the number of ethical Kampo extract formulations covered by health insurance has grown to 148. Japan's universal health insurance system [40, 41] does allow for simultaneous access to traditional Kampo preparations and western medicines. However, physicians in Japan cannot be licensed without passing a board examination on western medicine, which means that patients in this country receive health care with a high degree of safety. This is another factor that distinguishes the health care system in Japan from other countries. In Japan, physicians who have studied western medicine and Kampo medicine practice these approaches in their medical treatment of cancer with the aim of fusing eastern and western medicine into a unitary medical system (unlike the dual medical systems in China or Republic of Korea).

The goal of the system is to assist the functional maintenance and recovery of the living body complex, a host incorporating a nutrient state, mental balance, and so forth, rather than addressing direct anticancer efficacy. The present condition and stance of Kampo medicine in the medical treatment of cancer in Japan clearly diverges from development of an anticancer herbal medicine and formulation of an antitumor herbal tablet.

4. Juzentaihoto: A Typical Kampo Formula for Cancer Treatment

Juzentaihoto is an effective Kampo medicine for promoting restoration of physical strength after surgery and alleviating adverse effects of anticancer drugs or radiation therapy. While there are many other useful Kampo medicines, this discussion focuses on juzentaihoto. Table 1 and Figure 1 show juzentaihoto's composition and constituent analysis. Juzentaihoto is indicated for the relief of declined constitution after recovery from disease, fatigue and malaise, anorexia, perspiration during sleep, cold limbs, and anemia. Important precautions, (1) when this product is used, patient's "*sho*" (constitution and symptoms) should be taken into account. Patient's progress should be carefully monitored, and if no improvement in symptoms or findings is observed, continuous treatment should be avoided. (2) Since this product contains Glycyrrhiza, careful attention should be paid to the serum potassium level, blood pressure, and so forth, and if any abnormality is observed, administration should be discontinued. (3) When this product is coadministered with other Kampo preparations (Japanese traditional herbal medicines), attention should be paid to the duplication of the contained crude drugs.

Juzentaihoto itself has also been reported to prevent cancer occurrence and recurrence, as well as to suppress metastasis. A major report found that juzentaihoto stimulated activation of pluripotent hematopoietic stem cells in irradiated mice [42]. It has also been reported to have an antiangiogenic action in malignant glioma. There have been studies of its actions in suppressing carcinogenesis and metastasis [43–48].

Table 1: Composition of juzentaihoto.

Description	Juzentaihoto extract granules for ethical use	
Composition	7.5 g of juzentaihoto extract granules contains 5.0 g of a dried extract of the following mixed crude drugs	
	JP Astragalus Root	3.0 g
	JP Cinnamon Bark	3.0 g
	JP Rehmannia Root	3.0 g
	JP Peony Root	3.0 g
	JP Cnidium Rhizome	3.0 g
	JP Atractylodes Lancea Rhizome	3.0 g
	JP Japanese Angelica Root	3.0 g
	JP Ginseng	3.0 g
	JP Poria Sclerotium	3.0 g
	JP Glycyrrhiza	1.5 g
	Inactive ingredients	
	JP Magnesium Stearate	
	JP Lactose Hydrate	

Juzentaihoto's biological activity has also been widely reported. Among these reports are studies that found that it activates macrophages, enhances antibody production, induces cytokine production, and has other immunoenhancement actions, in addition to protection against disturbance of myelopoiesis and against immune suppression in anticancer drug and radiation therapy [49].

Muraishi et al. [50] examined the effect of juzentaihoto on immunological functions and antitumor activity in old mice. Juzentaihoto increased the number of T cells remarkably and NK cells slightly in the aged mice, while a significant increase was not observed in young mice.

Treatment with juzentaihoto increased NK activity in both young and old mice. Therefore, the combination of IFNs with juzentaihoto may provide a means to increase the therapeutic potential of IFNs and to decrease their toxicity for the treatment of metastatic renal cell carcinoma. Juzentaihoto increased regulatory activities in T cells by decreasing Foxp3 (+) Treg populations in advanced pancreatic cancer patients [51]. This effect can lead to immunoaugmentation for various combination therapies. Genetic analysis using microarrays has recently been used to indicate its effects in germ-free mice [52]. Ogawa et al. also report that myelosuppression due to TS-1 in mice may be improved with coadministration of juzentaihoto [53]. It is often used for adjunct therapy to cancer therapy as well as the main clinical goals of improving patients' quality of life and alleviating the side effects of anticancer agents and radiation therapy, anorexia, fatigue, and malaise and reduced physical strength following illness. Tokushima University Hospital introduced Kampo

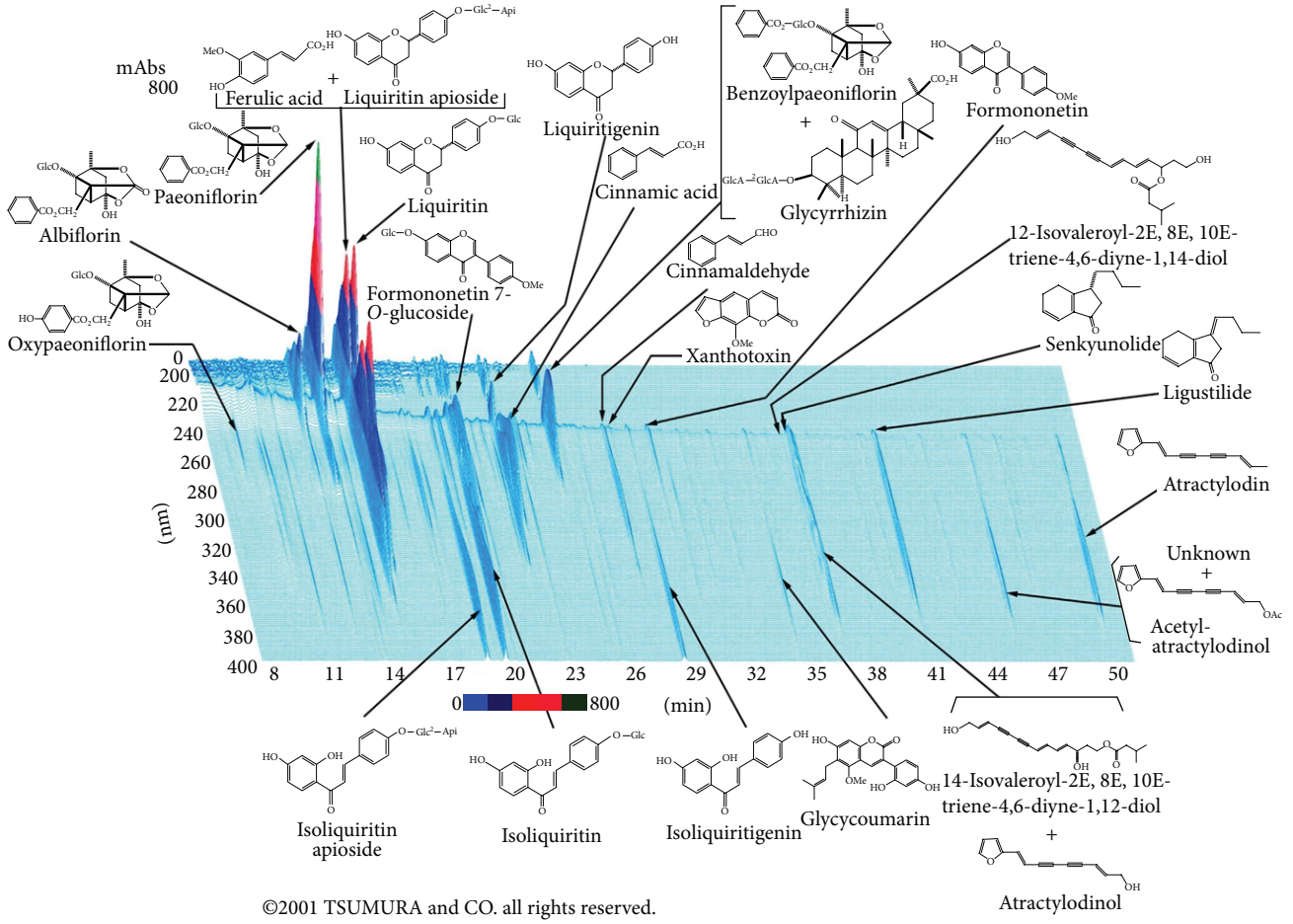

Figure 1: 3D-HPLC pattern of TJ-48 juzentaihoto.

medicine therapy to improve general malaise and the various side effects caused by chemotherapy and radiation therapy, which is used at the hospital for planocellular carcinoma of the uterine cervix [54]. The hospital reported that Kampo medicines are allowed for prolongation of the lives of cervical cancer patients. Satou and Arakawa investigated whether Kampo therapy based on traditional approaches is useful or not for inhibition of hepatic cell carcinogenesis (HCC) and reported that therapies based on traditional approaches are useful for HCC in chronic type C liver disease [55].

5. Summary

Several aspects of Kampo treatment in Japan have been introduced here with respect to holistic care, integrative therapy, and multidisciplinary treatment of cancer patients. The Kampo medicine approach is to control cancer by bringing out the natural healing power inherent to living bodies and emphasizing body's defense mechanism. In western medicine, ideas aimed at activation of organ functions and nourishment are scant, whereas Kampo medicine regards enhancement of self-defense mechanisms as the most important strategy. Integrative cancer therapy using Kampo medicine is expected to develop further in Japan.

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Research Article

Hemiparesis after Operation of Astrocytoma Grade II in Adults: Effects of Acupuncture on Sensory-Motor Behavior and Quality of Life

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To evaluate the effect of acupuncture on hemiparesis and quality of life for adults with brain astrocytoma grade II, we conducted a randomized, observer-blinded clinical trial. Fifty-eight patients were randomized to *standard rehabilitation (SR) therapy without acupuncture* ($n = 20$), *SR plus standard acupuncture (SA)* ($n = 19$), and *SR plus individualized acupuncture (IA)* ($n = 19$). SA points were *PC6*, *SP6*, *HT1*, *LU5*, *BL40*, and *ST36*, while a special concept called "*connecting and regulation Ren and Du*" and "*Jin-3-needling*" served as IA. This treatment was individualized according to the clinical syndrome. The outcome was measured by the Barthel Index (BI), the Fugl-Meyer scale (FM), and the EORTC Core Quality of Life Questionnaire (QLQ-C30) with the Brain Cancer Module (BCM20). IA + SR reached significantly higher BI scores than SA + SR, which reached significantly higher BI scores than SR. IA + SR was significantly superior to SA + SR and to SR at the 8th week for the scores of FM motor and sensory assessments and most QLQ-C30-BCM20 items. In conclusion, the individualized acupuncture concept of "*connecting and regulating Ren and Du*" combined with "*Jin-3-needling*" offers a promising possibility for the treatment of hemiparesis due to astrocytoma, but further evaluation is mandatory.

1. Introduction

Astrocytic tumors comprise a wide range of neoplasms of the central nervous system (CNS) which shows invasive and progressive growth. The following clinicopathological entities can be distinguished as pilocytic astrocytomas (grade I), fibrillary astrocytomas (grade II), anaplastic astrocytomas (grade III), and glioblastoma multiforme (grade IV) [1]. The most common site of these tumors is the brain [2]. They make up about 42% of all brain tumors including benign tumors and 80% of all malignant brain tumors [3]. These tumors tend to grow and infiltrate into the normal brain tissue, which makes complete surgical removal very difficult, or usually impossible, and complicates treatment [4]. The World Health Organization (WHO) classifies astrocytomas into the previously described four grades depending on how fast they are growing and the likelihood that they will spread (infiltrate) to nearby brain tissue [5]. Grade II astrocytomas are also called low-grade astrocytomas or diffuse astrocytomas and are usually infiltrating tumors. These tumors grow relatively slow and usually do not have well-defined borders. They are generally more common in men and are most common in the cerebral hemispheres of young adult patients [6].

Patients with brain tumors have many problems which relate to the consequences of neurological dysfunction caused by a destructive or invasive tumor mass in the central nervous system. Astrocytic tumors can cause several neurological symptoms such as sensorimotor deficit, ataxia, language problems, and cognitive decline [7]. Symptoms can arise from the disease process itself, which usually causes focal neurological damage, and the side effects of treatment (surgery,

radiation therapy, and chemotherapy) can cause more diffuse damage [6]. Nevertheless, it must be emphasized that although more than 60 percent of patients with astrocytic tumors suffer from hemiparesis at the time of diagnosis, but only 3 percent complain of weakness as the initial symptom. At the onset of their disease, patients with gliomas have relatively low rates of hemiparesis and hemianesthesia, by the time of diagnosis, some or all of these findings are present in the majority of patients [8].

For more than 3000 years, practitioners in China have used acupuncture to treat various diseases, including hemiparesis [9]. Acupuncture treatment also has become increasingly popular in western societies. Acupuncture, as a treatment of individuals with hemiplegia after stroke, has a long tradition in China and can improve functions and the quality of life. Recent studies showed benefits in the rehabilitation of strokes [10–12]. But while there are still limitations in the quality [13], further controlled trials have been recommended and recommendations for study designs have been published [14].

The reporting of the promising results of acupuncture on hemiparesis after a stroke to other causes of hemiparesis is unclear and has not been investigated on a large scale in patients with astrocytic brain tumors. Even though acupuncture is a common treatment option for brain tumors in China [15, 16], no randomized, controlled studies have yet explored the effectiveness of acupuncture on the treatment of hemiparesis due to astrocytoma type II.

Therefore, the aim of this study was to verify whether acupuncture can significantly improve sensory-motor behavior and the quality of life for hemiplegia patients with astrocytoma grade II. While new protocols for hemiparesis after a stroke recommend a standard concept for the selection of acupuncture points [14], the effect of the points on the basis of the prominent symptomatology for an individualized therapy might offer additional treatment effects.

In Chinese medicine theory hemiplegia, which is caused by brain tumors, has a similar pathogenesis to that described after a stroke by the Chinese medicine terms "wind", "phlegm", "blood stasis", "deficiency", and "*yang*-hyperactivity" as the main pathological factors. Previous studies showed that acupuncture treatment by needling the *Ren* and *Du* meridians can effectively improve motor disturbance for hemiplegia patients affected by a stroke and enhance the quality of life [17, 18]. Another unique therapy concept is called *Jin-3-needling* technique, introduced by Professor Jin Rui of the Guangzhou University of TCM. On the basis of the experiences of modern medicine, Jin Rui has drawn the development of traditional acupuncture prescriptions into a rational and scientific direction [19]. In his concept, groups of three different acupuncture points are combined for certain physical conditions. This concept is widely applied in various kinds of motor dysfunctions such as hemiplegia due to a stroke [9].

Thus, our study attempts to observe the effectiveness of acupuncture on the quality of life and functional gain and to assess whether a special individualized acupuncture concept is superior to a standard acupuncture concept.

2. Methods

2.1. Subjects

2.1.1. Design and Setting. The study was a randomized, observer-blinded, controlled clinical trial with three parallel groups carried out in the Acupuncture Department of the Rehabilitation Center in Shenzhen Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine. All study participants signed a written informed consent before enrollment. The protocols were approved by the Institutional Review Board and Ethics Committee at Shenzhen Hospital Affiliated to the Guangzhou University of Traditional Chinese Medicine (approval number SZSZYY2008/AC/04) and examined in accordance with the guidelines on good clinical practice and ethical standards for human experimentation established by the Declaration of Helsinki.

Patients who had been admitted to the Rehabilitation Center in Shenzhen Hospital Affiliated to the Guangzhou University of Traditional Chinese Medicine after a brain tumor operation of an astrocytoma between September 2008 and December 2012 had been screened (Figure 1).

Inclusion Criteria. After the initial screening evaluation, patients were enrolled in the study if they met all of the following criteria: (1) patients affected by hemiparesis with histological evidence of newly diagnosed WHO grade II brain astrocytoma; (2) subjects aged between 18 and 70 years; (3) minimum of 2 weeks after brain tumor surgery, minimum of 4 weeks since the last dose of chemotherapy (6 weeks since nitrosoureas), and at least 6 weeks after radiotherapy before entering the study; (4) Karnofsky performance status (KPS) [20] of 50~90; and a life expectancy of more than 3 months.

Exclusion Criteria. Patients were excluded from the study if the following criteria were true: (1) severe concomitant diseases reducing life expectancy or influencing neurological deficit; (2) pregnancy or breast-feeding women; (3) tumor crossed the midline; and (4) psychiatric disease or mental confusion.

In addition, patients were ineligible if they had a recurrent glioma at the beginning of the study or during the study (CT scan or MRI of the brain confirmed). A complete physical examination and neurological assessment and a thorough inspection on history of hemiplegia were conducted before the trial. The neurosurgeon classified the macroscopic extent of resection at the time of surgery as: biopsy, resection with less than 50% tumor removal, 50–89% tumor removal, or 90–100% tumor removal.

2.1.2. Randomization and Blinding. Centralized random allocation by telephone was performed immediately before the first treatment. Randomization lists were prepared from computer-generated random numbers through DAS (Drug And Statistics) soft 2.0 by staff unconnected with the study and held at a hospital pharmacy.

The rating scales were evaluated by an investigator who did not have access to the randomization code until all data had been entered. The clinician examined the subjects in a

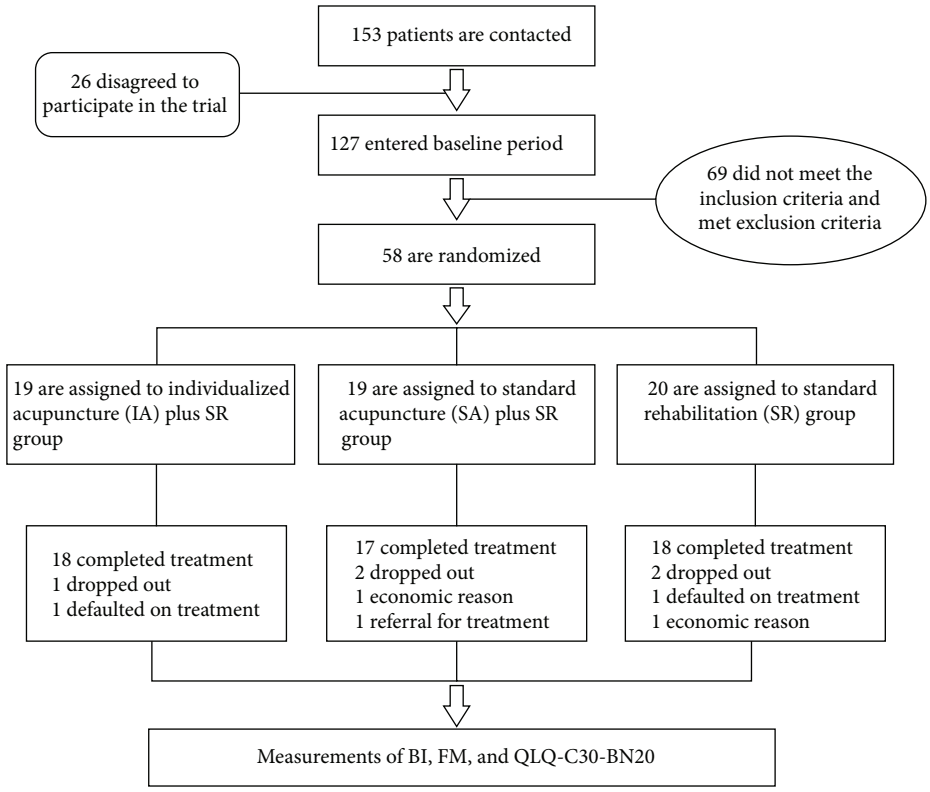


Figure 1: Trial flow diagram.

separate room and remained unaware of their group allocation. The consultation with the physician acupuncturist or rehabilitation therapist was standardized in terms of examination, treatment, and permitted discussion. Discussion with the acupuncturist about acupuncture or with the physiotherapist about rehabilitation was not permitted. Statisticians did not participate in the implementation process of the study either. During the preenrollment phase, principal investigators developed a manual of procedures that described the standardized data collection method for all outcome measurement tools, including the Barthel Index, the Fugl-Meyer scale, and the quality of life scale.

2.1.3. Interventions. Patients were randomized before the treatment to either a standard rehabilitation group (SR), a standard acupuncture plus standard rehabilitation group (SA + SR), or an individualized acupuncture plus standard rehabilitation group (IA + SR) by means of a random number table. While the study was conducted at the Acupuncture Department and Rehabilitation Center in Shenzhen Hospital Affiliated to the Guangzhou University of Traditional Chinese Medicine, subjects in the whole study received routine treatment from the Rehabilitation Center in Shenzhen Hospital including activating and passive range of motion (ROM) exercises of the upper and lower extremities for 40 minutes once a day during the full treatment period [21, 22] and received conventional physiotherapy to restore the normal movement and improve the muscle strength [23]. The treatment was customized by a qualified physiotherapist who was blinded to this study according to the individual health/recovery status. Participants received six 60-minute sessions per week of standard physiotherapy.

Routine medication included antihypertensive, antiepileptic, hypoglycemic, and antihyperlipidemic drugs which were taken according to the advice of a physician depending on different symptoms of each patient.

All subjects in our study were inpatients who had been hospitalized for 2 months. They received 2 months of treatment at the rehabilitation ward, so every patient received a weekly examination or evaluation in our hospital. Patients of the acupuncture groups were treated with acupuncture once a day, 6 times per week for 8 weeks.

Two appropriately qualified and experienced practitioners were involved in the study. They were physician acupuncturists of the Acupuncture Department in Shenzhen Hospital Affiliated to Guangzhou University of Traditional Chinese Medicine with 10 years of experience after graduation from the University of Traditional Chinese Medicine.

2.2. Acupuncture Treatment

2.2.1. Standard Acupuncture Treatment. Acupuncture therapy in the SA + SR group was given at acupoint *Neiguan (PC6)*, *Sanyinjiao (SP6)*, *Jiquan (HT1)*, *Chize (LU5)*, *Weizhong (BL40)*, and *Zusanli (ST36)*. This acupuncture protocol was developed according to traditional Chinese medicine theory and has been proved to be effective in treating patients with disabled states subsequent to a cerebrovascular lesion

[24–26]. Each point was performed on the affected limbs with the even reinforcing-reducing method [27]. The needle was maintained for 30 minutes. All acupuncture treatments were administered by the same physician acupuncturist in this group.

2.2.2. Individualized Acupuncture Treatment. Patients in IA + SR group were treated according to two specific treatment strategies called "*connecting and regulating Ren Du*" [28] and "*Jin-3-needling*" technique [9] as well as additional points selected on the basis of syndrome differentiation [29].

(1) Following the concept of "connecting and regulating *Ren and Du meridian*": *Guanyuan (RN 4)*, *Qihai (RN 6)*, *Zhongwan (RN 12)*, *Baihui (DU 20)*, and *Renzhong (DU 26)* were selected and acupuncture reinforcement manipulation [27] was applied to the acupoints of *Guanyuan (RN 4)*, *Qihai (RN 6)*, *Zhongwan (RN 12)*, and *Baihui (DU 20)*. The acupuncture reduction manipulation [27] was used at *the Renzhong (DU 26)* point.

(2) These were combined with points following the concept of "*Jin-3-needling*" [19].

Points of *Jin-3-needling* received ipsilateral acupuncture according to the affected limbs as described in Table 1. If there was spasticity, acupuncture reduction manipulation was used at the selected points. If there was no relevant increased muscle tone acupuncture reinforcement manipulation was used at the selected points.

(3) Additionally, points chosen on the basis of Chinese syndrome differentiation were added [29]. These syndromes and the additional points are described in Table 2. Points selected for deficiency syndromes (*Yin*-deficiency, *Qi*-deficiency) were stimulated with the acupuncture reinforcement manipulation and excess syndromes ("*Xue*-stasis", "phlegm", "Liver yang hyperactivity", and "endogenous liver wind") and with the acupuncture reduction manipulation.

The needles were manipulated by way of the respective method after insertion, after 15 minutes and before extraction after 30 minutes. All acupuncture treatments were administered by the same physician acupuncturist in IA + SR group. Point location and depth of insertion were performed as described in standard textbooks [30]. Disposable sterile steel needles of 0.30 × 30 mm were inserted to a depth of 10–30 mm.

2.3. Outcome Measurements

2.3.1. Primary Outcome Measurement

Barthel Index (BI). The Barthel Index (BI), also known as the Barthel Scale or Barthel ADL Index, is a scoring tool of a person's performance in the activities of daily life (ADL). It is used, in particular, to measure the self-care ability of patients with neurological disabilities such as those following a stroke. Patients were assessed in 10 areas of daily activities, including feeding, moving from a wheelchair to a bed and back, grooming, transferring to and from a toilet, bathing, walking on a level surface, going up and down stairs, dressing, and is continence of bowels and bladder. A score of 0–10 given for each of the 10 areas, depending on the patient's performance level, with a higher score indicating better performance, yielding a total score of 0–100. Besides being a quick and easy tool to provide an objective assessment of a patient's ADL, the Barthel Index is robust in that it has demonstrated high interrater reliability (0.95) and test retest reliability (0.89) as well as high correlations (0.74–0.8) with other measures of physical disability [31].

2.3.2. Secondary Outcome Measurements

FM Motor and Sensory Assessment. The Fugl-Meyer (FM) Motor and Sensory Assessments are used to measure the performance of voluntary limb movement (50 items) and the level of limb sensation (12 items), respectively. Each item is assessed and scored on a 3-point ordinal scale of 0–2 (0 for no performance/absent sensation, 1 point for partial performance/impaired sensation and 2 points for complete performance/normal sensation).

The FM motor assessment (50 items, total FM motor score of 100), in turn, consists of the upper extremity (UE) subscale (33 items, score range of 0–66) and the lower extremity (LE) subscale (17 items, score range of 0–34).

The FM sensory assessment (12 items, total FM sensory score of 24), in turn, consists of the light touch subscale (2 items each for UE and LE, score range of 0–8) and the proprioception subscale (4 items each for UE and LE, score range of 0–16) [32].

European Organization for Research and Treatment of Cancer (EORTC) Core Quality of Life Questionnaire (QLQ-C30) and Brain Cancer Module (BCM20). The standard Chinese version of the EORTC QLQ-C30 has been used to assess the quality of life (QOL) of patients. It is a 30-item questionnaire consisting of multi-item subscales and single items that cover various dimensions of QOL. Subscales include 5 functional subscales (physical, role, cognitive, emotional, and social); 3 symptom subscales (fatigue, pain, and nausea/vomiting); a global health/QOL subscale. Single items include items for the assessment of typical symptoms reported by cancer patients (dyspnea, appetite loss, sleep disturbance, constipation, and diarrhea) and an item related to the perceived financial impact of cancer and cancer treatment. The "yes/no-responses" are employed in the physical functional subscales. The "modified 7-point linear analogue scales" is employed in the global health/QOL subscales. The "4-point Likert-type scales", ranging from "1-not at all" to "4-very much", is employed in all other items. This questionnaire has demonstrated adequate reliability in studies related to patients with brain cancer [33]. It takes, on the average, 8 minutes to complete. Moreover, the compliance rates in multicenter and phase III clinical trials are very high for both the full questionnaire and individual item completion.

Table 1: The protocol of *Jin-3-needling* for hemiparesis.

Not relevantly increased muscle tone	Upper limb	*Quchi (LI 11), Hegu (LI 4), Waiguan (SJ 5)*
	Lower limb	*Zusanli (ST 36), Sanyinjiao (SP 6), Taixi (KI 3)*
Spasticity	Upper limb	*Neiguan (PC 6), Jiquan (HT 1), Chize (LU 5)*
	Lower limb	*Chongmen (SP 12), Weizhong (BL 40), Zhaohai (KI 6)*
Spasticity or not relevant increased muscle tone	Shoulder	*Jianyu (LI 15)* and 2 extrapoints [2 *cun* posterior and anterior of *Jianyu (LI 15)*]

Table 2: Additional acupuncture based on syndromes.

Syndrome [29]	Selected points	Operation
Qi deficiency	*Guanyuan (RN 4), Qihai (RN 6)*	Additional moxibustion with the reinforcing method
Xue stasis	*Xuehai (SP 10)*	Bilateral acupuncture with the reducing method
Phlegm	*Fenglong (ST 40)*	Bilateral acupuncture with the reducing method
Yin deficiency	*Taixi (KI 3)*	Bilateral acupuncture with the reinforcing method
Liver yang hyperactivity	*Fengchi (GB 20)*	Bilateral acupuncture with the reducing method

The BCM, a supplement to the QLQ-C30, is a new questionnaire developed for brain cancer patients, which addresses symptoms that are specific to brain cancer or its treatment. The 20-item EORTC brain cancer-specific questionnaire (QLQ-BN20) is organized into four scales: future uncertainty (3 items), visual disorder (3 items), motor dysfunction (3 items), and communication deficits (3 items), as well as 7 single items related to headache, seizures, drowsiness, hair loss, itching, weakness of both legs; and difficulties with bladder control. The "four-point Likert-type scales" ("not at all", "a little", "quite a bit" and "very much") is employed and is linearly transformed to a 0–100 scale, with higher scores indicating more severe symptoms [34].

FM and QLQ-C30-BN20 assessments were carried out before treatment and after an 8-week intervention. The Barthel scale was signed every week after intervention. An independent physician who performed the outcome assessments was blinded to treatment assignments.

2.4. Statistical Analysis. Statistical analysis was performed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). The analysis was on a per-protocol basis. Descriptive statistics are presented as arithmetic means, standard deviation (SD), and 95% confidence intervals (95% CI) for the sake of clarity. χ^2 test, t-test, one-way, and repeated measures data of ANOVA test which were used to compare the demographic characteristics and other variables of the 3 groups where appropriate. A value of $P < 0.05$ was considered significant. Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA) was used for the graphical presentation of the results. This analysis was restricted to the participants who fulfilled the protocol, known as per-protocol analysis.

We determined the sample size based on detecting the difference in the motor dysfunction of the BCM20 scale, with a power of 90% and a CI of 95%. The formula $n = 2[(Z_{\alpha/2} + Z_{\beta})\sigma/\delta]^2$ was used, with $\alpha = 0.05$, $\beta = 0.90$, $\delta = 38.7 - 11.1 = 27.6$, and $\sigma = 23.1$, which were chosen based on a previous study [35]. The estimated sample sizes were 20 (per group) in our study with a 15% dropout rate.

3. Results

3.1. Baseline Characteristics. For this pilot study a total of 58 patients were enrolled and randomized to either the IA + SR group ($n = 19$), the SA + SR group ($n = 19$), or the SR group ($n = 20$) in the present study. Patient baseline characteristics were comparable between the three groups (Table 3). There were no significant differences found between the three groups for age, gender, hemiplegia type, or extent of resection (all $P > 0.05$). None of the patients developed adverse effects caused by the acupuncture treatment.

Five patients dropped out during the trial, accounting for an 8.62% dropout rate. There were no deaths or protocol violators during the trial. Among the 5 dropouts, 2 patients discharged earlier because of economic problems; 2 patients defaulted on treatments; 1 patient withdrew and had a referral for radiotherapy or chemotherapy because the progressive tumor was growing. There was no statistical difference in between the dropouts from the 3 groups.

3.2. Barthel Index Scores. A repeated measure analysis of variance (ANOVA) model was used to compare the time-course profiles of the three groups with regard to the Barthel index scores.

The repeated measure analysis of variance between the groups showed significant differences ($F = 7.771$, $P = 0.001$). The LSD test showed significantly higher Barthel index scores in the IA + SR group than in the SR group from the 4th week to the 8th week after intervention ($P < 0.05$) and also higher scores, than in the SA + SR group from the 5th week to the 8th week ($P < 0.05$). It was also found that there was a statistically significant difference in the Barthel index score between the SA + SR group and the SR group at the 6th week ($P = 0.049$, 95% CI 0.04–19.56), while there were no significant differences between the two groups for BI scores at other weeks ($P > 0.05$ for all). By repeated measure analysis of variance, the Barthel index scores actually showed significant differences according to the sampling time ($F = 46.096$, $P < 0.001$). Similar results were, respectively, observed in the

Table 3: Characteristics of patients that completed the study in 3 groups.

Groups	IA + SR	SA + SR	SR
Characteristics	$N = 18$	$N = 17$	$N = 18$
Mean age (range), yr	44.21 ± 10.66 (25~65)	43.74 ± 11.26 (23~64)	43.99 ± 10.57 (20~62)
Gender (women/men)	6/12	7/10	7/10
Side of hemiplegia (right/left)	8/10	6/11	7/11
Extent of resection			
<50%	5	6	5
50–89% resection alone	4	4	5
Macroscopic removal (>90%)	9	7	8

IA + SR group ($F = 35.066$, $P < 0.001$), the SA + SR group ($F = 21.183$, $P < 0.001$), and the SR group ($F = 3.608$, $P = 0.005$). The Barthel index score in each group was the lowest in the pretreatment phase and then gradually increased after the intervention and reached a peak at the 8th week.

A group × time interaction was found between the group over time (ANOVA for Repeated Measure, Group-Time, $F = 3.643$, $P = 0.001$) (Figure 2).

3.3. Fugl-Meyer Assessment of Sensorimotor Function. By ANOVA we found that there were no significant differences at the baseline for the scores of FM (upper extremity), FM (lower extremity), FM (light touch), FM (proprioception), FM (total motor), and FM (total sensory) ($P > 0.05$, for all) between the three groups.

All groups showed significant changes on FM motor assessments (upper extremity and total motor) by comparison of scores at baseline and after 8 weeks (t test, $P < 0.05$, for all), while the score of FM motor assessment (lower extremity) significantly improved in the IA + SR group (t test, $P < 0.05$).

All scores of FM sensory assessments (light touch, proprioception, total sensory) significantly improved in the IA + SR group (t test, $P < 0.05$, for all), while in the SA + SR group, only the total sensory FM score significantly improved (t test, $P < 0.05$), while in the SR group, the scores of FM sensory assessments (light touch, proprioception, total sensory) did not significantly improve from the baseline to 8 weeks (t test, $P > 0.05$, for all) (Table 4).

Significant differences were observed at the 8th week for the scores of FM upper extremity ($F = 3.822$, $P = 0.029$), FM lower extremity ($F = 6.533$, $P = 0.003$), and FM total motor ($F = 6.463$, $P = 0.003$) between the three groups (Table 4). Figure 3 shows the comparison of the posttreatment motor scores of the three groups: significant differences were found in FM (upper extremity) scores in comparison between the IA + SR group and the SR group (Tamhane test, $P = 0.020$, 95% CI 1.52–21.82); in FM (lower extremity) scores in comparison between the IA + SR group and the SA + SR group (LSD's test, $P = 0.006$, 95% CI 1.99–10.97) and between the IA + SR group and SR group (LSD's test, $P = 0.002$, 95% CI 2.86–11.70); FM (total motor) scores comparison between IA + SR group and SA + SR group (Tamhane test, $P = 0.034$, 95% CI 1.00–30.97) and between IA + SR group and SR group (Tamhane test, $P = 0.001$, 95% CI 6.94–30.95). No significant differences were observed between the SR group and the SA + SR group in FM (upper extremity), FM (lower extremity), and FM (total motor) ($P > 0.05$, for all).

Significant differences between the three groups were observed at the 8th week for the scores of FM light touch ($F = 11.575$, $P = 0.000$), FM proprioception ($F = 6.346$, $P = 0.003$), and FM total sensory ($F = 16.594$, $P = 0.000$) (Table 4). Figure 4 shows the comparison of posttreatment sensory scores of the three groups by the LSD test: FM (light touch) scores are comparable between the IA + SR group and the SA + SR group ($P = 0.001$, 95% CI 0.76–3.01) as well as between the IA + SR group and the SR group ($P = 0.000$, 95% CI 1.45–3.66); FM (proprioception) scores are comparable between the IA + SR group and the SA + SR group ($P = 0.021$, 95% CI 0.42–4.91) and also between the IA + SR group and the SR group ($P = 0.001$, 95% CI 1.62–6.05); FM (total sensory) scores are comparable between the IA + SR group and the SA + SR group ($P = 0.000$, 95% CI 2.22–6.87) as well as between the IA + SR group and the SR group ($P = 0.000$, 95% CI 4.10–8.68). Meanwhile, there were no significant differences between the SR group and the SA + SR group in FM light touch, FM proprioception, and FM total sensory ($P > 0.05$, for all).

3.4. EORTC QLQ-C30-BN20. With respect to the QLQ-C30-BN20 scores there were no significant differences between the three groups in overall scores at the baseline (ANOVA, $P > 0.05$, for all) (Table 5).

Figure 5 summarizes significant differences between the three groups at 8 weeks after the intervention in the domains of physical function (ANOVA, $F = 5.404$, $P = 0.008$), emotional function (ANOVA, $F = 3.720$, $P = 0.031$), cognitive function (ANOVA, $F = 4.431$, $P = 0.017$), and global QOL (ANOVA, $F = 11.347$, $P < 0.001$) and in symptoms of fatigue (ANOVA, $F = 3.815$, $P = 0.029$), pain (ANOVA, $F = 6.632$, $P = 0.003$), insomnia (ANOVA, $F = 6.995$, $P = 0.002$), and anorexia (ANOVA, $F = 6.485$, $P = 0.003$). It also details significant differences of posttreatment through the posthoc tests: physical function between the IA + SR group and the SR group (LSD, $P = 0.005$, 95% CI 7.76–40.80) and between the SA + SR group and the SR group (LSD, $P = 0.009$, 95% CI 5.89–39.42); emotional function between IA + SR group and SR group (LSD, $P = 0.019$, 95% CI 2.20–23.58) as well as between the SA + SR group and the SR group (LSD, $P = 0.027$, 95% CI 1.49–23.18); cognitive function between the IA + SR

Table 4: Fugl-Meyer assessment of sensorimotor function at baseline and at 8th week.

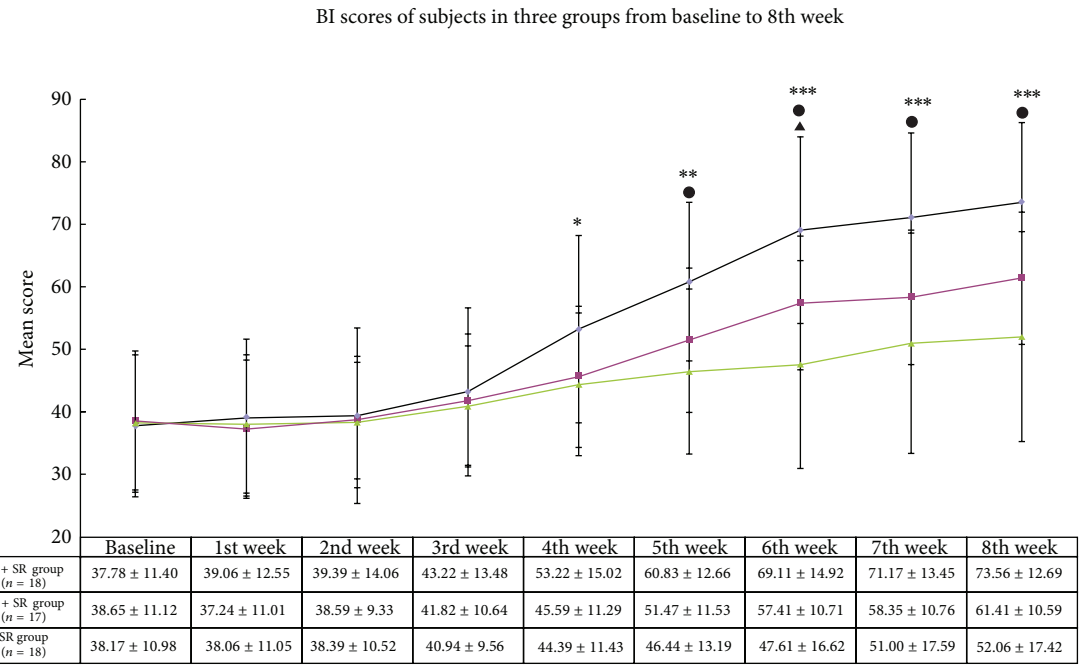
	IA + SR group ($n = 18$)		SA + SR group ($n = 17$)		SR group ($n = 18$)		P value (ANOVA)	
	Pretreatment	8th week later	Pretreatment	8th week later	Pretreatment	8th week later	Pretreatment	8th week later
FM motor assessment	35.56 ± 15.48	65.28 ± 10.45*	29.82 ± 16.21	49.29 ± 21.72*	29.94 ± 13.31	46.33 ± 17.12*	$P > 0.05$ for all	0.003#
Upper extremity subscore (0–66)	17.67 ± 12.01	39.44 ± 11.64*	16.47 ± 10.28	29.94 ± 15.88*	16.44 ± 8.72	27.78 ± 12.59*		0.029#
Lower extremity subscore (0–34)	13.89 ± 6.90	25.83 ± 5.28*	13.35 ± 6.38	19.35 ± 6.80	13.50 ± 5.83	18.56 ± 7.54		0.003#
FM sensory assessment	9.94 ± 2.73	16.67 ± 2.52*	9.53 ± 1.77	12.12 ± 3.41*	9.56 ± 2.01	10.28 ± 4.14		0.000#
Light touch subscore (0–8)	3.22 ± 2.16	5.94 ± 1.35*	3.18 ± 2.24	4.06 ± 1.71	3.28 ± 1.90	3.39 ± 1.85		0.000#
Proprioception subscore (0–16)	6.72 ± 2.93	10.72 ± 2.74*	6.35 ± 3.69	8.06 ± 3.23	6.28 ± 3.59	6.89 ± 3.85		0.003#

* $P < 0.05$, compared with pretreatment in each group by paired t-test, # $P < 0.05$, comparison between the three groups by ANOVA. The numbers are the means ± standard deviation.

Table 5: EORTC QLQ-C30-BN20 scores in 3 groups at baseline and 8th week.

Questionnaire	Scales/Items	IA + SR group ($n = 18$)		SA + SR group ($n = 17$)		SR group ($n = 18$)		P value (ANOVA)	
		Baseline	8th week	Baseline	8th week	Baseline	8th week	Baseline	8th week
QLQ-C30	*Function scales*							$P > 0.05$ for all	
	Physical function	70.78 ± 19.07	34.11 ± 30.36*	64.65 ± 25.64	56.76 ± 21.35	60.83 ± 28.45	58.39 ± 20.98		0.008△
	Role function	41.72 ± 17.76	40.50 ± 17.56	46.12 ± 14.72	44.35 ± 16.44	44.83 ± 15.25	43.94 ± 15.86		0.751
	Emotional function	65.22 ± 15.85	47.72 ± 17.57*	64.12 ± 17.48	60.06 ± 15.76	63.78 ± 17.02	60.61 ± 14.39		0.031△
	Cognitive function	47.78 ± 13.49	23.17 ± 16.26*	47.47 ± 18.36	38.41 ± 22.86*	49.39 ± 17.36	44.89 ± 26.96		0.017△
	Social function	60.00 ± 23.21	59.44 ± 23.48	53.47 ± 20.09	53.00 ± 20.62	55.56 ± 16.72	53.50 ± 20.64		0.617
	Global QL	56.78 ± 17.46	78.06 ± 18.68*	53.35 ± 15.34	65.35 ± 7.93*	50.28 ± 8.16	56.89 ± 11.02*		0.000△
	Symptom scales								
	Fatigue	46.94 ± 15.70	25.44 ± 21.56*	50.12 ± 20.11	37.06 ± 28.91*	51.33 ± 19.16	48.22 ± 23.42		0.029△
	Nausea/vomiting	22.33 ± 7.67	21.56 ± 8.91	21.71 ± 8.01	20.59 ± 9.13	21.89 ± 7.35	22.06 ± 11.00		0.903
	Pain	38.83 ± 8.07	15.06 ± 13.14*	37.94 ± 7.52	22.12 ± 13.17*	35.89 ± 9.54	31.72 ± 14.92		0.003△
	Dyspnea	26.89 ± 16.22	26.06 ± 16.67	34.35 ± 8.25	33.59 ± 9.82	31.72 ± 9.69	30.56 ± 11.73		0.241
	Insomnia	31.06 ± 7.76	13.83 ± 11.97*	32.06 ± 6.89	27.76 ± 12.03*	29.94 ± 7.55	27.28 ± 13.74		0.002△
	Anorexia	45.72 ± 12.89	15.72 ± 14.83*	46.71 ± 20.39	34.24 ± 27.20*	45.94 ± 19.86	41.94 ± 23.73		0.003△
	Constipation	23.56 ± 8.18	23.11 ± 8.70	25.35 ± 14.06	25.00 ± 14.35	24.39 ± 14.61	24.28 ± 16.53		0.917
	Diarrhea	20.22 ± 7.16	19.78 ± 7.79	21.53 ± 19.48	20.94 ± 19.87	22.06 ± 15.27	21.94 ± 19.80		0.927
	Financial impact	78.11 ± 10.35	64.72 ± 29.70	77.94 ± 10.78	76.65 ± 12.45	77.61 ± 12.25	78.06 ± 10.68		0.095
BN-20	*Scales*							$P > 0.05$ for all	
	Future uncertainty	51.06 ± 32.45	32.22 ± 19.77*	43.88 ± 31.98	28.94 ± 22.24	45.56 ± 30.26	30.39 ± 21.28		0.899
	Visual disorder	18.83 ± 20.71	17.17 ± 20.81	21.12 ± 21.66	18.76 ± 20.10	21.17 ± 19.71	19.28 ± 19.33		0.948
	Motor dysfunction	68.56 ± 16.48	32.94 ± 18.61*	64.12 ± 17.48	54.47 ± 21.14	65.67 ± 16.83	56.72 ± 17.79		0.001△
	Communication deficit	21.17 ± 23.51	15.06 ± 16.34	17.06 ± 16.98	16.35 ± 16.29	22.06 ± 21.71	21.11 ± 17.94		0.532
	Items								
	Headaches	17.28 ± 24.76	13.06 ± 19.88	14.35 ± 21.75	14.06 ± 20.24	14.61 ± 20.76	14.50 ± 19.62		0.975
	Seizures	12.06 ± 15.19	11.17 ± 12.90	10.71 ± 15.30	11.65 ± 15.15	12.56 ± 17.20	11.94 ± 14.75		0.987
	Drowsiness	33.22 ± 13.99	14.44 ± 11.75*	37.71 ± 19.94	24.29 ± 11.55*	36.50 ± 20.01	28.61 ± 10.37		0.001△
	Bothered by hair loss	9.39 ± 7.67	9.33 ± 7.59	9.29 ± 7.90	10.24 ± 7.77	8.89 ± 7.81	8.83 ± 7.34		0.858
	Bothered by itchy skin	10.44 ± 11.76	10.39 ± 11.54	9.94 ± 12.22	10.29 ± 12.11	10.28 ± 11.90	11.17 ± 12.42		0.972
	Weakness of both legs	64.78 ± 15.58	32.67 ± 13.46*	56.12 ± 24.07	47.71 ± 22.61	60.17 ± 19.19	50.67 ± 21.97		0.019△
	Trouble controlling bladder	18.11 ± 24.75	11.67 ± 19.01	12.06 ± 18.20	13.82 ± 20.41	18.39 ± 20.04	17.72 ± 19.37		0.646

*$P < 0.05$, compared with pretreatment in each group by paired t-test, △$P < 0.05$, comparison between the three groups by ANOVA. The numbers are the means ± standard deviation.



	Baseline	1st week	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week
IA + SR group ($n = 18$)	37.78 ± 11.40	39.06 ± 12.55	39.39 ± 14.06	43.22 ± 13.48	53.22 ± 15.02	60.83 ± 12.66	69.11 ± 14.92	71.17 ± 13.45	73.56 ± 12.69
SA + SR group ($n = 17$)	38.65 ± 11.12	37.24 ± 11.01	38.59 ± 9.33	41.82 ± 10.64	45.59 ± 11.29	51.47 ± 11.53	57.41 ± 10.71	58.35 ± 10.76	61.41 ± 10.59
SR group ($n = 18$)	38.17 ± 10.98	38.06 ± 11.05	38.39 ± 10.52	40.94 ± 9.56	44.39 ± 11.43	46.44 ± 13.19	47.61 ± 16.62	51.00 ± 17.59	52.06 ± 17.42

Figure 2: BI scores from the baseline to the 8th week. LSD post-hoc testing showed significant difference according to sampling time. "∗": comparison between the IA + SR group and the SR group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$); "•": comparison between the IA + SR group and the SA + SR group ($^{\bullet}P < 0.05$); "▲": comparison between the SA + SR group and the SR group ($^{\blacktriangle}P < 0.05$). The numbers are the means ± standard deviation.

group and the SR group (LSD, $P = 0.006$, 95% CI 6.69–36.76); global QOL between the IA + SR group and the SR group (Tamhane, $P = 0.001$, 95% CI 8.18–34.16) and between the IA + SR group and the SA + SR group (Tamhane, $P = 0.043$, 95% CI 0.35–25.06) as well as between the SA + SR group and the SR group (Tamhane, $P = 0.040$, 95% CI 0.31–16.62); fatigue between the IA + SR group and the SR group (LSD, $P = 0.008$, 95% CI 6.21–39.34); pain between the IA + SR group and the SR group (LSD, $P = 0.001$, 95% CI 7.44–25.89) and between the SA + SR group and the SR group (LSD, $P = 0.045$, 95% CI 0.24–18.97); insomnia between the IA + SR group and the SR group (LSD, $P = 0.002$, 95% CI 5.00–21.89) and between the IA + SR group and the SA + SR group (LSD, $P = 0.002$, 95% CI 5.36–22.50); anorexia between the IA + SR group and the SR group (Tamhane, $P = 0.001$, 95% CI 9.49–42.95).

Figure 6 shows that there were significant differences after intervention with respect to BN20 scores between the three groups in motor dysfunction (ANOVA, $F = 8.365$, $P = 0.001$), drowsiness (ANOVA, $F = 7.506$, $P = 0.001$), and weak legs (ANOVA, $F = 4.285$, $P = 0.019$). The post-hoc tests detected significant changes in motor dysfunction between the IA + SR group and the SR group (LSD, $P = 0.001$, 95% CI 10.93–36.63) and between the IA + SR group and the SA + SR group (LSD, $P = 0.002$, 95% CI 8.49–34.56); in drowsiness between the IA + SR group and the SR group (LSD, $P < 0.001$, 95% CI 6.65–21.69) and between the IA + SR group and the SA + SR group (LSD, $P = 0.012$, 95% CI 2.22–17.48); and in weak legs betweenthe IA + SR group and the SR group (Tamhane, $P = 0.018$, 95% CI 2.58–33.42).

The post-hoc tests show that there were no significant differences between the SA + SR group and the SR group for scores of items of QLQ-C30 and BCM20 scales ($P > 0.05$, for all) except with regard to the global quality of life and pain.

Compared with pretreatment in the IA + SR group tested by paired t-test, the scores significantly decreased after intervention in functional subscales of QLQ-C30 (physical, cognitive, and emotional) and symptom subscales (fatigue, pain, insomnia, and anorexia) ($P < 0.05$ for all) and in scales/items of BCM20 (future uncertainty, motor dysfunction, drowsiness, and weakness of both legs) ($P < 0.05$ for all). The score of a global QOL subscale, however, significantly improved after intervention in the IA + SR group and the SA + SR group ($P < 0.05$ for all). Significant changes were found in scores of cognitive function and symptom subscales (fatigue, pain, insomnia and anorexia) of QLQ-C30 and drowsiness of BCM20 for the SA + SR group ($P < 0.05$ for all).

4. Discussion

Our pilot study shows the first evidence that acupuncture may play a role in the rehabilitation of hemiparesis in patients with astrocytomas. The results show that either standard and individualized acupuncture or routine physiotherapy treatment can significantly improve motor functions in upper and lower extremities, but individualized acupuncture was more effective on the lower extremity and global motor functions than standard acupuncture and standard rehabilitation without acupuncture, which was confirmed by the motor function in BCM20 scales. The results also support

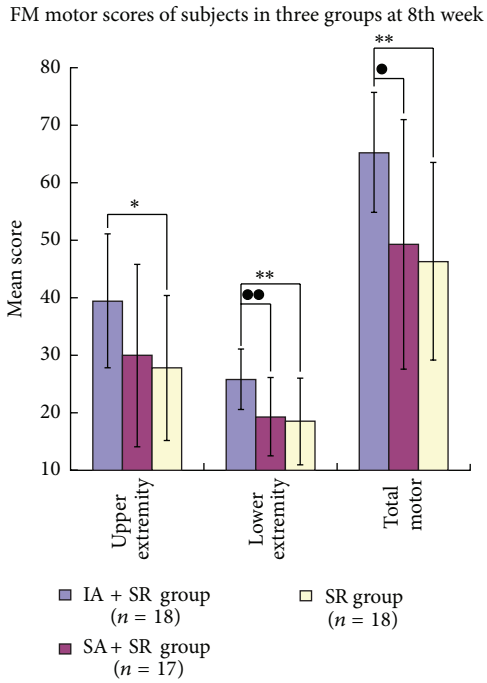


Figure 3: Comparison of FM motor scores of the 3 groups at 8th week. The post-hoc testing showed a significant difference: "∗": comparison between the IA + SR group and the SR group ($^{*}P < 0.05$; $^{**}P < 0.01$); "•": comparison between the IA + SR group and the SA + SR group ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$).

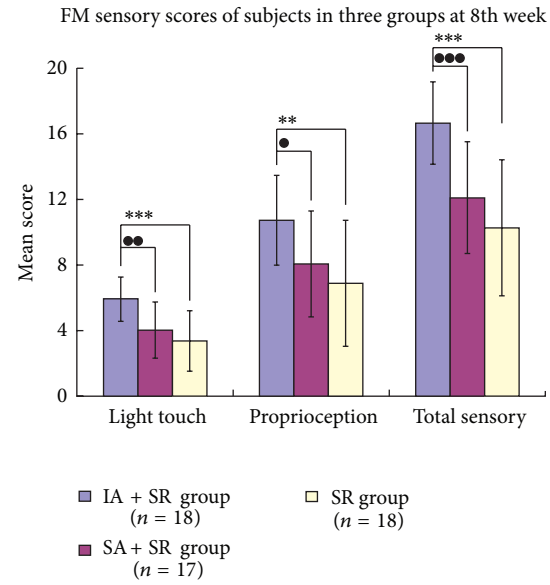


Figure 4: Comparison of FM sensory scores of the three groups at the 8th week. The post-hoc testing showed a significant difference: "∗": comparison between the IA + SR group and the SR group ($^{**}P < 0.01$; $^{***}P < 0.001$); "•": comparison between the IA + SR group and the SA + SR group ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$; $^{\bullet\bullet\bullet}P < 0.001$).

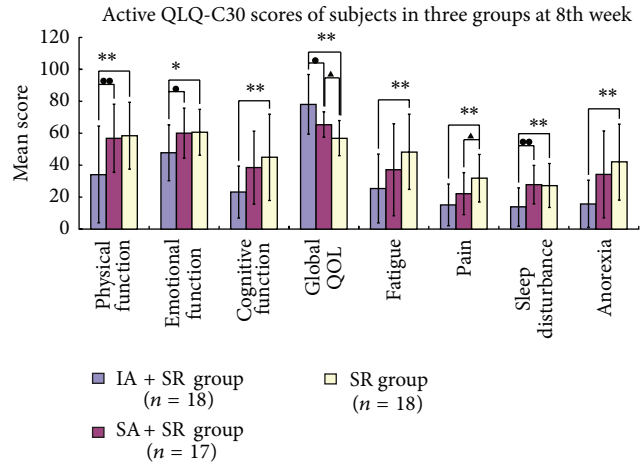


Figure 5: Active QLQ-C30 functions, symptoms, and global QOL scores at the baseline and at the 8th week. The post-hoc testing showed a significant difference: "∗": comparison between the IA + SR group and the SR group ($^{*}P < 0.05$; $^{**}P < 0.01$); "•": comparison between the IA + SR group and the SA + SR group ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$); "▲": comparison between the SA + SR group and the SR group ($^{\blacktriangle}P < 0.05$).

prior acupuncture studies on patients with hemiparesis after a stroke [10–12]. In Chinese medicine comparable concepts are used for treatment of hemiparesis after a stroke and due to brain tumors. This is not very surprising, because in western physiotherapy similar concepts for hemiparesis due to different causes are used. This might be an indication that certain parts of the mechanism of acupuncture in the rehabilitation of hemiparesis are, like western physiotherapy, the activation of healthy brain tissue around the lesions or activation of reciprocal inhibition [36] or perhaps even an enhancement of neuroplasticity [37, 38].

Placebo controlled studies in the rehabilitation of hemiparesis patients are almost impossible because all known therapies require an activation of the paralyzed limb. This includes acupuncture where, depending on the syndrome, reducing or reinforcing manipulation is necessary, while a neutral needling might not be stimulus enough [39]. A concept with placebo approaches like the Streitberger needle [40] seems inadequate for hemiparesis with differences of muscle tones. Randomization of patients, blinding the patients to the concept of acupuncture and blinding the independent observers to the therapeutic procedure as done in this study, seems adequate for studies on hemiparesis with acupuncture.

A general problem with acupuncture studies is the standardization of the procedures, while in traditional Chinese medicine an individualized approach with personalized acupuncture treatment is expected. We solved this problem by using two groups, one with a standardized concept that was promoted for studies on hemiparesis before [24–26] and the other with a unique, individualized concept taking the prominent area of symptoms and the status of the muscle tones into account. Our data showed the superiority of the individualized concept, which supports the necessity of individualized treatments and contradicts studies where there were not any different treatment effects found, regardless of where the needles were inserted [41]. One disadvantage of different treatment options in an individualized treatment

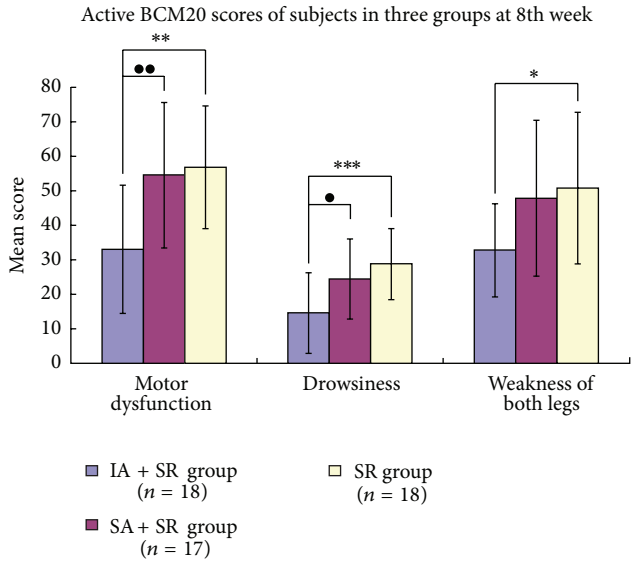


Figure 6: Active BCM20 scores at the baseline and the 8th week. The post-hoc testing showed a significant difference: "∗": comparison between the IA + SR group and the SR group ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$); "•": comparison between the IA + SR group and the SA + SR group ($^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$).

protocol is that it is hard to distinguish which acupoint has the specific effect. To study this, much bigger studies with the possibility of subgroup analysis are necessary. Prior studies of our group showed evidence that electroacupuncture at *Ren* and *Du* meridians can inhibit the overproliferation of hippocampal horizontal astrocytes after focal cerebral ischemia and promote the astrocytes differentiation in an animal model [42, 43]. Needling techniques on *Ren* and *Du* meridians had also been shown to improve brain stem auditory conducting function in patients with cerebral infarction by brain stem auditory evoked potential [44]. While activating of the *Ren* and *Du* meridians was part of the protocol of the individualized acupuncture group in this trial, similar mechanism of activation as well as inhibition of overproliferation might play a role especially in the treatment of spastic paresis, where we have a disbalance of activation and inhibition.

Our data shows that individualized acupuncture can improve the Barthel ADL index more than standard acupuncture and no acupuncture. Interestingly, significant differences between the tested groups just started after the treatment period of 4 weeks and became really significant after 8 weeks. This is similar to other clinical studies on the nervous system, where measurable improvements were seen in a period of more than 3 months [45]. This is not surprising, since activation of not completely damaged neural cells as well as activation of formerly nonactive cell takes time. This is important for future study designs: treatment as well as observation periods should be prolonged.

While in the nonacupuncture group no significant improvement of sensory function had been found, standard acupuncture improves only the global sensory function, and individualized acupuncture had a positive impact on global sensory impact, light touch, and proprioception. This is of importance since there is no other established treatment known having an effect on improving sensory function due to brain tumors beyond the natural improvement without specific treatment after intervention.

QLQ-C30 scales showed improvement after treatment with individualized acupuncture in functions such as physical, emotional, cognitive, and global QL, and in symptoms of fatigue, insomnia, and anorexia.

Our study also gave evidence that acupuncture can relieve pain in astrocytoma patients, but no significant difference between the standard and the individualized acupuncture was observed.

Future studies with bigger groups with the possibility of subgroup analysis and longer treatment and observation periods for astrocytomas grade 2 are necessary. Then the transferral of these data to other brain tumor types can be tested. While previous studies showed evidence of a positive impact of Chinese herbs on the quality of life of brain tumor patients [46, 47], it might be possible to develop a complex complementary concept for brain tumor patients.

5. Conclusion

This study gives first evidence that acupuncture might play a relevant role in the rehabilitation of astrocytoma grade II patients with hemiparesis. Individualized acupuncture plus standard rehabilitation was superior to standardized acupuncture plus standard rehabilitation and to standard rehabilitation alone. We conclude that the individualized acupuncture concept of "*connecting and regulating Ren and Du*" combined with "*Jin-3-needling*" offers a promising possibility for the treatment of hemiparesis due to astrocytoma, but further evaluation is mandatory.

Abbreviations

ADL:	Activities of daily life
ANOVA:	A repeated measures analysis of variance
BCM20:	20-item brain cancer module
BI:	Barthel index
BL:	*Taiyang* bladder meridian of the foot
CNS:	Central nervous system
DAS:	Drug and Statistics
DU:	*Du* meridian
EORTC:	European Organization for Research and Treatment of Cancer
FM:	Fugl-Meyer Motor and Sensory Assessment
HT:	*Shaoyin* heart meridian of the hand
IA:	individualized acupuncture
KPS:	Karnofsky performance status
LU:	*Taiyin* lung meridian of the hand
PC:	*Jueyin* pericardium meridian of the hand
QLQ-C30:	Core Quality of Life Questionnaire
QOL:	Quality of life

RN: *Ren* meridian
SA: Standard acupuncture
SP: *Taiyin* spleen meridian of the foot
SR: Standard rehabilitation
ST: *Yangming* stomach meridian of the foot.

Conflict of Interests

All authors certify that there is no conflict of interests with any financial organization regarding the material discussed in the paper.

Authors' Contribution

Haibo Yu and Sven Schröder contributed equally to the paper.

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Research Article

Integration of Different "-omics" Technologies Identifies Inhibition of the IGF1R-Akt-mTOR Signaling Cascade Involved in the Cytotoxic Effect of Shikonin against Leukemia Cells

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Hematological malignancies frequently have a poor prognosis and often remain incurable. Drug resistance, severe side effects, and relapse are major problems of currently used drugs, and new candidate compounds are required for improvement of therapy success. The naphthoquinone shikonin derived from the Chinese medicinal herb, *Lithospermum erythrorhizon*, is a promising candidate for the next generation of chemotherapy. The basal cellular mechanism of shikonin is the direct targeting of mitochondria. Cytotoxicity screenings showed that the compound is particularly effective against leukemia cells suggesting an additional cellular mechanism. mRNA and miRNA microarrays were used to analyze changes in gene expression in leukemia cells after shikonin treatment and combined with stable-isotope dimethyl labeling for quantitative proteomics. The integration of bioinformatics and the three "-omics" assays showed that the PI3K-Akt-mTOR pathway was affected by shikonin. Deregulations of this pathway are frequently associated with cancerogenesis, especially in a wide range of hematological malignancies. The effect on the PI3K-Akt-mTOR axis was validated by demonstrating a decreased phosphorylation of Akt and a direct inhibition of the IGF1R kinase activity after shikonin treatment. Our results indicate that inhibiting the IGF1R-Akt-mTOR signaling cascade is a new cellular mechanism of shikonin strengthening its potential for the treatment of hematological malignancies.

1. Introduction

Hematological malignancies comprise different types of cancers that affect blood cells, bone marrow, or lymph nodes. According to the National Cancer Institute, leukemia, lymphoma, and myeloma accounted for 9% of all deaths from cancer in the United States in 2011. In recent years improvements in radio therapy and new therapeutics including imatinib [1], rituximab [2], and lenalidomide [3] clearly improved the response and survival rate of these diseases [4]. Despite this considerable advances in chemotherapy, drug resistance and relapse remain major problems and several hematological malignancies remain incurable with standard treatments [4, 5]. This lack of cures requires novel targeted and less toxic therapies, and new candidate compounds need to be tested for the improvement of therapy success.

The naphthoquinone pigment shikonin is the most important pharmacologically active substance in the dried root of *Lithospermum erythrorhizon*. In traditional Chinese medicine (TCM) root extracts of *Lithospermum erythrorhizon* have been used to treat macular eruption, measles, sore-throat, carbuncles, and burns [6]. We recently showed that the natural compound shikonin has a strong cytotoxic effect against different cancer cell lines, especially sensitive and

resistant leukemia cells. Shikonin directly targets mitochondria of cancer cells causing an overproduction of reactive oxygen species (ROS), mitochondrial dysfunction, and ultimately apoptosis [7]. However, the question why shikonin is particularly effective against leukemia and lymphoma cells remains unresolved.

In the present study, we investigated the effect of shikonin on the myeloid leukemia cell line U937 by the integration of different quantitative "-omics" technologies, combining high-throughput techniques as a promising tool for elucidating molecular mechanisms of new drugs in a fast and precise manner [8]. The integration of genomic and pharmacological analysis significantly accelerates the identification of cancer-specific synthetic lethal targets [9]. We analyzed the mRNA, miRNA, and protein expression in U937 cells after shikonin treatment and integrated the results using a bioinformatic approach. Thereby, it was possible to identify cellular functions and signaling pathways strongly deregulated after shikonin treatment. The data obtained from the proteomic and transcriptomic studies confirmed previous findings indicating that shikonin has strong effects on cell proliferation, cell cycle progression, cellular movement, and DNA integrity of cancer cells [7]. Interestingly, our findings indicated that one of the most affected signaling pathways in U937 leukemia cells was the phosphatidylinositol 3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) cascade. Hence, we proposed that an inhibition of this signaling network is a reason for the strong activity of shikonin against leukemia cells. We validated the effect of shikonin on the PI3K-Akt-mTOR pathway by demonstrating a decreased phosphorylation and activation of Akt after shikonin treatment using phospho-specific antibodies and flow cytometric analysis. In addition, kinase activity tests revealed that shikonin inhibits the kinase activity of the insulin-like growth factor 1 receptor (IGF1R), which is an important trigger of the PI3K-Akt-mTOR signaling cascade.

Targeting of PI3K-Akt-mTOR signaling became an attractive therapeutic strategy for cancer chemotherapy over the last few years [10, 11]. The signaling pathway plays a central role in cellular growth and survival through the regulation of protein synthesis and ribosomal protein translation [12]. Deregulations of mTOR signaling are associated with tumorgenesis, angiogenesis, tumor growth, and metastasis [10, 13]. The mTOR signaling pathway has been found to be frequently deregulated, especially in a wide range of hematological malignancies [14]. The signaling cascade is activated by receptor tyrosine kinases (RTKs, e.g., IGF1R and epidermal growth factor receptor (EGFR)), integrins, and cytokine receptors coupling external signals from growth factors, cytokines and the availability of nutrients to cell growth and proliferation [15]. After binding of the corresponding ligands, the RTKs activate PI3K, which in turn causes the phosphorylation of Akt. Activated Akt inhibits the heterodimeric complex of tuberous sclerosis proteins 1 and 2 (TSC1/2) that negatively regulates the mammalian target of rapamycin complex 1 (mTORC1) [16]. This complex is a centerpiece of the signaling cascade that controls protein synthesis by phosphorylation of different effector proteins, for example, the S6 kinase 1 (S6K1) and the 4E-binding protein 1 (4E-BP1) [17]. Much less is known about the second mTOR complex mTORC2. This complex responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton [17]. Currently used drugs targeting this pathway are rapamycin and its derivatives (rapalogs) that directly target the mTORC1 complex [18, 19]. One weak point of these drugs is a resistance mechanism of cancer cells, which leads to an upregulation of IGF1R after mTORC1 inhibition [20–22]. This feedback mechanism causes an activation of the PIK3K-Akt-mTOR signaling cascade after initial inhibition resulting in only modest anticancer effects of rapalogs [14].

Ultimately, our results suggest that inhibition of IGF1R-Akt-mTOR signaling plays a key role in the cytotoxic effect of shikonin against U937 leukemia cells. Since this signaling network is frequently deregulated in hematological malignancies, shikonin is a promising candidate for the next generation of chemotherapy against these diseases.

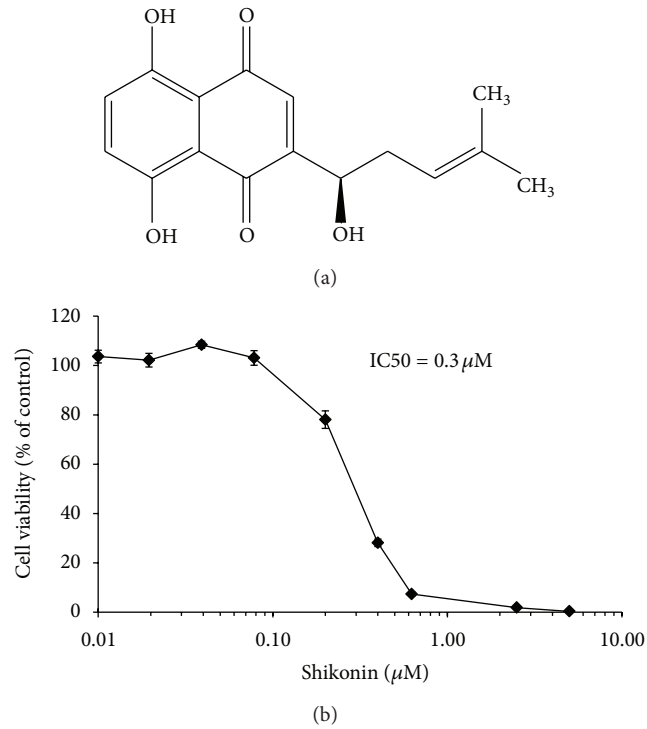

Figure 1: Cytotoxic effect of shikonin against U937 leukemia cells. (a) Chemical structure of shikonin. (b) Dose response curves of U937 cells after treatment with different concentrations of shikonin. Resazurin reduction assays were performed to determine dose response curve. Viability of U937 cells is represented by mean ± SEM of three independent experiments, and it is expressed as percentage survival of control.

2. Results

2.1. Cytotoxic Effect of Shikonin on U937 Leukemia Cells. The cytotoxic effect of shikonin against U937 leukemia cells was analyzed by resazurin reduction assay. The shikonin dose response curve was calculated after a 24 h treatment of subconfluent U937 cells (Figure 1). Shikonin inhibited

U937 proliferation reproducibly by 50% at a concentration of 0.3 μM, and this concentration was subsequently used in experiments for gene expression profiling and stable-isotope dimethyl labeling for quantitative proteomics.

2.2. Omics Data Reveals New Insights into Cellular Mechanisms of Shikonin. To get deeper insights into the molecular modes of action of shikonin in U937 cells, three "-omics" assays were performed (mRNA microarray, miRNA microarray, and stable-isotope dimethyl labeling for quantitative proteomics) quantifying transcriptomic and proteomic changes after shikonin treatment. The Venn diagram shows the number of deregulated molecules in each assay and the number of predicted mRNA targets of deregulated miRNAs (Figure 2). The intersections of the diagram indicate the number of corresponding molecules deregulated in different assays independently from the direction of their deregulated expression. The overlap of deregulated genes and proteins comprises 88 molecules (19%). Filtered to cases where the corresponding molecules are expressed in the same biological direction, for example up-regulated mRNA levels causing increased protein expression, the total overlap is limited to 52 molecules (11%). The top deregulated molecules are listed in Table 1.

Since each of the three "-omics" assays resulted in a plethora of information about shikonin's cellular mechanisms, we decided to evaluate the datasets in a comparative approach by extracting the most significant results. The data of the single assays were matched using the Ingenuity Pathway Analysis (IPA) comparative analysis tool, revealing cellular functions deregulated by shikonin treatment on the transcriptome as well as on the proteome (Figure 3(a)). The data confirms previous findings indicating that shikonin has strong effects on cell proliferation, cell cycle progression, cellular movement, and DNA integrity of cancer cells. Interestingly, shikonin also affects the posttranscriptional modification of RNA and disturbs cell-to-cell signaling and interaction. The data of the three "-omics" assays were compiled to one data set, and a pathway analysis was performed using IPA. This reanalysis identified a signaling network around the PI3K-Akt-mTOR axis, which was strongly affected by shikonin treatment (Figure 3(b)).

2.3. Effect of Shikonin on the PI3K-mTOR Signaling Cascade. We analyzed the PI3K-Akt-mTOR signaling pathway by a close examination of the upstream marker p-Akt and the downstream marker p-ribosomal protein S6 (pRiboS6) by flow cytometry. Samples with and without shikonin treatment were stained with directly conjugated antibodies against p-Akt and pRiboS6. Shikonin significantly inhibits the phosphorylation of Akt, while the phosphorylation of RiboS6 remained almost unchanged (Figure 4). This result indicates an effect of shikonin upstream of the PI3K-mTOR signaling cascade.

2.4. Virtual Screening. To identify possible targets of shikonin in the PI3K-mTOR pathway, we conducted a virtual screening approach. Binding energies of shikonin and key proteins

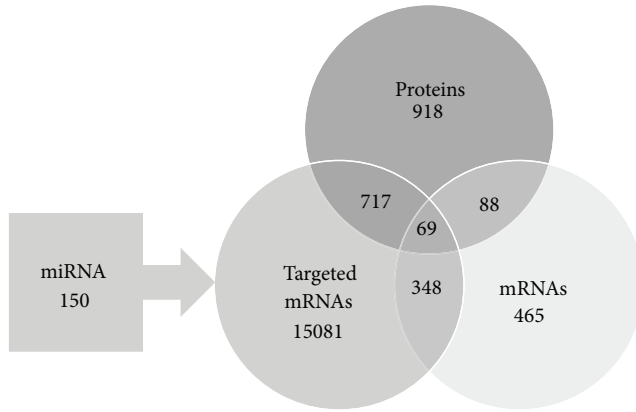

Figure 2: Results and relations of mRNA microarray, miRNA microarray, and stable-isotope dimethyl labeling for quantitative proteomics. Only molecules from the omics datasets that met the expression fold change cutoff of $\geq \pm 1.2$ are shown in this diagram. The intersections of the Venn diagram indicate the number of corresponding molecules deregulated in different assays independently from the direction of their deregulated expression.

of the signaling cascade—with available crystal structures—were calculated using the AutoDock Vina tool [23]. Proteins involved in the PI3K-mTOR pathway were selected according to a recent publication by Laplante and Sabatini [17]. Table 2 summarizes the ranking of the predicted target proteins of shikonin. Within the top potential binding partners, we found the receptor tyrosine kinases EGFR and IGF1R and the Serine/threonine-protein kinase Sgk1 (SGK1). Since the results of the Akt/RiboS6 phosphorylation by FACS showed no effect of shikonin on the phosphorylation status of ribosomal S6 protein, a target of SGK1, we did not further investigate the kinase as target of shikonin. EGFR and IGF1R are both important transmembrane receptors that trigger the PI3K-mTOR signaling cascade after binding to their respective growth factors like EGF, TGFα, and IGF. Previous studies showed that shikonin inhibits EGFR phosphorylation and modulates the EGFR signaling cascade [24], and we concentrated on the IGF1R for our further analysis of shikonin action, due to its known evolvement in drug evasion.

2.5. Shikonin Inhibits IGF1R kinase Activity. Since IGF1R showed a very high binding activity towards shikonin in the virtual screening experiment, we tested the inhibitory effect of shikonin on this kinase using a radiometric protein kinase activity assay. The dose response curve of shikonin on the IGF1R activity clearly indicates a dose-dependent inhibition of the kinase after shikonin application (Figure 5). An IC50 concentration of 2.6 μM was calculated for shikonin on the purified IGF1R kinase activity by nonlinear regression using Prism 5.04 (Graphpad, CA, USA). This IC50 of shikonin on the IGF1R kinase is very similar to the IC50 of about 3 μM observed for U937 cells suggesting that shikonin—besides inducing mitochondrial dysfunction—also exerts additional anticancer activities specifically by inhibiting IGF1R.

Table 1: Top up- and downregulated molecules in U937 cells after treatment with shikonin. Quantitative changes in the proteome were studied by stable-isotope dimethyl labeling. Microarray experiments were used to analyze changes in the mRNA and miRNA expressione.

	Proteins			mRNAs		miRNAS	
	Protein names	FC	Genes	Encoded proteins	FC	ID	FC
Top upregulated molecules	Heme oxygenase 1	+14.3	MLLT11	Protein AF1q	+6.2	miR-19b-2-5p	+5.2
	WD repeat-containing protein 3	+12.1	S100A8	Protein S100-A8	+4.7	miR-20b-3p	+3.7
	Histone H4	+11.1	LY96	Lymphocyte antigen 96	+4.5	miR-155-3p	+3.4
	Cell differentiation protein RCD1 homolog	+10.0	S100A9	Protein S100-A9	+4.2	miR-181a-2-3p	+3.2
	Sorting nexin-17	+9.1	CCL2	C-C motif chemokine 2	+4.2	miR-33b-5p	+3.1
	Mannose-6-phosphate isomerase	+7.7	VIM	Vimentin	+4.2	miR-30a-3p	+2.9
	Histone H2B type 2-E	+7.1	HMOX1	Heme oxygenase 1	+4.1	miR-3907	+2.8
	Schlafen family member 11	+6.7	ANXA1	Annexin A1	+4.1	miR-223-5p	+2.7
	LIM domain and actin-binding protein 1	+6.7	MAFB	Transcription factor MafB	+3.5	miR-193b-3p	+2.7
	Neuronal-specific septin-3	+6.7	JUN	Transcription factor AP-1	+3.2	miR-92a-3p	+2.7
Top downregulated molecules	Hexokinase-1	−20.5	ALDOA	Fructose-bisphosphate aldolase A	−17.6	miR-4299	−30.6
	Vacuolar protein sorting-associated protein 11 homolog	−20.0	ACTG1	Actin, cytoplasmic 2	−11.9	miR-1915-3p	−13.8
	60S ribosomal protein L24	−7.5	CCT7	T-complex protein 1 subunit eta	−7.8	miR-2861	−9.8
	5′-Nucleotidase domain-containing protein 2	−5.4	ARPC1B	Actin-related protein 2/3 complex subunit 1B	−7.8	miR-1207-5p	−6.3
	Heterogeneous nuclear ribonucleoprotein H2	−4.5	PSMC4	26S protease regulatory subunit 6B	−7.7	miR-1290	−6.2
	ATP-dependent RNA helicase DDX3Y	−4.3	ACTB	Actin, cytoplasmic 1	−6.8	miR-638	−6.1
	Methylosome protein 50	−4.0	HSPD1	60 kDa heat shock protein, mitochondrial	−6.6	miR-1246	−6.0
	Golgi phosphoprotein 3	−3.5	SLC39A3	Zinc transporter ZIP3	−6.2	miR-1185-5p	−5.2
	Leucine-rich repeat-containing protein 58	−3.5	ERP29	Endoplasmic reticulum resident protein 29	−6.0	miR-630	−5.1
	Rho GTPase-activating protein 4	−3.1	NCF1	Neutrophil cytosol factor 1	−5.9	miR-513a-5p	−4.7

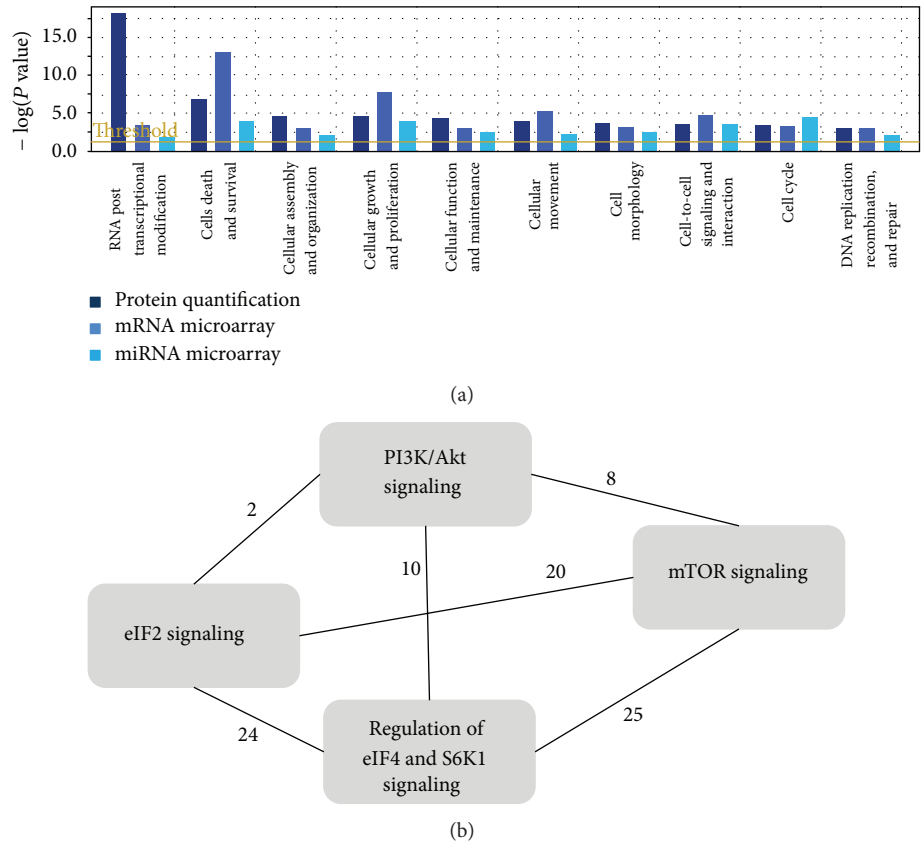

Figure 3: Complementarity of various "-omics" technologies provides a system-level understanding of shikonin's effects in U937 cells. (a) Comparison analysis of molecular and cellular functions affected by shikonin in three "-omics" assays. The bar graph displays only functions disturbed in all three assays. Right-tailed Fisher's exact test was used to calculate a P value determining the probability that each biological function assigned to the datasets is due to chance alone. (b) Overlapping signaling pathways deregulated after shikonin treatment. Numbers of identical molecules deregulated in overlapping pathways are shown.

3. Discussion

The combination of "-omics" data is a powerful tool to investigate the cellular effects and mechanisms of small molecules [25]. In the present study, we used this experimental approach to show that the natural naphthoquinone shikonin strongly deregulates the IGF1R-Akt-mTOR signaling cascade in U937 myeloid leukemia cells. Three different "-omics" assay indicated that the signaling pathway is disturbed at transcriptomic and proteomic level. Functional validation studies showed that shikonin indeed inhibits two central signaling nodes of the pathway: the kinase activity of IGF1R (a central receptor of the signaling cascade) was inhibited, and in addition the phosphorylation of Akt was significantly decreased upon shikonin application.

The PI3K-Akt-mTOR cascade is the predominant signal transduction pathway after IGF1R activation [15]. Our results are consistent with previous findings indicating that an inhibition of IGF1R causes a decreased phosphorylation and activation of Akt [26]. The reduced Akt phosphorylation in turn causes a decreased activation of the mTOR complexes (mTORC1/2) [12]. mTORC1 regulates cellular protein synthesis by phosphorylation of S6K1 and 4E-BP1, and mTORC2 controls cellular growth and survival through the phosphorylation of many AGC kinases including Akt, SGK1, and PKC-α [17]. It was shown that inhibition of critical signaling nodes of this pathway induces cell cycle arrest and apoptosis in leukemia cells [27]. These findings corroborate our results indicating that the cytotoxic effect of shikonin against leukemia cells is reinforced by a direct inhibition of IGF1R and a deregulation of the IGF1R-Akt-mTOR signaling cascade.

The signaling network around the mTOR kinase has been shown to be frequently deregulated in a wide range of hematological malignancies, especially in different types of leukemia [14]. For example, in acute myelogenous leukemia (AML), the mTORC1-signaling pathway is constitutively activated in almost 100% of all patients [28, 29]. Blocking this pathway could be an effective new treatment strategy for leukemia and other hematological malignancies. At the moment, rapamycin and its derivatives (rapalogs), for example, RAD001 (everolimus), CCI-779 (temsirolimus), and AP23573 (deforolimus), are used to partially inhibit the signaling pathway by directly binding the mTORC1 complex [18, 19]. However, results of clinical trials were mostly disappointing and showed only modest anticancer effects

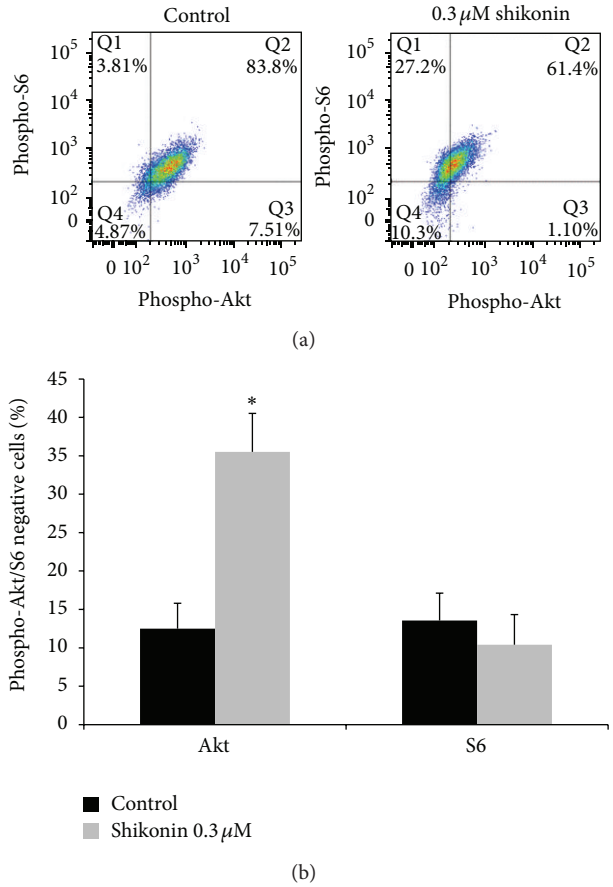

Figure 4: Effect of shikonin on the PI3K-mTOR signaling cascade. (a) U937 cells were treated with 0.3 μM shikonin for 24 h and subsequently stained with phospho-specific antibodies against p-Akt and p-ribosomal protein S6. Shikonin treatment significantly decreased the amount of phosphorylated Akt (left shift), but no effect on the phosphorylation status of ribosomal protein S6 was detected. (b) Statistical quantification of p-Akt or p-ribosomal protein S6 negative cells after shikonin treatment. Data points represent mean ± SD of at least three independent experiments. (*Significantly different according to Student's t-test, $P < 0.05$).

Table 2: Calculation of binding energies of shikonin and key proteins of the IGF1R-Akt-mTOR signaling pathway. Virtual screening was performed using the AutoDock Vina software.

Symbol	Description	Binding energy [kcal/mol]
EGFR	Epidermal growth factor receptor	−8.8
SGK1	Serine/threonine-protein kinase Sgk1	−8.8
IGF1R	Insulin-like growth factor 1 receptor	−8.6
GSK3B	Glycogen synthase kinase-3 beta	−8.5
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1	−8.5
S6K1	Ribosomal protein S6 kinase beta-1	−8.4
PKCA	Protein kinase C alpha type	−8.4
PIK3C3	Phosphatidylinositol 3-kinase catalytic subunit type 3	−8.3
PDK1	3-phosphoinositide-dependent protein kinase 1	−8.3
eIF4E	Eukaryotic translation initiation factor 4E	−8.3
RND3	Rho-related GTP-binding protein RhoE	−8.2
AKT2	RAC-beta serine/threonine-protein kinase	−8.1
RSK1	Ribosomal protein S6 kinase alpha-1	−8.1
Rheb	GTP-binding protein Rheb	−7.9
AMPK2	5′-AMP-activated protein kinase catalytic subunit alpha-2	−7.9
RHOA	Transforming protein RhoA	−7.9
AKT1	RAC-alpha serine/threonine-protein kinase	−7.8
RRAGD	Ras-related GTP-binding protein D	−7.8
mTOR	Serine/threonine-protein kinase mTOR	−7.5
RHOC	Rho-related GTP-binding protein RhoC	−7.3
RHOB	Rho-related GTP-binding protein RhoB	−7.2
ERK1	Extracellular signal-regulated kinase 1	−7.1
RHOD	Rho-related GTP-binding protein RhoD	−6.9
IRS1	Insulin receptor substrate 1	−6.4
IKKB	Inhibitor of nuclear factor kappa-B kinase subunit beta	−5.1

of these drugs [14]. A combination of rapalogs with other targeted molecules was more successful, and clinical trials have shown that rapalogs synergize with different conventional chemotherapeutics to overcome resistance [14, 30, 31]. Recent findings showed that a positive feedback loop causes the rapamycin-resistance phenotype: mTOR inhibition by rapalogs leads to an upregulation of IGF1R signaling, which in turn activates the PI3K-Akt-mTOR cascade again [20–22]. Thus, combining an mTOR inhibitor and an IGF-1R inhibitor may be an appropriate strategy to enhance mTOR-targeted anticancer therapy [22]. Since shikonin strongly deregulates the mTOR signaling pathway and in addition directly inhibits the kinase activity of IGF1R, it is a promising candidate for a cotreatment with rapalogs.

Our results are consistent with recent findings indicating that shikonin also modulates cell proliferation by inhibiting EGFR signaling [24]. EGFR is a further RTK that triggers the PI3K-Akt-mTOR cascade. Results showed that simultaneous targeting of EGFR and mTOR inhibits the growth of cancer cells [32]. An additional inhibition of EGFR signaling makes shikonin even more valuable for targeting the PI3K/mTOR cascade since a second important starting point of the pathway is eliminated.

Besides the deregulation of the PI3K-Akt-mTOR signaling cascade, we recently showed that shikonin directly targets the mitochondria of cancer cells and thereby triggers apoptosis [7]. Mitochondria targeting drugs were suggested to synergize with the common and clinically established

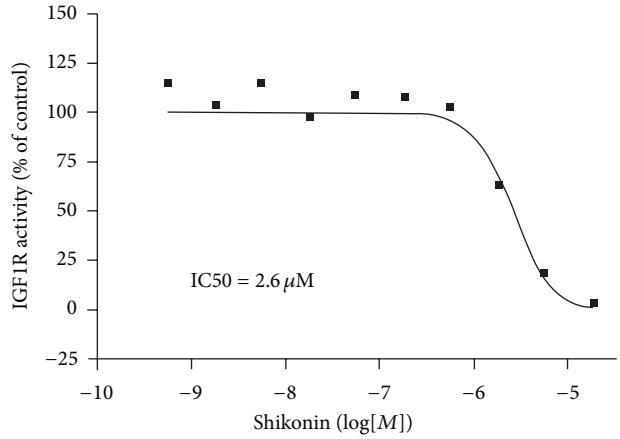

Figure 5: Dose response curve of shikonin in an IGF1-R kinase assay. A radiometric protein kinase assay (33PanQinase Activity Assay) was used for measuring the kinase activity of IGF1R. IC50 values were calculated by nonlinear regression using Prism 5.04 (Graphpad, CA, USA).

antileukemic drug arsenite trioxide [33]. This makes shikonin even more interesting for the treatment of leukemia. On the one hand, it inhibits the PI3K-mTOR signaling cascade, which is frequently deregulated in leukemia, and in addition it is directly targeting mitochondria of cancer cells, which makes it to a promising candidate for a combined treatment with established antileukemic drugs.

Ultimately, the direct targeting of mitochondria and the simultaneous deregulation of the IGF1R-Akt-mTOR signaling cascade make shikonin a promising compound for the treatment of hematological malignancies.

4. Materials and Methods

4.1. Chemicals. Shikonin was purchased from Enzo Life Sciences (Lausen, Switzerland) and a 50 mM stock solution was prepared by dissolving it in DMSO. Triethylammonium bicarbonate (TEABC), sodium cyanoborohydride, ammonium hydroxide, formaldehyde (37% solution in H_2O), formaldehyde-13C, d2 (20% solution in D_2O), tris (2-carboxyethyl) phosphine hydrochloride (TCEP), ammonium persulfate (APS), methyl methanethiosulfonate (MMTS), Triton X-100 (TX-100), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), sodium bicarbonate, N,N,N′,N′-Tetramethylenediamine (TEMED), 40% acrylamide/bis-acrylamide (37.5 : 1) solution, trifluoroacetic acid (TFA), formic acid (FA), bovine serum albumin (BSA), and bovine beta casein were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol, methanol, acetonitrile (ACN), and sodium dodecyl sulfate (SDS) were purchased from J. T. Baker (Phillipsburg, NJ). Trypsin (modified, sequencing grade) was from Promega (Madison, WI). Deionized water (18.1 MΩ·cm resistivity) from Milli-Q system (Millipore, Bedford, MA) was used throughout this work.

4.2. Cell Cultures. U937 cells were obtained from the German Cancer Research Center (DKFZ, Heidelberg, Germany). The original source of the cell line is the American Type Culture Collection (ATCC, USA). U937 cells were maintained in complete RPMI 1640 medium with 2 mM L-glutamine (Invitrogen, Germany) supplemented with 10% FBS (Invitrogen, Germany) and 1% penicillin (100 U/mL)-streptomycin (100 μg/mL) (Invitrogen, Germany). Cells were maintained in a humidified environment at 37°C with 5% CO_2 and subcultured twice per week. All experiments were performed on cells in the logarithmic growth phase.

4.3. Resazurin Reduction Assay. Resazurin reduction assay [34] was performed to assess cytotoxicity of shikonin toward U937 cells. The assay is based on reduction of the indicator dye, resazurin, to the highly fluorescent resorufin by viable cells. Nonviable cells rapidly lose the metabolic capacity to reduce resazurin and thus produce no fluorescent signal. Briefly, aliquots of 2×10^4 U937 cells per well were seeded in 96-well plates in a total volume of 100 μL. Shikonin was immediately added in varying concentrations in an additional 100 μL of culture medium to obtain a total volume of 200 μL/well. After 24 h, 20 μL resazurin (Sigma-Aldrich, Germany) 0.01% w/v in ddH_2O was added to each well and the plates were incubated at 37°C for 4 h. Fluorescence was measured on an Infinite M2000 Pro plate reader (Tecan, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was done at least two times, with six replicates each. The viability was evaluated based on a comparison with untreated cells. IC_{50} value represents the shikonin concentrations required to inhibit 50% of cell proliferation and was calculated from a calibration curve by linear regression using Microsoft Excel.

4.4. mRNA Microarray. Total RNA from U937 cells after 24 h of treatment with shikonin at IC_{50} concentration or DMSO solvent control was isolated using RNeasy Kit from Qiagen (Hilden, Germany) according to the manufacture's instruction. The quality of total RNA was checked by gel analysis using the total RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany). Only samples with RNA index values greater than 9.3 were selected for expression profiling. Microarray experiments were performed in duplicates for treated and untreated samples. Biotin-labeled cRNA samples for hybridization on Illumina Human Sentrix-HT12 Bead Chip arrays (Illumina, Inc.) were prepared according to Illumina's recommended sample labeling procedure based on the modified Eberwine protocol [35]. Biotin-16-UTP was purchased from Roche Applied Science (Penzberg, Germany). The cRNA was column purified with TotalPrep RNA Amplification Kit and eluted in 60 μL of water. Quality of cRNA was controlled using the RNA Nano Chip Assay on an Agilent 2100 Bioanalyzer and spectrophotometrically quantified (NanoDrop). Hybridization was also performed according the manufacturer's recommendations. Microarray scanning was done using a Beadstation array scanner, setting adjusted to a scaling factor of 1 and PMT settings at 430.

Data was extracted for each bead individually, and outliers were removed when the MAD (median absolute deviation) was greater than 2.5. Data analysis was performed by using the quantile normalization algorithm without background subtraction, and differentially regulated genes were defined by calculating the standard deviation differences of a given probe in a one-by-one comparison of samples or groups. The expression data obtained was filtered with Chipster data analysis platform. These steps include filtering of genes by two times standard deviation of deregulated genes and a subsequent assessment of significance using empirical Bayes t-test ($P < 0.05$).

4.5. Real-Time Reverse Transcription PCR. The same RNA samples used in the microarray experiments were also used for RT-PCR experiments. Total RNA samples were converted to cDNA by reverse transcriptase (Invitrogen) with random hexamer primers. Quantification of cDNA was performed by real-time PCR using a Taq-polymerase master mix (Roche) containing the fluorescent dye SYBR Green (Biozol) and the CFX384 Real-Time PCR Detection System (Bio-Rad). The efficiency of all primer pairs used for real-time PCR expression was better than 90%. PCR was performed with an initial denaturation at 95°C for 5 min followed by 50 cycles consisting of strand separation at 95°C for 30 s and annealing and extension at 60°C for 40 s. After PCR product amplification, melting curves were computed. Expression levels were normalized to the transcription level of G6PD. All samples were run in triplicates.

4.6. miRNA Microarray. miRNA from U937 cells after 24 h of treatment with shikonin at IC_{50} concentration was isolated using miRNeasy Kit from Qiagen (Hilden, Germany) according to the manufacture's instruction. The quality of miRNA was checked by gel analysis using the Small RNA Nano chip assay on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Germany). The miRNA microarray was performed at the Institute for Molecular Biology (IMB). Microarray experiments were performed in duplicates for treated and untreated samples. Human miRNA microarray chips (8 × 60 K, Agilent Technologies) were used. Probe labeling and hybridization were carried out following the miRNA microarray system with miRNA complete labeling and hyb kit protocol (Agilent Technologies). Briefly, extracted RNA was treated with phosphatase. Dephosphorylated RNA was fluorescently labeled by ligation of Cyanine 3-pCp molecules to the $3'$ end of RNA molecules using Agilent's miRNA Complete Labeling and Hyb Kit. The labeled RNA was desalted and hybridized for 20 hours at 55°C and 20 rpm. Microarray slides were washed and scanned with Agilent Microarray Scanning system. Images were analyzed and data were extracted, background subtracted, and normalized using the standard procedures of Agilent Feature Extraction Software. The expression data obtained was filtered with Chipster data analysis platform. These steps include filtering of miRNAs by two times standard deviation of deregulated genes and a subsequent assessment of significance using empirical Bayes t-test ($P < 0.05$).

4.7. Proteomics Analysis Using Dimethyl Labeling. To examine quantitative changes in the proteome of U937 leukemia cells after shikonin treatment, the dimethyl labeling proteomics analysis was performed. Briefly, 5×10^6 U937 cells were seeded in 10 mL RPMI 1640 medium in a 25 cm^2 culture flask. Subsequently, cells were treated with 0.3 μM shikonin (IC50) or DMSO as solvent control for 24 h. After incubation cells were washed with PBS and protein extraction was performed using the M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Germany) containing a protease inhibitor cocktail (Roche, Germany), cells were incubated with the extraction reagent for 40 min at 4°C. After incubation, cellular debris was removed by centrifugation at 14000 ×g for 15 min. Supernatants were transferred into new tubes and an acetone precipitation of the proteins was performed. After precipitation, the acetone was removed and the protein pellet in each of the tube was further diluted to 1 μg/μL with 50 mM TEABC and reduced with 5 mM TCEP for 1 h at 37°C, followed by alkylation using 2 mM MMTS for 45 min at room temperature. For the proteolytic digestion, the modified tube-gel digestion protocol was applied and the detergent residue was checked using the method described previously [36]. Two 200 μg proteolytic protein mixtures from DMSO and shikonin treated cells were first dissolved in 200 μL of 100 mM TEABC (pH 8.5) and, respectively, mixed with 20 μL of formaldehyde and formaldehyde-13C, d2 (4%, diluted with H_2O). After vortexing (5 min) and centrifugation, each of the sample solutions was mixed with 20 μL of 600 mM sodium cyanoborohydride solution. The sample solutions were vortexed (10 min) and centrifuged again and then allowed to react for 30 min at 25°C. To quench the reaction, ammonium hydroxide (7% in water, 10 μL) was added to each sample solution. Finally, 16 μL of formic acid was added to acidify each of the sample solutions and two samples were further combined for strong cation exchange (SCX) fractionation.

For SCX fractionation, the buffer SCX-A (5 mM KH_2PO_4 in 25% ACN at pH 3) and SCX-B (5 mM KH_2PO_4 and 350 mM KCl in 25% ACN at pH 3) were used as the mobile phase. The peptide mixtures were reconstituted in buffer SCX-A and then loaded into a PolySULFOETHYL A column (200 × 2.1 mm, 5 μm, 300Å, PolyLC, Columbia, MD) for 10 min at the flow rate of 0.2 mL/min. Peptides were fractionated using a 75 min gradient from 0 to 100% of buffer SCX-B. Fractions were collected every three minutes from the retention time of 10 to 55 min using a fraction collector (BioFrac Fraction Collector, BioRad Laboratories, Hercules, CA). The peptide mixtures in each of the fraction were further analyzed by the LC-MS/MS.

LC-MS/MS analysis was performed with a nanoUHPLC system (nanoACQUITY UPLC, Waters, Millford, MA) coupled online to the nanoelectrospray source of a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF-MS) (SYNAPT HDMS G2, Waters, Manchester, UK). For LC-MS/MS analysis, water with 0.1% FA and ACN with 0.1% FA were used as the mobile phase. The sample was injected into a trap column (Symmetry C18, 5 μm, 180 μm × 20 mm, Waters, Milford, MA) and separated online with a reverse

phase column (BEH C18, 1.7 μm, 75 μm × 250 mm, Waters, Milford, MA) at the flow rate of 300 nL/min using either a 95 min gradient with 5–90% ACN/water ratio. The mass spectrometry instruments were all operated in the positive ion mode, and data-dependent acquisition methods were applied for all experiments. The acquisition settings were set to one full MS scan (350–1600 m/z) with a scan time of 1 second and switched to six product ion scans (50–1900 m/z) with 0.4 second scan time when a precursor ion charge was 2+, 3+, or 4+, and the intensity was higher than 200 counts. The data files generated from LC-MS/MS were processed by UniQua and further analyzed by the MASCOT and Trans Proteomics Pipeline (TPP) 1 version 4.4 rev. 1, and the details and criteria of data processing were described previously [37].

4.8. Ingenuity Pathway Analysis. "-omics" datasets were analyzed through the use of ingenuity pathway analysis (IPA, Ingenuity Systems, CA, USA; http://www.ingenuity.com/). This software integrates the experimental results to known biological relationships, mechanisms, and functions using the regularly updated Ingenuity Knowledge Base, a giant database of biological findings and relations gathered from the literature. Only molecules from the "-omics" datasets that met the expression fold change cutoff of $\geq \pm 1.2$ were used for analysis with IPA. Briefly, filtered molecules were fed into Ingenuity Pathway Analysis software, and a separate core analysis was performed for each of the three "-omics" assays to assign the deregulated mRNA, miRNA, and proteins to cellular networks, functions, and pathways. Furthermore, the Ingenuity microRNA Target filter tool was used to associate deregulated miRNAs from the miRNA microarray with experimentally observed and predicted mRNA targets. The results of the core analysis were further studied in a comparison analysis to identify cellular functions affected in all three assays. Finally, the results of the "-omics" assays were merged in one dataset that was screened for overlapping cellular signaling pathways using the core analysis tool. For molecules that showed a nonconsistent deregulation of mRNA and protein expression (36 cases), the stronger deregulated expression values were considered for analysis.

4.9. FlowCellect PI3K-mTOR Signaling Cascade Assay. The FlowCellect PI3K-mTOR signaling cascade assay kit (Merck Millipore, Germany) was used to analyze the effect of shikonin on the PI3K-mTOR signaling pathway. The assay is based on two directly conjugated phospho-specific signaling antibodies against phosphorylated Akt and phosphorylated ribosomal S6 protein, which are both important signaling nodes of the mTOR cascade. The phosphorylation of Akt is indicative of the upstream PI3K signaling, marking the cells initiation into proliferation or cell survival. Phosphorylated-ribosomal S6 protein is indicative of downstream mTOR and p70S6K signaling leading to protein translation. The assay was performed according to the manufacture's protocol. Briefly, 1.5×10^6 U937 cells were treated with 0.3 μM shikonin or DMSO solvent control and incubated for 24 h. Subsequently, cells were fixed on ice for 20 min using the supplied fixation buffer. After fixation, cells were washed twice and treated with permeabilization buffer on ice for 20 min. After two further washing steps, cells were resuspended in a final volume of 90 μL assay buffer and incubated with 5 μL 20x anti-phospho-ribosomal protein S6 (Ser235) PerCP conjugated monoclonal antibody and 5 μL 20x anti-phospho-Akt1/PKBα (Ser473) Alexa Fluor 488 conjugated monoclonal antibody in the dark on ice for one hour. After antibody incubation, cells were centrifuged and resuspended in 500 μL assay buffer. Subsequently, cells were measured in a LSR-Fortessa FACS analyzer (Becton-Dickinson, Germany) and shikonin fluorescence was compensated for the respective channels. For each sample, 1×10^4 cells were counted. The PerCP signal was measured with 488 nm excitation and detected using a 670/30 nm bandpass filter. The Alexa Fluor 488 signal was analyzed with 488 nm excitation and detected using a 530/30 nm bandpass filter. All parameters were plotted on a logarithmic scale. Cytographs were analyzed using FlowJo software (Celeza, Switzerland). All experiments were performed at least in triplicate. Student's t-test was used for statistical analysis.

4.10. Virtual Screening. Virtual screening represents an approach to identify possible binding interactions between ligands and proteins without any previous knowledge of interactions or binding sites. Since shikonin deregulates the PI3K-mTOR signaling cascade, a virtual screening approach was used to identify proteins of the mTOR pathway which are most likely targeted by shinonin. Virtual screening was performed using AutoDock Vina with the graphical user interface AutoDock Tools [23]. X-ray structures of proteins involved in signaling cascade were downloaded from the "Protein Data Bank" (http://www.pdb.org/). The three-dimensional structure of shikonin was downloaded from the PubChem compound library (http://pubchem.ncbi.nlm.nih.gov/).

4.11. IGF1R Kinase Inhibition Assays. The kinase inhibition assay was performed by ProQinase GmbH (Freiburg, Germany). Shikonin was provided as 1.85×10^{-3} M stock solution in DMSO. In the process, shikonin was serially diluted in semi-log steps with 100% DMSO in a 96-well microtiter plate. Directly before use, shikonin was further diluted 1 : 10 with water. Shikonin was tested at 10 final assay concentrations in the range from 1.85×10^{-5} M to 5.55×10^{-10} M. The final DMSO concentration in the reaction cocktails was 1% in all cases. A radiometric protein kinase assay (^{33}PanQinase Activity Assay) was used for measuring the kinase activity of IGF1R. All kinase assays were performed in 96-well FlashPlates from Perkin Elmer (Boston, MA, USA) in a 50 μL reaction volume. The reaction cocktail was pipetted in 4 steps in the following order: 10 μL of nonradioactive ATP solution (in H_2O), 25 μL of assay buffer/[γ-^{33}P]-ATP mixture, 5 μL shikonin in 10% DMSO, and 10 μL of IGF1R/Poly(Glu, Tyr)4 : 1 mixture. The reaction cocktails were incubated at 30°C for 60 minutes. The reaction was stopped with 50 μL of 2% (v/v) H_3PO_4, and plates were aspirated and washed two times with 200 μL 0.9% (w/v) NaCl. Incorporation of $^{33}P_i$

(counting of "cpm") was determined with a Wallac Micro-Beta scintillation counter (Perkin Elmer). All assays were performed with a Beckman Coulter Biomek 2000/SL robotic system. Kinase activity was evaluated based on a comparison with experiments containing the complete reaction cocktail but no shikonin. A complete reaction cocktail without kinase served as control for unspecific binding of radioactivity to the plate in the absence of protein kinase but in the presence of shikonin. IC50 values were calculated by nonlinear regression using Prism 5.04 (Graphpad, CA, USA).

Abbreviations

ACN:	Acetonitrile
AML:	Acute myelogenous leukemia
AP23573:	Deforolimus
APS:	Ammonium persulfate
BSA:	Bovine serum albumin
CCI-779:	Temsirolimus
EGFR:	Epidermal growth factor receptor
4E-BP1:	4E-binding protein
FA:	Formic acid
FC:	Fold change
HEPES:	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
IC50:	Half maximal inhibitory concentration
IGF1R:	Insulin-like growth factor 1 receptor
IPA:	Ingenuity Pathway Analysis
LC-MS:	liquid chromatography-mass spectrometry
MMTS:	Methyl methanethiosulfonate
MS:	Mass spectrometry
mTOR:	Mammalian target of rapamycin
mTORC1/2:	Mammalian target of rapamycin complex 1 and 2
PI3K:	Phosphatidylinositol 3-kinase
Q-TOF-MS:	Quadrupole time-of-flight mass spectrometer
RAD001:	Everolimus
RiboS6:	Ribosomal protein S6
ROS:	Reactive oxygen species
RTK:	Receptor tyrosine kinase
S6K1:	S6 kinase 1
SCX:	Strong cation exchange
SDS:	Sodium dodecyl sulfate
SGK1:	Serine/threonine-protein kinase Sgk1
TCM:	Traditional Chinese medicine
TEABC:	Triethylammonium bicarbonate
TCEP:	Tris (2-carboxyethyl) phosphine hydrochloride
TEMED:	N,N,N′,N′-Tetramethylenediamine
TFA:	Trifluoroacetic acid
TPP:	Trans-proteomics pipeline
TSC1/2:	Tuberous sclerosis proteins 1 and 2
TX-100:	Triton X-100.

Conflict of Interests

The authors declare that there are no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this paper.

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Review Article

Significance of Kampo, Traditional Japanese Medicine, in Supportive Care of Cancer Patients

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The current standard treatment for cancer is a multidisciplinary therapy whereby various types of treatment are properly combined. Chemotherapy with multiple anticancer drugs is now common, and traditional, complementary, and alternative therapies are adopted as supportive measures. Medical care in Japan is distinguished by the ability for patients to access both Western and Kampo medical cares at the same time. There is a high degree of trust in the safety of Kampo therapies because they are practiced by medical doctors who are educated with fundamental diagnosis of Western medicine. Highly reliable clinical studies are being published, demonstrating that palliative or supportive care for cancer patients using Kampo preparations alleviates adverse effects of chemotherapy or radiotherapy. This paper reports the circumstances around cancer care in Japan where traditional therapeutic Kampo formulas are used for patients undergoing cancer treatment with cutting-edge chemotherapy, specifically to alleviate adverse effects of anticancer drugs.

1. Background

1.1. Historical Background of Cancer Treatment in Japan. Surgery, radiotherapy, and chemotherapy are the main medical treatments for cancer. Chief among those is surgery. In recent years, advances have been made in a range of treatments that target specific characteristics and stages of cancer. By its nature, cancer develops after gene mutations in the body's cells, and the difficulty in treating cancer lies in the fact that cells metastasize. Surgery and radiation are local therapies, which leave the problem of how to treat the invisible remaining cancer cells. What is then required is not a localized treatment but a systemic treatment such as chemotherapy.

But until progress was made in the development of anticancer drugs, there was no effective treatment against cancer once it had spread throughout the body. A combination of surgery with chemotherapy is generally used. And sometimes radiotherapy is also used. Nowadays, the standard treatment is "multidisciplinary treatment [1–5]," a comprehensive form of treatment that efficiently combines a variety of treatments.

1.2. Anticancer Drugs. Chemotherapy now occupies an important position in the treatment of cancer. Anticancer drugs have greatly changed cancer treatment. Excellent therapeutic effects have recently been achieved by combining radiation with anticancer drugs, even for solid cancers. The Achilles heel of anticancer drugs has been the strength of the adverse reactions [6–18]; however, these have been alleviated with the development of administration methods and supportive care to control nausea, vomiting, and so forth; therefore, patients do not suffer as much as before. Yet, the history of chemotherapy is still short. Surgery has been available for about 100 years and radiotherapy for about 50,

but anticancer drugs have only been used to treat cancer for the last 35 years.

Anticancer drugs have completely different effects depending on the type of cancer. While chemotherapy may be effective for some cancers, it is virtually ineffective for others. The effects of anticancer drugs also differ according to the way they are used. Potent effects are demonstrated when using drugs in combination, even if each anticancer drug does not promise sufficient effect when used alone. Nowadays, two to four types of anticancer drugs are used in combination to enhance their effectiveness, even at a modest amount. Such multidrug therapy is now being widely used and offers the hope of synergistic or additive effects.

1.3. Historical Background of Kampo Medicine. Traditional, complementary, and alternative therapies [19–25] are widely used and researched in the USA. Underlying this is the high cost of health care in that country and the common use of cheap folk remedies as well as traditional therapies and supplements against illness. The same situation exists in Europe and is becoming more widespread in Asia, where governments are promoting integrative medicine. There is a universal health insurance system which enables everybody in Japan to receive advanced health care at low cost. Therefore, alternative medicine did not attract attention. Japan's universal health insurance system [26, 27] is held in high regard across the country, and it means patients receive standard care for cancer at any medical service provider under this insurance system. However, if you prefer complementary or alternative therapies, you must pay a private provider out of your own pocket.

Yet, another characteristic of medical care in Japan is that patients can access Western and Kampo medical cares at the same time. Kampo medicine [28–30] is a unique medical system that originated from ancient China, was gradually imported to Japan since approximately 1500 years ago, and has been improved and refined by many excellent physicians especially since the 17th century (Edo periods in Japanese era). Now, most Kampo preparations (Japanese traditional herbal medicines) are available as extract formulations of high quality, which are greatly different from herbal medicines used in China, Taiwan, and Korea, where most preparations are herbal decoctions.

Four ethical Kampo extract formulations were approved in 1967 in Japan. Since then, the number of ethical Kampo extract formulations covered by health insurance has grown to 148. Much Japanese herbal extract preparation is used in Japan. Kampo extract preparation is mostly used in Japan. These Kampo extracts are the combination of herbal medicines from the viewpoint of Kampo theory. Standard examinations are done, and quality control of index ingredient is displayed. Kampo formulation for prescription is used for cancer medical treatment. Japan's universal health insurance system does allow simultaneous access to traditional Kampo preparations and Western medicines. However, doctors in Japan cannot be licensed without passing a board examination of Western medicine, which means patients in this country receive health care with a high degree of safety. This is another factor that distinguishes the health care system in Japan from other countries. In Japan, physicians who have studied Western medicine and Kampo medicine practice these approaches in their medical treatment of cancer with the aim of fusing Eastern and Western medicines into a unitary medical system, unlike the dual medical systems in China or Korea.

1.4. Supportive Care for Cancer Patients Using Kampo Preparations. Some people involved in the treatment of cancer reject Kampo therapy. The biggest reason they give is the scarcity of evidence. Kampo medicine is fundamentally a tailor-made type of treatment, and Kampo prescription is changed according to the patients' condition and symptoms. Therefore, the benefits of Kampo preparations cannot be fully evaluated using the criteria of the randomized clinical trials as in Western medicine. Objective data and proof of action mechanisms are required. Most of the studies on the actions of Kampo preparations have been animal trials and small-scale clinical trials. Little research has been done that offers highly reliable evidence, although progress has been made in this area recently [31–37]. The use of Kampo preparations for palliative and supportive care of cancer patients in combination with anticancer drugs or radiotherapy may offer alleviation of adverse effects and survival benefits, and the number of such research papers being published in international journals is increasing.

2. Kampo for Chemotherapy-Induced Peripheral Neuropathy

2.1. Cancer Chemotherapy-Induced Peripheral Neuropathy. A drawback of most anticancer drugs currently in use is that they are not cancer cell-specific: their actions affect all multiplying cells. They interfere with cancer cell division by interfering with DNA replication and the functioning of the proteins necessary for cell division, but they also damage normal cells. Myeloid cells, immune cells, gastrointestinal mucosal cells, and hair root cells are particularly susceptible to damage and are prone to adverse effects such as bone marrow suppression, immunodeficiency, digestive symptoms, and alopecia. Since nerve and muscle cells do not undergo cell division, they are thought to be robust against such damage. However, some anticancer drugs are known to cause peripheral neuropathy. While it is only certain anticancer drugs that has this side effect, we know that patients who take the following drugs develop peripheral neuropathy: taxane-based drugs [38–43] such as paclitaxel and docetaxel; vinca alkaloids such as vincristine sulfate; and platinum-based drugs [41, 44–55] including cisplatin and oxaliplatin. The causes involve injury to axonal microtubules and direct injury to nerve cells. Microtubules are necessary for the transfer of chromosomes when cells divide. If the formation of microtubules is disturbed, cell division is inhibited. In addition, microtubules are also found within axons, which transmit nerve cell signals, and are involved in axonal development and material transportation. Vinca alkaloids and taxanes, in particular, act on the microtubules within cancer cells but cause neuropathy because they simultaneously damage the

microtubules in normal nerve cells. Platinum-based drugs directly damage nerve cells and are thought to lead to nerve cell axon disorder.

2.2. Medical Treatment of Peripheral Neuropathy. Peripheral neuropathy symptoms include limb extremity numbness, as well as sensory motor ataxia, deep tendon reflex decline, and decreased muscle strength. There is great variation among sufferers of such complaints because sensation of these symptoms is extremely subjective. Patients may variously feel a tingling or stinging numbness or pain in the toes or fingers; an electric, shooting pain; loss of sense of touch; loss of heat/cold sensation; loss of power in the arms/legs; difficulty in grasping objects; or they may fall when walking. There are few effective remedies once peripheral neuropathy appears as a result of chemotherapy. In some cases the neuropathy may be almost irreversible. If symptoms are severe, the anticancer drug treatment must be discontinued or the prescription should be changed. In most cases, neuropathy persists as long as chemotherapy continues, and the symptoms do not disappear completely even after treatment ends, and complete recovery may take a long time. Treatment for peripheral neuropathy is not yet well established. Common medications including the combined use of calcium and magnesium or vitamin B6 and B12 have been reported to be effective to relieve numbness. The main symptomatic treatments for neuropathic pain include antidepressants, NSAIDs, or serotonin and norepinephrine reuptake inhibitors. If pain is severe, morphine and other narcotic analgesics may also be prescribed [56].

2.3. Indications and Evidence for Kampo Therapy for Chemotherapy-Induced Peripheral Neuropathy. The use of the Kampo preparation goshajinkigan [57–67] as drug therapy for peripheral neuropathy symptoms has been widely reported in Japan. Goshajinkigan extract preparation has been reported to relieve symptoms such as numbness or pain in 80% of cases in which it is used for peripheral neuropathy caused by paclitaxel for breast cancer [68]. Goshajinkigan also improves subjective symptoms of peripheral neuropathy due to the combined use of paclitaxel and carboplatin for ovarian or uterine cancers. Neuropathy is a characteristic adverse effect of oxaliplatin, the core drug for colorectal cancer. A high incidence of symptoms such as extremity numbness and cold sensation has been observed with the continued therapeutic use of oxaliplatin, especially at a cumulative dose over 500 mg/m^2. Treatment can be continued if symptoms are mild, but the dosage is decreased or the administration is discontinued in some severe cases. On the other hand, research has found that goshajinkigan can alleviate such symptoms. Nishioka et al. [69] and Kono et al. [70] conducted a retrospective comparison and examination of the effects of goshajinkigan for peripheral neuropathy associated with oxaliplatin in advanced or recurrent colorectal cancer patients. They found that the group which was administered goshajinkigan from the start of chemotherapy tolerated the largest dosage until onset of peripheral neuropathy. Goshajinkigan's efficacy differs according to the causal anticancer drug. It promises some effectiveness for numbness caused by paclitaxel, and so forth, but it is virtually ineffective for oxaliplatin. Since it might be effective for prevention of oxaliplatin-induced neuropathy, it would be better to administer goshajinkigan from the start of chemotherapy. It has been reported that administration of Kampo preparations promises an increase in the frequency of administration during the FOLFOX regimen, which centers on oxaliplatin, before onset of numbness as an adverse effect [58, 70].

Table 1: Goshajinkigan extract granules for ethical use.

Description	Goshajinkigan extract granules for ethical use	
Composition	7.5 g of TSUMURA goshajinkigan extract granules contains 4.5 g of a dried extract of the following mixed crude drugs:	
	JP *Rehmannia* root	3.0 g
	JP *Achyranthes* root	3.0 g
	JP *Cornus* fruit	3.0 g
	JP *Dioscorea* rhizome	3.0 g
	JP *Plantago* seed	3.0 g
	JP *Alisma* rhizome	3.0 g
	JP poria sclerotium	3.0 g
	JP moutan bark	1.0 g
	JP cinnamon bark	1.0 g
	JP powdered processed aconite root	5.0 g
	Inactive ingredients	
	JP magnesium stearate	
	JP lactose hydrate	
	Sucrose esters of fatty acids	

(JP: The Japanese Pharmacopoeia.)

2.4. Goshajinkigan. Goshajinkigan's Kampo constituents and HPLC fingerprint appear in Table 1 and Figure 1.

Goshajinkigan is indicated for the relief of the following symptoms in patients with decreased urine volume or polyuria, occasional dry mouth, proneness to fatigue, and sensitivity to cold in the extremities: leg pain, low back pain, numbness, blurred vision (elderly), pruritus, dysuria, and edema. Goshajinkigan consists of 10 constituent crude drugs (Table 1) and is a prescription with fortified effectiveness against swelling, numbness, and arthralgia, in addition to the beneficial effects of hachimijiogan. Specifically, goshajinkigan is a Kampo preparation that improves blood circulation, has a body warming analgesic action, and reduces swelling. It is used for patients with remarkable edema tendency, severe arthralgia, and persistent low back pain. It is frequently used for symptoms in which peripheral vascular disease is suspected of being involved, such as sciatica and diabetic neuropathy, and has demonstrated effectiveness for these conditions. The usefulness of goshajinkigan is conjectured to be aconitine [71]. Shakuyakukanzoto is a Kampo preparation used for various types of myalgia including menstrual pain and cramp [72]. Shakuyakukanzoto has been reported to demonstrate effectiveness for arthralgia and

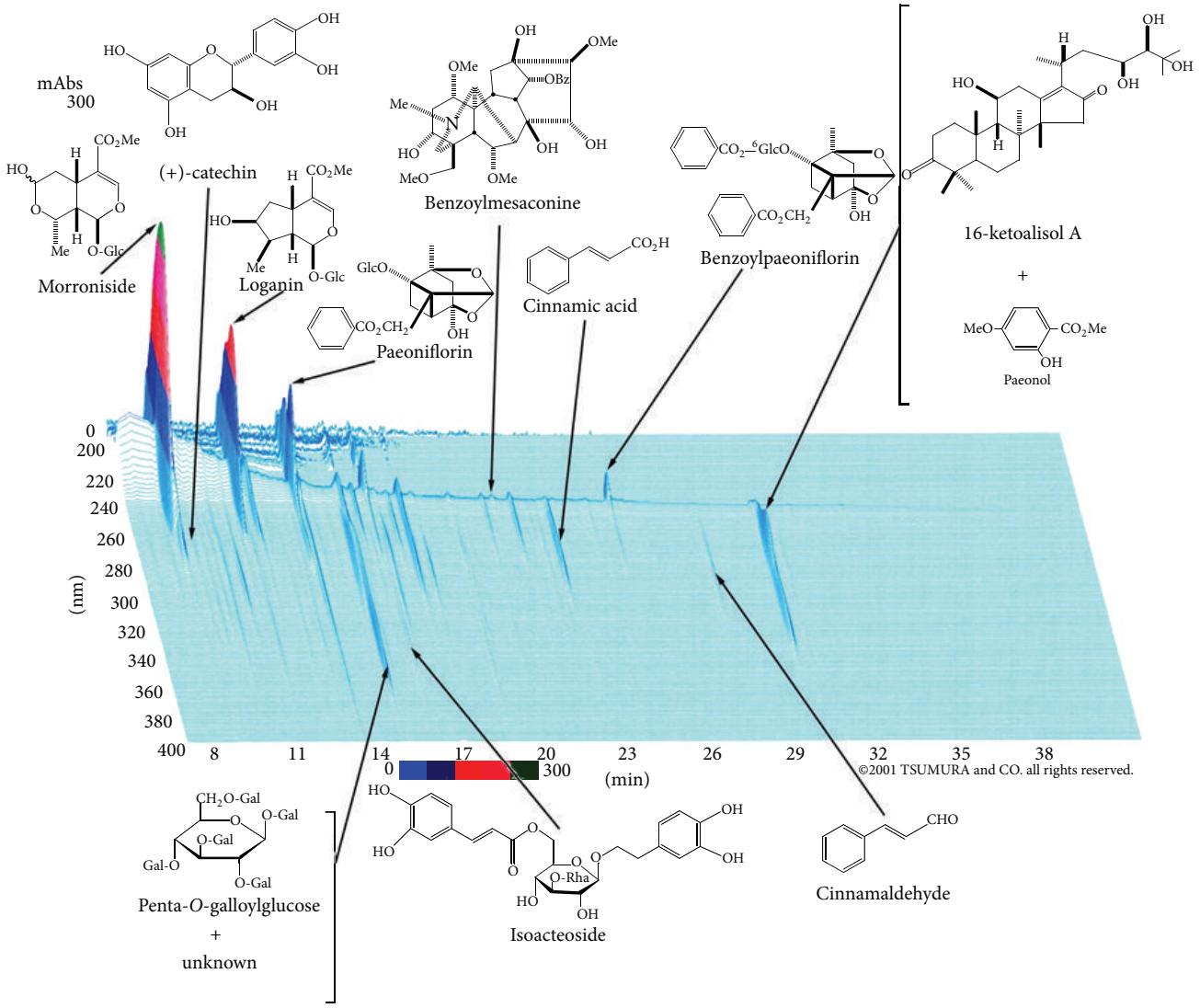

Figure 1: 3D-HPLC pattern of TJ-107 goshajinkigan (this 3D-HPLC was created in 2001 by TSUMURA and CO.).

myalgia due to paclitaxel [73]. While it is impossible to completely control peripheral neuropathy and myalgia caused by anticancer drugs, the combined use of goshajinkigan and shakuyakukanzoto may enhance improvement of subjective symptoms.

3. Kampo for Chemotherapy-Induced Diarrhea

3.1. Development of a Camptothecin Derivative—Irinotecan. Irinotecan is an anticancer drug classified as a plant alkaloid. It inhibits cancer cell proliferation by breaking DNA during cell division through its inhibition of the enzyme topoisomerase, which is required when DNA replicates. Wall et al. extracted and isolated camptothecin (CPT) in 1966 from *Camptotheca acuminate*, a plant native to China, and found that it has a powerful antineoplastic effect [74]. Subsequent development was undertaken by the National Cancer Institute (NCI) in the USA but was abandoned following the emergence of adverse effects. Pharmaceutical manufacturers in Japan vigorously pursued synthetic research into derivatives to preserve CPT's activity while reducing its toxicity, resulting in the CPT derivative irinotecan, which has been subsequently used as a potent anticancer drug. Irinotecan has demonstrated usefulness for various types of cancer, including colon and lung cancers, and its applications have been widening. Irinotecan suppresses the action of topoisomerase 1, which is involved in DNA replication, thereby demonstrating a strong antitumor effect; however, it can cause severe adverse effects including leukopenia and diarrhea [16, 17, 75–94].

3.2. Adverse Effects of Irinotecan and Their Frequency. The chief adverse effects are severe myelosuppression and intractable diarrhea. There have been reports of death following severe infection due to myelosuppression, intractable diarrhea, and intestinal perforation due to intestinal paralysis or bowel obstruction. Irinotecan undergoes metabolism

in the liver where it is converted into the active metabolite SN-38, setting off an antitumor action. SN-38 is then deactivated by conjugation reaction by uridine diphosphate glucuronosyltransferase (UGT) and excreted into the duodenum through the biliary tract. However, individual variability in the UGT activity is thought to be a reason for individual variation in the adverse effects of irinotecan. Many reports [95–99] in recent years mention the relation between UGT1A1 genetic polymorphism and onset of adverse effects of irinotecan. UGT1A1 is a molecular species of UGT in the liver and is the enzyme that metabolizes irinotecan. UGT1A1*28 and *6 are variants of UGT1A1, and reports cite an increase in the incidence of severe adverse effects of irinotecan due to reduced UGT1A1 activity.

The most troublesome adverse effect of CPT-11 is severe delayed diarrhea, which is caused by reactivation in the digestive tract by enteric bacterial β-glucuronidase. CPT-11 is a prodrug firstly decomposed by carboxyl esterase in the liver into SN-38, which has a powerful anticancer action and is then transported throughout the body. The SN-38 formed in the liver is glucuronidated by a glucuronidation enzyme also present in the liver. At this point, the SN-38 is deactivated, losing its injurious effect. However, after being excreted into the digestive tract via the biliary tract, the SN-38 is decomposed by enteric bacterial β-glucuronidase, thereby reforming SN-38. It is surmised that this SN-38 formed in the digestive tract then damages intestinal mucosal epithelial cells, giving rise to delayed diarrhea.

3.3. Irinotecan Hydrochloride-Induced Diarrhea and Kampo. The flavonoid glycoside baicalin may control irinotecan hydrochloride-induced diarrhea because it actively inhibits β-glucuronidase of intestinal flora and suppresses reformation of the active form (SN-38) in the digestive tract. Large amounts of baicalin are contained in *Scutellaria* root, a constituent crude drug of Kampo preparations. Researchers have tested hangeshashinto for diarrhea as it is a Kampo preparation containing *Scutellaria* root. It has been reported in human clinical trials and animal experiments that administration of hangeshashinto [37, 100–104] extract formulation two to three days before irinotecan hydrochloride administration effectively prevents or reduces diarrhea. It has been ascertained that this does not affect the antitumor action.

If the preventative effect against irinotecan hydrochloride-induced diarrhea is contained solely in the action mechanism of the flavonoid glycoside-induced β-glucuronidase inhibition, single administration of a flavonoid glycoside or *Scutellaria* root, rather than a Kampo formulation, may also be effective. Nevertheless, the comprehensive actions of the other crude drugs contained in hangeshashinto improve effectiveness. Specifically, it has been reported that hangeshashinto suppresses elevation of enteric prostaglandin E2, promotes repair of damaged intestinal mucosa, and improves intestinal water absorption [37, 100]. A particular characteristic of Kampo preparations is that they give greater efficacy through the synergistic effect of multiple crude drugs compared to one constituent alone. Loperamide hydrochloride is often administered for irinotecan hydrochloride-induced diarrhea, yet in some cases it is ineffective, maybe because loperamide does not cure intestinal mucosal damage. The Tochigi Cancer Center Research Group published the results of a clinical study that compared the degree of diarrhea in 41 patients with advanced non-small-cell lung cancer following anticancer drug treatment with irinotecan hydrochloride and cisplatin. Eighteen patients were administered hangeshashinto while the control group (without hangeshashinto administration) consisted of 23 patients. No significant difference in diarrhea frequency or interval was observed between the hangeshashinto group and the nonadministration group; however, the frequency of severe grade three and four diarrheas was lower in the hangeshashinto group [37].

Table 2: Hangeshashinto extract granules for ethical use.

Description	Hangeshashinto extract granules for ethical use	
Composition	7.5 g of TSUMURA hangeshashinto extract granules contains 4.5 g of a dried extract of the following mixed crude drugs:	
	JP *Pinellia* tuber	5.0 g
	JP *Scutellaria* root	2.5 g
	JP processed ginger	2.5 g
	JP *Glycyrrhiza*	2.5 g
	JP jujube	2.5 g
	JP *Ginseng*	2.5 g
	JP *Coptis* rhizome	1.0 g
	Inactive ingredients	
	JP magnesium stearate	
	JP lactose hydrate	
	Sucrose esters of fatty acids	

(JP: The Japanese Pharmacopoeia.)

3.4. Hangeshashinto. The Kampo constituents and HPLC fingerprint appear in Table 2 and Figure 2.

Hangeshashinto is indicated for the relief of the following symptoms in those patients with blocked feeling in the stomach pit and occasional nausea, vomiting, anorexia, borborygmus, and a tendency of loose stool or diarrhea. The targeted diseases and symptoms are as follows: acute or chronic gastrointestinal catarrh, fermentative diarrhea, dyspepsia, gastroptosis, nervous gastritis, gastrasthenia, hangover, belching, heartburn, stomatitis, and neurosis.

The reference sources for hangeshashinto are "*Shokanron*" and "*Kinki-yoryaku*". Hangeshashinto consists of seven crude drugs. *Pinellia* Tuber clears fluid retention in the stomach and stops vomiting, while together with *Coptis* rhizome and *Scutellaria* root, it clears gastrointestinal inflammation. *Coptis* rhizome and *Scutellaria* root are bitter, and they are good for the stomach and have anti-inflammatory actions. *Ginseng* and ginger improve gastrointestinal blood flow and promote the recovery of gastrointestinal function. *Glycyrrhiza* and jujube harmonize crude drugs and enhance their cooperative effects.

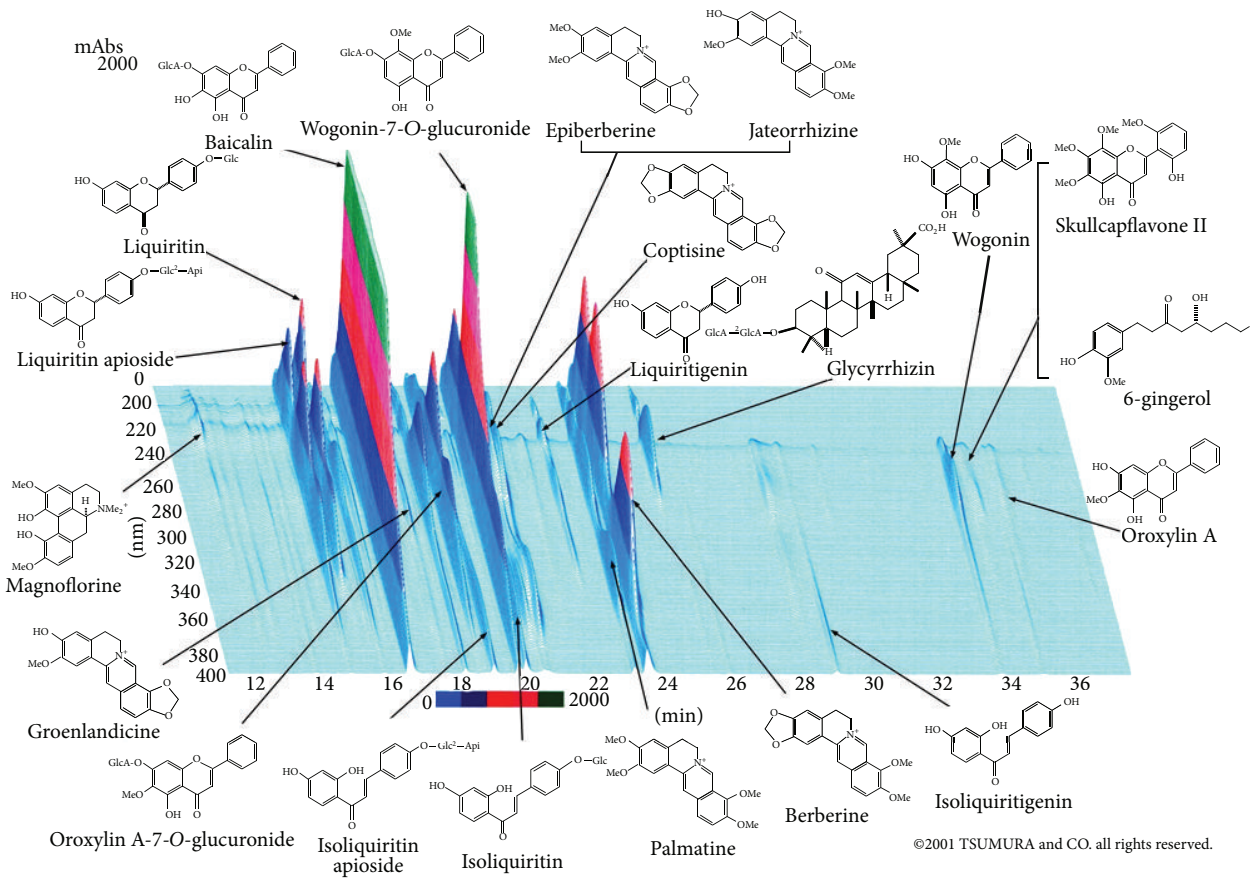

Figure 2: 3D-HPLC pattern of TJ-14 hangeshashinto (this 3D-HPLC was created in 2001 by TSUMURA and CO.).

4. Conclusions

Steady progress is being made in the treatment of cancer. However, highly invasive treatment can cause patients' distress. It would seem to be a natural progress that traditional, complementary, and alternative forms of medical care are now being adopted to alleviate the attendant suffering. Some express opposition to the combined use of Kampo preparations with anticancer drugs or surgery. Yet, if appropriate Kampo preparations alleviate adverse effects of cancer treatment, improve QOL, enhance therapeutic efficacy, and prolong life, the importance of treatment that includes Kampo preparations as palliative or supportive care for cancer will go on growing. Western and Kampo medicines coexist in Japan as a characteristic form of medical care. This combination needs to be further promoted and also to be established as integrative medicine based on scientific evidence.

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Research Article

Cucurbitane Triterpenoid from *Momordica charantia* Induces Apoptosis and Autophagy in Breast Cancer Cells, in Part, through Peroxisome Proliferator-Activated Receptor γ Activation

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Although the antitumor activity of the crude extract of wild bitter gourd (*Momordica charantia* L.) has been reported, its bioactive constituents and the underlying mechanism remain undefined. Here, we report that 3β,7β-dihydroxy-25-methoxycucurbita-5,23-diene-19-al (DMC), a cucurbitane-type triterpene isolated from wild bitter gourd, induced apoptotic death in breast cancer cells through peroxisome proliferator-activated receptor (PPAR) γ activation. Luciferase reporter assays indicated the ability of DMC to activate PPARγ, and pharmacological inhibition of PPARγ protected cells from DMC's antiproliferative effect. Western blot analysis indicated that DMC suppressed the expression of many PPARγ-targeted signaling effectors, including cyclin D1, CDK6, Bcl-2, XIAP, cyclooxygenase-2, NF-κB, and estrogen receptor α, and induced endoplasmic reticulum stress, as manifested by the induction of GADD153 and GRP78 expression. Moreover, DMC inhibited mTOR-p70S6K signaling through Akt downregulation and AMPK activation. The ability of DMC to activate AMPK in liver kinase (LK) B1-deficient MDA-MB-231 cells suggests that this activation was independent of LKB1-regulated cellular metabolic status. However, DMC induced a cytoprotective autophagy presumably through mTOR inhibition, which could be overcome by the cotreatment with the autophagy inhibitor chloroquine. Together, the ability of DMC to modulate multiple PPARγ-targeted signaling pathways provides a mechanistic basis to account for the antitumor activity of wild bitter gourd.

1. Introduction

In Asia, bitter gourd (*Momordica charantia* L.) is widely used as a functional food to prevent and treat diabetes and associated complications [1]. In addition to the hypoglycemic effect, the antitumor activity of crude bitter gourd extract has also been reported in various types of cancer cells *in vitro* and *in vivo* [2–5]. Reported chemical constituents of bitter gourd include, but are not limited to, glycosides, saponins, alkaloids, fixed oils, triterpenes, polypeptides, and steroids (for review, see [6]). Wild bitter gourd, a wild species of *M. charantia* L., (*M. charantia* Linn. var. *abbreviate*), is native to several tropical areas of Asia, including southern Taiwan, where it is consumed not only as a vegetable but also as a herbal medicine. Recent studies have demonstrated that wild bitter gourd extracts exhibited multiple pharmacological activities associated with anti-inflammation and antidiabetics, including those of suppressing inflammatory response in macrophages [7], overcoming insulin resistance in skeletal muscle in fructose-fed rats [8], enhancing insulin

signaling in skeletal muscle in high-fat-diet-fed mice [9], and upregulating mRNA expression of peroxisome proliferator-activated receptor (PPAR) α, PPARγ, and their target genes in mice [10]. Together, these activities might account for the ability of wild bitter gourd to improve metabolic syndrome in humans [11].

PPARγ regulates the expression of genes involved in the control of lipid metabolism and insulin sensitivity via the ligand-activated transcriptional activity [12–15]. PPARγ ligands include naturally occurring fatty acids, 15-deoxy-delta12,14-prostaglandin J2 (PGJ2), and thiazolidinediones (TZDs), such as troglitazone and rosiglitazone [15]. Many of these PPARγ agonists exhibit antiproliferative activities against many types of cancer cells including those of colon, prostate, and breast, suggesting the potential use of these agents in cancer therapy or prevention [16]. Evidence suggests that PPARγ activation leads to the transcriptional suppression of a series of signaling effectors associated with tumorigenesis, including cell cycle regulators (cyclin D1, cyclin E, and cyclin-dependent kinase (CDK) 6) [17], antiapoptotic proteins (Bcl-2 and XIAP) [18], and cyclooxygenase- (COX-) 2 [19], thereby facilitating apoptotic death in cancer cells [20, 21]. Therefore, PPARγ is recognized as a therapeutically relevant target for cancer therapy [22, 23].

Although the health benefits of wild bitter gourd have been associated with PPARγ activation [10], bioactive constituents that contribute to this pharmacological effect, however, remain undefined. In this study, we investigated the mechanism underlying the antitumor effect of 3β,7β-dihydroxy-25-methoxycucurbita-5,23-diene-19-al (DMC; structure, Figure 1(a)), a cucurbitane triterpenoid isolated from wild bitter gourd with antileukemic activity [24]. We obtained first evidence that DMC induced apoptotic death in breast cancer cells, at least in part, through a PPARγ-dependent mechanism.

2. Materials and Methods

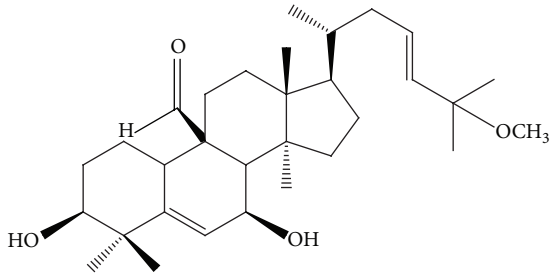
2.1. Plant Materials. DMC was isolated from the whole plant of *M. charantia* Linn. var. *abbreviate* collected in Pingtung County, Taiwan, in October 2008, and a voucher specimen (2008) has been deposited in the Department of Biological Science and Technology, China Medical University (Taichung, Taiwan). The identity and purity of purified DMC were verified by proton nuclear magnetic resonance (NMR) spectroscopy, high-resolution mass spectrometry, and 2D NMR spectrometry (Supplementary Material available online at http://dx.doi.org/10.115/2013/935675) using reported spectral data [25]. For *in vitro* experiments, DMC was dissolved in DMSO and was added to culture medium with a final DMSO concentration of less than 0.1%. Rabbit polyclonal antibodies against various biomarkers were obtained from the following sources: p-^{473}Ser Akt, p-^{308}Thr Akt, PARP, caspase-9, cyclin-dependent kinase (CDK) 6, p-^{2448}Ser mTOR, mTOR, p-^{216}Ser p70S6K, p-^{79}Ser acetyl-CoA carboxylase (ACC), ACC, p70S6K, LC3, Atg7, GADD153, XIAP, GRP78, COX-2, Beclin 1, cyclin D1, p-^{172}Thr adenosine monophosphate protein kinase (AMPK), AMPK, ERα, HIF1α, and NF-κB (Cell Signaling Technologies, Beverly, MA, USA); Akt and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β-actin (Sigma-Aldrich, St. Louis, MO, USA). The enhanced chemiluminescence (ECL) system for detection of immunoblotted proteins was from GE Healthcare Bioscience (Piscataway, NJ, USA). The GFP-LC3 and peroxisome proliferator-activated receptor response element (PPRE) x3-TK-Luc plasmids were kindly provided by Dr. Ching-Shih Chen at The Ohio State University. GW9662, chloroquine, and other chemical and biochemical reagents were obtained from Sigma-Aldrich unless otherwise is mentioned.

2.2. Cell Culture. MCF-7 and MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Manasas, VA, USA). Cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 5 mg/mL of penicillin, 10 mg/mL of neomycin, and 5 mg/mL streptomycin at 37°C in a humidified incubator containing 5% CO_2.

2.3. Cell Viability Analysis. Effect of test agents on cell viability was assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays [26] in 6 replicates. Cells (5×10^3) were seeded and incubated in 96-well, flat-bottomed plates in 10% FBS-supplemented DMEM/F12 for 24 h and were exposed to test agents at indicated concentrations for different time intervals. The medium was removed and replaced by 200 μL of 0.5 mg/mL MTT in 5% FBS-DMEM/F12, and cells were incubated at 37°C for 2 h. Medium was removed and the reduced MTT dye was solubilized in 200 μL/well DMSO. Absorbance was determined with a Synergy HT spectrophotometer (BioTek) at 570 nm.

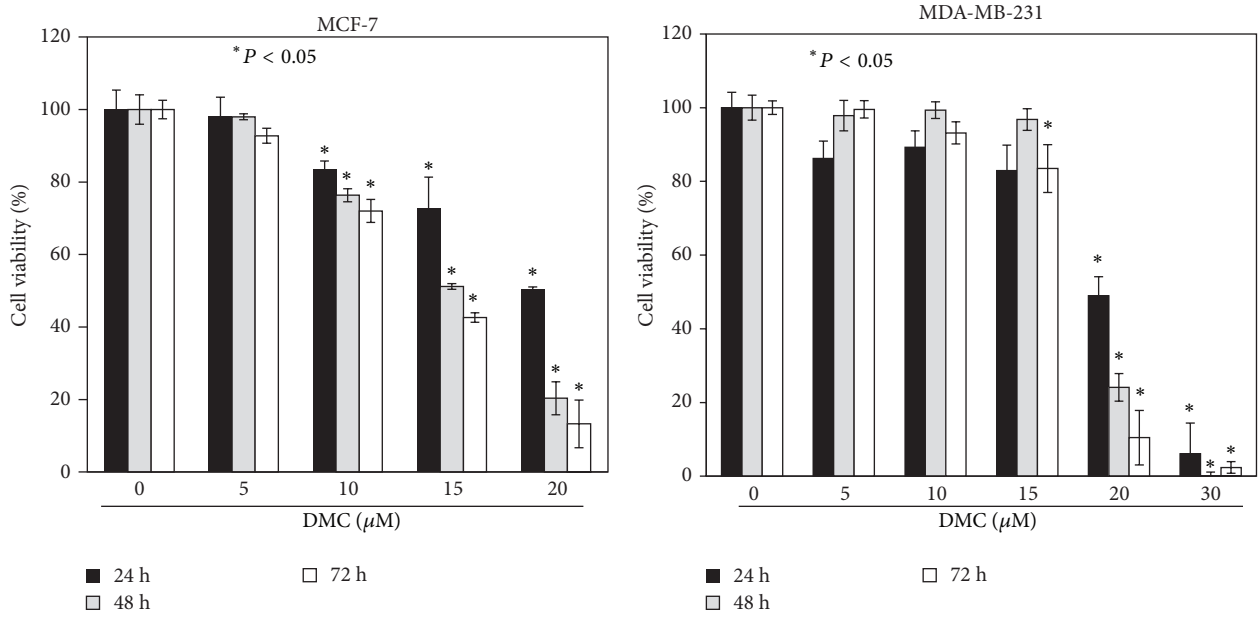
2.4. Flow Cytometry. For assessment of apoptosis, 5×10^4 cells were plated and treated with DMC at indicated concentration in 5% FBS-supplemented DMEM/F12 for 72 h. Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and fixed in 70% cold ethanol for 4 h at 4°C, followed by spinning at 1200 rpm for 5 min and resuspending in ice-cold PBS containing 2% FBS. The cells were stained with Annexin V-FITC and propidium iodide according to the vendor's protocols (BD Pharmingen, San Diego, CA, USA) and analyzed by using BD FACSAria flow cytometer (Becton, Dickinson and Company). Caspase-3 activation was assessed using a FITC Rabbit Anti-Active Caspase-3 kit (BD Pharmingen) according the manufacturer's protocol.

2.5. Immunoblotting. Drug-treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer, consisting of 20 mM Tris-HCl (pH 8), 137 mM NaCl, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 100 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, leupeptin at 10 μg/mL, and aprotinin at 10 μg/mL. Soluble cell lysates were collected after centrifugation at 1500 ×g for 5 min, and equivalent amounts of protein (60–100 μg) were resolved in 10% SDS-polyacrylamide gels. Bands were transferred to nitrocellulose membranes and blocked with



3β, 7β-dihydroxy-25-methoxycucurbita-5, 23-diene-19-al

(a)



(b) (c)

Figure 1: Antiproliferative effects of DMC in breast cancer cells. (a) The chemical structure of 3β,7β-dihydroxy-25-methoxycucurbita-5,23-diene-19-al (DMC). (b) Dose- and time-dependent suppressive effects of DMC on the viability of MCF-7 breast cancer cells. (c) Dose- and Time-dependent suppressive effects of DMC on the viability of MDA-MB-231 breast cancer cells. Cells were treated with DMC at indicated concentrations in 5% FBS-supplemented DMEM/F12 medium for 24, 48, and 72 h, and cell viability was determined by MTT assays. *Points*, mean; *bars*, SD ($n = 6$). $^*P < 0.05$.

5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) and incubated overnight with the corresponding primary anti body at 4°C. After washing with PBST three times, the memb rane was incubated at room temperature for 1 h with the secondary antibody with PBST and visualized by enhanced chemiluminescence.

2.6. Transient Transfection. Plasmids were transiently transfected into cells by using the Fugene HD reagent (Roche) according to the manufacture's protocol. After 24 h, the transfected cells were treated with DMC or DMSO control and subjected to fluorescent analysis or Western blotting.

2.7. Confocal Imaging. MCF-7 cells expressing GFP-LC3 (2×10^5/3 mL) were seeded in each well of a six-well plate and treated with DMC at the indicated concentration for 3 h. Cells were fixed in 2% paraformaldehyde (Merck) for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min. Cells were washed with PBS and then subjected to examination on a Leica TCS SP2 confocal microscope (Leica Biosystems Nussloch GmbH, Heidelberg, Germany) examination.

For PPARγ immunostaining, cells were fixed in 2% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min. After blocking with 1% bovine serum albumin (BSA), cells were incubated with mouse anti-human PPARγ antibody at 4°C overnight, followed by anti-mouse IgG at room temperature for 1 h, washed with PBS, and subjected to fluorescent microscopic examination.

2.8. Transmission Electron Microscope. Cells were fixed in a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.2 M sodium cacodylate for 1 h. Fixed cells were

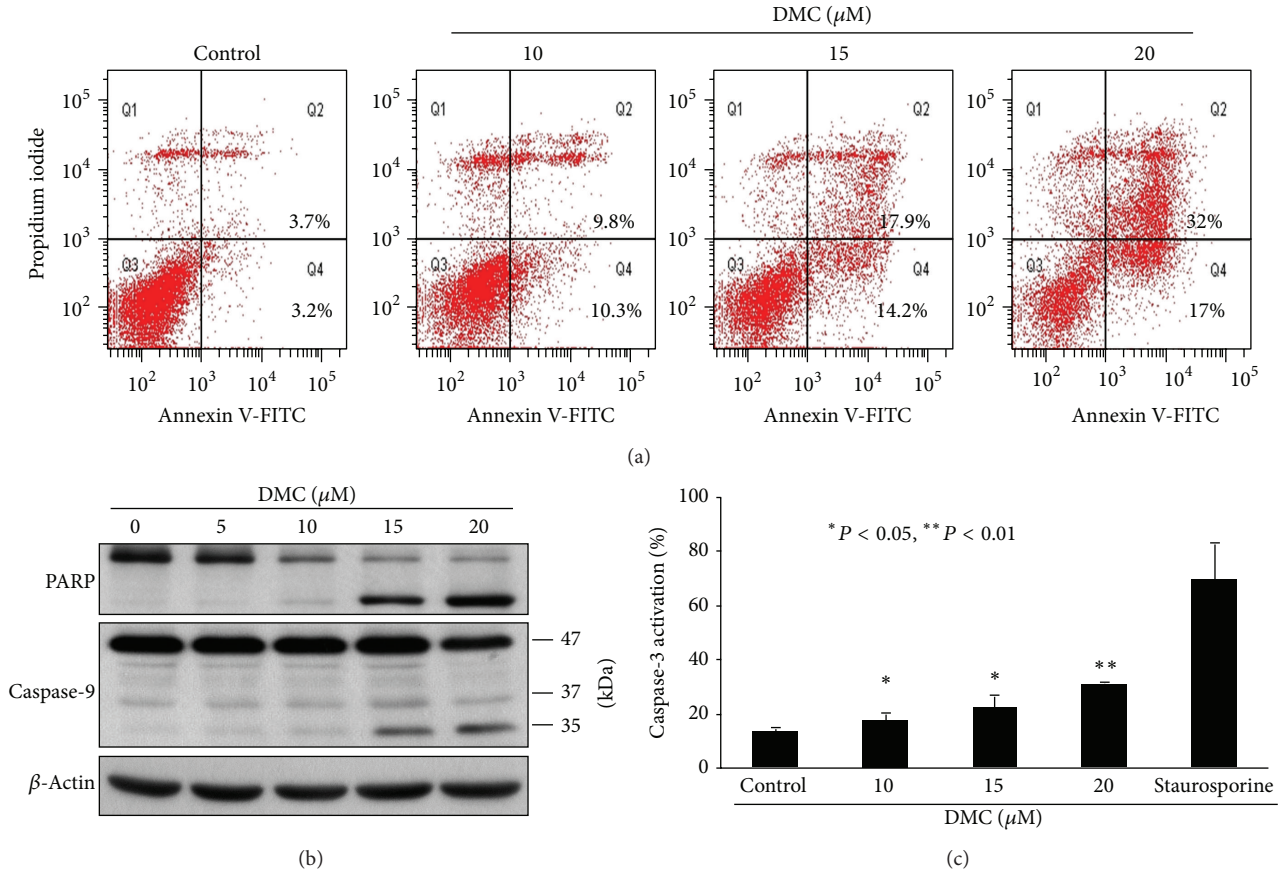


FIGURE 2: DMC induces apoptosis in MCF-7 cells. (a) Histograms showing the dose-dependent effect of DMC on Annexin V/PI staining. Cells were treated with DMC at indicated concentrations for 72 h, followed by Annexin V-PI staining. Data are representative of three independent experiments. (b) Dose-dependent effect of DMC on PARP cleavage and caspase-9 activation in MCF-7 cells after 72 h exposure in 5% FBS-supplemented DMEM/F12 medium. (c) Flow cytometry analysis of caspase-3 activity in MCF-7 cells after treatment with DMSO vehicle or the indicated concentrations of DMC for 72 h. 10 μM staurosporine as the positive control. $^{*}P < 0.05$, $^{**}P < 0.01$.

suspended in a buffered solution containing 1% osmic acid for 1 h, followed by dehydration in a graded ethanol series, washing with acetone, and embedding into EPON epoxy resin. Ultrathin sections (60–80 nm) were prepared on an ultramicrotome and double-stained with uranyl acetate and lead citrate. All sections were examined and photographed with a Hitach H-600 transmission electron microscope.

2.9. Statistical Analysis. All data are presented as means ± SD obtained from three independent experiments. Statistical differences were calculated using Student's *t*-test, with the following symbols of significance level: $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results and Discussion

3.1. Antiproliferative Effect of DMC in Breast Cancer Cells. The dose- and time-dependent effects of DMC on cell viability were assessed in 2 human breast cancer cell lines, ERα-positive MCF-7, and ERα-negative MDA-MB-231, by MTT assays (Figures 1(b) and 1(c)). Relatively, MCF-7 cells were more sensitive to the antiproliferative effect of DMC than MDA-MB-231 cells, with IC_{50} values of 14.3 and 17.6 μM, respectively (Figures 1(b) and 1(c)).

3.2. DMC Induces Apoptosis through Caspase Activation. We obtained several lines of evidence that DMC suppressed the proliferation of MCF-7 cells by inducing apoptosis. First, annexin V-propidium iodide staining reveals a dose-dependent increases in the proportion of apoptotic cells (defined as annexin V+ cells) after DMC treatment (Figure 2(a)). Second, Western blot analysis showed that DMC induced PARP cleavage and caspase-9 activation in a dose-dependent manner in MCF-7 cells (Figure 2(b)). Third, flow cytometric analysis shows a dose-dependent increase in the expression of activated caspase-3 in response to DMC (Figure 2(c)), confirming the involvement of caspase activation in DMC-mediated apoptosis. Treatment with DMC at 10, 15, and 20 μM for 72 h increased the level of activated caspase-3, from 13% in the control group to 18%, 22%, and 30.5%, respectively ($^{*}P < 0.05$ compared to the DMSO control).

3.3. DMC Acts as a PPARγ Agonist. It has been reported that cucurbitane- and oleanane-type triterpenoids and saponins

are potential active constituents of wild bitter gourd and that wild bitter gourd crude extracts activated PPARγ signaling in mice [10]. We hypothesized that the antitumor activity of DMC was associated with its ability to facilitate PPARγ activation. To corroborate this premise, MCF-7 cells were transiently transfected with PPRE-TK-Luc and Renilla plasmids and exposed to DMC at different concentrations or the positive control troglitazone at 50 μM for 24 h. As shown, the PPRE-luciferase reporter assay indicates that DMC at 15 and 20 μM increased the activity in PPARγ transactivation by 2.2- and 5.2-fold, respectively ($^{*}P < 0.05$), while troglitazone at 50 μM gave rise to a 2.8-fold increase (Figure 3(a)). Equally important, cotreatment with GW9662, a PPARγ inhibitor, protected cells from the suppressive effect of DMC on cell viability (Figure 3(b)). Although DMC did not alter the expression level of PPARγ (Figure 3(c), upper panel), two lines of evidence support the effect of DMC on PPARγ activation in MCF-7 cells. First, immunocytochemical analysis demonstrates the ability of DMC to facilitate the nuclear localization of PPARγ in a manner similar to that of troglitazone (Figure 3(c), lower panel).

Second, DMC was effective in modulating the expressions of various PPARγ-targeted gene products associated with cell cycle progression and apoptosis in MCF-7 cells, including cyclin D1, CDK6 [17], Bcl-2, XIAP [18], and COX-2 [19] (Figure 3(d)). In addition, the drug effect on the expression of NF-κB/p65 was interrogated in light of a recent report that PPARγ acts as an E-3 ligase targeting p65 degradation [27]. As shown, DMC suppressed the expression of NF-κB/p65 in a dose-dependent manner (Figure 3(d)). Moreover, the suppressive effect of DMC on estrogen receptor (ER) α expression is noteworthy (Figure 3(d)) as PGJ2 and TZD PPARγ agonists have been shown to disrupt ERα signaling by facilitating ERα degradation [28].

Previously, it was reported that PGJ2 and thiazolidinedione PPARγ agonists induce endoplasmic reticulum stress [29]. The ability of DMC to trigger this stress response was borne out by increased expression of two endoplasmic reticulum stress-associated markers, growth arrest- and DNA damage-inducible gene (GADD)153 (also known as CHOP), and GRP78 in MCF-7 cells (Figure 3(d)). GADD153 is a well-recognized endoplasmic reticulum stress-inducible transcription factor [30], and GRP78, a major endoplasmic reticulum chaperone, maintains endoplasmic reticulum integrity to mediate cell death when endoplasmic reticulum stress is beyond the tolerance of cell adaption [31].

3.4. DMC Induces Autophagy. In light of a recent report that PPARγ activation induces autophagy in breast cancer cells [15], we examined the effect of DMC on autophagy in MCF-7 cells. By using transmission electron microscopy, we found that exposure of MCF-7 cells to DMC at 10 or 15 μM for 24 h led to autophagosome formation in the cytoplasm (Figure 4(a)). During autophagy, the cytoplasmic form of microtubule-associated protein 1 light-chain (LC3-I) is processed and recruited to the autophagosomes, where LC3-II is generated via site-specific lipidation. Thus, to confirm this drug-induced autophagosome formation, we assessed the effect of DMC on LC3-II conversion in two ways. First, MCF-7 cells were transiently transfected with GFP-tagged LC3 (GFP-LC3) and exposed to DMSO, 15 μM DMC, or 100 nM rapamycin as a positive control. As observed by confocal fluorescence imaging, DMC induced the accumulation of LC3-positive puncta in the cytoplasm in a manner similar to that of rapamycin (Figure 4(b)). Second, Western blot analysis indicates that DMC treatment led to dose- and time-dependent increases in the abundance of endogenous LC3-II expression (Figures 4(c) and 4(d)). Moreover, our data show that this DMC-induced autophagy was associated with elevated expression levels of autophagy-related protein (Atg) 7 and Beclin 1 (a component of the class III phosphatidylinositol 3-kinase complex) (Figure 4(c)), both of which play a pivotal role in autophagy induction [32, 33].

3.5. DMC Targets mTOR Signaling via PPARγ and AMPK Activation. Interruption of mTOR signaling is known to stimulate autophagy [34]. To investigate the mechanistic link between mTOR and DMC-induced autophagy, we assessed the activation status of the Akt/mTOR signaling axis by examining the phosphorylation status of Akt at Thr308, Ser473, and mTOR, and the mTOR target p70S6K in DMC-treated MCF-7 cells. As shown, DMC decreased the phosphorylation levels of all of these kinases in a dose-dependent manner (Figure 5(a), left panel). It was also reported that thiazolidinedione PPARγ agonists induce autophagy through the activation of HIF1α [15], which led us to examine the expression of HIF1α. The result showed that DMC dose-dependently upregulated the expression of HIF1α (indicated by the 120 kDa band; Figure 5(a), left panel). To provide a mechanistic link among DMC-induced mTOR inhibition, PPARγ activation, and autophagy, we examined the effect of the PPARγ inhibitor GW9662 on these drug effects. Western blotting indicates that treatment of DMC or troglitazone, each at 15 μM, decreased phosphorylation of p70S6K, indicative of mTOR inhibition, accompanied by increased accumulation of LC3-II as compared to the vehicle control (Figure 5(a), right panel). Although GW9662 alone had no appreciable effect on either cellular response, it was able to reduce the activities of DMC and troglitazone to induce p70S6K dephosphorylation and the conversion of LC3-I to LC3-II (Figure 5(a), right panel).

In addition, it is well recognized that AMPK activation promotes autophagy via mTOR inhibition [35]. Western blot analysis indicated that DMC facilitated concentration-dependent increases in the phosphorylation levels of AMPK and its downstream target acetyl-CoA carboxylase (ACC) in both MCF-7 and MDA-MB-231 cells (Figure 5(b)). The ability of DMC to activate AMPK in MDA-MB-231 cells is noteworthy as this cell line is deficient in liver kinase B1 (LKB1) [36], an upstream kinase of AMPK. Interestingly, DMC treatment led to a concentration-dependent increases in the expression level of ACC in MDA-MB-231 cells, which plateaued at 15 μM, followed by a decrease at 20 μM. This change, however, was cell line-specific as it was not noted in

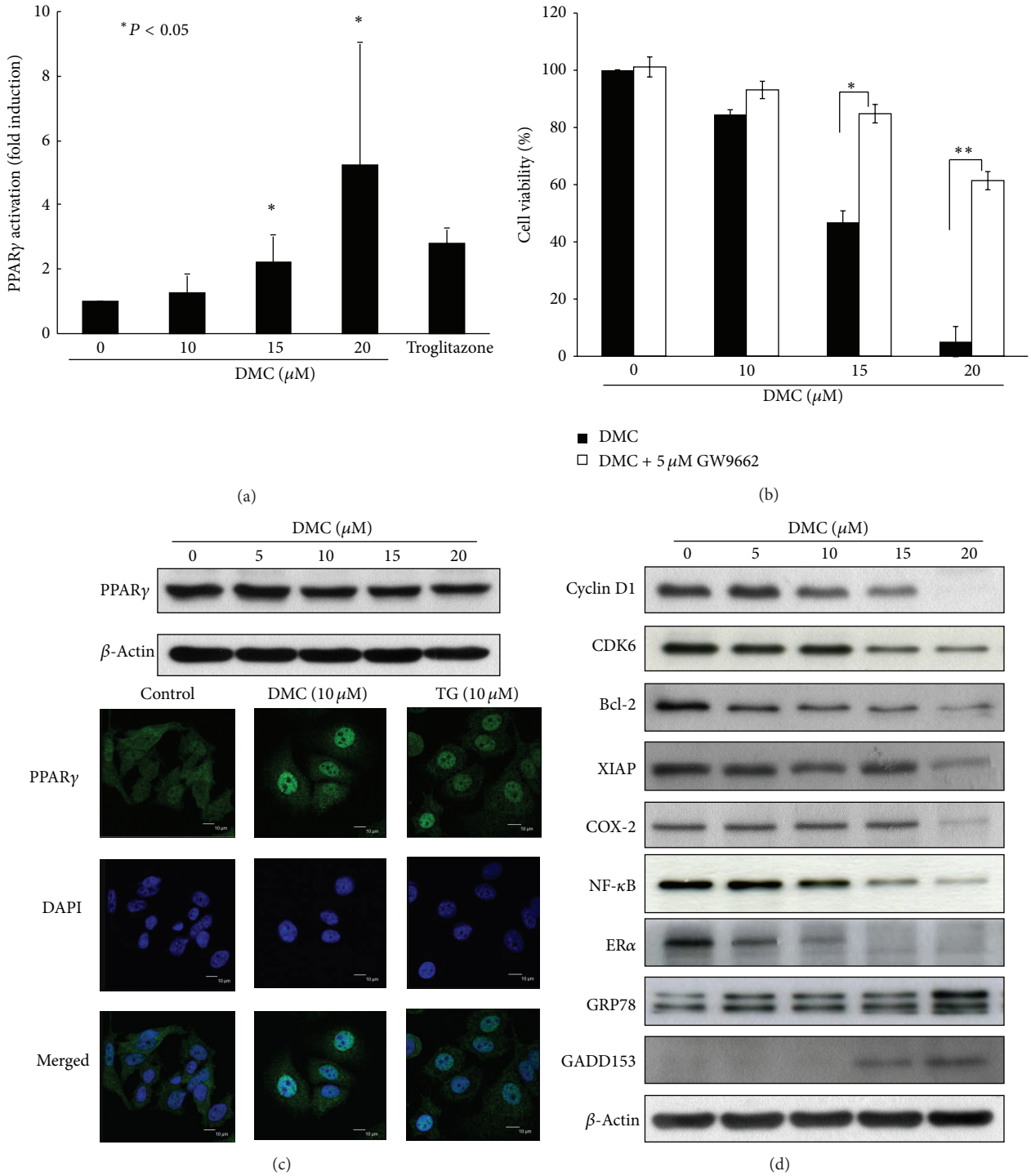


Figure 3: The PPARγ-inducing activity of DMC. (a) Dose-dependent effect of DMC on PPARγ activation in MCF-7 cells. Cells were transfected with PPRE x3-TK-Luc and Renilla plasmids for 24 h before treatment with DMC at indicated concentrations for 24 h. Data are expressed as percentage of the respective PPARγ activity to the control. Normalized luciferase activity was determined and is shown as the fold activation relative result in DMSO-treated cells. Troglitazone at 50 μM was used as positive control. Values are means ± S.E.M. of three independent experiments. $^{*}P < 0.05$. (b) DMC-inhibited cell growth could be blocked by GW9662, an inhibitor of PPARγ. Cells were treated with DMC at indicated concentrations in the presence of 5 μM GW9662 or DMSO control for 72 h, and cell viability was determined by MTT assays. *Points*, mean; *bars*, SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$. (c) Effect of DMC on the protein expression and nuclear translocation of PPARγ. MCF-7 cells were treated with DMC at the indicated concentrations for 72 h and detected PPARγ by Western blotting (upper panel). MCF-7 cells were treated with 10 μM DMC or 10 μM troglitazone (TG) for 24 h, stained with anti-PPARγ, and examined by confocal microscopy (lower panel). (d) Dose-dependent effect of DMC on the expression of various PPARγ-targeted proteins in MCF-7 cells after 72 h exposure in 5% FBS-supplemented DMEM/F12 medium.

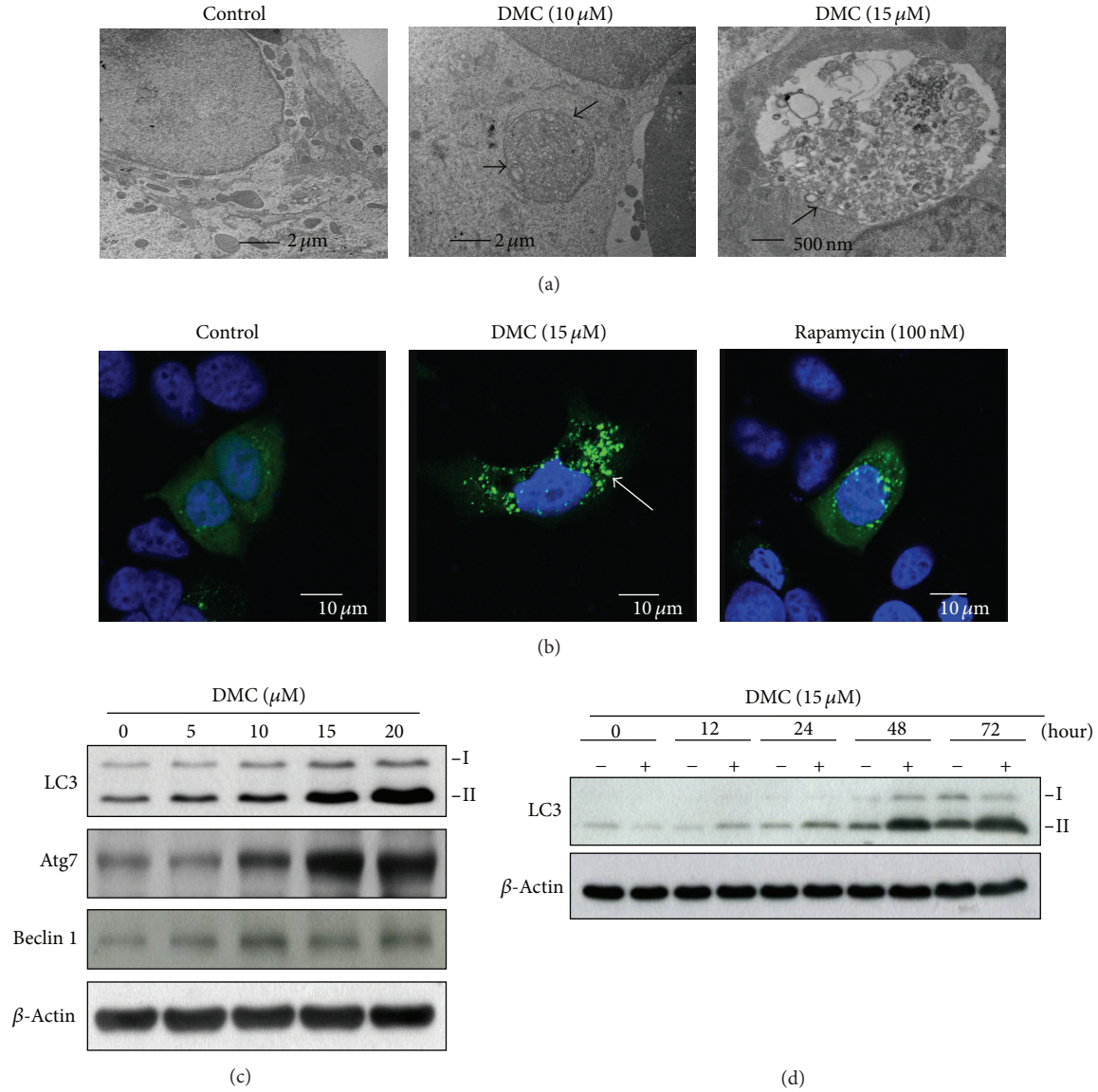


Figure 4: Induction of autophagy in MCF-7 cells by DMC. (a) Electron microscopic analysis of autophagosome formation in vehicle- or drug-treated MCF-7 cells as described in Section 2. Arrow: autophagosomes. (b) Fluorescent confocal microscopic analysis of drug-induced autophagosome formation in MCF-7 cells ectopically expressing GFP-LC3. The arrow indicates LC3-positive puncta in DMC-treated cells. MCF-7 cells transiently transfected with GFP-LC3 plasmids were treated with DMSO, 15 μM DMC, or 100 nM rapamycin for 24 h and then fixed by 3.7% paraldehyde and examined by confocal microscopy. (c) Dose- and (d) time-dependent effects of DMC on the conversion of LC3-I to LC3-II and/or the expression of Atg7 and Beclin 1. MCF-7 cells were treated with DMC at indicated concentrations or DMSO for 72 h.

MCF-7 cells, of which the underlying mechanism remained unclear.

Together, these findings suggest the ability of DMC to suppress mTOR signaling through the concerted action of Akt dephosphorylation and AMPK activation, which underlies the effect of DMC on autophagy induction.

3.6. Pharmacological Inhibition of Autophagy Enhances DMC-Induced Apoptotic Death. Autophagy has been reported to mediate a protective or enhancing effect on drug-induced cell death [37]. In light of the finding that autophagy acts as a survival signal in response to inhibitors of the PI3K-Akt-mTOR signaling axis [38], we examined the effect of chloroquine, an autophagy inhibitor, on DMC-mediated inhibition of MCF-7 cell proliferation. Chloroquine (CQ) inhibits autophagy at a later step in the pathway by blocking the fusion of autophagosome with lysosome and the subsequent lysosomal protein degradation [39]. As a consequence, treatment of CQ leads to increased LC3-II accumulation. As shown in Figure 6(a), exposure of MCF-7 cells to CQ, alone or in combination

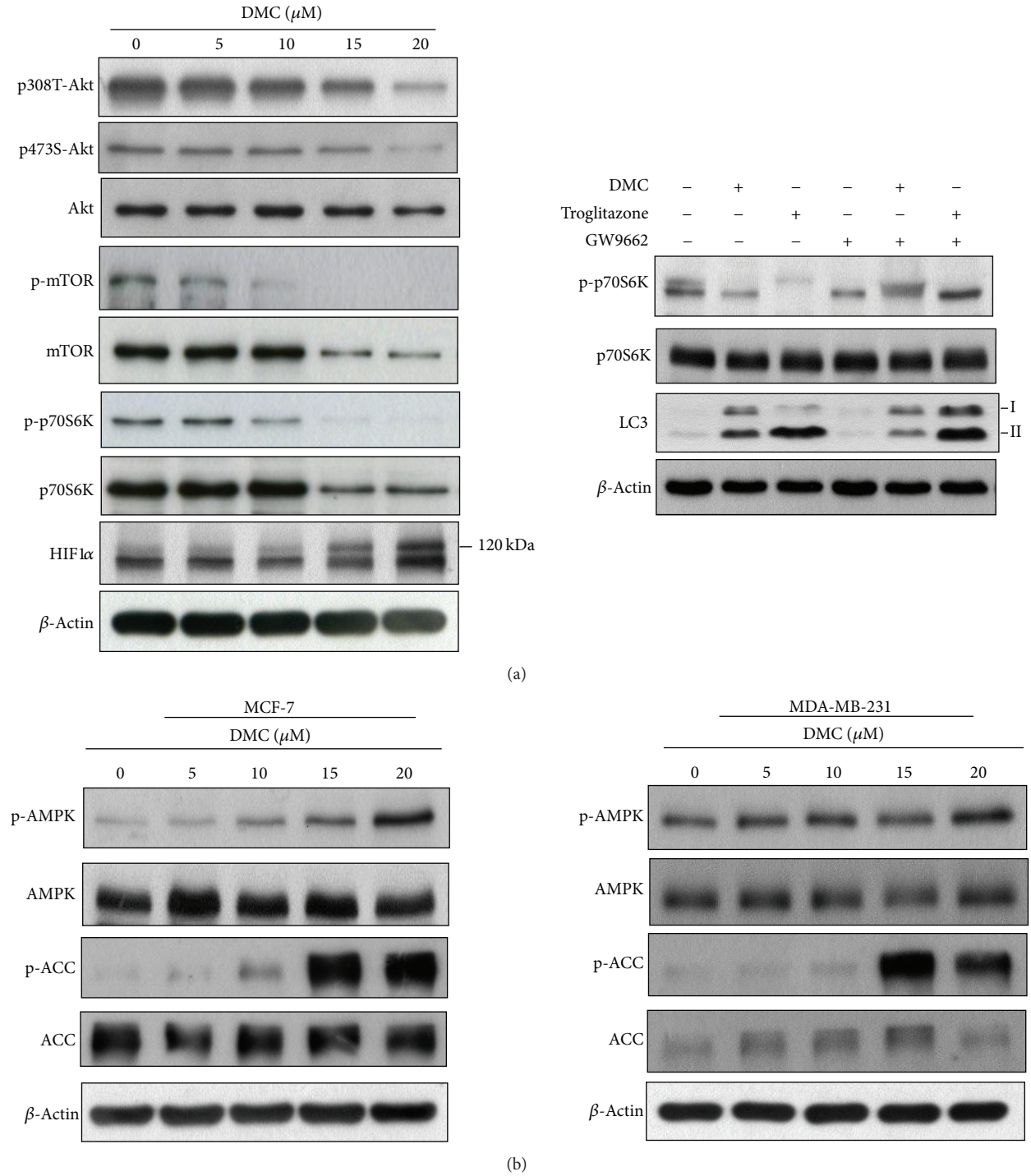


Figure 5: Effects of DMC on the activation status of the Akt-mTOR signaling axis and AMPK. (a) Dose-dependent of DMC on the phosphorylation/expression of Akt, mTOR, p70S6K, and HIF1α in MCF-7 cells after 72 h exposure in 5% FBS-DMEM/F12 (left panel). Effects of 15 μM DMC, 15 μM troglitazone, or 5 μM GW9662 or the drug combination relative to DMSO control in MCF-7 cells after 72 h exposure in 5% FBS-DMEM/F12 (right panel). (b) Dose-dependent effects of DMC on the phosphorylation of AMPK and ACC in MCF-7 (left panel) and MDA-MB-231 (right panel) cells after 72 h exposure in 5% FBS-DMEM/F12.

with DMC, showed a significantly higher accumulation of converted LC3-II relative to that of vehicle- or DMC-treated cells. This LC3-II accumulation is indicative of the inhibition of autophagy by CQ in MCF-7 cells. It is noteworthy that while the drug showed no antiproliferative activity against MCF-7 cells, chloroquine at 10 μM significantly enhanced the suppressive effect of DMC on viability of MCF-7 cells (Figure 6(b)). Annexin V-staining and Western blot analyses

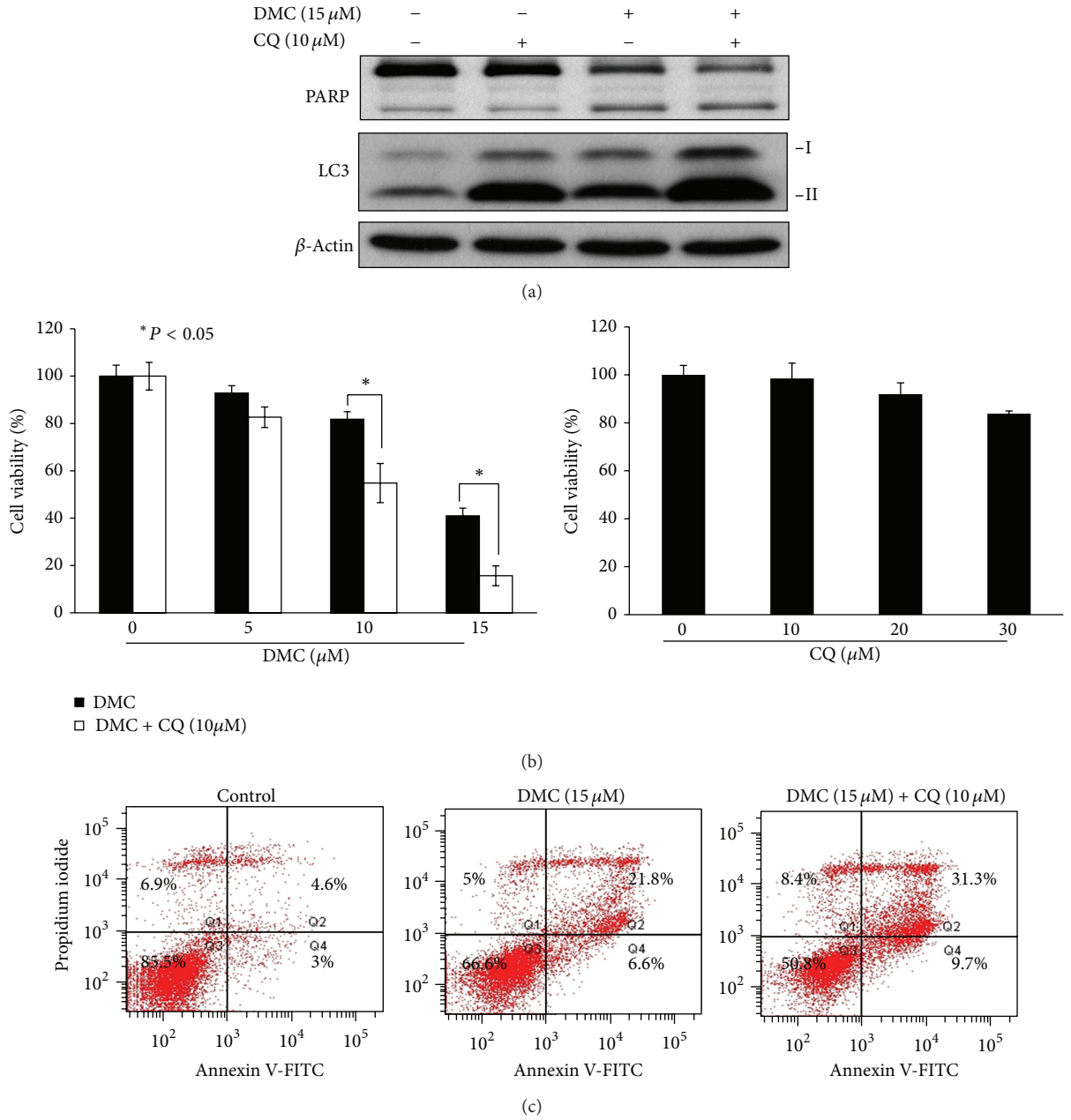


FIGURE 6: Effects of chloroquine (CQ) on DMC-induced autophagy and cytotoxicity. (a) Effects of 15 μM DMC, 10 μM chloroquine, or the drug combination relative to DMSO control in MCF-7 cells after 72 h exposure in 5% FBS-DMEM/F12. (b) MCF-7 cells were treated with chloroquine (CQ) alone (right panel) or in combination with DMC at indicated concentrations (left panel) for 72 h, and cell viability was determined by MTT assays. *Points*, mean; *bars*, SD ($n = 6$). $^*P < 0.05$. (c) MCF-7 cells were treated with DMSO or DMC (15 μM) alone or in combination with 10 μM chloroquine (CQ) for 72 h exposure in 5% FBS-DMEM/F12, followed by Annexin V-propidium iodide staining. Data are representative of three independent experiments.

suggest that this increase in cytotoxicity was attributable to increased apoptosis as a result of autophagy inhibition (Figures 6(a) and 6(c)).

4. Discussion

Wild bitter gourd has been reported to exert anti-inflammatory [7], antioxidant [40], and hypoglycemic effects [41] in various experimental animal models. However, its active constituents contributing to these pharmacological effects have not been fully characterized. In this study, we demonstrated that DMC, a cucurbitane-type triterpene isolated from wild bitter gourd, acts as a PPARγ agonist. Although the activity of other cucurbitane-type triterpenes in modulating PPARγ activity remains to be investigated, the ability of DMC to activate PPARγ might underlie the hypoglycemic effect of wild bitter gourd crude extracts in diabetic mice [41].

Several lines of evidence indicate that PPARγ plays a pivotal role in mediating DMC's antiproliferative activity in breast cancer cells. First, inhibition of PPARγ by the pharmacological inhibitor GW9662 protected cells from the inhibitory effect of DMC on the viability of MCF-7 cells (Figure 3(b)). Second, DMC suppressed the expression of a series of PPARγ-targeted signaling effectors that govern cell cycle progression, proliferation, and survival, including cyclin D1, CDK6, Bcl-2, XIAP, COX-2, NF-κB, and ERα (Figure 3(d)). Particularly, the downregulation of ERα expression is noteworthy since ERα has been shown to bind to PPRE and negatively interfere with PPARγ signaling in breast cancer cells [42]. Thus, this ERα-ablating effect might account for the higher sensitivity of MCF-7 cells relative to MDA-MB-231 cells to DMC (Figure 1(b)). Third, DMC induced autophagy through mTOR inhibition (Figures 4 and 5), which is reminiscent with that reported with TZD PPARγ agonists in breast cancer cells [15].

mTOR, a central cell growth regulator that integrates growth factor and nutrient signals [43], is positively and negatively regulated by Akt and AMPK, respectively. This DMC-facilitated mTOR downregulation is likely attributable to the concerted action of DMC to facilitate Akt dephosphorylation and AMPK activation (Figure 5). The ability of DMC to activate AMPK is noteworthy as AMPK is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [44]. Previously, TZD PPARγ agonists were reported to induce PPARγ-independent AMPK activation through changes in cellular energy state [45, 46]. However, the finding that DMC was equally effective in activating AMPK in LKB1-deficient MDA-MB-231 and MCF-7 cells suggests that this activation might be independent of the cellular metabolic status as LKB1 is activated in response to environmental nutrient changes [47]. Thus, the mode of AMPK activation by DMC warrants further investigat ion.

The Akt/mTOR pathway is often dysregulated in malignant cells, thus representing an important target for cancer prevention and therapy [48]. The concurrent suppressive effect of DMC on Akt and mTOR activation is noteworthy, because it circumvents the feedback activation that results from mTOR inhibition, a problem associated with rapamycin-based mTOR inhibitors [49]. From a clinical perspective, the ability of DMC to concurrent target Akt and mTOR underlies its translational potential as a chemopreventive agent. Nonetheless, consistent with the reported effect of mTOR inhibitors and many other anticancer agents, DMC induced a cytoprotective autophagy that decreased DMC's antiproliferative potency, which, however, could be overcome by the co-treatment with the autophagy inhibitor chloroquine (Figure 6). Further *in vivo* studies are needed to better understand the role of DMC, alone or in combination with other agent, in breast cancer prevention and treatment.

5. Conclusion

There has been growing interest in the use of wild bitter gourd as a dietary supplement for the treatment of various illnesses in light of its diverse pharmacological effects, including hypoglycemic, anti-inflammatory, antibacterial, antiviral, and antitumor activities. However, despite recent advances in gaining understanding of the health beneficial effects of this herbal medicine, information regarding the mode of action of its bioactive ingredients is lacking or fragmentary. In this study, the activity of DMC isolated from wild bitter gourd in PPARγ activation was characterized for the first time. As the role of PPARγ in regulating lipid metabolism, insulin sensitivity, apoptosis, and cell differentiation is well recognized, the ability of DMC to modulate multiple PPARγ-targeted signaling pathways provides a molecular basis to account for the hypoglycemic and antitumor activities of wild bitter gourd. Today, processed bitter gourd in the form of capsules or tablets is used by natural health practitioners, which like many other herbal medicine suffers from difficulty in quality control due to high variability of bioactive compounds involved. From a clinical perspective, wild bitter gourd extracts enriched in DMC and related compounds, of which the contents could be quantified by chromatographic fingerprint analysis, will provide a better alternative to capsules or tablets for disease management.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgments

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Review Article

Herbal Medicine and Acupuncture for Breast Cancer Palliative Care and Adjuvant Therapy

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Breast cancer is a life-threatening disease among women worldwide with annual rates of reported incidence and death increasing alarmingly. Chemotherapy is a recommended and effective treatment option for breast cancer; however, the narrow therapeutic indices and varied side effects of currently approved drugs present major hurdles in increasing its effectiveness. An increasing number of literature evidence indicate that complementary and alternative medicine (CAM) used in treatment-related symptom control and alleviation of side effects plays an important role in increasing survival rate and quality of life in breast cancer patients. This review focuses on the use of herbal medicines and acupuncture in palliative care and as adjuvants in the treatment of breast cancer. Herbal medicinal treatments, the correlation of clinical use with demonstrated *in vitro* and *in vivo* mechanisms of action, and the use of certain acupoints in acupuncture are summarized. The aim of this review is to facilitate an understanding of the current practice and usefulness of herbal medicine and acupuncture as adjuvants in breast cancer therapy.

1. Introduction

Breast cancer remains to be the leading cause of cancer death among women worldwide with the rate of reported incidence and mortality increasing annually [1, 2]. In the past decade, women with tumors between stages I and II increased from 41% to 65%, 80% of which are invasive tumors originating from ductal carcinoma and its variants [3]. Current early detection methods allow breast cancer to be diagnosed at an early stage when successful treatment is more likely. Multiple agencies and organizations around the world support mammography as the most reliable way to detect breast cancer at an early stage, particularly in women aged 50 years and older [4, 5]. About 70% of breast cancers express estrogen hormone receptor (ER) and/or progesterone receptor (PR), and these markers along with human epidermal growth factor receptor 2 (HER-2) and proliferation marker Ki-67 provide information about tumor grade and possible response to different treatments [6]. Although several treatment options are currently available including surgery, radiation therapy, and chemotherapy, specific treatment strategies depend on characteristics such as tumor grade, hormone receptor status, metastatic potential, and molecular and patient profile [7]. Chemotherapy is still the most commonly used and recommended treatment option for breast cancer, either by using a single compound or combination therapy with multiple drugs [8]. However, chemotherapeutic drugs have narrow therapeutic indices resulting in nonselective toxic effects on normal tissues, thus increasing the risk of infection. Although chemotherapy and radiotherapy are effective against breast cancer, they are accompanied by varied side effects including vasomotor syndrome (occurring in up to 80% of patients), nausea and vomiting (75%), postmastectomy edema (30–60%), arthralgia (over 40%), neutropenia, cachexia, fatigue, pain, hair loss, hot flushes, and psychological stress, which present major hurdles in increasing the effectiveness of cancer therapy [7–10].

Palliative care is an important aspect of cancer therapy that centers on the relief of pain and other symptoms related to cancer and its treatment. It aims to improve the

patient's quality of life (QOL) and can be administered along with curative treatment. Pharmacological interventions that reduce or prevent adverse side effects and increase chemosensitivity may have a substantial impact on cancer treatment and palliative care. Though the use of complementary and alternative medicine (CAM) by cancer patients is not part of conventional cancer palliative care regimens in some countries, according to the World Health Organization (WHO), 80% of cancer patients use CAM, in one form or another, for these purposes [11]. According to the WHO definition, the term CAM is used interchangeably with "traditional medicine" and refers to a broad set of health care practices, including traditional Chinese medicine (TCM), acupuncture, herbal preparations, vitamins, homeopathic remedies, music therapy, and other psychological, physical, and spiritual techniques [12–14]. The effectiveness of CAM is primarily based on empirical evidence and case studies however, in the recent years, the increasing amount of supporting data from controlled clinical trials relating CAM use to overall quality of life and safety has dramatically increased [15–17]. These supportive measures are supposed to control symptoms, improve QOL, boost the immune system, decrease cytotoxicity to normal cells, and possibly prolong life [17–20]. It may also be important to note that the integration of CAM into palliative care and cancer treatment regimens is influenced by culture [21]. One multicenter study that reported oncology professionals' attitude towards CAM concluded that in European countries, for example, CAM therapies commonly include mistletoe extracts, vitamin supplementation, and phytoestrogens, and only an approximately 4% of Scandinavian health practitioners, in contrast to the 20% German doctors, believe that CAM use has a positive role in adjuvant treatment of cancer patients [22]. Traditional oriental medicine systems (Chinese, Japanese, Korean, or Ayurvedic), spiritualism, hypnosis, aromatherapy, and acupuncture represent the widespread use of these CAM practices in the region [23]. In particular, China, Japan, Republic of Korea, and Taiwan operate a two-tiered medical system of integrative medicine, and CAM is fully integrated into national health, education, and insurance policies [16, 24, 25]. On the other hand, though not integrated in current oncological practice, 16% to 63% of North American cancer patients are reported to commonly use acupuncture, hypnosis, and spiritualism, as well as vitamin therapies and botanicals. In one population survey, 75% agreed that combining conventional medical treatment and CAM was preferable to using either alone [26, 27]. The apparent widespread use of CAM worldwide and its (erroneous or otherwise) association with minimal or zero risk means that there is a significant need to do further studies to gain an understanding of the pharmacodynamic interactions between chemotherapeutic drugs and herbal components and the effects of either component and dosing regimens in cancer treatment and palliative care [28, 29].

Among cancer patients, CAM is used more frequently by breast cancer patients with an estimated use by 45% of patients across different treatment stages [30, 31]. In one survey done among long-term breast cancer survivors (on average, 8.7 years after-diagnosis), more than 50% believed that CAM use could prevent cancer recurrence (69%), play an active role in recovery (67%), and help to manage stress (64%) [32]. Evidence gathered from recent randomized control trials (RCTs) demonstrates that herbal medicines and chemopreventive phytochemicals in combination with chemotherapeutic agents are effective in sensitizing cancer cells to treatment and minimizing the side effects arising from conventional therapy, thus increasing patient survival rate and QOL [18, 33–40]. In addition to herbal medicine, acupuncture has also become a popular complementary treatment in oncology, particularly as patients seek non-pharmacological alternatives to provide symptom control. A review of recent RCTs of acupuncture in oncology suggests that it has a promising role in controlling a wide variety of cancer and treatment-related symptoms. The evidence currently available suggests that acupuncture is a safe, low cost, and effective therapy, which further permits cancer patients to actively participate in their own care plan [41].

Several reviews had been done in the past on the use of either herbal medicine or acupuncture [42–47]; however, a comprehensive review of clinical trials utilizing either of these CAM methods in palliative care of breast cancer patients had not been done yet. This review focuses on the use of herbal medicine treatments, either as single herbs or combinations, and acupuncture in palliative care and as adjuvants in combination with chemo- or radiotherapy in the treatment of breast cancer based on recently conducted or completed RCTs. The correlation between clinical use, *in vitro* mechanistic and *in vivo* animal studies of herbal medicine, and the effectiveness of acupuncture with the use of certain acupoints in breast cancer patients is summarized and is aimed at facilitating an understanding of current practices involving the use of herbal medicine and acupuncture as adjuvants in breast cancer therapy.

2. Methods

An electronic search for previously published articles was conducted in PubMed, the Cochrane Database, the US National Center for Complementary and Alternative Medicine (NCCAM) (http://www.nccam.nih.gov/), and the US National Institutes of Health (http://www.clinicaltrials.gov/) databases to find relevant studies published up until February 2013 (inclusive). The search included the following specific medical subject heading (MeSH) terms: breast cancer, AND/OR breast neoplasms, AND/OR adjuvant chemo/radiotherapies, AND/OR herbal medicine, AND/OR acupuncture, AND/OR acupuncture points in addition to relevant text keywords comprising the following words in combination: cancer palliative care, traditional Chinese medicine, herbal formulation, phytoagent, and acupoints. The article or study types were limited to clinical trials (Phases I to IV), controlled clinical trials, and randomized controlled trials (RCTs). The titles and abstracts of all retrieved citations were read and analyzed. In total, 90 RCTs, either completed or ongoing, were included regardless of blinding. The most common phytoagents, single herbal treatments, herbal formulations, and acupoints used in the retrieved RCTs were singled out. Moreover, an exhaustive

search for references regarding *in vivo* and *in vitro* studies pertaining to mechanistic actions, acupuncture practices, individual acupoints, treatment-related symptoms, and associated effects was conducted.

Furthermore, the herbal medicine and acupuncture practices included in the most recent RCTs are highlighted in this review because these are generally accepted as constituting the most reliable evidence of treatment effects [48, 49]. RCTs include experiments wherein individuals are randomly allocated to receive or not receive experimental preventive, therapeutic, or diagnostic procedure; they are then followed over a given time period to determine the effects. The RCTs included in this review were either completed or ongoing and are assumed to have complied with health and ethics regulations in the countries where they were conducted. In the following sections, we summarize and describe the results and discuss in some detail the related mechanisms of action and therapeutic effects of these CAM practices as applied in the adjuvant treatment and palliative care of breast cancer patients.

3. Results and Discussion

3.1. Herbal Medicines as Adjuvant Treatment in Breast Cancer Chemotherapy. The most common complaint among patients receiving chemotherapy treatment is fatigue, which is experienced by 80% to 96% of the patient population [50]. Chemotherapy-induced mucositis and myelosuppression, experienced by almost 40% of patients, are the common, dose-limiting, and costly side effects of cancer therapy [51]. Moreover, cytotoxic chemotherapy suppresses the hematopoietic system, impairing the immune system and limiting the doses of drugs that can be tolerated by the patient [52]. Table 1 summarizes the conventional/approved drugs used in breast cancer chemotherapy together with their major mechanisms of action and most commonly observed side effects [50–63]. Several drugs have been used in combination, for example, cisplatin-methotrexate fluorouracil (CMF), fluorouracil-epirubicin-cyclophosphamide (FEC), and FEC-tamoxifen (FEC-T), supposedly to increase efficacy and reduce side effects. Patients, however, still experience fatigue, phlebitis, alopecia, nausea, mucositis, anemia, and myelosuppression alongside long-term side effects including ovarian failure, weight gain, cardiac dysfunction, and, in some cases, leukemia [64, 65]. Herbal medicines and natural supplements are widely used in cancer chemoprevention in the clinic and are also studied *in vitro* and *in vivo* [66, 67].

3.1.1. Traditional Chinese Medicines Composed of Multiple Herbs. In traditional medicinal systems, herbal medicines are used often to treat the symptoms associated with cancer and the side effects of cancer treatment [68]. Herbal formulations used in TCM include mixtures of herbal compounds constituted as decoctions, tea, injections, or capsules, which are purported to possess anticancer compounds and are used alone or as adjuvants to existing chemotherapy regimens to improve efficacy and/or reduce drug-induced toxicity [69]. Although TCM is commonly used to counteract the side effects of chemotherapy, scientific evidence for its use in women with breast cancer still is being collected. Among the most common Chinese medicinal herb formulations used in preclinical and clinical practice for breast cancer treatment are Danggui (*Angelica sinensis*-radix) and Ren Shen (*Panax ginseng*-radix), which are reported to have potential beneficial synergistic effects that include decreasing treatment-associated toxicity, psychosocial stress, and fatigue [70]. Jia-wei-xiao-yao-san, commercially known as "Augmented Rambling Powder," a Chinese medicinal herb formulation containing Danggui, is the most frequently prescribed formula for treating breast cancer and chemotherapy-related symptoms by TCM practitioners in Taiwan. This formulation has a long history of use for alleviation of blood toxicity and sleep disturbance. It is also used to relieve hot flushes and lower serum levels of inflammatory cytokines, IL-6, IL-8, and macrophage protein 1-β [71, 72].

LSC101, an encapsulated homogenized mixture of dry powdered extracts from a combination of medicinal herbs, including *Astragalus membranaceus*, *Poriae cocos*, *Atractylodes macrocephala*, *Lycium chinense*, *Ligustrum lucidum*, *Paeonia lactiflora*, *Paeonia obovata*, *Citrus reticulata*, *Ophiopogon japonicus*, *Millettia reticulata*, *Oldenlandia diffusa*, *Scutellaria barbata*, *Prunella vulgaris*, and *Glehnia littoralis,* is used widely by breast cancer patients. Its efficacy in attenuating the hematological complications of chemotherapy has been tested in clinical settings [70]. In mouse breast cancer models, the use of LSC101 together with doxorubicin led to significantly higher neutrophil, splenic erythrocyte, and leukocyte counts [71]. In addition, the use of LCS101 together with conventional chemotherapy regimens provided protection against mild to moderate chemotherapy-induced anemia and neutropenia, supporting its use for decreasing hematological toxicity but not cancer prevention [73]. Though it is not yet clear how the compounds in LCS101 reduce hematological toxicity, it is suspected that the interactions and synergistic effects of the active compounds from the combination of herbs may be responsible for the pronounced efficacy [74]. Some of the component herbs, for example, *Ophiopogon japonicus* and *Astragalus membranaceus,* in LSC101 have been independently shown to stimulate the production of erythroid progenitor cells in mice and promote recovery of hematopoietic function in patients with chronic aplastic anemia [75]. A TCM formulation composed of five herbs, commonly known as "Ruyiping" and "Runing II," is used as treatment for detoxification and preventing relapse, recurrence, and metastasis in breast cancer patients after mastectomy [76]. Clinical evidence suggests that the mechanism of action of this herbal formulation is via inhibition of angiogenesis and downregulation of vascular endothelial growth factor (VEGF) and VEGF receptor as well as microvessel count (MVC) and micro-vessel area (MVA) [75, 76].

Shenqi Fuzheng Injection (SFI), a TCM formulation used in repairing immune function at the cellular and molecular levels, is also effective in alleviating myelosuppression and GI tract reaction induced by chemotherapy and surgical operation [77]. In clinical evaluations, the protein expressions

Table 1: Summary of major mechanisms of action and common side effects of chemotherapeutic drugs approved for breast cancers.

Chemotherapeutic agent	Mechanism of action	Side effect	References
Cyclophosphamide	Apoptotic cell death	Pulmonary toxicities, weight gain	[50, 52]
Cisplatin	DNA damage, apoptosis	Nephrotoxicity	[54]
Doxorubicin	DNA damage	Impaired cognitive function, anemia	[55, 56]
Docetaxel	Mitotic inhibition	Pulmonary toxicities, colitis, diarrhea	[57]
Epirubicin	DNA damage	Nausea, cardiotoxicity	[58–60]
Fluorouracil	Thymidylate synthetase inhibition; DNA synthesis inhibition	Cardiotoxicity, anemia, GI tract toxicity	[61]
Gemcitabine	Nucleic acid synthesis inhibition	GI tract toxicity	[62]
Methotrexate	Cell cycle arrest	Anemia, weight gain, jaundice, diarrhea, loss of bone density	[50]
Mitomycin	DNA alkylating agent	Myelotoxicity, fatigue, systemic toxicity	[63]
Mitoxantrone	Topoisomerase inhibition	Alopecia, systemic toxicity	[63]

of CD83, CD80, and CD86 in patients' tumor tissue and auxiliary lymph nodes were detected before and after treatment. Results suggest that SFI could help repair immunity impaired by cancer and cancer treatment by activating dendritic cells and upregulating costimulatory molecules [77, 78]. Clinical studies using a natural dietary supplement composed of a combination of medicinal mushrooms *Coriolus versicolor*, *Ganoderma lucidum*, and *Phellinus linteus* and medicinal herbs *Scutellaria barbata*, *Astragalus membranaceus*, and *Curcuma longa* suggested that the formula can alleviate chemotherapy-induced toxicity in liver, spleen, kidney, lung, and heart tissue [79]. *In vitro* studies also elucidated the mechanism of action of this mushroom-herbal formulation in inhibiting proliferation and lowering the invasive behavior of a highly metastatic human cancer cell line, MDA-MB-231, by the inhibition of cyclin A1 expression and by the downregulation of CXCR4 [79–81].

A combination of rose geranium (*Pelargonium graveolens*, Geraniaceae), *Ganoderma tsugae* (Ganodermataceae), *Codonopsis pilosula* (Campanulaceae), and *Angelica sinensis* (Apiaceae) (RG-CMH) has been used in TCM treatments for breast cancer and is associated with immunomodulation based on anti-inflammatory and wound-healing properties attributed to the synergistic activity of the components of the herbs [82]. In one RCT, RG-CHM intervention improved the immune cell count of cancer patients receiving chemotherapy and/or radiotherapy preventing leukopenia and immune impairment associated with a decrease in levels of T cells, helper T cells, cytotoxic T cells, and natural killer cells compared with the group receiving placebo treatment. However, the differences between the two groups were not statistically significant [83]. The results did show, however, that the administration of RG-CMH to patients receiving chemotherapy/radiotherapy delayed the reduction in levels of leucocytes and neutrophils experienced by patients undergoing cancer treatment [83, 84].

Yunzhi-Danshen (*Coriolus versicolor* and *Salvia miltiorrhiza*) capsules have been shown to benefit the circulatory system through vasodilation, immunomodulation, and antidementia activities [85]. Results of a recent RCT showed that the absolute counts of T-helper lymphocytes (CD4+), the ratio of T-helper (CD4+)/T suppressor and cytotoxic lymphocytes (CD8+), and the percentage and the absolute counts of B lymphocytes were significantly elevated in patients after taking Yunzhi-Danshen capsules. These clinical findings imply that regular oral consumption of Yunzhi-Danshen capsules could be beneficial for promoting immunological function in breast cancer patients after chemotherapy [85, 86]. These findings were also supported by *in vitro* results showing that Yunzhi-Danshen treatment inhibited cancer cell proliferation by cell-cycle arrest and downregulation of Akt phosphorylation in MCF7 cells, a human breast cancer cell line, and by inducing apoptosis [87, 88].

3.1.2. Single Herbs and Medicinal Mushrooms. Black cohosh (*Cimicifuga racemosa*) is known in TCM to reduce hot flushes in menopausal women and to have low toxicity. Several clinical studies have backed up this claim [89–92]. Since breast cancer chemotherapeutics such as cytostatics, aromatase inhibitors, or antiestrogens frequently induce or aggravate preexisting menopausal symptoms, extracts of *C. racemosa* are currently being explored as an adjuvant. The mechanisms of action of this phytotherapeutic herb are still not totally understood, but there is growing interest in its usefulness in the treatment of vasomotor symptoms and hot flushes and in preventing the decrease in bone density associated with menopause [93, 94]. The use of black cohosh in clinical trials as an adjuvant to chemotherapy was observed to help patients improve their QOL through relief of vasomotor symptoms [95, 96]. Moreover, *in vitro* studies using MCF7 cells showed the high antitumor activity of *C. racemosa* extracts and their involvement in induction of apoptosis [97].

Coriolus versicolor (Yunzhi) also known as *Trametes versicolor* is a popular component in TCM mushroom preparations. Several clinical trials with patients receiving chemotherapy or radiotherapy have found that encapsulated Yunzhi preparations significantly improve appetite, alleviate weakness, anorexia, vomiting, dryness of the throat, and

spontaneous or night sweats and pain, increase weight, stabilize white blood cell counts, NK cells, IL-2 levels, and CD4/CD8 ratio, and demonstrate a 9% absolute reduction in 5-year mortality rate [98–100]. Polysaccharide-K (PSK), also known as krestin, is one of the active compounds found in Yunzhi [83]. It is a unique protein-bound polysaccharide, which has been used as a chemoimmunotherapy agent. Several RCTs have demonstrated the efficacy of PSK as an adjuvant in cancer therapy, with positive results seen in the adjuvant treatment of gastric, esophageal, colorectal, breast, and lung cancers. PSK is a biological response modifier (BRM) that improves the ability of cancer patients to fight off tumor progression through different mechanisms, most probably by leukocyte activation, regulation of IFN-γ and IL-2 levels, and inhibition of metalloproteinases and other enzymes involved in metastatic activity [101–105]. PSK has further been shown to have antioxidant activity which may allow it to play a role as a normal tissue chemo- and radioprotector when used in combination with adjuvant or definitive chemotherapy and/or radiotherapy in the treatment of cancers and may also enable it to defend the host from oxidative stress [106].

Ganoderma Lucidum, also known as Lingzhi, is used in TCM to promote health and increase life expectancy [137]. Clinically, the spore powder is used to treat cancer-related fatigue in breast cancer patients undergoing endocrine therapy. Patients given the treatment reported improved physical well-being, less fatigue, less anxiety and depression, and overall better QOL. Comparative evaluation of TNF-α, IL-6, and liver and kidney function before and after interventions showed a statistically significant effect [138]. The wide spectrum of biological effects reported for *G. lucidum* in the prevention of chronic diseases, such as hepatitis, hepatopathy, and hypertension, makes it a viable adjuvant for hepatoprotection in cancer therapy [139]. Among the active compounds present in *G. lucidum* extracts, triterpenoids are one of the main components responsible for the pharmacological activities including immunomodulatory, antioxidative, antimetastatic, and antitumor effects [140]. *In vitro* and *in vivo* assays have revealed that the mixtures of triterpenoids in *G. lucidum* exerted antiproliferative effects by inducing apoptosis and cell-cycle arrest [141].

Ginseng (*Panax Ginseng*) is one of the most well-known herbal remedies and is used in TCM to proactively promote health, vitality, and longevity. Ginseng is ranked as the fourth top-selling herbal medicine globally [142, 143]. In recent years, ginseng has been included in the pharmacopoeias of Germany, Austria, the United Kingdom, and the United States. In adjuvant breast cancer therapy, ginseng has been used to maintain natural energy, increase physical and psychomotor performance, and improve mood and general health [144, 145]. *In vitro* experiments and *in vivo* animal studies have reported that ginsenosides, a group of bioactive compounds identified in ginseng, have a variety of beneficial effects, including immunomodulatory, antistress, antifatigue, and anticarcinogenic effects [146].

Accumulating evidence from epidemiologic, clinical, and laboratory studies has revealed an inverse-relationship between increased intake of green tea (*Camellia sinensis*) and relative risk for breast cancer [147]. Green tea extract polyphenon E (PPE) containing bioactive compound epigallocatechin gallate (EGCG) was supplemented as decaffeinated green tea capsules for 2 months in a double-blind, randomized, and placebo-controlled intervention study. Results suggest the beneficial effects of EGCG on LDL-cholesterol concentrations and glucose-related markers [148]. Since green tea has been associated with weight control and cardiovascular disease prevention, its effect on weight gain after breast cancer treatment was also investigated [149]. A slight reduction in body weight and improved HDL and glucose homeostasis was seen in overweight breast cancer survivors. These clinical findings, together with substantial *in vitro* and *in vivo* evidence, suggest that tea polyphenols can be used as chemopreventive agents and as adjuvant treatments for breast cancer [149–151].

Mistletoe (*Viscum album*) extracts have been used for cancer therapy since the early 1920s, most commonly in central Europe [152, 153]. Most recent clinical studies have focused on the use of mistletoe extracts as adjuvants for chemotherapy specifically for nausea/vomiting and the side effects of systemic therapy [154]. The active compounds in mistletoe treatment are the recently identified mistletoe lectins (ML I, II, and III) that consist of two polypeptide chains: a carbohydrate-binding B chain that can bind to cell surface receptors enabling the protein to enter the cell, and the catalytic A chain, which can subsequently inhibit protein synthesis, due to its ribosome-inactivating properties [155, 156]. Other pharmacologically relevant compounds found in mistletoe are viscotoxins and other low molecular proteins, oligo- and polysaccharides, flavonoids, and triterpene acids, which have been found to act synergistically resulting in the cytotoxic and apoptosis-inducing effects of the whole plant extract [157, 158]. One RCT showed that mistletoe preparations boosted the immune system in low doses, helping to improve the QOL and survival of some cancer patients by as much as 40% alongside cotreatment with chemo- and radiotherapy. These results are attributed to the overregulation of genes responsible for immune defense, stress response, apoptosis, and cell-cell adhesion pathways [159–162].

Rhodiola algida is widely used in TCM to stimulate the immune system. Oral ulcerative mucositis, a common adverse effect of mainstream cytotoxic drugs, limits the nutritional intake of cancer patients. One clinical study demonstrated the effects of *R. algida* in alleviation of the occurrence of oral ulcers after four cycles of chemotherapy using 5-fluorouracil, epirubicin and cyclophosphamide, and postmasectomy [163]. Lymphocyte proliferation was induced and serum levels of IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor (GMC-SF) were increased by taking *R. algida* extracts. While white blood cell (WBC) levels returned to the normal range a week after every cycle of chemotherapy, WBC count increased faster in patients using *R. algida*. Patients also presented fewer and smaller oral ulcers and no liver or renal complications were observed in any of the patients involved in the study. Thus *R. algida* has the potential to be used concurrently with chemotherapy to alleviate the occurrence of oral ulcers [163–165].

Several flavonoids with cytotoxic activity have been isolated from the aqueous extract of the aerial part of *Scutellaria barbata*. Despite identification of several active chemical compounds, none demonstrated more potent cytotoxic activity than the whole plant extract. Thus, the whole herb extract is being used and studied clinically [166–168]. In one multicenter, open-label, and dose-escalation phase 1B clinical trial, *S. barbata* extract was administered orally, once or twice daily on a continuous basis to women with advanced metastatic breast cancer (MBC) receiving chemotherapy. Dose-limiting side effects were decreased including aspartate transferase (AST) elevation, diarrhea, fatigue, and pain, proving this herb to be effective and safe and thus showing promise in the treatment of side effects related to the treatment of women with MBC [169]. Most notably, the components of the whole herb extract work in synergy to inhibit cell proliferation, induce cell-cycle arrest, stimulate ROS production and hyperactivation of poly(ADP-ribose) polymerase(PARP), and inhibit glycolysis [170].

Curcumin, the principal active component of turmeric (*Curcuma longa*), has potential therapeutic activities against breast cancer through multiple signaling pathways [171]. It has been widely reported to reverse chemoresistance and sensitize cancer cells to chemotherapy and targeted therapy in breast cancer [172, 173]. In cell models, curcumin could suppress expression of progrowth and antiapoptosis molecules, induce inactivation of NF-κB, Src, and Akt/mTOR pathways, and downregulate the key epigenetic modifier EZH2 [174–178]. One clinical study reported that when curcumin was used as an adjuvant with docetaxel, dose-limiting toxicity effects were significantly decreased [175].

Uncaria tomentosa, commonly known as Utor Cat's Claw, is a medicinal herb used in the treatment of different diseases including cancer, arthritis, gout, and epidemic diseases [179]. Whole plant extracts were reported to have cytostatic and anti-inflammatory activity, and patients who use Cat's Claw along with chemotherapy and radiation report fewer adverse effects [180]. The use of *U. tomentosa* helps in the restoration of cellular DNA, preventing mutations and cell damage caused by chemotherapy drugs [181]. In addition to its antioxidant properties, *U. tomentosa* modulates the activity of the immune system by proliferation of normal T and B lymphocytes and modulation of certain cytokines, including IL-1, IL-6, and TNF-α [182–184].

3.2. Use of Acupuncture in Breast Cancer

3.2.1. Definition and Concept of Acupuncture. The National Institutes of Health (NIH), USA, has defined acupuncture as a family of procedures involving stimulation of anatomical locations on the skin by a variety of techniques. The most studied mechanism of stimulation of acupuncture points uses penetration of the skin by thin, solid, and metallic needles, which are manipulated manually or by electrical stimulation [185].

The general theory of acupuncture is based on the premise that bodily functions are regulated by an energy called "qi" which flows through the body; disruptions of this flow may cause disease [186]. Traditional acupuncturists understand qi as circulating between the organs along channels called meridians, which are classified as yin or yang meridians [187]. Yin meridians include the lung, spleen, heart, kidney, pericardium, and liver, while yang meridians include the stomach, large intestines, small intestines, bladder, triple energizer, and gall bladder [188, 189]. Qi energy must flow in the correct strength and quality through each of these meridians and organs for health to be maintained. Throughout the history of Chinese medicine and descriptions in *Huangdi Neijing*, the concept of balancing yin and yang had been extensively applied in the application of combination of meridians, corresponding organs, and acupuncture points or acupoints [188, 189]. Acupoints are mainly (but not always) found at specific locations along the meridians which provide one means of altering the flow of qi. There are also a number of acupuncture points with specified locations outside the meridians; these are called "extraordinary" points and are often credited with special therapeutic properties. A third category of acupuncture points called "A-shi" points have no fixed location but represent tender or reflexive points appearing in the course of pain syndromes [188]. Acupuncture points are thought to correspond to conventional (Western) physiological and anatomical features, such as the peripheral nerve junctions, and are known to stimulate the release of neurotransmitters, partially explaining its effect particularly in pain management [189].

3.2.2. Scientific Exploration into Acupuncture. Acupuncture is aimed at correcting imbalances in the flow of qi by stimulation of acupoints by a variety of techniques which involves the insertion of fine needles into the skin and underlying tissues at specific points, for therapeutic or preventative purposes. Evidence of the neurophysiological mechanisms underlying acupuncture now exists [190–193]. For example, the release of a number of endogenous substances including β-endorphin, met-enkephalin, and dynorphins was observed during treatment [194–196]. Moreover, acupuncture can alter gene expression, upregulating opioid production [197, 198]. Acupuncture works by modulating noradrenergic and serotonergic pathways to give extra segmental pain relief, that is, analgesia throughout the body [199]. Acupuncture releases serotonin [200], oxytocin [201], and endogenous steroids [202], which may further contribute to analgesia. In functional MRI studies, acupuncture induced brain activation in the hypothalamus and nucleus accumbens and deactivated areas of the anterior cingulate cortex, amygdala, and hippocampus. In terms of analgesia, it was suggested that acupuncture modulated the affective-cognitive aspect of pain perception [199]. Furthermore, correlations between signal intensities and analgesic effects have been reported [203]. Further work using PET scanning showed that acupuncture induced extra effects in the ipsilateral insula beyond the sham needle, which also had greater effects on activation patterns than the control group [107].

Recent advances in clinical research on acupuncture suggest that acupuncture provides clinical benefit for breast oncology patients in symptom control and supportive care.

Symptoms that respond to acupuncture treatment include pain, gastrointestinal side effects, hot flushes, fatigue, anxiety, depression, and insomnia. Patients welcome a supportive therapy that can reduce symptoms without the need for long-term medication. The strength of current scientific evidence has made acupuncture more acceptable to Western-trained doctors and given rise to Western medical acupuncture [108].

3.3. Effectiveness of Acupuncture in Breast Cancer Patients and Suggested Acupoints

3.3.1. Cancer-Related Hot Flushes. Twelve trials explored the effect of acupuncture on vasomotor syndrome (summarized in Table 2) [109–116, 204, 205], including eight RCTs and four single-group pre-post comparisons. Daily flush frequency was the main outcome measure. All the studies used self-administrated questionnaires to measure this effect. Some trials also used the Kupperman Index (KI) to score climacteric symptoms. Most studies used six or more acupoints of which SP6 was the most commonly used. A course of acupuncture treatment has been found to reduce hot flushes associated with normal menopause and also from hormonal treatments for cancer. Studies found that acupuncture reduced hot flushes by up to 60% in women treated with tamoxifen for breast cancer [108, 110, 111, 205]. Those in the acupuncture group additionally reported improved libido, increased energy, and improved clarity of thought and sense of well-being. Furthermore, the acupuncture group reported no adverse side effects. An algorithm has been developed for the long-term treatment of hot flushes, with the observed effects of the initial course of treatment maintained for up to 6 years by weekly self-needling at SP6 or by using semipermanent needles [117]. For self-needling, patients require clear demonstration of cleansing, insertion, and safe disposal [118].

3.3.2. Nausea and Vomiting. Ten studies included in Table 2 investigated the antiemetic effect of acupuncture on distress symptoms induced by chemotherapy [119–126]. Participants received intervention over a treatment period of 5 days to 3 weeks. These studies, including three high quality studies [118, 119, 121], reported that acupuncture could significantly improve emesis caused by breast cancer therapy. Acupuncture stimulation at points PC6 and ST36 has repeatedly been shown to be a clinically useful antiemetic treatment for postoperative nausea and vomiting and chemotherapy-induced emesis. In 1998, the US NIH stated that "acupuncture is a proven effective treatment modality for nausea and vomiting" [185]. A three-arm RCT comparing conventional antiemetics alone with antiemetics plus either electroacupuncture or minimal acupuncture demonstrated that the electroacupuncture plus antiemetics arm was the most effective for preventing nausea and vomiting associated with high-dose chemotherapy [121]. Ezzo and colleagues reviewed eleven trials in 2006 and concluded that electroacupuncture has demonstrated benefit for chemotherapy-induced acute vomiting, and self-administered acupressure appears to have a protective effect against acute nausea and can readily be taught to patients [206]. Since then, two multicenter longitudinal RCTs have shown the beneficial effect of acupressure in significantly reducing the severity of both acute and delayed vomiting [118, 119]. These studies also demonstrate that acupuncture and acupressure are simple to administer and merit wider consideration.

3.3.3. Pain. Up to 70% of cancer patients still suffer significant pain which adversely impacts their QOL [207]. Bone pain is the most common type of cancer-associated pain, and bone metastases are common in advanced breast cancers. Current pain-relieving strategies include the use of opioid-based analgesics, bisphosphonates, and radiotherapy. The pharmacological failure to control pain alone has led to the use of nondrug treatments including acupuncture. The analgesic effects of acupuncture may permit a decrease in the requirement and side effects of pharmaceuticals. It can also help those who are sensitive to normal doses of analgesics and those who have pain despite analgesic dose titration [196]. Although acupuncture is used in palliative care settings for all types of cancer pain, the evidence base is still insufficient and inconclusive and there is very little evidence to show its effectiveness in relieving cancer-induced pain [208, 209].

Three trials used acupuncture to manage postmastectomy pain (Table 2) [127–129, 208, 209]. Acupoint L14 was used in all the three trials. Two studies demonstrated a significant effect favoring the acupuncture group [128, 129], but one high quality RCT [127] found no significant difference between the intervention group and the control group. Although reviews vary in their conclusions, acupuncture was found to be superior to no treatment or waiting list control in most studies.

Finally, emerging evidence demonstrates the analgesic effectiveness of both acupuncture and electroacupuncture in breast cancer patients experiencing joint pain as a result of adjuvant aromatase inhibitor treatment. Four trials have included investigation of arthralgia, and all explored the effect of acupuncture therapy on aromatase inhibitor-related joint pain and functional ability (Table 2). Positive results were obtained including enhanced postoperative analgesic efficiency, relief of postoperative pain, and significant improvement in joint and muscle stiffness [128, 130–132, 210].

3.3.4. Fatigue. Fatigue is an extremely common symptom in cancer patients [211]. Fatigue is also an adverse side effect of chemotherapy and radiotherapy, which can persist long after the cessation of treatment. In a prospective phase II study on patients with persistent fatigue who had previously completed chemotherapy, acupuncture resulted in a significant reduction in baseline fatigue scores [212]. Further four RCTs showed that acupuncture was associated with a significant improvement in general fatigue scores [213–216].

3.3.5. Anxiety, Depression, and Insomnia. The two anxiety, sickness, and dyspnoea (ASAD) points located at the upper left and right sternal regions are used extensively in the UK to control dyspnea and also anxiety. Patients can massage

Table 2: Summary of the effectiveness of acupuncture (acupoints) used in breast cancer patients for cancer-related syndromes or side effects caused by treatments.

Symptoms	Acupoints used	References
Hot flush	LIV3, GB20, LU7, KI3, SP6, REN4, P7, LIV8 DU14, GB20, BL13, PC7, H6, K7, ST36, SP6, Ear shen men, Ear sympathetic point, BL23, BL32, HT7, SP6, SP9, LR3, PC6, GV20 KI6, SP6, BL23, CV4, GB35, H5 BL62, LR14, KI3, HT7, TE6, SP6, LI11, ST36, GV20, LI4	[107–116]
Nausea and vomiting	P6, ST36, LI4	[117–126]
Postmastectomy pain	LI4, SP6, auricular points, GB6, SJ6, PC2, PC3, LE14, MP19, DI14, BL17, LU2, RE6, RE17	[127–129]
Arthralgia	TB5, GB41, GB34, LI4, ST41, KD3, LI15, SJ14, SI10, SJ4, LI5, SI5, SI3, LI3, Du3, Du8, UB23, GB30, GB39, SP9, SP10, ST34	[130–132]
Lymphedema	CV2, CV3, CV12, LI15, TE14, LU5, TE5, LI4, ST36, ST6, SP9, SJ5, SJ14, REN2, REN3, REN12	[133, 134]
Leukopenia	ST36	[135, 136]

acupuncture studs for 1-2 min on demand to provide anxiolysis [196]. This method has the added benefit of empowering the patient to control these distressing symptoms in the event of a panic attack. In a systematic review of RCTs of acupuncture in the treatment of depression, Leo and Ligot Jr. stated that although the odd ratios derived from comparing acupuncture with control conditions in the existing literature suggest a role for acupuncture, the evidence is thus far inconclusive [217]. More recent evidence suggests that acupuncture when combined with antidepressant therapy has a faster therapeutic onset rate than pharmacotherapy alone, coupled with a reduction in the side effect profile of the antidepressant medication [218]. An additional RCT examining the treatment of hot flushes revealed that compared to women taking venlafaxine, those receiving acupuncture felt they had more energy, improved clarity of thought, increased libido, and a greater sense of well-being [219]. In one study done by Mehling and colleagues, massage and acupuncture in postoperative cancer patients who were also receiving usual care resulted in a significant improvement in their depressed mood with short-lived significant improvement in tension and anxiety when compared to patients receiving usual care alone [220]. A subsequent meta-analysis revealed that the rate of improvement in insomnia produced by auricular acupuncture was significantly higher than that achieved by taking Diazepam [221]. Although a Cochrane systematic review of acupuncture for insomnia in 2007 concluded that acupuncture or its variants were not more effective than the control groups [222], five clinical studies showed significant improvement in anxiety and depression over time in patients who underwent acupuncture treatment. QOL measures of pain severity and interference, physical and psychological distress, life satisfaction, and mood states also showed improved scores after acupuncture treatment [223–227].

3.3.6. Lymphoedema and Leukopenia. Lymphoedema is a distressing problem that affects many women after breast cancer surgery. In the United States, needling and even lifting objects using the affected arm has been prohibited, resulting in a limited number of publications on acupuncture and lymphoedema [133]. However, recent results of two studies demonstrated that traditional acupuncture after breast cancer surgery was associated with improvements in movement amplitude of the shoulder, symptoms of heaviness and tightness in the arm, and the degree of lymphedema [133, 134].

Two trials conducted in China found that dexamethasone injected at the ST36 intra-acupoint was effective in preventing bone marrow suppression-related leukopenia in breast cancer patients undergoing chemotherapy or radiotherapy (Table 2) [135, 136]. The main body of evidence comes from China where a systematic review of RCTs was positive for increasing WBC in patients undergoing chemotherapy [228]; however, the quality of trials was considered poor, and the authors suggest that the positive meta-analysis should be considered as exploratory.

3.4. Safety of Acupuncture. With an increasing number of positive evidence-based acupuncture trials, more cancer patients may seek acupuncture treatment. While closely monitored clinical trials often report low incidences of adverse events of acupuncture, many physicians remain concerned about its safety. Serious adverse events are exceedingly rare—roughly five in one million [229]—and are usually associated with poorly trained, unlicensed acupuncturists [230]. The vast majority of adverse events from acupuncture

are minor; those most commonly reported occur at the site of needle insertion: minor bleeding (3%), hematoma (2-3%), and pain from needling (up to 3%). Dizziness is reported in about 1% of treatments [229, 231, 232]. Serious adverse effects including pneumothorax, spinal lesions, and hepatitis *B* transmission have been reported in the literature for acupuncture, but these are rare and are generally associated with poorly trained unlicensed acupuncturists [233]. Acupuncture for oncology should be administered by a suitably qualified practitioner who can maintain a constant dialogue with the oncology team treating the patient. The contraindications and cautions for acupuncture in an oncology setting are outlined in Table 3.

In general, acupuncture can be considered a safe method of treatment, with a low side effect profile, which in part adds to its popularity among patients [234–236]. Establishing an eligibility guideline for cancer patients before receiving acupuncture would add another layer of safety. Lu and Rosenthal suggested that cancer patients should not be recommended for acupuncture if they have one of the following conditions: (a) absolute neutrophil count (ANC) less than 500/μL, (b) platelet count less than 25,000/μL, (c) altered mental state, (d) clinically significant cardiac arrhythmias, and (e) other unstable medical conditions (case-by-case consideration). Guidelines for safe practice within this field have previously been published [237]. Before the first visit, approval is required from the primary oncologist based upon these guidelines [238].

Table 3: Contraindications and cautions for the use of acupuncture in breast cancer patients.

(1) Extreme needle phobia, very "strong reactors" to acupuncture
(2) Coagulopathy
(3) Immunocompromised patients or neutropenia (less 500/mm^3)—risk of infection
(4) In young patients (age less than 18 years old) postsplenectomy —risk of infection
(5) Avoid directly onto a tumor nodule or into an ulcerative area
(6) In areas of spinal instability—risk of cord compression secondary to acupuncture's muscle relaxing properties
(7) Into a prosthesis—risk of leakage of saline/silicone
(8) Over intracranial deficits following neurosurgery
(9) Pregnancy
(10) Confused patients

4. Conclusions and Future Prospects

Research on CAM as adjuvants in chemotherapy and/or radiotherapy, particularly that on herbal medicine and acupuncture, has gained momentum over the past few years. This development paves the way toward understanding their efficacy and modes of action in alleviating cancer or cancer treatment-related conditions. Evidence from various *in vitro*, *in vivo* studies and RCTs support the use of herbal medicine or acupuncture in boosting the immune system, in relieving pain, fatigue, cyto- and hepatotoxicity, and in inhibiting gastrointestinal toxicity, angiogenesis, and other side effects from chemo- and radiotherapy. The inclusion of selected herbal medicines from well-designed RCTs in this review provides evidence-based knowledge to strengthen the rationale for the use of herbal medicines in controlling breast cancer in the clinical setting. Further, considering the assessment of benefit : risk ratio of the presented results, acupuncture is seen as a valuable nonpharmaceutical treatment option for symptom management in cancer patients. Although current evidence from basic science and clinical research on herbal medicines or acupuncture is still not sufficient to change oncological practice in general, the quality and design of clinical trials have significantly improved over the last few years which can provide patients with the most effective protocols or treatment types and safety profiles. Despite all the evidence presented, key challenges still exist including quality control of herbal medicinal materials, standardization of practices and current methodology in acupuncture, and pharmacokinetic interaction between drug components or between chemotherapy and herbal medicine. Further research addressing these challenges in the form of rigorously designed clinical trials accompanied by comprehensive and in-depth laboratory studies is needed to improve the quality of the existing evidence base and support the use of CAM.

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Research Article

Juzentaihoto Failed to Augment Antigen-Specific Immunity but Prevented Deterioration of Patients' Conditions in Advanced Pancreatic Cancer under Personalized Peptide Vaccine

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Juzentaihoto (JTT) is a well-known Japanese herbal medicine, which has been reported to modulate immune responses and enhance antitumor immunity in animal models. However, it is not clear whether JTT has similar effects on humans. In particular, there is little information on the effects of JTT in antigen-specific immunity in cancer patients. Here we conducted a randomized clinical study to investigate whether combined usage of JTT could affect antigen-specific immunity and clinical findings in advanced pancreatic cancer patients undergoing personalized peptide vaccination (PPV), in which HLA-matched vaccine antigens were selected based on the preexisting host immunity. Fifty-seven patients were randomly assigned to receive PPV with ($n = 28$) or without ($n = 29$) JTT. Unexpectedly, JTT did not significantly affect cellular or humoral immune responses specific to the vaccine antigens, which were determined by antigen-specific interferon-γ secretion in T cells and antigen-specific IgG titers in plasma, respectively. Nevertheless, JTT prevented deterioration of patients' conditions, such as anemia, lymphopenia, hypoalbuminemia, plasma IL-6 elevation, and reduction of performance status, which are frequently observed in advanced cancers. To our knowledge, this is the first clinical study that examined the immunological and clinical effects of JTT in cancer patients undergoing immunotherapy in humans.

1. Introduction

Juzentaihoto (JTT) is a well-known Kampo (Japanese herbal) medicine, which consists of 10 different herbs and has been used as a supplementary therapy in patients with various types of chronic diseases/symptoms, such as fatigue, loss of appetite, night sweats, circulatory problems, and anemia [1]. JTT has also been frequently used for cancer patients, since it was reported to have anti-tumor effects [1–7] and diminish the side effects caused by cancer treatments, such as chemotherapy and radiotherapy [8–12]. In addition, JTT was shown to possess immune-modulating properties, such as enhancement of phagocytosis, cytokine production, antibody production, and NK, NKT, and T-cell functions, in animal experiments [1–7, 13–21]. However, only limited information is available on the immunological and clinical effects of JTT in humans.

Pancreatic cancer, the fourth largest cause of cancer death in the world, is one of the most aggressive cancers [22, 23]. Although there have been substantial advances in the therapeutic modalities for pancreatic cancer, including systemic chemotherapies using gemcitabine (GEM), S-1 (tegaful, gimeracil, and oteracil potassium), and/or molecular-targeted agents, the prognosis of advanced pancreatic cancer patients still remains dismal [22, 23]. Therefore, development

of new therapeutic approaches, including immunotherapy, is needed.

We have developed a novel immunotherapeutic approach, personalized peptide vaccination (PPV), in which HLA-matched peptides were selected and administered, based on the pre-existing host immunity before vaccination [24–28]. Recent clinical trials of PPV have demonstrated feasibility and safety of this new therapeutic approach in various types of advanced cancers [24–28]. For example, in our previous clinical trials, immune responses boosted by vaccination were well associated with overall survival (OS) in advanced pancreatic cancer patients undergoing PPV in combination with GEM as the first-line therapy [28]. In the current study, we conducted a randomized phase II study of PPV to investigate whether combined usage of JTT could show immunological and/or clinical effects in advanced pancreatic cancer patients undergoing PPV.

2. Patients and Methods

2.1. Patients. Patients with pathological and/or clinical diagnosis of pancreatic cancer, who were refractory to conventional treatments, such as surgery, chemotherapy, and radiotherapy, were eligible for inclusion in the current study, if they showed positive IgG responses to at least 2 of the 31 different vaccine candidate peptides, as reported previously [24–28]. Other inclusion criteria were as follows: age of more than 20 years; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; positive status for the HLA-A2, -A24, -A3 supertype (A3, A11, A31, or A33), or -A26; expected life expectancy of at least 12 weeks; and adequate hematologic, hepatic, and renal function. Exclusion criteria included pulmonary, cardiac, or other systemic diseases; an acute infection; a history of severe allergic reactions; regular use of herbal medicines; pregnancy or nursing; and other inappropriate conditions for enrollment as judged by clinicians. The protocol was approved by the Kurume University Ethical Committee and was registered in the UMIN Clinical Trials Registry (UMIN 000006295). After a full explanation of the protocol, a written informed consent was obtained from all patients before enrollment.

2.2. Clinical Protocol. This was an open-label, randomized phase II study. The patients were randomly assigned to receive PPV with or without oral administration of JTT (PPV plus JTT group versus PPV alone group), according to age and performance status. The primary and secondary objectives were to compare cellular and humoral immune responses to the vaccine antigens and safety between the PPV plus JTT group and the PPV alone group, respectively. Thirty-one peptides, whose safety and immunological effects had been confirmed in previously conducted clinical studies [24–28], were employed for vaccination (12 peptides for HLA-A2, 14 peptides for HLA-A24, 9 peptides for HLA-A3 supertype (A3, A11, A31, or A33), and 4 peptides for HLA-A26) (Supplementary Table 1) (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/981717). The peptides were prepared under the conditions of Good Manufacturing Practice (GMP) by PolyPeptide Laboratories (San Diego, CA, USA) and the American Peptide Company (Vista, CA, USA).

The peptides for vaccination to individual patients were selected in consideration of the pre-existing host immunity before vaccination, by assessing the titers of IgG specific to each of the 31 different vaccine candidates, as reported previously [24–28]. A maximum of 4 peptides (3 mg/each peptide), which were selected based on the results of HLA typing and peptide-specific IgG titers, in mixture with incomplete Freund's adjuvant (Montanide ISA51; Seppic, Paris, France), were subcutaneously administered once a week for 6 consecutive weeks. In the PPV plus JTT group, JTT (TJ-48, 15 mg/day; Tsumura Co., Tokyo, Japan) was orally administered for 35 days during the first cycle of 6 vaccinations. After the first cycle of 6 vaccinations, up to 4 vaccine peptides were reselected according to the titers of peptide-specific IgG and administered every 2 weeks. The vaccine peptides were re-selected at every cycle of 6 vaccinations until the discontinuation of PPV. Adverse events were monitored according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. Complete blood counts and serum biochemistry tests were performed before and after every cycle of 6 vaccinations.

2.3. Measurement of T-Cell Responses to the Vaccine Peptides. T-cell responses specific to the vaccine peptides were evaluated by interferon (IFN)-γ ELISPOT assay (MBL, Nagoya, Japan). Briefly, peripheral blood mononuclear cells (PBMCs) (2×10^5 cells/well) were cultured in U-bottomed 96-well microculture plates (Nunc, Roskilde, Denmark) with 200 μL of medium (OpTmizer T-Cell Expansion SFM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (MP Biologicals, Solon, OH, USA), IL-2 (20 IU/mL; AbD Serotec, Kidlington, UK), and each peptide (10 μM). Half of the medium was replaced with new medium containing the corresponding peptides (20 μM) at day 3. After incubation for the following 4 days, the cells were harvested and tested for their ability to produce IFN-γ in response to the corresponding specific peptides. The cells were also tested for IFN-γ production in response to negative control peptides from human immunodeficiency virus (HIV), which might activate nonspecific immune cells, including non-specific CD8 or CD4 T cells and NK cells. IFN-γ secretion after 18-hour incubation was determined by ELISPOT assay with an ELISPOT reader (ImmunoSpot S5 Versa Analyzer; Cellular Technology Ltd., Shaker Heights, OH, USA). All assays were carried out in quadruplicate. The two-tailed Student's t-test was used for statistical evaluation. Antigen-specific T-cell responses were considered positive, when the spot numbers in response to the specific peptides were significantly higher ($P < 0.05$) than those in response to the control HIV peptides, which were supposed to reflect the numbers of immune cells nonspecifically producing IFN-γ. Peptide-specific T-cell responses were shown as the differences between the spot numbers per 1×10^5 PBMCs in response to the specific peptides and those in response to the control peptides.

Table 1: Characteristics of the enrolled patients.

Factor	PPV + JTT ($n = 28$)	PPV alone ($n = 29$)	P value
Age (years)			0.389
Median (range)	66 (50–83)	65 (45–79)	
Gender			0.922
Male	18	19	
Female	10	10	
Performance status			0.706
0	19	22	
1	9	7	
HLA type			0.753
A24	18	15	
A2	12	13	
A3 supertype	10	17	
A26	5	7	
Clinical stage			0.845
IV	19	20	
Recurrence	9	9	
Location of the main tumor			0.182
Head	6	12	
Body-tail	22	17	
Number of previous chemotherapy regimens			0.843
0	1	1	
1	11	13	
2	13	10	
>3	3	5	
Number of vaccinations			0.443
Median (range)	9 (3–17)	10 (3–18)	
Combination chemotherapy			0.640
None	4	0	
Gemcitabine	10	13	
S-1	5	7	
Gemcitabine + S-1	7	8	
Others	2	1	

2.4. Measurement of Humoral Immune Responses to the Vaccine Peptides. The humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay with the Luminex 200 system (Luminex, Austin, TX, USA), as reported previously [29]. In brief, plasma (×100 diluted) was incubated with 100 μL of peptide-coupled color-coded beads for 1.5 hours at 30°C, followed by washing and incubation with 100 μL of biotinylated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA) for 1 hour at 30°C. The beads were washed and incubated with 100 μL of streptavidin-PE (Invitrogen) for 30 min at 30°C. After washing, the fluorescence of the beads was detected using the Luminex 200 system. If peptide-specific IgG titers in the postvaccination plasma were more than 2-fold higher than those in the prevaccination plasma, the changes were considered to be significant. If a significant increase was observed in at least one of the vaccine peptides, the antigen-specific humoral immune response was considered to be augmented.

2.5. Measurement of Laboratory Markers. ELISA kits were used to measure serum amyloid A (SAA) (Invitrogen), IL-6 (eBioscience, San Diego, CA, USA), IL-18 (MBL), and C-reactive protein (CRP), IL-12 and TGF-β1 (R&D systems, Minneapolis, MN, USA). Bead-based multiplex assays were used to measure Th1/Th2 cytokines, including IFN-γ, IL-2, IL-4, IL-5, and IL-10 (Human Th1/Th2 5-Plex, Invitrogen), with the Luminex 200 system (Luminex). Frozen plasma samples were thawed, diluted, and assayed in duplicate in accordance with the manufacturer's instructions. The mean of duplicate samples was used for statistical analysis.

Free-radical elective evaluator (Wismerll, Tokyo, Japan) was used to measure biological antioxidant potential (BAP) and derivatives of reactive oxidative metabolites (d-ROM), an index of oxidative stress. Frozen plasma samples were thawed, diluted, and assayed in accordance with the manufacturer's instruction.

2.6. Flow Cytometric Analysis of a Suppressive Immune Cell Subset in PBMCs. A suppressive immune cell subset, myeloid-derived suppressor cells (MDSCs), in PBMCs was examined by flow cytometry. For analysis of MDSCs, PBMCs (0.5×10^6) were incubated for 30 min at 4°C with monoclonal antibodies (mAbs) against lineage markers (CD3, CD14, CD19, and CD56), CD33, and HLA-DR. After washing, the samples were run on a FACSCanto II (BD biosciences, San Diego, CA, USA), and data were analyzed using the Diva software (BD biosciences). All mAbs were purchased from Biolegend (San Diego, CA). Granulocytic MDSCs were identified as CD33 positive in the cell subset negative for both the lineage markers and HLA-DR. Monocytic MDSCs were identified as CD14 positive and HLA-DR negative. The frequency of MDSCs in the mononuclear cell gate defined by the forward scatter and side scatter was calculated.

2.7. Statistical Methods. The Wilcoxon signed-rank test, Student's t-test, the chi-square test, or Fisher's exact test was used to compare differences between measurements. OS was calculated from the first date of peptide vaccination until the date of death or the last date when the patient was known to be alive. Curves for OS were estimated by the Kaplan-Meier method, and the log-rank test was conducted for the comparison of survival curves. Two-sided P values of <0.05 were considered as statistically significant. All statistical analyses were conducted using the JMP version 10.0 software (SAS Institute Inc., Cary, NC, USA).

Table 2: Adverse events.

Adverse events	PPV + JTT (n = 28)				Total (%)	PPV alone (n = 29)				Total (%)
	G1	G2	G3	G4		G1	G2	G3	G4	
Injection site reaction	15				15 (54%)	20				20 (69%)
Blood/bone marrow										
Leukopenia	3	2			5 (18%)	4				4 (14%)
Lymphopenia	3	2			5 (18%)	3	1			4 (14%)
Anemia	3	4			7 (25%)	2	4			6 (21%)
Thrombocytopenia	1	1			2 (7%)	2		1		3 (10%)
Laboratory										
AST increased	2	1			3 (11%)	4	2			6 (21%)
ALT increased	4	1			5 (18%)	3	2			5 (17%)
Bilirubin increased		1			1 (4%)	1				1 (3%)
GGT increased	1	8	1	1	11 (39%)	1	4	3		8 (28%)
ALP increased	2	1	1		4 (14%)	1	2			3 (10%)
Creatinine increased	1				1 (4%)					0 (0%)
Hypoalbuminemia	6	1			7 (25%)	6	2			8 (28%)
Glucose intolerance			1		1 (4%)					0 (0%)
Hyponatremia		1			1 (4%)	2				2 (7%)
Hyperkalemia		1			1 (4%)	1				1 (3%)
Gastrointestinal disorders										
Nausea	1				1 (4%)	1	1			2 (7%)
Diarrhea	2				2 (7%)					0 (0%)
Constipation		1			1 (4%)		1			1 (3%)
Abdominal pain	1				1 (4%)	1	2			3 (10%)
Gastroesophageal reflux disease		1			1 (4%)					0 (0%)
Ascites			1		1 (4%)					0 (0%)
Biliary tract infection			1		1 (4%)		1			1 (3%)
Anorexia		3			3 (11%)	1	1	1		3 (10%)
Fever		1			1 (4%)	3				3 (10%)
Pain		2			2 (7%)	2		1		3 (10%)
Edema limbs	1				1 (4%)		2			2 (7%)
Insomnia					0 (0%)		1			1 (3%)
Rash acneiform					0 (0%)	1				1 (3%)

3. Results

3.1. Patients' Characteristics. Between September 2011 and December 2012, a total of 57 advanced pancreatic cancer patients, who were refractory to conventional treatments, were enrolled in this study. The patients were randomly assigned in a 1 : 1 ratio to receive PPV with or without oral administration of JTT (PPV plus JTT, n = 28; PPV alone, n = 29). The demographic and baseline disease characteristics of the enrolled patients are given in Table 1. There were no significant differences between the two groups in the clinicopathological characteristics, including age, gender, performance status, HLA-type, clinical stage, location of the main tumor, and numbers of previous chemotherapy regimen(s). The median number of vaccinations was 9 (range 3–17) in the PPV plus JTT group and 10 (range 3–18) in the PPV alone group. Five and 2 patients did not complete the first cycle of 6 vaccinations due to disease progression in the PPV plus JTT group and the PPV alone group, respectively. In the PPV plus JTT group, PPV was combined with GEM (n = 10), S-1 (n = 5), GEM and S-1 (n = 7), or other combinations of chemotherapeutic agents (n = 2). Four patients received PPV alone because they could not tolerate chemotherapy. In the PPV alone group, PPV was combined with GEM (n = 13), S-1 (n = 7), GEM and S-1 (n = 8), or other combination of chemotherapeutic agents (n = 1).

3.2. Adverse Events. Adverse events occurring in the patients are listed in Table 2. The most frequent adverse event was injection site reactions in both groups. Severe adverse events (grade 3 or grade 4) were as follows: gamma-glutamyl transpeptidase (GGT) increase (n = 2), alkaline phosphatase (ALP) increase (n = 1), glucose intolerance (n = 1), ascites (n = 1), and biliary tract infection (n = 1) in the PPV plus JTT group; GGT increase (n = 3), thrombocytopenia (n = 1), anorexia (n = 1), and pain (n = 1) in the PPV alone group. There were no significant differences in the overall rates of adverse events between the PPV plus JTT group

Table 3: Cellular and humoral immune responses to the vaccine antigens.

	PPV + JTT	PPV alone	*P* value
Cellular immune responses to the vaccine antigens*			
Before vaccination	2/27 (7.4%)	4/28 (14.3%)	0.669
After vaccination	5/22 (22.7%)	11/26 (42.3%)	0.260
Humoral immune responses to the vaccine antigens†			
Augmented	10/23 (43.5%)	10/27 (37.0%)	0.643

*Antigen-specific T-cell responses were evaluated by IFN-γ ELISPOT assay before and after the first cycle of vaccination.

†Antigen-specific IgG titers in plasma were evaluated before and after the first cycle of vaccination. If peptide-specific IgG titers in the postvaccination plasma were more than 2-fold higher than those in the prevaccination plasma in at least one of the vaccine peptides, the antigen-specific humoral immune response was considered to be augmented.

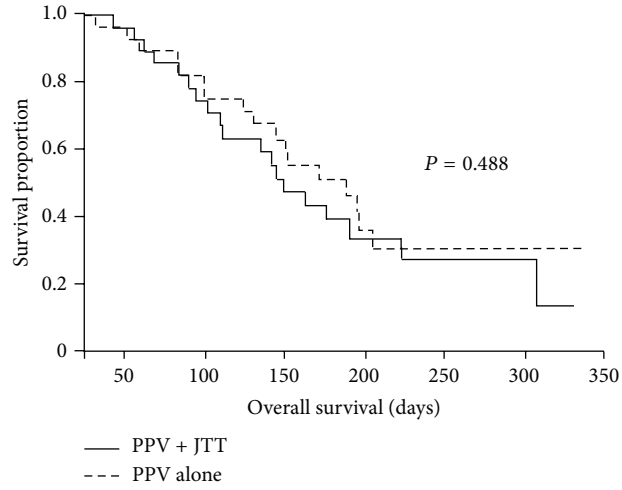


Figure 1: Kaplan-Meier survival analysis in advanced pancreatic cancer patients undergoing PPV with or without JTT. Curves for overall survival were estimated in the PPV plus JTT group ($n = 28$) and the PPV alone group ($n = 29$) by the Kaplan-Meier method, and a difference between survival curves was statistically analyzed using the log-rank test.

and the PPV alone group. According to assessment by the independent safety evaluation committee in this trial, all of these severe adverse events were due to cancer progression or other causes, such as side effects related to combined chemotherapies, rather than to the administration of peptide vaccines or JTT.

3.3. Cellular and Humoral Immune Responses to the Vaccine Peptides. Cellular and humoral immune responses specific to the vaccine peptides were analyzed in blood samples before and after the first cycle of vaccination (Supplementary Table 2 and Supplementary Table 3). Since 5 and 2 patients did not complete the first cycle of 6 vaccinations due to disease progression in the PPV plus JTT group and the PPV alone group, respectively, post-vaccination samples of these patients were unavailable.

T-cell responses to the vaccine peptides were measured by IFN-γ ELISPOT assay with PBMCs. PBMCs were available for this assay in 27 and 22 patients before and after the first cycle of vaccination in the PPV plus JTT group, respectively (Supplementary Table 2). In this group, antigen-specific T-cell responses were detectable in 2 of 27 patients (7.4%) and 5 of 22 patients (22.7%) before and after vaccination, respectively. In the PPV alone group, PBMCs were available in 28 and 26 patients before and after the first cycle of vaccination, respectively (Supplementary Table 3). In this group, antigen-specific T-cell responses were detectable in 4 of 28 patients (14.3%) and 11 of 26 patients (42.3%) before and after vaccination, respectively. There were no significant differences between the PPV plus JTT group and the PPV alone group in the antigen-specific T-cell responses both before and after vaccination ($P = 0.669$ and $P = 0.260$, resp.) (Table 3).

In addition, the humoral immune responses specific to the vaccine peptides were determined by peptide-specific IgG titers using a bead-based multiplex assay. Plasma samples both before and after the first cycle of vaccination were available in 23 and 27 patients in the PPV plus JTT group and the PPV alone group, respectively (Supplementary Table 2 and Supplementary Table 3). The IgG responses specific to at least one of the vaccine peptides were augmented in 10 of 23 patients (43.5%) and in 10 of 27 patients (37.0%) in the PPV plus JTT group and the PPV alone group, respectively. There was no significant difference in the augmentation of antigen-specific humoral immune responses between the two groups ($P = 0.643$) (Table 3).

3.4. Clinical Outcome. All the 57 patients were analyzed for OS. Median followup was 148 (95% confidence interval (CI), 123 to 176) days. The median survival times (MST) from the first vaccination were 148 (95% CI, 109 to 222) days and 187 (95% CI, 129 to undefined) days in the PPV plus JTT group and the PPV alone group, respectively. There was no significant difference in OS between groups ($P = 0.488$, log-rank test) (Figure 1).

In the PPV alone group, 6 of 29 patients showed reduced ECOG performance status during or after the first cycle of vaccination. In contrast, in the PPV plus JTT group, performance status was reduced during or after the first cycle of vaccination in only 3 of 28 patients. A significant change in performance status was observed between before and after (or during) vaccination in the PPV alone group ($P = 0.0156$, paired Wilcoxon signed-rank test) but not in the PPV plus JTT group ($P = 0.125$, paired Wilcoxon signed-rank test).

3.5. Laboratory Markers. Laboratory data both before and after the first cycle of vaccination were available in 23 and 27 patients in the PPV plus JTT group and the PPV alone group, respectively. Complete blood counts and serum biochemistry tests were compared between the two groups. There were no significant differences in complete blood counts, such as hemoglobin and lymphocyte counts, and serum biochemistry tests, such as albumin, total bilirubin, and creatinine, before vaccination (Table 4). In the PPV alone group, hemoglobin, lymphocyte counts, and albumin were significantly decreased after the first cycle of vaccination, whereas they did not change significantly after vaccination in the PPV plus JTT group (Figures 2(a), 2(b), and 2(c)). Of note, these results were consistent, even if 4 patients without combined chemotherapies were excluded from the PPV plus JTT group for statistical analysis. This finding suggested that combined usage of JTT prevented the decrease in hemoglobin, lymphocyte counts, and albumin in pancreatic cancer patients undergoing PPV.

In addition, other markers, including cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-18, IFN-γ, and TGF-β1), inflammation markers (CRP and SSA), and oxidative stress markers (d-ROM and BAP), were compared between the PPV plus JTT group and the PPV alone group. There were no significant differences between the two groups in all of these markers examined before vaccination (Table 4). Inflammatory cytokine IL-6 was significantly increased after the first cycle of vaccination in the PPV alone group, but not in the PPV plus JTT group, suggesting that combined usage of JTT inhibited plasma IL-6 elevation in pancreatic cancer patients undergoing PPV (Figure 2(d)). There were no significant changes in other markers between before and after vaccination in the PPV plus JTT group or in the PPV alone group (data not shown). In addition, there were no significant changes in suppressive immune cell subsets, granulocytic and monocytic MDSCs, in PBMCs between before and after vaccination in the PPV plus JTT group or in the PPV alone group (data not shown).

Table 4: Laboratory markers in peripheral blood before vaccination.

Factor	PPV + JTT (n = 28)	PPV alone (n = 29)	P value
Hemoglobin (g/dL)	11.2 ± 1.4*	11.4 ± 1.6	0.4821
Lymphocyte count (/mm^3)	1469.8 ± 482.6	1493.3 ± 409.8	0.8732
Albumin (g/dL)	3.9 ± 0.4	4.1 ± 0.5	0.0895
Creatinine (mg/dL)	1.05 ± 1.90	0.72 ± 0.20	0.6791
Total bilirubin (mg/dL)	0.646 ± 0.473	0.583 ± 0.309	0.7829
IL-2 (pg/mL)	6.17 ± 4.45	4.92 ± 4.42	0.3800
IL-4 (pg/mL)	5.247 ± 15.169	0.662 ± 2.117	0.3160
IL-5 (pg/mL)	0.938 ± 3.887	0.098 ± 0.314	0.8965
IL-6 (pg/mL)	5.037 ± 3.786	4.612 ± 4.089	0.5134
IL-10 (pg/mL)	0.000 ± 0.000	0.062 ± 0.284	0.3415
IL-12 (pg/mL)	0.711 ± 0.793	0.637 ± 0.686	0.5433
IL-18 (pg/mL)	580.9 ± 269.5	571.5 ± 236.6	0.9731
IFN-γ (pg/mL)	2.87 ± 5.48	2.29 ± 6.66	0.4495
TGF-β1 (ng/mL)	5.68 ± 3.08	5.01 ± 1.87	0.7278
C-reactive protein (mg/dL)	1.90 ± 3.50	1.30 ± 1.92	0.2015
Serum amyloid A (μg/mL)	100.66 ± 75.47	69.31 ± 81.49	0.1505
d-ROM (U.CARR)†	267.6 ± 51.4	242.2 ± 86.5	0.2424
BAP‡ (μmol/L)	973.3 ± 261.0	979.3 ± 183.1	0.7442

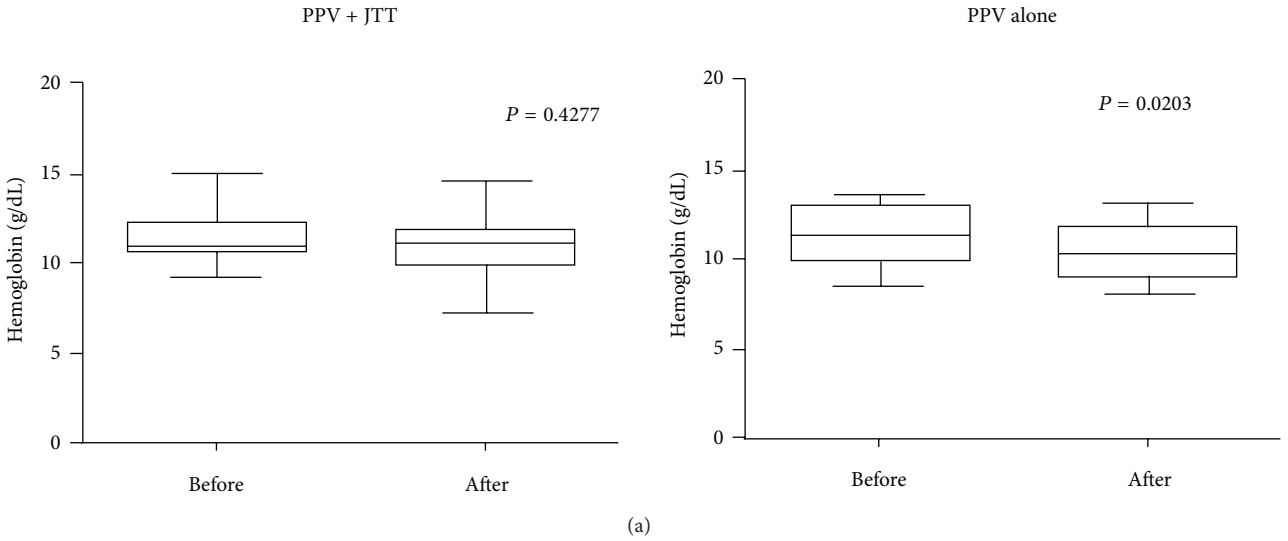
*Values are means ± standard deviations.
†d-ROM: derivatives of reactive oxidative metabolites: U.CARR, Carratelli unit (1 Carratelli unit = 0.8 mg H_2O_2/L).
‡BAP: biological antioxidant potential.

4. Discussion

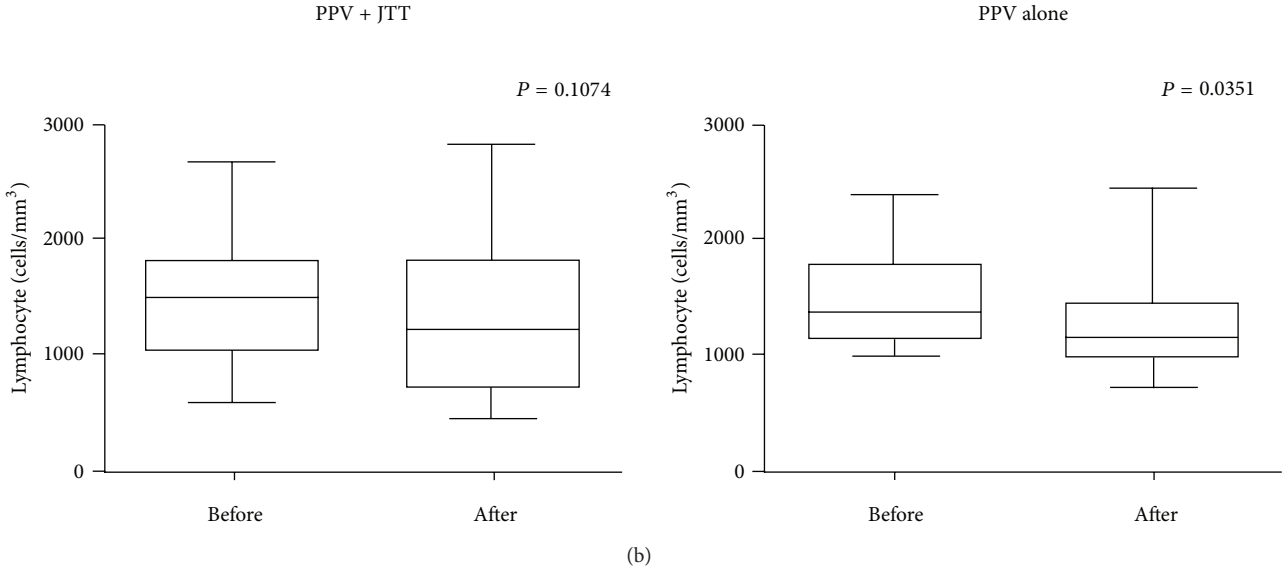
JTT is a well-known Kampo (Japanese herbal) medicine and has been shown to possess immune-modulating and antitumor properties in animal experiments [1–7, 13–21]. However, only limited information is available on the immunological and clinical effects of JTT in cancer patients. To our knowledge, this is the first clinical study that examined the immunological and clinical effects of JTT in cancer patients undergoing immunotherapy in humans.

JTT has been reported to modulate antigen-specific adoptive immune responses in mice [2, 15]. For example, Dai et al. demonstrated that oral administration of JTT induced cytotoxic T cells specific to tumor cells and prevent tumor development in the RET-transgenic mouse model [2]. Iijima et al. reported that JTT induced Th1-skewed immune responses and Th1-dependent antibody responses in aged mice [15]. However, the current study showed that combined usage of JTT did not significantly affect cellular or humoral immune responses to the vaccine antigens after PPV. JTT has also been shown to enhance production of cytokines, such as IL-12 and IL-18, in mice [17, 18]. But, in the current study, there were no significant differences in production of several different cytokines, except IL-6, between the PPV plus JTT group and the PPV alone group. Furthermore, there were no significant differences in suppressive immune cell subsets, granulocytic and monocytic MDSCs [30, 31], in PBMCs between the two groups. Based on our results, combined usage of JTT had no significant immune-modulating effects in advanced cancer patients undergoing PPV, in disagreement with the results of previous animal experiments. In addition, although JTT was reported to inhibit immune cell-mediated oxidative stress [6, 19], the current study showed no significant effects of JTT in redox status, which was determined by oxidative stress markers (d-ROM and BAP) in plasma, in advanced cancer patients undergoing PPV.

Several previous reports demonstrated that JTT showed antitumor effects through various mechanisms [1–7]. Ohnishi et al. showed that oral administration of JTT before tumor inoculation resulted in dose-dependent inhibition of liver metastasis of colon 26-L5 carcinoma cells [5]. Matsuda et al. also reported that oral administration of JTT before tumor cell injection significantly inhibited lung metastasis



(a)



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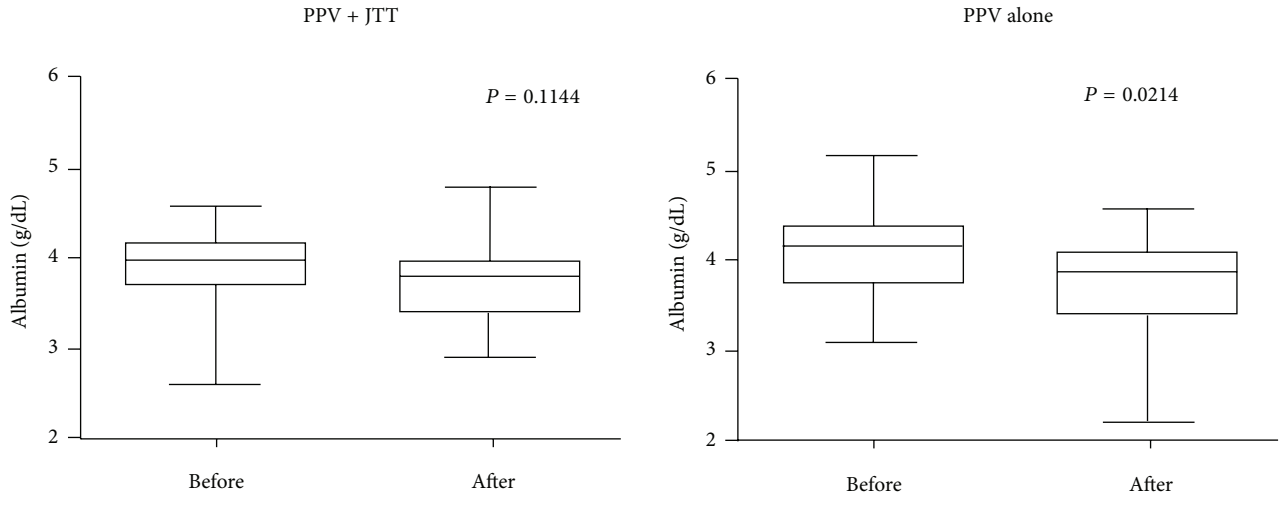

(c)

Figure 2: Continued.

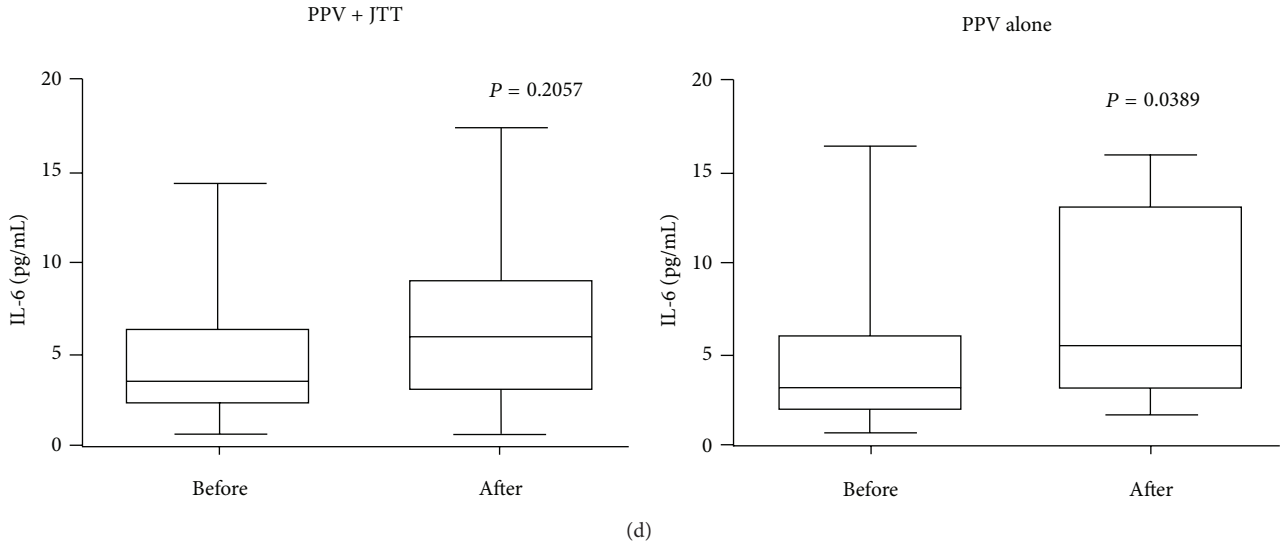

Figure 2: Laboratory markers before and after vaccination in advanced pancreatic cancer patients undergoing PPV with or without JTT. Laboratory markers were compared between before and after the first cycle of 6 vaccinations in the PPV plus JTT group ($n = 23$) and the PPV alone group ($n = 27$) by the paired Wilcoxon signed-rank test. The levels of hemoglobin (a), lymphocyte counts (b), albumin (c), and IL-6 (d) in peripheral blood before and after vaccination are shown. The results are represented by box-and-whiskers graphs. The box plots show median and interquartile range. The whiskers go down to the lowest value and up to the highest value.

of B16 melanoma cells in mice [4]. In addition, in humans, JTT supplementation was shown to result in considerable improvement in intrahepatic recurrence-free survival in hepatocellular carcinoma (HCC) patients after surgical treatment [6]. Although these results suggested the preventive effects of JTT in tumor development in mice and humans, the therapeutic effects of this agent for advanced stage of tumors are not well defined. The current study showed that combined usage of JTT conferred no survival benefits in patients with pancreatic cancer undergoing PPV.

Combined usage of PPV and JTT was well tolerated. The most frequent adverse event was injection site reactions, and all of the severe adverse events observed were due to cancer progression or other causes rather than to the vaccinations or JTT administration. Of note, JTT administration induced some beneficial effects in pancreatic cancer patients undergoing PPV. Although the patients treated with PPV alone showed decrease in hemoglobin, lymphocyte counts, and albumin after vaccination possibly due to side effects of combined chemotherapies and/or malnutrition mediated by disease progression, those treated with PPV in combination with JTT maintained a stable level of these factors, as previously suggested [1, 12, 32]. Consistent with these findings, a significant change in performance status was observed between before and after (or during) vaccination in the PPV alone group but not in the PPV plus JTT group. These results suggest that JTT has the potential to prevent deterioration of patients' conditions without severe adverse events even in advanced cancer patients undergoing immunotherapy. Other clinical data, such as patients' quality of life (QOL), were unavailable in this study, but they might be worthy of assessment in future clinical trials.

It should also be noted that the elevation of the proinflammatory cytokine IL-6 was inhibited by combined usage of JTT. IL-6 is a multifunctional cytokine that regulates various aspects of immune responses, acute phase reactions, and hematopoiesis. In particular, IL-6 has been reported to be deeply involved in inflammation associated with cancer development and progression [33, 34]. Indeed, there have been many studies describing the correlation between IL-6 elevation and poor prognosis in various types of cancers, including pancreas cancer [35–38]. In addition, IL-6 has recently been reported to be one of the critical cytokines for inducing suppressive immune cell subsets, such as MDSCs and Th17, which are known to negatively affect anti-tumor immunity [39–41]. Therefore, the inhibitory effect of JTT on IL-6 elevation might be beneficial for controlling cancer progression.

5. Conclusion

In summary, we for the first time examined the immunological and clinical effects of JTT in cancer patients undergoing cancer vaccination in humans. Our randomized clinical trial of PPV with or without JTT suggested that combined usage of JTT revealed a potential to prevent deterioration of patients' conditions but had no effects in antigen-specific immunity in advanced pancreatic cancer patients. Since all of the enrolled patients had rapidly progressive advanced tumors, it might be possible that JTT supplementation for a limited, short period was not sufficient to elicit beneficial immune responses in the treated patients. A next step of randomized clinical trials of PPV with or without JTT would thus be recommended in

cancer patients in the adjuvant setting or in those with more slowly growing tumors.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Therapeutic Effects of Saireito (Chai-Ling-Tang), a Traditional Japanese Herbal Medicine, on Lymphedema Caused by Radiotherapy: A Case Series Study

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Despite the development of radiotherapy machines and technologies, a proportion of patients suffer from radiation-induced lymphedema. Saireito (SRT) is a traditional Japanese herbal medicine that has been used for treating edema and inflammation in conditions such as nephritic disease. This study investigated the effect of SRT on lymphedema caused by radiotherapy. Four patients were treated with SRT at a dose of 9 g/day. The severity of lymphedema was evaluated using the Common Terminology Criteria for Adverse Events version 4 and Numerical Rating Scale before and after SRT treatment. After the treatment with SRT, 2 of 4 patients (50%) showed apparent improvement in lymphedema. One of the cases had difficulty in wearing the custom-made thermoplastic cast, but after SRT administration, he could wear the mask easily. One case decided to stop taking SRT 3 days after initiation because cough and fever appeared. In conclusion, it is important to control the side effects of radiotherapy, which leads to improved tumor control rates. Prospective randomized studies are necessary to confirm the findings of this case series study.

1. Introduction

With the aging of the population, radiotherapy is taking an increasingly important role for preserving organ function. With the development of machines and technologies for radiotherapy, adverse effects have decreased. However, some patients still suffer from irreversible adverse events associated with radiotherapy. One of the most serious complications is lymphedema, which is a chronic, debilitating, refractory, or incurable condition. No consensus has been reached on the standard therapy for lymphedema. It is difficult to control lymphedema in Western medicine, and the long-term use of the applied medications has been limited due to documented hepatotoxicity [1–3]. The only effective types of primary care for lymphedema have been reported to be elastic clothing, multilayer lymphedema bandaging (MLLB), lymph drainage, skin care, and complex physical therapy. Complex physical therapy includes elastic clothing, MLLB, lymph drainage, skin care, and exercise [4–8]. However, the problem is that elastic clothing or MLLB cannot be used on the head and neck, so it is difficult to control lymphedema in these areas.

Kampo, Japanese traditional herbal medicine, is the most frequently used type of alternative and complementary medicine in Japan. The aim of Kampo therapy is to improve patients' condition whatever their disease is. Kampo medicine is playing an increasingly important role in closing the gap between modern Western medicine and the demands of patients. Kampo applications for cancer therapy were developed by trial and error because of dissatisfaction with Western medicine in dealing with problems such as the adverse effects of radiotherapy, chemotherapy, and various types of general malaise [9]. For example, Daikenchuto is

effective on postoperative adhesive small bowel obstruction requiring long-tube decompression [10] and Goshajinkigan has been used for peripheral neurotoxicity of oxaliplatin in patients with advanced or recurrent colorectal cancer [11]. The Japanese herbal medicine Saireito (SRT) has been used empirically in the treatment of various edematous disorders (nephritic syndrome, cirrhosis, pregnancy, swelling, and lymphedema after surgery and macular edema) [12, 13]. It is composed of 12 crude drugs in fixed proportions: 7.0 g of *Bupleurum root*, 5.0 g of *Pinellia tuber,* 5.0 g of *Alisma rhizome,* 3.0 g of *Scutellaria root,* 3.0 g of *ginseng,* 3.0 g of *Poria sclerotium,* 3.0 g of *Polypoms sclerotium,* 3.0 g of *Astractylodes lancea rhizome,* 3.0 g of *jujube,* 2.0 g of *Glycyrrhiza*, 2.0 g of *cinnamon bark,* and 1.0 g of *ginger.* Saikosaponin, which is derived from the medicinal plant *Bupleurum root,* exhibits a variety of pharmacological and immune-modulatory activities including anti-inflammatory responses [14]. In light of the purported activities of SRT for various edematous disorders in experimental models, we investigated whether SRT had beneficial effects in terms of the reduction of lymphedema in patients after or during radiotherapy.

2. Materials and Methods

2.1. Preliminary Study

2.1.1. Patients. Between December 2010 and January 2013, 625 patients underwent radiotherapy at Ishikawa Prefectural Central Hospital. We prescribed SRT to 5 patients who suffered from lymphedema among them. They had already undergone some treatment for lymphedema or had received no treatment because of its location in the head and neck. We excluded 1 patient whose lymphedema had been caused by an operation. Therefore, we evaluated 4 patients with lymphedema caused by radiotherapy.

2.1.2. Radiotherapy Planning. Patients were treated by radiotherapy using 6-MV X-rays from the Novalis Tx image-guided radiotherapy system (Varian, CA, USA, and Brainlab, Feldkirchen, Germany) or 4-MV or 10-MV X-rays from Clinac 21EX (Varian, CA, USA). Stereotactic radiotherapy and intensity-modulated radiotherapy (IMRT) were performed with Novalis Tx, and CLINAC 21EX was used for conventional radiotherapy. Patients were immobilized using a custom-made thermoplastic body cast (Hip-Fix, Med-Tec, Orange City, IA, USA), a Type-S head and shoulder thermoplastic mask (CIVCO, Iowa City, IA, USA), or a customized Vac-Lok (Med-Tec, Orange City, IA, USA) bag that conformed to both the patient's body contours and the treatment box. They were used for head and neck treatment using CLINAC 21EX and for treatment anywhere in the body using Novalis Tx.

2.1.3. Target Localization and Treatment Delivery. On the treatment day, the patients were again appropriately immobilized on the custom-made thermoplastic cast and Vac-Lok bag box. Initial setup was based on bone anatomy using two-dimensional orthogonal kV images registered to digitally reconstructed radiographs. In the case of IMRT, additional shifts for accurate setting up within the treatment field were performed using three-dimensional cone beam computed tomography (CBCT): 200° gantry rotation, 100 kV, 20 mA, and 20 mS for the head and neck; 200° gantry rotation, 125 kV, 80 mA, and 25 mS for the body. Treatment was then delivered, with the entire process of setting up and treatment taking approximately 15–30 minutes per patient.

2.1.4. Grading of Lymphedema. The severity of lymphedema was graded using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 and the Numerical Rating Scale (NRS). The NRS is a symptom-rating scale from 0 to 10. Zero indicates no symptoms, and 10 indicates the worst symptom that a patient can imagine. Clinical evaluation was performed before and after the administration of SRT.

2.1.5. SRT Application. SRT (Tsumura Co., Tokyo, Japan) at a dose of 3.0 g was administered orally as a solution three times daily immediately before meals during radiotherapy. None of the patients received diuretic drugs or steroid drugs during the study.

3. Results

3.1. Preliminary Results in 4 Patients. Table 1 shows the patient characteristics. The mean age of the 4 patients was 64 years (range 33–69). Three of them had a cancer (in the tongue, breast, and mesopharynx, resp.), and one had Kimura disease. Two patients were treated with radiotherapy using Novalis Tx, and the other two cases were treated using CLINAC 21EX. We delivered 2 or 3 Gy per fraction, and the total dose of radiotherapy was 30 to 66 Gy. Three patients had undergone an operation before radiotherapy. They had undergone their last operation 1–15 years ago. Three patients received chemotherapy before radiotherapy and 1 of them received concurrent chemotherapy. Two received SRT after 12 Gy. Two patients received SRT after they finished the total course of radiotherapy (63 or 66 Gy). The compliance was good. Prior to SRT treatment, all patients had grade 2 lymphedema according to the CTCAE, and all patients had symptoms scored as 4 to 10 according to the NRS. After 2 or 3 days of SRT treatment, lymphedema of 2 patients treated with SRT after they received 12 Gy of a score of 10 improved to 0, but lymphedema of the other 2 patients treated with SRT after they finished the entire radiotherapy of a score of 4 or 8 did not improve. Patient 3 decided to stop taking SRT after 3 days because cough and fever appeared. After radiotherapy, in 2 patients who took SRT from the time when they received 12 Gy, lymphedema of a score of 2 improved to a score of 1 according to the CTCAE, but the other two cases showed no change (Table 2).

3.2. Case Report. A 69-year-old man (patient 1) received radiotherapy using Novalis Tx for cervical lymph node metastases after an operation for tongue cancer. He presented with bleeding from the tongue and visited a local hospital in

Table 1: Patient characteristics.

Case	1	2	3	4
Age/sex	69/M	33/M	68/F	60/M
Performance status	2	0	0	0
Area for radiotherapy	Neck	Neck	Axilla	Neck
Primary lesion	Tongue cancer	Kimura disease	Breast cancer	Mesopharyngeal carcinoma
Total dose (Gy)	50	30	63	66
Machine for radiation therapy	Novalis Tx	CLINAC 21EX	Novalis Tx	CLINAC 21EX
Type of radiation therapy	IMRT	Conventional	IMRT	Conventional
Past operations	Yes	Yes	Yes	No
Chemotherapy	Yes	No	Yes	Yes
Past	Yes	No	Yes	Yes
Concurrent	No	No	No	Yes
Total dose at start of medication (Gy)	12	12	63	66

Table 2: Scores for lymphedema before and after medication.

Case	1	2	3	4
Total dose at start of medication (Gy)	12	12	63	66
NRS				
Before	10	10	8	4
After	0	0	8	4
CTCAE				
Before	2	2	2	2
After	1	1	2	2
Results	Improved	Improved	Not changed	Not changed

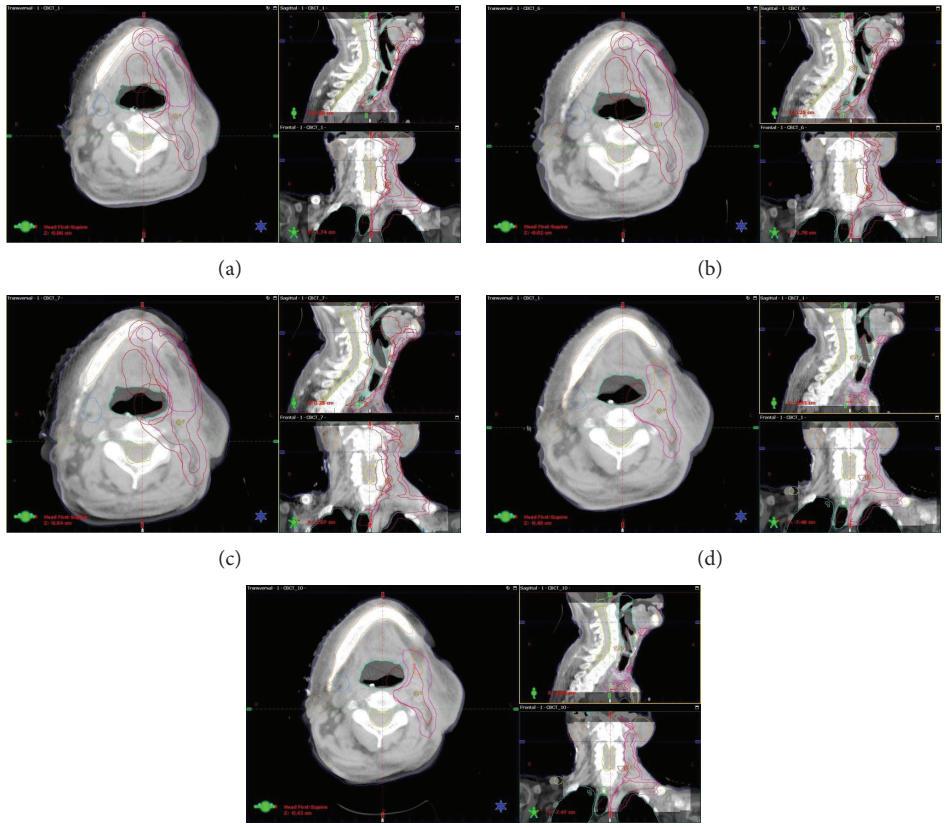
NRS: Numerical Rating Scale; CTCAE: Common Terminology Criteria for Adverse Events.

May 2012. He was referred to Ishikawa Prefectural Central Hospital and was diagnosed with tongue cancer with lymph metastasis (T2N1M0, stage III). He was initially treated by radiotherapy with arterial infusion chemotherapy for only tongue cancer. Next, he was treated by tongue cancer surgery with neck lymph node dissection. Pathologic examination revealed invasion to three regional lymph nodes with extracapsular extension. These findings indicated a high probability of local recurrence and distant metastases and a high risk of death. Therefore, an oral surgeon suggested radiotherapy of the neck region. Two weeks after the start of radiotherapy (12 Gy), lymphedema of the cheek progressed due to the influence of operation and radiotherapy, and the wearing of the custom-made thermoplastic cast became difficult. Therefore, 9.0 g/day of SRT was administered. After 3 days, he demonstrated a dramatic improvement in lymphedema of the cheek and we could easily fit the mask on him. Figure 1 shows CBCT demonstrating the improvement of lymphedema.

4. Discussion

Lymphedema is a troublesome complication of radiotherapy, especially for patients wearing a custom-made thermoplastic cast. Massive lymphedema leads to temporary discontinuation of radiotherapy because the custom-made thermoplastic cast and treatment planning (adapted plan) need to be made again to ensure accuracy of the treatment. Therefore, it is important to control lymphedema, especially for the patients treated with a custom-made thermoplastic cast. Complications of radiotherapy have decreased with the development of machines and technology for radiotherapy. One of these technological advances is IMRT. The intensity of radiation beams can be changed in IMRT during treatment to spare more adjacent normal tissues than during conventional radiotherapy [15]. Owing to this, an increased dose of radiation can be delivered to the tumor using IMRT. IMRT is a type of conformal radiation, in which radiation beams are shaped to ensure close approximation to the shape of the tumor. When it is applied, improvement in the tumor control rates and reduction of complications can be expected. Nevertheless, some patients suffer from irreversible adverse effects of radiotherapy like lymphedema, for which Western medicine is not useful. In this study, lymphedema in patient 1 progressed during radiotherapy and it became difficult to fit the custom-made thermoplastic cast. Kishida et al. [16] concluded that SRT is useful for the prevention and early recovery of postoperative leg edema after total hip arthroplasty with an association of rapid CRP reduction.

The mechanism of lymphedema due to radiotherapy has been postulated as follows [17]. The initial fractional doses of irradiation would destroy cells in the vegetative intermitotic cell and differentiating intermitotic cell compartments and reduce the production of cells that normally flow into the



(a) (b) (c) (d) (e)

Figure 1: CBCT images of patient 1 on the first day of radiotherapy (a), first day of SRT medication (before medication) (b), 3 days later (c), 14 days later (adaptive radiotherapy) (d), and 28 days later (last day of radiotherapy) (e). Lymphedema improved 3 days later, but it got worse on one occasion. It improved 28 days later.

postmitotic compartment. The lining or mucous membrane thins and, as the dose builds, the connective tissue becomes edematous; this causes expansion or stenosis of blood vessels. These disorders are typically recovered immediately. However, in the chronic clinical period, cells are sloughed away and fibrosis increases, and blood vessels and lymphatic duct become stenosed and obstructed. These microcirculation disorders cause repeated edema. SRT exhibits pharmacological effects via the following actions. (1) Diuretic actions: SRT shows diuretic actions due to an antagonistic action on the mineralocorticoid receptor, exerted by saikosaponin H, which is a principal component of SRT [13]. (2) Anti-inflammatory actions: there have been several reports about the relationship between SRT and inflammation [13, 18], which are discussed below. Aldosterone, whose function was reportedly suppressed by SRT, produced reactive oxygen species and activated nuclear factor kappa-B (NF-κB) [19, 20]. NF-κB activation leads to increased expression of chemokine and proinflammatory cytokine genes, including tumor necrosis factor-α, interleukin-6, inducible nitric oxide synthase, and vascular endothelial growth factor [21–24]. In addition, saikosaponin inhibited T-cell activation via the suppression of NF-κB [13]. Thus, both direct and indirect actions of suppression of NF-κB activity might have contributed to a change in the normalization of CRP early in the study. Oral administration of SRT to rats increased the plasma ACTH level and the expression of proopiomelanocortin (an ACTH precursor in the anterior pituitary lobe) mRNA [25]. Furthermore, these actions were inhibited by antiserum corticotropin-releasing factor [26].

SRT has not been investigated (drug use investigations, etc.) to determine the incidence of adverse reactions. There have been several reports of adverse reactions including interstitial pneumonitis, pseudoaldosteronism, myopathy, fulminant hepatitis, hepatic dysfunction, and jaundice [27]. In this study, one patient decided to stop taking SRT after 3 days because cough and fever appeared. Fever, cough, dyspnea, abnormal pulmonary sound (fine crackle), and ground-glass-like and reticular opacities predominantly in the bilateral lower lung fields on chest computed tomography were observed when interstitial pneumonitis occurred. The pathogenesis of drug-induced pneumonitis is not completely understood; intolerance to side effects, secondary effects, and idiosyncratic allergic reactions is thought to be the most important factor [28]. Pneumonitis generally develops 1-2 weeks after the start of administration, and bronchoalveolar lavage and histologic examination of lung biopsies reveal the features of eosinophilic pneumonitis [29, 30]. Allergic

pneumonitis is particularly mediated via types III and IV allergic reactions [31]. The patient who stopped taking SRT after 3 days visited our hospital 1 month later, so it was too late to examine the cause of her disease and it was too early for interstitial pneumonitis by SRT to have developed. The Japanese Ministry of Health and Welfare had approved 148 types of Kampo drug by 1999. Nakagawa et al. [32] conducted national surveillance of drug-related pneumonitis in Japan and reported that cases of Kampo drug-related pneumonitis accounted for as much as 10% (75 cases due to 13 kinds of Kampo drug) of all cases of drug-related pneumonitis in Japan from 1984 to 1996. Using their data, 84% of cases of Kampo drug-related pneumonitis were associated with drugs containing Ougon (*Scutellaria* root, dried root of *Scutellaria baicalensis* Georgi), that is, 49 cases for Shosaikoto, 9 for saibokuto, 4 for SRT, 1 for scihaito, 1 for daisaikoto, 1 for saikokeishito, and 1 for saikokeishikanshouto. Including these drugs, the Japanese Ministry of Health and Welfare has approved 29 kinds of Kampo drug that contain Ougon. Ougon is thought to be the most important cause of pneumonitis because of previously reported highly positive lymphocyte stimulation tests [33].

Tsumura & Co. investigated reports on Shosaikoto drug use from October 1995 to March 1997, including abnormal laboratory test results, and 88 adverse reactions were observed in 69 of 2,495 patients (2.8%). The incidence of interstitial pneumonitis was less than 0.1% [34]. Nakagawa et al. [32] showed that the rate of interstitial pneumonitis as an adverse reaction of SRT was lower than the rate for Shosaikoto. Therefore, it is rare for interstitial pneumonitis to develop as an adverse reaction of SRT.

5. Conclusions

It is important to control lymphedema caused by radiotherapy because it leads to improvement of the tumor control rate. SRT can easily be taken continuously for a long period with few side effects. SRT also has pharmacologic action in patients with lymphedema caused by radiation. In this study, we investigated the effectiveness of SRT in only 4 cases, so further research with much more patients and, preferably, prospective randomized studies are needed to confirm our findings.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Lessons Learnt from Evidence-Based Approach of Using Chinese Herbal Medicines in Liver Cancer

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This paper is a systematic review of evidence-based studies of the effectiveness of Chinese herbal medicine (CHM) in the treatment of liver cancer. After a detailed analysis of the literature, five animal studies and four human clinical trials met the criteria for inclusion. Analysis revealed that results of the clinical trials, whilst encouraging, need to be interpreted with caution as problems with study designs may lead to apparent benefits being attributable to various forms of bias. However, as each of the CHM agents used in these studies appeared to be potentially beneficial, further well-designed and controlled randomized clinical trials are warranted. The second part of this review focused on the lessons learned from the relationships between Traditional Chinese Medicine (TCM) theory, TCM Syndrome Differentiation, and modern scientific understanding of mechanisms of action of CHM agents. The understanding of TCM Syndrome Differentiation may allow identification of different patterns of disharmony and may provide important guidance to the prescription of CHM. Furthermore, quality control using both biological and chemical fingerprinting of CHM is important to ensure batch-to-batch consistency to deliver sustained therapeutic benefit. Also, careful assessment of herb-drug interactions is paramount for safety and integrative use of western chemotherapeutic and CHM agents.

1. Introduction

This review aimed to examine the evidence for using Chinese herbal medicine (CHM) in cancer treatment in terms of the benefits and potential mechanisms of action. As the same CHM formulae may be used for different cancer types with different effectiveness, in this review, liver cancer or hepatocellular carcinoma (HCC) has been used as the specific focus for discussion. Liver cancer is the second leading cause of cancer death in men and the sixth in women worldwide with an estimated 748,300 new liver cancer cases and 695,900 liver cancer deaths occurring in 2008 [1]. The highest liver cancer rates are found in East and South-East Asia and in Middle and Western Africa. In the United States between 1990 and 2007, the increase in death rates for liver cancer accounted for nearly 70% of the total increase in the cancer death rates in men and for almost 23% of the increase in women [2].

Liver cancer has a poor prognosis, and the 5-year survival rate reported between 1973 and 2007 remained below 12% in the United States [3]. Early stage liver cancer is currently difficult to diagnose due to the lack of a sensitive screening test. As a result, only 30% to 40% of patients with liver cancer are candidates for potentially curative treatments at the time of their diagnosis [4]. Treatment modalities such as surgical resection, liver transplantation, and local ablation are only considered for patients with preserved liver function. However, most newly diagnosed liver cancer patients are already at an advanced stage. For these intermediate or late stage liver cancer patients, the therapeutic options are limited to palliative approaches using transcatheter arterial chemoembolization (TACE) or chemotherapeutic agents [3]. However, many patients are either not suitable for TACE or suffer from poor outcomes with conventional systemic cytotoxic chemotherapy [5].

A meta-analysis summarized the results of 193 randomized controlled trials (RCTs) of a range of medical modalities for HCC treatment reported during 2005–2010 indexed by MEDLINE, CANCERLIT, Embase databases, and the Cochrane Library [6]. From the 32 studies that met their inclusion criteria, only 17 studies were eventually selected based on the strength of the trials. Of these, one study used chemoembolization, three used tamoxifen hormonal treatment, and a further three employed systemic chemotherapy treatment modalities such as doxorubicin and PIAF—cisplatin/interferon alpha 2b/doxorubicin/fluorouracil. The researchers summarized the effects of all these treatment modalities for HCC in the category of "No survival benefit."

New conventional medicine treatment approaches for HCC now rely on molecular targeted therapies such as the multikinase inhibitor Sorafenib. In a recent randomized multicenter placebo-controlled phase III trial, Sorafenib exhibited a benefit in advanced liver cancer patients by extending overall survival, but this was only by 2 to 3 months compared with the placebo [7]. Therefore in view of the poor clinical effectiveness of a broad range of medical modalities including the molecular targeted approach, new therapeutic agents with low cost and high effectiveness, such as herbal medicines, are urgently needed.

Natural products represent a rich reservoir of potential bioactive compounds exhibiting anticancer properties [8]. Several compounds have been found to have powerful antitumor effects, such as taxol, which was identified by screening approximately 114,000 plant extracts and obtained therapeutic approval from the Food and Drug Administration of the United States [9]. Although herbal medicines have been used in clinical applications for centuries, their present use lacks stringent supporting scientific evidence in terms of double-blind placebo-controlled clinical trials. In addition, since there are usually multiple compounds in a single herb, the actual active compounds and their sites of action and mechanisms of action are generally unknown. The situation is further complicated by the use of herbal medicines as a formula with multiple components [10–12], which represents a "polychemical" approach in contrast to the "single-chemical" approach of classical chemotherapeutics.

CHM has been practiced for centuries by local physicians caring for a huge population in China and in East Asia including Korea and Japan and has developed a comprehensive set of well-documented medical theories. CHM usually requires the use of multiple herbs, minerals, or even compounds derived from animal parts. In light of CHM theory, management of health can be characterized as holistic with the emphasis on regulating the integrity of the human body functions and the interaction between various organs and the internal environment. Likewise, liver cancer is a systemic disease associated with a local tumor. Therefore, CHM therapies not only focus on eliminating the local malignancy, but also aim to restore the homeostasis of the whole body.

This review focuses on the current understanding of CHM as a therapy for liver cancer and explores approaches for its future development as an evidence-based complementary and alternative medicine (CAM) for liver cancer management. Based on the premise that a successful CHM cancer treatment would require the clinical efficacy substantiated by clinical trials in humans, this review aims to first present a critical analysis of the clinical studies and the related preclinical animal *in vivo* studies, followed by experimental *in vitro* results which can help to delineate the underlying mechanisms of action of CHM treatment. This review highlights some important lessons from critical analysis of the CHM anti-HCC research, including inadequacies in the reported clinical trials, possible CHM candidates for future clinical trials, and quality control of CHM to ensure batch-to-batch consistency. These considerations may contribute to enhancing the development of an evidence-based cancer research platform using CHM.

2. Preclinical and Clinical Studies of CHM in Liver Cancer

2.1. Study Selection. An extensive search was performed in four electronic databases (PubMed, EMBASE (Excerpta Medica Database), China Biological Medicine database, and CNKI (Chinese Journal Full-Text Database for clinical trials and animal studies of CHM-based therapy) targeting liver cancers. The database search period covered 1980 to February 2012. The search terms were comprised of the following combinations: "Chinese herbal medicine," "Chinese herb," "herbal medicine," "CHM," "traditional Chinese medicine," "TCM," "liver cancer," "hepatocellular carcinoma," "cholangiocarcinoma," and "hepatic carcinoma." No other restrictions were imposed. In addition, the reference lists of recent reviews related to this topic were examined.

The flow chart demonstrating the selection process of the systematic analysis is shown in Figure 1. An initial screen of identified abstracts or titles was performed followed by a thorough reading of selected full-text articles. Studies were considered eligible if they met the following criteria: (1) the study design was a clinical trial or animal study; (2) the language of publication was English; (3) the main exposure of interest was CHM; (4) the outcome of interest was treatment effects for liver cancer. Using the previous criteria, 30 clinical trials in the area of integrative CHM with western medicine for treating liver cancer reported in Chinese were excluded from this review [13]. This review focused on the evaluation of four CHM formulas or extracts in human trials and five related preclinical animal experiments targeting liver cancer. We attempted to critically analyze the reports of use of these CHM extracts or formulas in clinical trials (216 liver cancer patients in total). A meta-analysis was not performed because of the small number of identified studies.

2.2. Preclinical Studies in Animal Models and Anticancer Mechanism of CHM. Effects of five CHM extracts of Bufalin, *Scutellaria barbate D. Don*, Kanglaite injection, PHY906, and Ganfujian or formulas on liver cancer have been reported in animal models (Table 1).

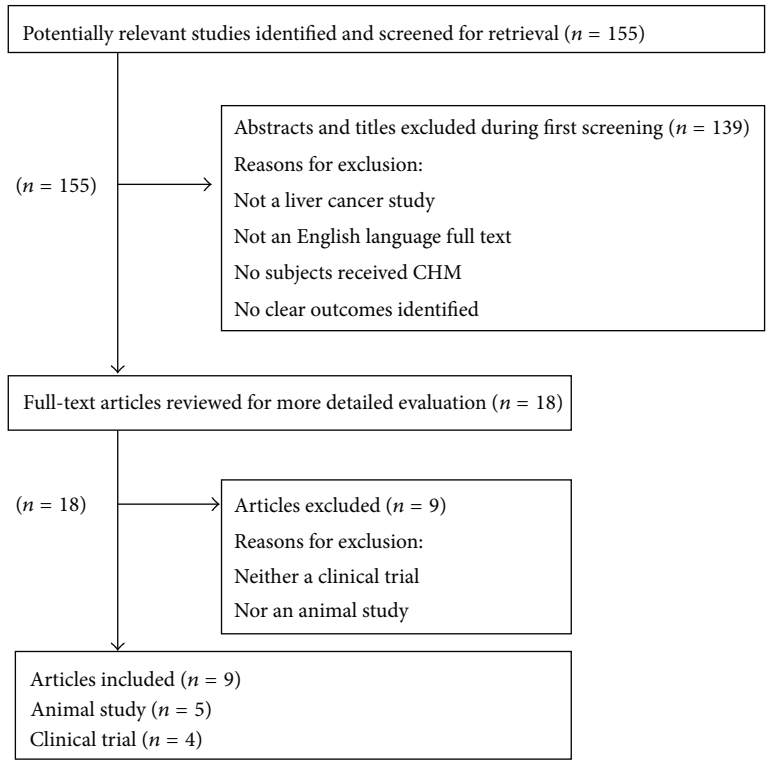

Figure 1: Process of study selection.

2.2.1. Bufalin. Bufalin, a cardiotonic steroid isolated from the skin and parotid venom glands of Bufo toad (*Bufo bufo gargarizans Cantor*), is a major active component of Huachansu [20]. The molecular formula of bufalin is $C_{24}H_{34}O_4$ with a relative molecular weight of 386.5 g/mol. To investigate the antitumor activity and apoptosis-regulating mechanism of bufalin in an animal model, human BEL-7402 tumors were implanted into the liver of 75 nude mice to establish orthotopic transplantation tumor models [14]. This simulation used a novel intrahepatic tunnel implantation to establish the *in situ* hepatoma. Compared to the murine tumor model, in which cancer cells are subcutaneously transplanted in immune-deficient animals, this *in situ* transplantation model is closer to the clinical situation of liver cancer. The results indicated that bufalin alone could inhibit growth, leading to tumors with a significantly smaller size ($35.21 \pm 12.51\ cm^3$) compared with those in the normal saline (NS) ($170.39 \pm 25.29\ cm^3$; $P < 0.01$) and Adriamycin (ADM) groups ($55.17 \pm 16.13\ cm^3$; $P < 0.05$). Bufalin also prolonged survival times of hepatoma-bearing mice compared with those in the NS group and ADM groups (31.8 ± 4.2 d versus 23.4 ± 2.1 d, $P < 0.05$, and 31.8 ± 4.2 d versus 22.2 ± 1.6 d, $P < 0.05$, resp.).

In addition to these effects, apoptosis was induced by bufalin which regulated the expression of an apoptosis-related gene, Bax, and the ratio of Bcl-2/Bax. Apoptotic characteristics such as cell shrinkage, cytoplasm concentration, and apoptotic corpuscles were also determined by ultrastructure observation. These results suggested that bufalin treatment can alleviate the tumor burden and prolong survival in the mouse model. Clinical trials should be inaugurated to test the efficacy of bufalin in liver cancer patients.

2.2.2. Kanglaite. Kanglaite is an acetone extract of Coix seed (*Semen Coicis*) that is widely used in clinical cancer therapy in China. CHM theory states that *Semen Coicis* increases the energy of the body and reduces or eliminates nodes, as well as benefiting digestive disorders [21].

Effects of Kanglaite injection were investigated using rat models of transplanted Walker-256 hepatoma [15]. Forty hepatoma-bearing Wistar rats were treated with intratumor injections of Kanglaite and compared with a control group receiving saline treatment. The hepatoma volumes of rats in the Kanglaite group were significantly smaller than those in NS group ($235.4\ mm^3$ versus $464.6\ mm^3$, $P < 0.05$). The tumor inhibitory rate in the Kanglaite group was 49.4%, and the serum levels of alanine aminotransferase, related to hepatic function, were significantly lower. Inhibition of proliferating cell nuclear antigen (PCNA) expression indicated that one of the antitumor mechanisms of Kanglaite could be inhibition of karyokinesis and propagation of cancer cells. The results indicated that intratumor injection of Kanglaite could effectively inhibit hepatoma in rats. Kanglaite was also reported to induce *in vitro* apoptosis of HepG2 cells through the activation of the Fas/FasL pathway [16]. Although further studies of the mechanisms are needed, these findings have contributed to the understanding of Kanglaite's anticancer activity.

Table 1: Preclinical studies of CHM in liver cancer.

Name	Composition	Biological action	Preclinical study
Bufalin [14]	*Secretia bufonis*	Anticancer	Inhibited growth of tumor in *in situ* transplantation tumor model of nude mice Prolonged survival time of hepatoma bearing mice Induced apoptosis through regulating expression of apoptosis-related gene *bax* and the ratio of bcl-2/bax *in situ* hepatoma transplantation model and apoptotic characteristics determined by ultrastructure observation
Kanglaite injection [15, 16]	Semen Coicis	Anticancer	Prohibited growth of transplant hepatoma in rats by intra-tumor injection Inhibited expression of PCNA of transplanted hepatoma in rats Reduced liver toxicity compared to ethanol therapy Induced apoptosis in cancer cell through activation of the Fas/FasL pathway
Banzhilian [17]	*Scutellaria barbate D. Don*	Anticancer, Immune modulation	Inhibited growth of tumors in hepatoma bearing mice Improved phagocytic function of macrophages determined by chicken-red cell phagocytic rate Inhibited proliferation of cancer cells *in vitro* Induced apoptosis determined by ultrastructure observation of apoptotic cancer cells
PHY906 [18]	*Scutellaria baicalensis Georgi, Glycyrrhiza uralensis Fisch, Paeonia lactiflora Pall, Ziziphus jujube Mill*	Anticancer	Inhibiting growth of tumors in mice bearing human HepG2 tumor and enhancement of chemotherapeutic efficacy when combined with anticancer agents such as CPT-11, capecitabine, doxorubicin, and thalidomide
Ganfujian granule [19]	*Rhizoma Dioscoreae, Fructus Crataegi, Fructus Ziziphi Jujubae, etc.*	Anticancer	Reduced and delayed the incidence of hepatocellular carcinoma in rats Prolonged survival time of tumor bearing rats Affected process of cancer cell cycle through suppressing overexpression of related modulators

2.2.3. Scutellaria Barbate D. Don. *Scutellaria barbate D. Don*, a perennial herb growing throughout southern China, is known as *Banzhilian* in CHM. According to CHM theory, it can be used to eliminate toxicity, promote blood circulation, and reduce tumor nodes. It has been widely used as a therapy for cancers of the liver, lung, stomach, and breast, as well as colorectal cancer [22]. In an *in vivo* study of hepatoma investigating the antitumor effect and the mechanisms of a crude extract of *Scutellaria barbate* (SB) [17], 60 ICR H22 hepatoma-bearing mice were treated with SB, fluorouracil (5-FU), and NS. SB significantly inhibited tumor growth compared to use of NS (1.67 ± 0.76 g, 2.65 ± 1.12 g, resp.; $P < 0.05$). In addition, SB significantly improved the phagocytotic function of macrophages, which was analyzed by comparing the chicken-red cell phagocytic rate, with both 5-FU and NS groups ($P < 0.05$). The phagocytotic function of macrophages can reasonably reflect the antitumor immune function. The study demonstrated the antitumor activity of SB in H22-bearing mice and suggested that a potential mechanism was the improvement of immune function. This study also demonstrated that SB was able to inhibit the proliferation of H22 cells *in vitro* in a dose- and time-dependent manner by use of MTT assays. Observation of ultrastructure revealed apoptosis of H22 cells induced by SB. Further detailed investigation of the mechanisms involved in the observed antitumor effects of SB is warranted.

2.2.4. PHY906. PHY906 is derived from the formulation known as Huang Qin Tang, which was first described in CHM documents dating approximately 1,800 years ago and is used for the treatment of various gastrointestinal symptoms, including diarrhea, nausea, and vomiting [23]. PHY906 consists of four herbs: *Scutellaria baicalensis Georgi*, *Glycyrrhiza uralensis Fisch*, *Paeonia lactiflora Pall*, and *Ziziphus jujube Mill* in the ratio of 1.5 : 1.0 : 1.0 : 1.0.

Researchers from Yale investigated if use of PHY906 could reduce the nonhaematological side effects of chemotherapy in particular GI problems. Surprisingly, in the preclinical mouse cancer model, NCr-nude mice bearing human HepG2 tumor and treated with herb-drug combinations including PHY906/CPT-11, PHY906/Capecitabine, PHY906/Doxorubicin, and PHY906/Thalidomide showed that the integrated treatment not only greatly alleviated side effects of the chemotherapy, but that PHY906 could actually potentiate tumor inhibition in a significant way. However, the nude mice used in the model have limited host cellular immunity and reduced humoral immunity, and so the model

Table 2: Clinical studies of CHM in liver cancer.

Name	Composition	Biological action	Clinical study
Huachansu injection [25]	*Bufo bufo gargarizansCantor*	Anticancer Anti-HBV	11 patients with stage III or IV 6 had response of prolonged SD SD time is 5.5 to 11.1 months 1 had a 20% regression of tumor Patents with SD had improved quality of life No dose-limiting toxicities found No drug-related toxicity greater than grade II
Kanglaite capsule [26]	*Semen Coicis*	Anticancer Immune modulation	65 patients with stage II or III Tumor RR for combination and TACE alone were 40% and 25% AFP RR for combination and TACE alone were 73.1% and 0.7% TTP for combination and TACE alone were 7.0 and 5.5 months Improved immune function evaluated by $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ Improved QoL Reduced TACE-induced adverse reaction of liver damage
PHY906 [27, 28]	*Scutellaria baicalensis Georgi, Glycyrrhiza uralensis Fisch, Paeonia lactiflora Pall, Ziziphus jujube Mill*	Anticancer, Anti-inflammatory Immune modulation	18 advanced liver cancer patients were enrolled in phase I study to determine a safe and tolerable dose 39 advanced liver cancer patients were enrolled in phase II study to observe tumor response, OS, TTP, and QoL No patient experienced drug-related grade 4 or 5 toxicities Disease control rate was 65.2% : 8.7% MR and 56.5% SD OS was 9.2 months TTP was 3.4 months The 12-month survival rate was 44.5% OS for HBV/HCV and non-HBV/HCV subgroups were 13.8 and 7.6 months OS for Child-Pugh A and Child-Pugh B patients were 10.9 and 6.5 months OS for Asian and non-Asian subgroups were 16.5 and 6.2 months Patients' QoL did not deteriorate significantly and changes in score did not exceed 25%
Jinlong capsule [29]	*Gekko japonicas Dumeril et Bibron, Bungarus Parvus, Agkistrodon, etc.*	Anticancer	98 liver cancer patients RR (CR + PR) was 60.38% when combined with TACE, while TACE alone was 40.00% KPS scores were higher than TACE alone No adverse reaction Jinlong capsule found Level of serum OPN was lower than that of TACE alone

TACE: transcatheter arterial chemoembolization; OS: overall survival; TTP: time to disease progression; QoL: quality of life; SD: stable disease; CR: complete response; PR: partial response; RR = CR + PR: total response rate.

does not completely reflect human cancer patients who have intact but progressively defective immunity [24].

Encouraged by the *in vivo* findings, attempts were made to determine if the mouse model findings could be replicated in human clinical trials. Four clinical trials using different combinations of PHY906 and chemotherapeutic agents have been reported [18] showing promising results and are described in Section 2.3 and Table 2.

Other preclinical animal studies have shown that the use of PHY906 is not restricted to targeting HCC but is also active in other tumor models such as colorectal cancer and pancreatic cancer [27, 30]. In summary, although PHY906 used alone had no significant effect on tumor weight loss, it can work as an effective broad-spectrum adjuvant to enhance the chemotherapeutic efficacy of a variety of anticancer agents. In addition, other animal experiments of colorectal cancer indicated that PHY906 could reduce chemotherapy-induced toxicities by exhibiting anti-inflammatory effects and promoting the regrowth of intestinal progenitor/stem cells [23, 30] while not affecting the metabolism and antitumor activity of commonly used chemotherapeutic drugs.

2.2.5. Ganfujian. Ganfujian is another CHM formula commonly used as a therapy for liver diseases. The main components are three herbs *Rhizoma Dioscoreae*, *Fructus Crataegi*, and *Fructus Ziziphi Jujubae*. These herbs are not toxic and may be used as dietotherapy for prolonged periods [31]. A preclinical study to investigate the inhibitory effect of Ganfujian granule on diethylnitrosamine- (DEN-) induced hepatoma in SD rats involved the use of 165 rats with free access to water containing 0.1 g/L DEN for 16 weeks, which were assigned into two groups to receive either normal diet or Ganfujian, respectively [19]. Thirty rats from each group were sacrificed at week 20 to observe incidence rate of liver cancer. Observation of the remaining animals continued to

determine survival until week 28. At week 20, all 30 rats in the normal diet group had developed liver cancer, in comparison with 24 of 30 rats in the Ganfujian group ($P < 0.05$). The longest survival time of rats was 28 weeks in the Ganfujian group and 20 weeks in the control group ($P < 0.05$). The results suggested that Ganfujian could reduce and delay the incidence of hepatoma in rats and prolong survival time of hepatoma-bearing rats. The researchers found that Ganfujian could affect the cancer cell cycle by suppressing overexpression of related modulators such as cyclin D1 and CDK4, which is a potential antitumor mechanism [19]. Ganfujian granule is a promising liver cancer chemical preventive agent, with potential for clinical application.

2.3. Clinical Trials. The selected articles included clinical trials investigating 4 CHM extracts or formulas and involving a total of 216 liver cancer patients. The CHM medications tested were Huachansu injection, Kanglaite capsule, PHY906, and Jinlong capsule (Table 2).

2.3.1. Huachansu. Huachansu (Cinobufacini), which is a water extract of Bufo toad skin, is used in CHM to treat conditions including swelling, pain, and heart failure [32]. Huachansu is commercially prepared for injection and is widely used at oncology clinics in China [33]. It is reported to have good effects in eliminating toxicity, as well as relieving swelling and pain. An *in vitro* pharmaceutical study of Huachansu conducted in China identified bufalin as a major anticancer component of Huachansu [34].

In a clinical trial of 11 HCC patients with stage III or IV disease who received Huachansu as a single agent, 6 patients were found to have stable disease with a response duration of 5.5 months to 11.1 months [25]. One patient whose response lasted for 11.1 months had a 20% reduction in tumor mass. Patients with stable disease had improved quality of life as assessed by using the M.D. Anderson Symptom Inventory (MDASI) scores. There were no drug-related toxicity greater than grade II and no dose-limiting toxicities reported. There was a dose-dependent increase in bufalin levels, with a maximum level of bufalin reached two hours after infusion. Further clinical studies of Huachansu with larger sample sizes and including appropriate control arms are needed. An NIH-funded project on pancreatic cancer using Huachansu has recently been completed and the report will soon be available [35]. This may shed light on the mechanism of action of clinical efficacy of Huachansu on solid tumors.

2.3.2. Kanglaite. Animal experiments with Kanglaite, an acetone extract of CHM *Semen Coicis,* were described in Section 2.2 and Table 1. In a randomized controlled trial in China, 65 unresectable stage II or III HCC patients were enrolled to receive either Kanglaite with TACE (30 patients) or TACE alone (32 patients) [26]. The method of randomization was not disclosed, and it was unclear whether the trial had used a blinded method to assess the outcome. It was reported that three subjects withdrew because of financial problems. The response rates of tumors in the combination group and the TACE group were 40% and 25%, respectively ($P > 0.05$). Serum alpha fetoprotein (AFP) levels in the combination group and the TACE group were 73.1% and 60.7% lower following therapy ($P > 0.05$). Kanglaite combined with TACE had a higher median time to progression (TTP) (7.0 months) than TACE alone (5.5 months) ($P < 0.05$). Compared with TACE alone, Kanglaite plus TACE improved immune function significantly by increasing the indexes of $CD3^+$, $CD4^+$, and $CD4^+/CD8^+$ ($P < 0.05$). The TACE-induced adverse reaction of liver damage was less serious in the combination group than that in the TACE group. Further trials are needed with improved methodological quality so that definite conclusions on the clinical benefits of using Kanglaite in HCC can be assessed.

2.3.3. PHY906. To ensure reproducible clinical efficacy, the researchers first attempted to determine the batch-to-batch consistency among different batches of PHY906 through strict quality control Good Manufacturing Practices (GMP) production measures [27]. They introduced a comprehensive technology platform termed "PhytomicsQC," which is a multifaceted approach integrating chemical and biological fingerprinting. Together with a novel statistical analysis, the researchers showed that PhytomicsQC was useful to evaluate different batches of PHY906 and provide a robust platform to determine the batch-to-batch consistency of the four-herb CHM formula PHY906 effectively [18]. The researchers also suggested that *in vivo* animal testing can be viewed as the ultimate quality control platform, if there is a discrepancy in the chemical and biological fingerprinting results.

The clinical trials of PHY906 not only demonstrated reduced chemotherapy-induced gastrointestinal toxicity but also reported an overall stronger protective effect of global toxicity [30]. Two open-labeled clinical studies of PHY906 with capecitabine were conducted in patients with unresectable HCC [28]. The first study was a phase I/II study with 93% of patients being enrolled in the US, whereas the second study was a phase II study in Taiwan. The phase I/II trial was a multicenter, open-label, dose-escalation, safety and efficacy study of PHY906 plus capecitabine. Only 3 patients from the Taiwan site were enrolled in the phase II study, and these Taiwanese patients were excluded from the data analysis as the enrollment criteria were slightly different from those for US subjects. Of the 18 US patients enrolled in a phase I trial to determine a safe and tolerable dosing regimen, it was found that the combination of PHY906 800 mg bid and capecitabine 750 mg/m^2 bid was well tolerated.

Subsequently, 39 patients using the recommended dose were enrolled in a phase II trial to determine whether PHY906 enhances the response rate of capecitabine, overall survival time (OS), time to disease progression (TTP), and quality of life (QoL) of patients. One of the major limitations of this trial is that there was no control arm for comparison. The disease control rate was 65.2% with 8.7% having a moderate response (MR) and 56.5% exhibiting stable disease (SD). The median TTP was 3.4 months and median OS was 9.2 months. The 12-month survival rate was 44.5%. No patients experienced drug-related grade 4 or 5 toxicities. Patients'

QoL evaluated by functional assessment of cancer therapy-hepatobiliary (FACT-Hep) did not deteriorate significantly and changes in score did not exceed 25%. In subgroup analysis, the median OS of patients with hepatitis B or C was 13.8 months and nonhepatitis patients exhibited a median OS of 7.6 months. Median OS values for Child-Pugh A and Child-Pugh B patients were 10.9 and 6.5 months, respectively. The researchers stated that no formal statistical analysis was performed in this study.

The same study reported that, surprisingly, Asian patients had a higher median OS (16.5 months) than non-Asian patients (6.2 months) ($P = 0.03$). This significant survival benefit in Asian patients may be associated with their genetic phenotype of PHY906-sensitive, but further investigation with a larger patient sample is needed. The previously mentioned PHY906 clinical trials had very good quality control with consistently prepared 4-herb products, but some lacked appropriate control arms and involved relatively small numbers of patients. Therefore, future phases II and III double blind, randomized, placebo-controlled studies with sufficient patient populations are required to determine the efficacy of PHY906 in liver cancer therapy.

2.3.4. Jinlong. Jinlong capsules have been commonly used as a CHM therapy for liver cancer. The major constituents are three CHM products *Gekko japonicas Dumeril et Bibron*, *Bungarus Parvus*, and *Agkistrodon*, which are all derived from reptiles. CHM theories maintain that some animal-derived medicines, especially from reptiles, can produce stronger and more deeply penetrating effects compared with those derived from plants [36]. Such CHMs are used to reduce or eliminate toxicity, activate meridians, and reduce or eliminate tumor nodes.

A randomized controlled trial (RCT) conducted in China enrolled 98 patients with liver cancer [29], of whom 53 received TACE plus Jinlong capsules and the other 45 received TACE alone. Randomization was achieved by using a table. The total response rate including both complete responses and partial responses of Jinlong in combination with TACE was 60.38% and RR of TACE alone was 40.00% ($P > 0.05$). The QoL of all patients before and after treatment was evaluated using Karnofsky (KPS). The KPS score of the combination group was higher than the TACE group after treatment (84.35 ± 12.19 versus 69.86 ± 11.58; $P < 0.05$). After treatment, the level of serum osteopontin (OPN) which relates with tumorigenesis, invasion, and metastasis was lower in the combination group than in the TACE group ($117.69 \pm 78.50\ \mu$g/L, $151.09 \pm 83.90\ \mu$g/L, resp.; $P < 0.01$). The researchers did not report any adverse reactions. So, this trial suggests that short-term clinical efficacy and QoL in liver cancer can be improved by Jinlong capsules combined with TACE. Primary long-term outcomes such as overall survival time should be observed to provide further evidence of including Jinlong for liver cancer therapy.

3. Lessons Learned

Evidence-based clinical efficacy is the most important key criterion to be evaluated to establish whether CHM can benefit liver cancer management. Ideally the Chinese herbs chosen should comply with Chinese medicine theory in a holistic way (Section 3.1). To ensure consistent desirable clinical effects as well as safety and batch-to-batch consistency, quality control of CHM preparations is crucial (Section 3.2). As most regimens require use of CHM together with other chemotherapeutic agents, herb-drug interaction is another key factor in evidence-based CHM cancer management (Section 3.3). This section also acknowledges the multitargeted, multidimensional features of CHM by highlighting the related mechanisms of action and some possible active ingredients (Section 3.4). Other factors such as the ethical and regulatory issues of CHM products and the safe usage of health products have been addressed by other reviews [37] and will not be further discussed here.

3.1. Nature of the Chosen CHM. Why did the researchers choose the herbs described earlier for preclinical and clinical studies? Generally, the reason for choice of herbs originates from CHM theory. The herbs can be classified into several groups in terms of CHM thinking. *Semen Coicis*, *Rhizoma Dioscoreae*, *Glycyrrhiza uralensis Fisch, Paeonia lactiflora Pall*, and *Ziziphus jujube Mill* belong to herbs thought to strengthen healthy Qi. In contrast, *Scutellaria baicalensis Georgi*, *Bufo bufo gargarizans* Cantor, and *Scutellaria barbate D. Don* are herbs able to clear heat and remove toxins, while *Gekko japonicas Dumeril et Bibron*, *Bungarus Parvus*, and *Agkistrodon* reduce phlegm and soften hard solid mass. In the studies reviewed, herbs for strengthening healthy Qi appear to modulate immunity or affect the process of cancer cell cycle, while the heat-reducing and detoxifying herbs modulated immunity or induced cancer cell apoptosis. The anticancer mechanisms of herbs of reducing phlegm and softening hard solid mass were not reported in the animal studies. Choice of herbs used should also be in accordance with TCM Syndrome Differentiation, a theory that involves the categorization of patients into different patterns of unbalanced homeostasis, which is a vital element of TCM practice [38].

3.2. Quality Control of CHM to Ensure Batch-to-Batch Consistency of Clinical Efficacy. Quality control is crucial for ensuring the safety and efficacy of CHM [39]. Consistent CHM batch preparation is essential for the reliability of clinical and preclinical studies. However, many studies including some described in this review fail to mention the quality control aspects of the CHM herb of interest or the combined complex multiple-herb CHM formula. This is understandable as CHM formulas may contain hundreds of different components. In addition, the majority of active ingredients related to the effective use of CHM in disease treatment are currently unknown. Thus, to control the production of such complex matrices of diverse compounds in single herb or herbal formulae with predictable clinical efficacy is extremely difficult or even not possible.

At present, identification of the major components in the CHM preparations is by means of an array of fingerprint technologies accredited by the World Health Organization

as evaluation tools [40]. These fingerprinting techniques comprise high-performance liquid chromatography, capillary electrophoresis, gas chromatography, X-ray diffraction, and DNA fingerprinting [41]. However, it should be noted that major "peaks" or "features" may not have any relationship with the bioactivities or the desirable clinical effects of that particular herb or formula.

The studies of PHY906 highlight the use of "PhytomicsQC" that integrates both chemical and biological fingerprinting to evaluate different batches of PHY906 in order to effectively provide a robust quality control platform of batch-to-batch consistency of the four-herb CHM formula [21]. Another approach advocated by Chau and his team was the use of "Quantitative-Pattern-Activity-Relationship (QPAR) [42, 43]. Using whole herbal medicines chromatographic/fingerprint profiles and their corresponding total biological activities as input, by means of sophisticated chemometrics computation, the QPAR approach can be used to explore and exploit the relationship between the CHM whole fingerprint profiles and their biological activities. Firstly, QPAR can reveal the important multiple features in the chromatographic profiles responsible for the biological activities, secondly, build a model for activities prediction simply using the chemical fingerprinting profiles, and thirdly, discover the active ingredients of the HM by identifying the multiple genuine "active" regions on the chromatogram, which are not necessarily the major components. In summary, future clinical trials using CHM should consider use of either "QPAR" or "PhytomicsQC" or similar platforms for the quality control of the batch to batch consistency to ensure reproducible clinical efficacy.

Other than quality control issues mentioned earlier, a few other important areas also require stringent control and management in order to obtain excellent batch-to-batch consistency. These areas comprise good manufacturing process conditions for high standard production and good agricultural practice to safeguard standardized plant cultivation.

3.3. Herb-Chemotherapeutics Interactions. Since CHMs have commonly been used in an integrative way with standard chemotherapeutics in cancer management protocols, it is pertinent to investigate if any herb-drug interaction is occurring. From both the physicians' and patients' points of view, it is important to understand if the CHMs affect the pharmacokinetics of the chemotherapeutic agent by reducing the level of chemotherapeutics in the patients' blood leading to failure to provide enough dosage inside the cancer cells. Conversely, herb-drug interaction may also potentially relate to the drug safety, if the herb unexpectedly enhances absorption of the chemotherapeutic agent. For instance, in a clinical trial of lung cancer, an astragalus-based herbal formula, Jinfukang, was shown to alter the pharmacokinetics of docetaxel, with most patients experiencing increase in docetaxel levels by at least 33%, although no clear trend was evident [44].

Currently, there are few reports describing this key aspect of herb-drug interaction. One study has addressed the effects of PHY906 on the pharmacokinetics of CTP-11, capecitabine, gemcitabine, and sorafenib in colorectal cancer, HCC, and pancreatic cancer, respectively, in animal models [21]. Importantly, results indicated that PHY906 did not affect the metabolism of these chemotherapeutic agents or their corresponding metabolites. However, it has to be noted that these findings were based on animal studies only, and it is therefore important that a similar approach should be adopted for human clinical trials. There is a clear need for more systematic coordinated herb-chemotherapeutic interaction studies to provide an in-depth knowledge base about use of combination therapy.

3.4. Mechanistic Evaluation of CHMs Leading to Personalized Integrative Medicine. Traditional Chinese Medicine theory proposes CHM formulae may provide holistic, multitargeted, multidimensional pharmacological therapies leading to effective cancer management. Accordingly, the mechanisms of action of CHM for liver cancer are also likely to be multifactorial. As shown in Tables 1 and 2, the potential mechanisms of action include anti-inflammation, antiangiogenesis, antivirus, apoptosis-induction, cell cycle arrest, modification of tumor microenvironment, and immune-modulation.

To date, relatively few studies have examined the mechanistic effects of CHM on the immune response, and the specific target proteins or the underlying related signal transduction pathways affected by CHM have also not been addressed. This may be due to the current poor understanding of the actual targets of the multiple active compounds of the CHM responsible for the clinical effects. A coordinated network of laboratories collaborating to determine these effects is urgently needed.

Methodologies to approach these multitargeted, multidimensional pharmacological activities of CHM have been proposed by several research groups [45–47]. These platforms make use of recent advances in "Omics" bioinformatics, pharmacogenomics, and systems biology, which have all been found to be useful for the analysis of diverse complex data [48].

Furthermore, as CHM targets the underlying disturbed homeostasis, studies concerning the mechanisms related to the characteristics of TCM Syndrome Differentiation that reflect the inner health status of the body should be conducted [49]. Recent reports discovering "phenotypes" based on the systems biology approach may be the beginning of a new frontier of scientific research into this important aspect of TCM Syndrome Differentiation [50, 51]. A sophisticated and fuller understanding of Syndrome Differentiation may lead to TCM-based personalized treatment strategies for HCC CHM or integrative treatment, or it may provide the rationale for patient stratification of groupings with similar homeostasis imbalances for interpretation of clinical trials.

4. Discussion

This review focused on some CHM therapies reported to have significant effects for liver cancer. These therapies have not yet gained wide acceptance as part of integrative liver cancer management. The explanation for this is multifaceted.

First, many publications of the use of CHMs in liver cancer management are written in Chinese; of the 139 articles excluded from this review, 85 were rejected due to this language criterion. These papers could potentially include useful information; however, they may suffer from the severe inadequacy of lack of independent assessment for veracity and reproducibility by western scientists [52].

Second, this review highlights preclinical studies using various laboratory experimental platforms and animal cancer studies, which are important for providing evidence of the efficacy and mode of action of CHMs in liver cancer clinical trials. This approach illustrates the potential of reverse-translational research. Laboratory experimental platforms are used to study the mechanisms of action and the potential "active ingredients" in the CHMs in relation to the effective clinical trials results. Such knowledge can lead to modifications of treatment and assist in the quality control of the CHM using defined amounts of those "active ingredients." This knowledge can then be applied to the patients, leading to improvement and consistency of both clinical efficacy and safety.

Third, although some of the clinical studies included in this review suffered from problems of study design or other quality issues, they nevertheless provide credible evidence suggesting effectiveness of some selected CHMs. Further trials ensuring high quality control of production of CHM agents to reduce the batch-to-batch variations and rigorously designed randomized, controlled, multicentre trials are required.

In this review, most clinical studies examined the effectiveness of CHM therapies for liver cancer. In TCM, the medicines are prescribed according to syndrome. Syndrome differentiation remains the essence of CHM treatment and is the key to evaluating a patient's disease state and developing an efficient, individualized treatment strategy. Therefore, syndrome differentiation should be applied to the treatment of liver cancer by CHM, possibly using a personalized treatment plan or a set of plans for a few defined syndrome profiles. We believe that the application of TCM syndrome differentiation to disease diagnosis can yield improved therapeutic efficacy. We also envision that TCM syndrome will be able to be assessed by modern technologies in the future. The diagnosis and efficacy of CHM therapies for liver cancer can then be evaluated on a molecular and Systems biology basis.

5. Conclusions

In this study, we systematically reviewed some CHMs for the treatment of liver cancer. We first summarized the CHM extracts or formulas in preclinical studies and clinical trials and evaluated these studies with focus on the mechanisms of action and TCM theory. Analysis of these clinical trials revealed the need for cautious interpretation of results as apparent benefits may actually attributable to various forms of bias inherent in the study designs. Overall these CHM agents appeared to be potentially beneficial, and further well-designed and controlled randomized clinical trials should be performed to enhance the creditability of CHM treatment. This critical analysis of the CHM anti-HCC research covered methodology in clinical trials, TCM Syndrome Differentiation and prescription, quality control for therapeutic consistency, assessment of herb-drug interactions, and the outlined shortcomings. The learned lessons based on the suggested improvements may contribute to the enhancement of developing a comprehensive evidence-based liver cancer CHM research platform.

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Research Article

Tetrandrine Suppresses Cancer Angiogenesis and Metastasis in 4T1 Tumor Bearing Mice

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Metastasis remains the most deadly aspect of cancer and still evades direct treatment. Thus, there is a great need to develop new treatment regimens to suppress tumor cells that have escaped surgical removal or that may have already disseminated. We have found that tetrandrine (TET) exhibits anticolon cancer activity. Here, we investigate the inhibition effect of TET to breast cancer metastasis, angiogenesis and its molecular basis underlying TET's anticancer activity. We compare TET with chemotherapy drug doxorubicin in 4T1 tumor bearing BALB/c mice model and find that TET exhibits an anticancer metastatic and antiangiogenic activities better than those of doxorubicin. The lung metastatic sites were decreased by TET, which is confirmed by bioluminescence imaging *in vivo*. On the other hand, laser doppler perfusion imaging (LDI) was used for measuring the blood flow of tumor in 4T1-tumor bearing mice. As a result, the local blood perfusion of tumor was markedly decreased by TET after 3 weeks. Mechanistically, TET treatment leads to a decrease in p-ERK level and an increase in NF-κB levels in HUVECs. TET also regulated metastatic and angiogenic related proteins, including vascular endothelial growth factor, hypoxia-inducible factor-1α, integrin β5, endothelial cell specific molecule-1, and intercellular adhesion molecule-1 *in vivo*.

1. Introduction

Tetrandrine (TET), a bisbenzylisoquinoline alkaloid isolated from the dried root of *Stephania tetrandra* (or *hang fang ji*) of the Menispermaceae, is a bioactive alkaloid with a molecular weight of 622.76 g/mol. Many reports indicated that TET exhibits very broad pharmacological actions, including immunomodulating, antihepatofibrogenetic, antiinflammatory, antiarrhythmic, antiportal hypertension, anticancer, and neuroprotective activities [1]. The beneficial effects of TET on tumor cell cytotoxicity and radiosensitization, multidrug resistance, normal tissue radioprotection, and angiogenesis are most promising and deserve great attention [2, 3]. Several investigations indicated that TET generally presents its anticancer effects in the micromolar concentrations on clone, leukemia, bladder, hepatoma, and lung cancer *in vitro* [4–8].

In our previous research, we have found that TET exhibits significant anticancer activity in colon cancer line HCT116. Mechanistically, the inhibitory effect of TET on colon cancer cells may be at least in part mediated by targeting β-catenin activity, and the sensitivity of cancer cells to TET may be determined by the functional status of β-catenin [9].

Nevertheless, with the development of clinical surgical treatment, the growth of primary tumor was no longer the critical element influencing the overall survival in cancer patients. Prevention of metastasis and more effective treatment of cancer metastasis are necessary for cancer therapy. Thus, antimetastasis therapy that targets tumor cells escaped

surgical removal or already disseminated, and tumor angiogenic process has a better chance of success. Although several rational lines of evidence support the application of TET as an anticancer metastatic agent [10], the cellular mechanisms underlying the antiangiogenic and antimetastatic effects of TET activation in tumor cells remain elusive.

In this study, we use a mouse model for stage IV breast cancer (4T1 tumor bearing BALB/c mice model) for evaluating the antimetastatic effect of TET in lower concentration (10 mg/kg/d, which could not change the growth of tumor mass). Meanwhile, laser Doppler imager (LDI) was used for measuring blood perfusion of the tumor bearing area, which could measure the local blood flow and vascular network and reflect the angiogenic activity of the tumor bearing mice indirectly. Our results show that TET could significantly inhibit endothelial cell (EC) proliferation, adhesion, migration, invasion, and tube formation by targeting angiogenic factors, namely vascular endothelial growth factor (VEGF) and hypoxia-Inducible factor-1α (HIF-1α), as well as adhesion factors, such as integrin β5, endothelial cell specific molecule-1 (ESM-1), and intercellular adhesion molecule-1 (ICAM-1), and by interfering with the ERK pathway, leading to the suppression of tumor metastasis and tumor angiogenesis.

2. Materials and Methods

2.1. Cell Culture and Animals. Mouse breast cancer 4T1 and HEK-293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in the DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 50 U penicillin/streptomycin. Human umbilical vein endothelial cell line (HUVEC) was immortalized as described in [11] and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% FBS, 2 mM L-glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37°C in a 5% CO_2 incubator.

Female BALB/c mice (4-5 weeks old) were purchased from Shanghai Lab. Animal Research Center and maintained at the animal facility of Experimental Animal Research Center of Zhejiang Chinese Medical University. All procedures were performed according to protocols following the guidelines for the Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996).

2.2. Chemicals and Drug Preparations. TET and doxorubicin (Dox) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These compounds were dissolved in DMSO to make stock solutions and were kept at −20°C as aliquots. The solution was diluted with Milli-Q water into 2 mg/mL and ultrasonificated into a fine suspension before *in vivo* use. 0.1% DMSO was used in vehicle group in *in vitro* assays.

2.3. Establishment of Stably Tagged 4T1-Luc Cell Lines. The 4T1 cells were stably transduced with firefly luciferase by using a retroviral vector expressing firefly luciferase as described in [12]. Briefly, recombinant retrovirus was packaged in HEK-293 cells by cotransfecting cells with pSEB-Luc and pAmpho packaging plasmid using LipofectAMINE (Invitrogen). Pooled stable cells were selected with blasticidin S (6 μg/mL) for 7 days. The firefly luciferase activity was confirmed by using Promega's Luciferase Assay kit (Promega, Madison, WI, USA).

2.4. MTT Proliferation Assay. A modified MTT assay was used to examine the cell proliferation as described in [13]. Briefly, iHUVEC (1×10^4 cells/well, 50–70% density) cells were seeded in 96-well plates. Drugs were added to the cells at variable concentrations or solvent control (0.1% DMSO). At 48 h after treatment, 15 μL MTT dye solution was added to each well and incubated for additional 4 h. Subsequently, the cell culture medium was removed, and 100 μL/well DMSO was added to dissolve formazan crystals in a humidified atmosphere overnight. Absorbance at 570 nm was measured using a 96-well microplate reader.

2.5. Crystal Violet Viability Assay. Crystal violet assay was conducted as described in [14]. Experimentally, iHUVEC cells were treated with drugs. At 24 h after treatment, cells were carefully washed with PBS and stained with 0.5% crystal violet formalin solution at room temperature for 20–30 min. The stained cells were washed with tape water and air-dried for taking macrographic images. For quantitative measurement, the stained cells were dissolved in 10% acetic acid (1 mL per well for 12-well plate) at room temperature for 20 min with shaking. Absorbance at 570–590 nm was measured.

2.6. Cell Adhesion Assay. For the cell adhesion model, HUVECs and 4T1 cells were used to study the adhesive ability between two different kinds of cell types. Briefly, HUVECs (2×10^4 each well) were grown to confluence on fibronectin-coated wells of 96-well plates. The plates were blocked with Hank's balanced salt solution (HBSS) containing 1% bovine serum albumin (BSA) (HBSS-BSA) for 30 min before the adhesion assay. BSA-coated wells serve as a negative control.

The 4T1 cells were trypsinized and suspended in HBSS-BSA and then labeled with 10 μL Hoechst 33258 for 30 min at 37°C followed by washing with HBSS-BSA. The labeled 4T1 cells were then suspended in HBSS-BSA to a final density of 4.0×10^5 cells/mL, and different dosages of TET were added. Cell suspension (100 μL/well) was incubated with HUVECs at 37°C for 30 min. Cultures were carefully washed three times with PBS to remove nonadherent cells. Three random views were photographed in each well at 100x magnification with an inverted fluorescence microscope (Olympus Corporation, Japan). The image was analyzed with Image-Pro Plus 6 software (Media Cybernetics, USA).

2.7. Cell Migration Assay. A wound-healing model was used for evaluation of cell migration ability [15]. Cells treated with 0.1% DMSO were used as the vehicle control. Three random views along the scraped line were photographed in each well at 100x magnification before and after 10 h drug treatment

with an inverted fluorescence microscope. The image was analyzed with Image-Pro Plus 6 software. Average scraped width of each well was measured and compared with control.

2.8. Boyden Chamber Transwell Cell Invasion Assay. Cell invasive ability was measured on a transwell system with a polycarbonate membrane (8 μm pores) as previously described in [15]. The upper and lower sides of the membrane were precoated with 1 : 30 (v/v) and 1 : 100 (v/v) matrigel, respectively. The iHUVECs (50,000 cells) were seeded into culture inserts. Low-serum medium containing different concentrations of TET was added into the plate wells. After 12 h, the inserts were washed with PBS; upper surface cells were removed by cotton swabs and the lower side was fixed in 3.7% paraformaldehyde. The invasive cells were then stained with propidium iodide (PI) and mounted on microscope slides. Images were captured at 200x magnification with an inverted fluorescence microscope. Invasive cells were quantified by Image-Pro Plus 6 software. The number of migrate cells per fields was determined by averaging nine randomly counted fields.

2.9. Tube Formation Assay. The effects of the drugs on HUVEC differentiation were examined by their *in vitro* tube formation ability on matrigel [15]. HUVECs were harvested and diluted to 2×10^5 cell/mL in low-serum medium (0.5% FBS) containing 20 ng/mL Vascular Endothelial Growth Factor (VEGF) and different concentrations of drugs. The cells were then seeded onto 1 : 1 matrigel (v/v) coated 24-well plates at 37°C for 8 h. Cells treated with 0.1% DMSO were used as the vehicle control. The branch points of the capillary-like tubes were counted under light microscopy (100x field).

2.10. Cell Cycle Analysis by Flow Cytometry. Flow cytometry was used for quantitatively detecting the cell-cycle distribution [16]. Cells (1×10^5/well) were plated into 6-well plates 1 day before treatment with TET at various concentrations. After treatment for 24 and 48 h, cells were harvested, washed with PBS, fixed in cold 3.7% paraformaldehyde overnight at 4°C for at least 2 h, and stained with 50 ng/mL PI in the presence of 200 μg/mL RNase A by incubation at 37°C for at least 30 min. The stained cells were analyzed by flow cytometry (Becton-Dickinson). The red fluorescence (PE) representing the DNA content was collected through a 585 nm filter. Data were analyzed using Mod Fit LT 3.0 software.

2.11. Protein Extraction and Western Blotting Analysis. Western blotting was performed as previously described in [17]. Briefly, cells were collected and lysed in RAPI buffer. After treatment on ice for 30 min, cell lysates were clarified by centrifugation at 11,419 ×g for 20 min at 4°C to remove cell debris and the protein content was measured using a BCA protein assay kit (Beyotime, Jiangsu, China). Cleared total cell lysate was denatured by boiling, and aliquots of the lysates were loaded onto a 10% gradient SDS-PAGE. After electrophoretic separation, proteins were transferred to an Immobilon-P membrane. Membrane was blocked with SuperBlock Blocking Buffer and probed with the primary antibody, anti-NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ERK1/2, and p-ERK1/2 (Cell Signaling Technology, Vancouver, Canada), followed by incubation with a secondary antibody conjugated with biotin. Then the PVDF membrane was incubated with streptavidin HRP. The proteins of interest were detected by using SuperSignal West Pico Chemiluminescent Substrate kit.

2.12. Gelatin Zymography. Gelatin zymography was performed on 7.5% polyacrylamide gels containing 0.1% gelatin as previously described in [15]. Cells were treated as indicated in 0.5% FBS RPMI1640 (containing 20 ng/mL VEGF) for 24 h. The cell culture medium was then centrifuged at 350 ×g for 4 min at 4°C, and the total protein of the supernatant was normalized with BCA protein assay kit. The supernatant was mixed with 5x nonreducing sample buffer and loaded onto 10-well gels (20 μL/sample), and electrophoresis was performed at 100 V for 1.25 h. After electrophoresis, the gel was rinsed with 1x renaturing buffer for 1.5 h at room temperature. The buffer was then changed to 1x developing buffer and incubated for 48 h at 37°C. Gelatin gel was stained with Coomassie blue and then destained with 10% acetic acid. The unstained bands correspond to the areas of gelatin digestion.

2.13. 4T1 Tumor Bearing Mice Model. Female BALB/c mice (4 weeks old, 18–20 g, 10 mice per group) were used. Subconfluent 4T1-Luc cells were harvested and resuspended in PBS to a final density of 1×10^7 cells/mL. Before injection, cells were resuspended in PBS and analyzed by 0.4% trypan blue exclusion assay (viable cells, >90%). For cancer cell injection, approximately 5×10^5 4T1-Luc cells in 100 μL of PBS were injected into the mammary fat pad (MFP) of each mouse using 27 gauge needles [18]. At 48 h after tumor cell injection, TET was administered at 10 mg/kg body weight to mice once every 2 days orally, and Dox was administered by intraperitoneal injection at 1 mg/kg/2 days to mice as a positive control.

2.14. Xenogen Bioluminescence Imaging. Small animal whole-body optical imaging was carried out as described previously in [9]. In brief, mice were anesthetized with isoflurane attached to a nose-cone mask equipped with Xenogen IVIS 200 imaging system (Caliper Life Sciences, Hopkinton, MA, USA) and subjected to imaging weekly after MFP injection.

For imaging, mice were injected intraperitoneally with D-luciferin sodium salt (Gold Biotechnology, St. Louis, MO, USA) at 100 mg/kg body weight in 0.1 mL of sterile PBS. Acquired images were obtained by superimposing the emitted light over the grayscale photographs of the animal. Quantitative analysis was done with Xenogen's Living Image V2.50.1 software as described previously in [14]. Animals were taken *in vivo* images for both untreated and treated groups and sacrificed after 4 weeks. Tumor, lung, and vascular samples were retrieved for histological examination.

2.15. High-Resolution Laser Doppler Perfusion Imaging. Microvascular blood flow was assessed by laser Doppler

with a moorFLPI V2.1 software (Moor instruments Ltd, UK) [19, 20]. Mouse hair was carefully removed and mice were anesthetized with isoflurane attached to a nose-cone mask. With a distance of 10 cm between the scanner and the skin surface, three examined areas (1.4 ∗ 1.4 cm) were chosen so that tumor (Flux 1), adjacent healthy skin around tumor (Flux 2), and the heart of mice (Flux 3) were covered. The laser beam is reflected by the erythrocytes, which allows recording of the returning signal by a detector positioned in the scanner head and thus conversion to an electrical signal, proportional to the tissue perfusion. The underlying intensity of perfusion values is expressed on a scale of different colours extending from blue (low perfusion values) over green and yellow to red (highest perfusion values). The related perfusion values were calculated as follows: perfusion rate (tumor) = $F1/F3 * 100\%$; perfusion rate (vascular) = $F2/F3 * 100\%$.

2.16. Histological Evaluation and Immunohistochemical Staining. Retrieved tumor tissues were fixed in 10% formalin and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin. For immunohistochemical staining, slides were deparaffinized and then rehydrated in a graduated fashion [21]. The deparaffinized slides were subjected to antigen retrieval and probed with anti-ICAM-1, anti-HIF-1α, anti-integrin β5, anti-ESM-1, or anti-VEGF antibody (Santa Cruz Biotechnology) or isotype IgG control, followed by incubation with biotin secondary antibodies and streptavidin-horseradish peroxidase. The presence of the expected protein was visualized by DAB staining and examined under a microscope. Stains without the primary antibody were used as negative controls.

2.17. Statistical Analysis. Data were expressed as mean ± S.D. Statistical significances between vehicle group versus drug treatment groups were determined by one-way analysis of variance. The IC_{50} of the TET was calculated by SPSS software. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. TET Exhibits a Significant Growth-Inhibitory Effect in HUVECs. To assess the antiangiogenic property of TET *in vitro*, we examined the inhibitory effects of TET on cell viability in HUVECs using MTT assay and crystal violet staining. As shown in Figures 1(a) and 1(b), TET (<10 μM) does not have any remarkable effect on HUVEC proliferation. However, TET can significantly inhibit cell viability at a much higher concentration with a half-maximal inhibition at 16.76 μM (by MTT assay) or 29.31 μM (by crystal violet staining assay). To examine the possible mechanism behind TET's inhibition effect on HUVEC's proliferation, we performed cell cycle analysis by FACS, and the result revealed that when HUVECs were treated with TET for 24 h, TET in 10 μM induced a depletion of cells in the G_2-M phase, from 10.70% to 4.21%, and a concomitant accumulation of cells in S phase, from 34.17% to 38.35%. These data suggested that TET could arrest endothelial cell proliferation. Furthermore, we examined the effects of TET on NF-κB, ERK1/2, and p-ERK1/2 expressions, and the results suggested that the inhibition of TET in HUVECs was related to the upregulation of NF-κB and suppression of the phosphorylation of ERK1/2.

3.2. TET Inhibits Cell Adhesion, Cell Migration, and Cell Invasion of HUVECs. The antiadhesion ability of TET between 4T1 and HUVEC cells was investigated. As a result, TET could suppress the 4T1 and HUVEC adhesion after 30 min treatment (Figure 2(a)). The adhesive cells were decreased to 245 compared with 592 of the control group.

Moreover, the VEGF-induced migration of HUVEC cells was significantly suppressed by TET (Figure 2(b)). The migratory distance of HUVEC was significantly decreased by 10 μM TET-contained medium when compared to that of control ($P < 0.01$). We further tested whether TET could affect chemotactic HUVECs invasion. Using the Boyden chamber transwell assay, we found that when HUVECs were treated with 10 μM TET the numbers of migrated cells across the extracellular matrix protein-coated membranes significantly decreased (Figure 2(c)). Quantitatively, TET was shown to inhibit the numbers of migrated HCT116 cells by approximately 33% over that of the control treatment.

Notably, this inhibitory effect of TET on EC invasion was potentially related to the activity of proteinases (MMPs). To determine the effect of TET on the production of proteinases by HUVEC, culture supernatants were collected and subjected to gelatin zymography. As shown in Figure 2(d), the presence of proteinases (MMPs) digested the gelatin-containing gel and resulted in a clear band at 66 kDa, which was assigned to MMP-2, and TET reduced the gelatinolytic activities of secreted MMP-2 in a dose-dependent manner which corresponded to the inhibition of EC invasion.

In addition, we examined the expression of adhesion and invasion related factors, including integrin β5, ESM-1, and ICAM-1 *in vivo*. The whole cell staining intensities of integrin β5 and ESM-1 protein were markedly reduced in TET treated tumors, compared with those of the tumors from the control group (Figure 2(e)). On the contrary, the ICAM-1 level in cell was significantly decreased by TET treatment (Figure 2(e), the middle column). These results have demonstrated that TET can effectively inhibit EC adhesion, migration, and invasion through mechanisms of changing the integrin β5, ESM-1, and ICAM-1 expressions.

3.3. TET Inhibits Tubeformation of HUVECs. The effect of TET on the capillary tube formation of ECs was examined. In the absence of VEGF, there was no tube network structure in ECs, whereas the addition of VEGF (20 ng/mL, positive control) induced the formation of tube or cordlike structure and tube network on GFR matrigel. Cultured with TET resulted in shorter and less blunted tubes of ECs than those of VEGF control group (Figure 3(a)). Quantitative measurements showed that TET caused an increase in mean tube branch point formation as compared to VEGF 20 ng/mL group (positive control) (Figure 3(b)). The number of branch

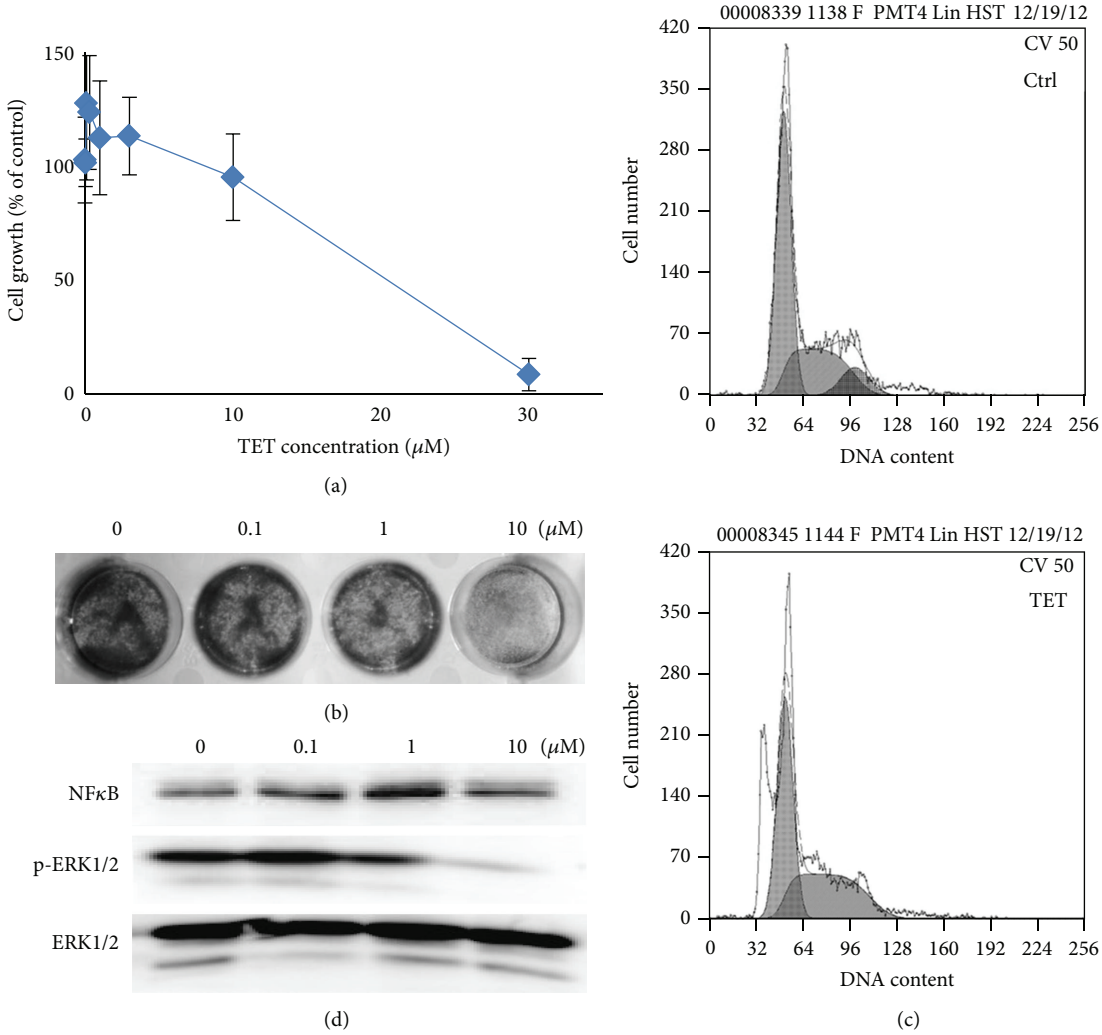

Figure 1: Antiproliferative activity of TET in HUVECs. (a) MTT assay. Subconfluent HUVECs were treated with indicated concentrations of TET for 48 h. The cells were then subjected to MTT assay. Each assay condition was done in triplicate ($n = 12$). (b) Crystal violet assay. HUVECs were treated with TET at the indicated concentrations for 24 h. The cells were subjected to crystal violet assay as described in Section 2. (c) Cell cycle analysis. Cell cycle distribution of HUVECs was analyzed by flowcytometry. Cells were treated with 1 μM TET for 24 h and fixed, and then nuclear DNA was labeled with PI. Histogram display DNA content (x-axis [PE-A]: PI-fluorescence) versus cell counts (y-axis). (d) ERK, p-ERK, and NFκB expressions in HUVECs. The expression of ERK, p-ERK, and NFκB was detected by western blot.

was reduced from 15.7 per area in the control group to 3.6 in the group treatment with TET (10 μM) ($P < 0.01$).

3.4. TET Inhibits In Vivo Tumor Metastasis in Mouse Breast Tumor Model. We next investigated the *in vivo* antimetastatic activity of TET using a mouse breast cancer model. Briefly, exponentially growing firefly luciferase-tagged 4T1 cells were injected into the MFP of BALB/c mice, and TET was orally administered (10 mg/kg body weight, once every two days). As shown in Figure 4(a), the Dox and TET treatment groups exhibited significantly decreased Xenogen imaging signal in lung, when compared with the control group four weeks after treatment. At sacrifice, lung metastases were counted. In keeping with the *in vitro* data, the number of metastasis sites on the lung surface was remarkably decreased by TET, from 6.2 to 2.6 for each mouse, rather than that of Dox treated mouse (Figures 4(b) and 4(c)). Moreover, histological analysis (H & E staining) indicated that TET treatment group exhibited a decreased metastatic tumor mass in lung (Figure 4(d)).

3.5. TET Inhibits In Vivo Angiogenesis in 4T1 Tumor Bearing Mice. The effect of TET on angiogenesis *in vivo* was also examined in the animal model. As shown in Figure 5(a), four weeks after MFP injection of 4T1 cells into mice, the diameter of the blood vessels in the tumor implanted side is increased, rather than in the other side, but TET could significantly inhibit the increase of the blood vessel diameter from control levels. Consistent with gross observations, solid tumor sections further indicated that TET inhibited neovascularization in tumor mass *in vivo*, and the average

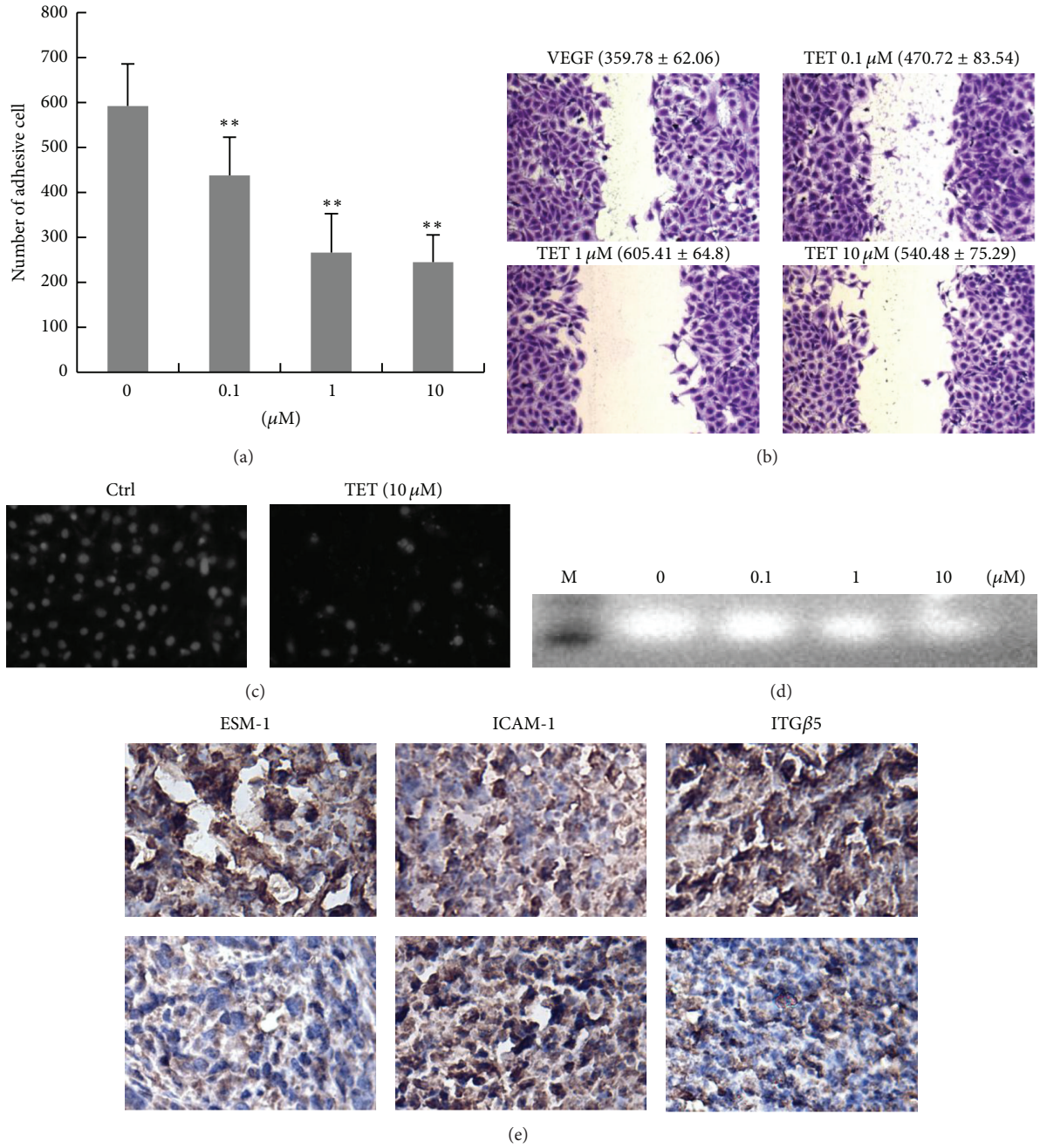

FIGURE 2: TET inhibits cell adhesion (a), migration (b), and invasion (c) in HUVECs. (a) Cell adhesion assay. HUVECs and 4T1 cells were used to study the cell adhesive ability. $^{**}P < 0.01$ versus vehicle control. (b) Cell migration assay. Wound-healing model was used for evaluation of cell migration ability. Three random views were photographed along the scraped line in each well at 100x magnification. (c) Cell invasion assay. Cell invasive ability was measured with a transwell system with a polycarbonate membrane (8 μm pores). Images were captured at 200x magnification. (d) Effects of TET on secretion of matrix metalloproteinase-2 (MMP-2). Gelatin zymography was carried out in an SDS-PAGE gel that contained 0.1% gelatin. (e) Effect of TET on ESM-1, ICAM-1, and integrin β5 expressions in tumor tissues. 4T1 tumor bearing mice (top panel) and TET treated tumor bearing mice (bottom panel) (magnification ×400).

number of new capillaries blood vessel in control group was more than that in TET treated group (Figure 5(b)). These results demonstrated that TET is a potent inhibitor of vascularization and angiogenesis.

We sought to further investigate the mechanism behind the TET mediated inhibition of angiogenesis activity. As shown in Figure 5(c), we examined the expression of metastatic and angiogenic related proteins, including VEGF and HIF-1α *in vivo*. The whole cell and nuclear staining intensities of VEGF and HIF-1α were markedly reduced in TET treated tumors, compared with those of the control group.

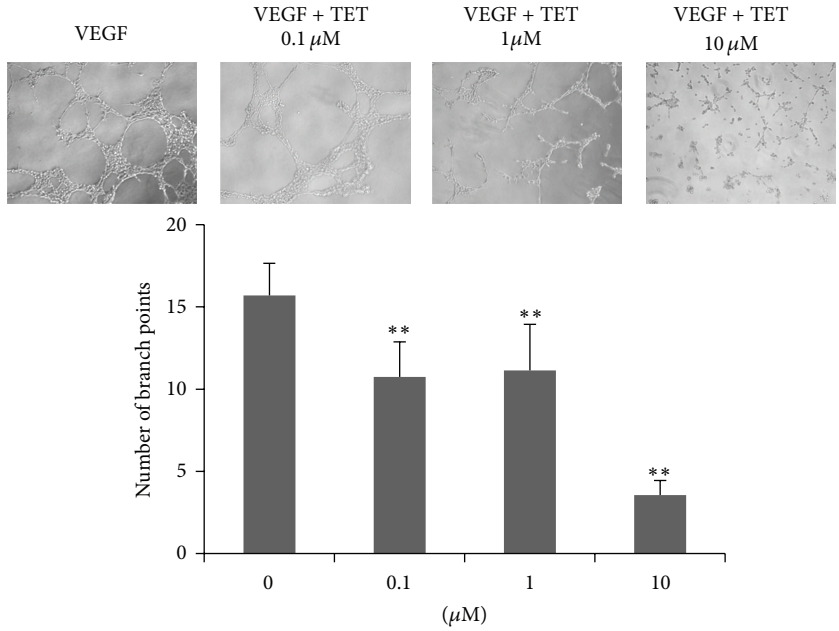

Figure 3: TET inhibits tube formation in HUVECs. The effects of TET on HUVEC differentiation were examined by their *in vitro* tube formation ability on matrigel. The branch points of the capillary-like tubes were counted under light microscopy (magnification 100x). Each value represents the mean ± S.D. of triplicate samples in each case. $^{**}P < 0.01$ versus the VEGF-only group.

Lastly, to investigate the blood perfusion change of TET treated tumor bearing mice in tumor surface and skin around tumor area, we took advantage of the laser Doppler perfusion imaging (LDPI) to measure the local perfusion pattern. In the LDPI, the related perfusion of the mice abdomen was decreased after tumor being implanted for 3 weeks, especially the tumor site (Figure 6(a)). The related perfusion of tumor site (Flux 1) in TET group was decreased to 49% lower than that in healthy mice, but with no difference from that of the model group. It suggested that TET could slightly improve the necrosis of tumor 4 weeks after TET administration (Figure 6(b)). However, the related perfusion around tumor site (Flux 2) in TET group was significantly decreased to 82.54% of the model group ($P < 0.05$), which indicated that TET could markedly decrease the local blood perfusion of tumor 3 weeks after TET administration (Figure 6(c)) and suggested that the tumor angiogenesis was suppressed by TET treatment.

Taken together, these *in vivo* results strongly suggest that TET may inhibit the tumor metastasis of breast cancer, possibly by reducing angiogenesis activity and related protein level of breast cancer cells, although further investigation is required.

4. Discussion

The growth and progression of solid tumors are usually limited by the nutrient supply for tumor. Thus, the blockage of microvessels formation and local blood perfusion in tumor might be useful in cancer therapy. Recently, more than 20 antiangiogenic drugs including TNP-470, thalidomide, and endostatin are subjected to different phases of clinical trials. In addition, phytochemicals such as resveratrol, salvianolic acid B, and ginseng saponins were found to exert inhibitory effect on the vascularization [22].

TET has been shown to exhibit anticancer activity in many *in vivo* models [5, 9, 10]. TET-treated mice (10 mg/kg/day) have fewer metastases than vehicle treated mice, and no acute toxicity or obvious body weight changes [23]. Recent studies showed that TET induces cell cycle arrest and also induces apoptosis in many human cancer cells. In our previous study, we found that inhibition of Wnt/beta-catenin signaling might contribute to the anticancer effects of TET [9]. Nonetheless, it is conceivable that other signaling pathways may also participate in TET's anticancer activity. For example, activation of glycogen synthase kinase 3β (GSK-3β), generation of ROS, activation of p38 mitogen-activated protein kinase (p38 MAPK), and upregulation of p53, p21, p27, and Fas might contribute to the anticancer effects of TET [5, 24–29].

As mentioned previously, TET exhibits significant anticancer activity both *in vitro* and *in vivo*, as well as its inhibitory effect on tumor metastasis and angiogenesis. Chen et al. found that TET inhibits the expression of VEGF in glioma cells, has cytotoxic effect on ECV304 HUVECs, and suppresses *in vivo* angiogenesis in rat [10]. However, the tumor related angiosuppressive property of TET and the molecular mechanism that underlies its activity are not fully understood. In the present study, we used a new LDPI method combined with different angiogenesis assays that are related to proliferation, adhesion, migration, invasion, and tube formation of EC during angiogenic process, to assess the angiosuppressive activity of TET.

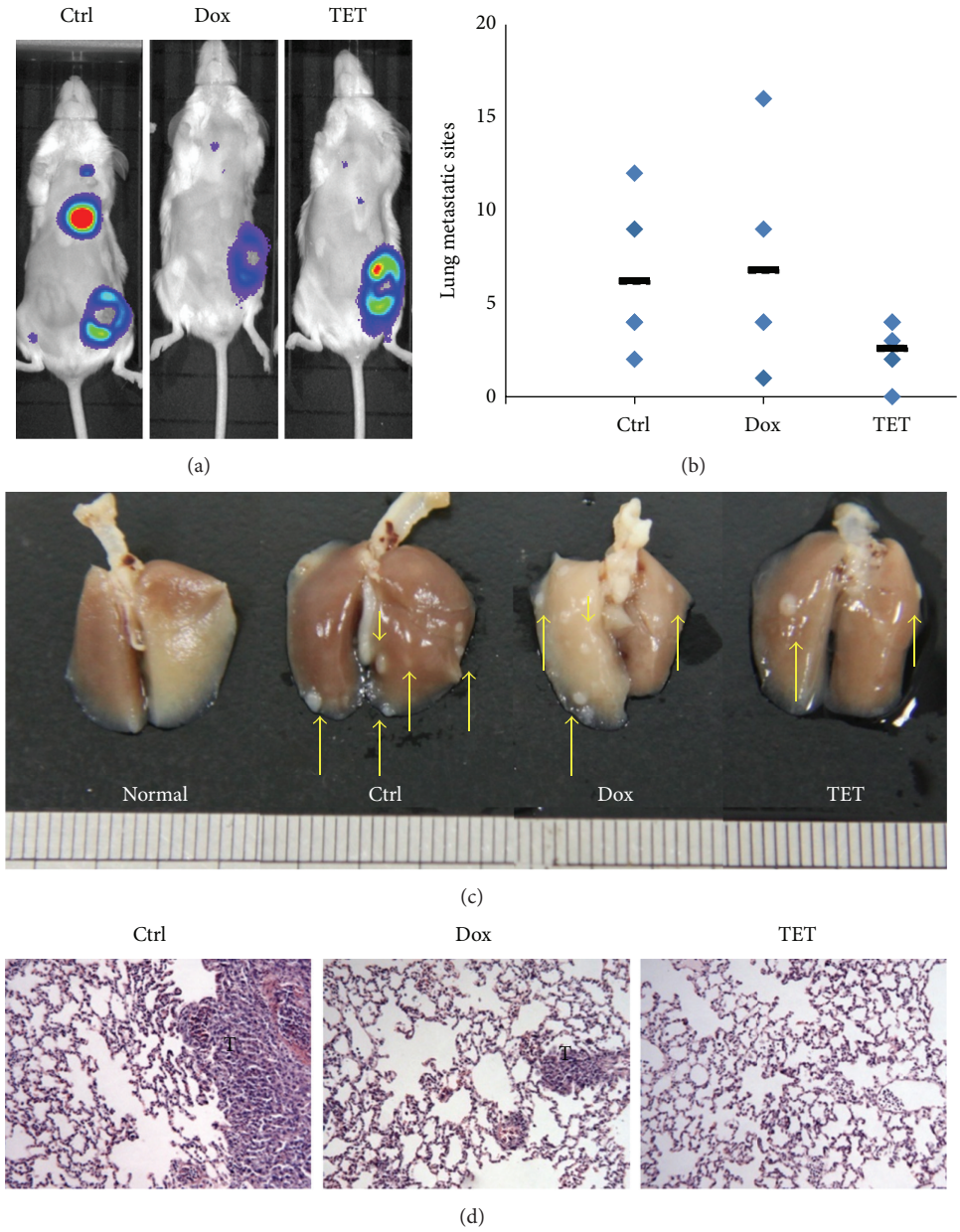

FIGURE 4: TET inhibits *in vivo* tumor metastasis in mouse breast cancer model. (a) Bioluminescence imaging of TET treated tumor bearing mice. Firefly luciferase-tagged 4T1 cells were injected into the MFP of BALB/c mice and TET was orally administered (10 mg/kg body weight, once every two days); images were obtained by using IVIS 200 imaging system. Representative Xenogen imaging results at week 4 are shown. (b) TET reduced tumor lung metastasis sites in 4T1 tumor-bearing mice. Picture shows the number of lung metastasis sites on the lung surface for each mouse. The black line shows the average number of metastasis sites for each group. (c) Photograph of pulmonary metastases. Animals were sacrificed after 4 weeks, and the lungs were dissected and photographed. (d) Histological examination of lung samples. Lung tissues sections were stained with H&E stain, and photographs were made under a microscope at a magnification of 400x (T: tumor).

Results from the present study demonstrated that TET exerted inhibitory effect on proliferation, adhesion, and capillary tube formation of ECs in a dose-dependent manner. Interestingly, the blood perfusion of the periphery of tumors was significantly reduced by TET treatment. Owing to that the blood perfusion is usually proportional to the body's blood vessels density [30], this result implied that the antimetastasis effect of TET was passably related to the angiosuppressive activity. Similar cases were also observed in the tumor mass of TET treated 4T1-tumor bearing mice. TET was found to effectively suppress the formation of micro vessels in tumor. Furthermore, since tube formation of HUVEC involves EC attachment, migration, and production of ECM degrading enzymes, data in the presented paper indicated that all these steps were interfered by TET and resulted in the attenuation of angiogenesis *in vitro* and *in vivo*. Thus, TET may be useful in cancer metastasis by acting as a specific and effective angiosuppressive agent.

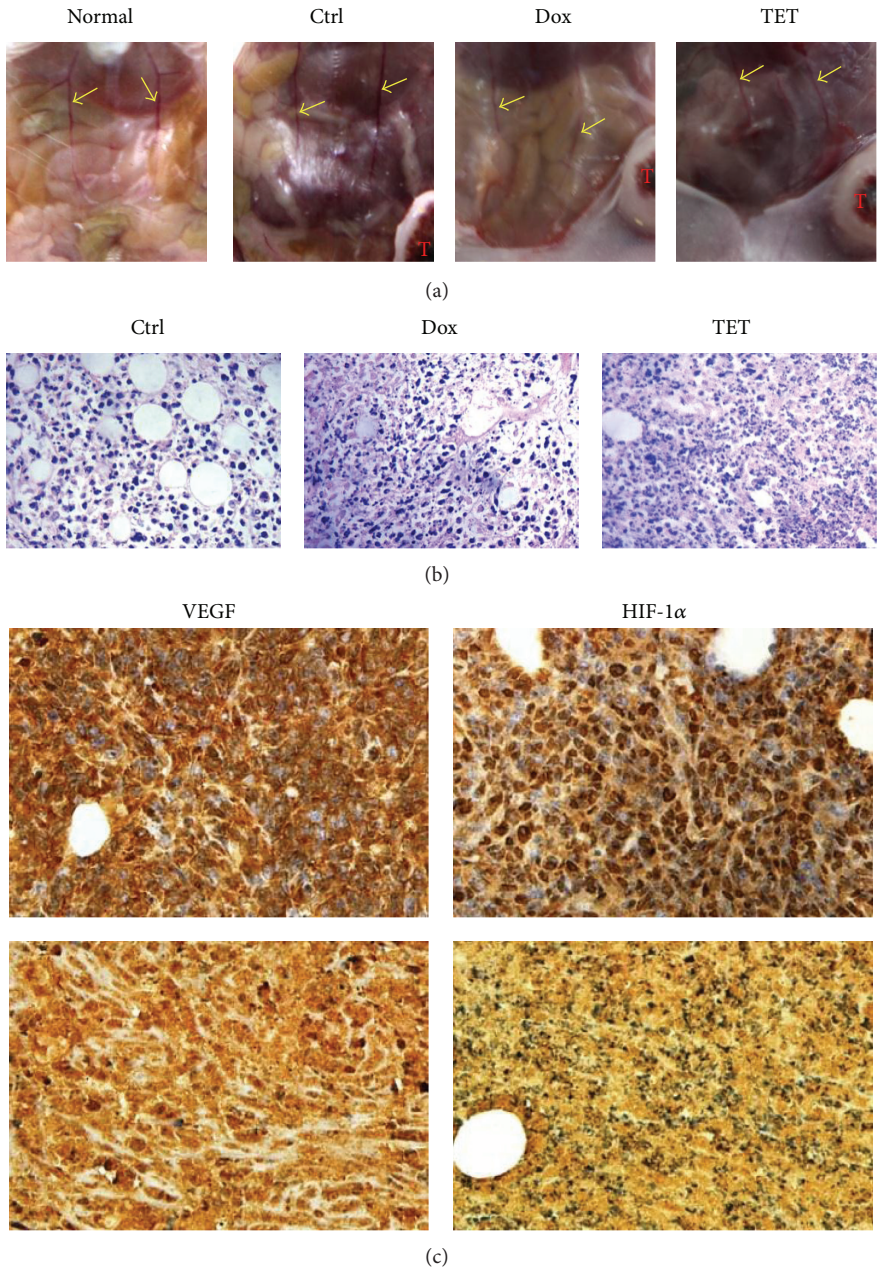

FIGURE 5: TET suppressed tumor angiogenesis in 4T1 tumor bearing mice. (a) The blood vessel diameter of TET treated tumor bearing mice. Picture shows the diameter of blood vessel on mice abdomen for each mouse. (b) Hematoxylin & Eosin staining of tumor tissues. Retrieved tumor samples were fixed, embedded, and subjected to H&E staining. Representative images are shown (magnification, ×400). (c) VEGF and HIF-1α expressions in tumor tissues. Representative staining results are shown: 4T1 tumor-bearing BALB/c mice (top panel) and TET treated mice (bottom panel) (magnification, ×400).

We investigated the molecular mechanism underlying the antivascularization activity of TET in breast cancer. TET treated group exhibited a decreased level of VEGF, HIF-1α, ESM-1 and Integrin β5 protein but upregulation of ICAM-1 level. These *in vivo* results strongly suggest that the inhibitory effect of TET on breast cancer metastasis may be at least in part mediated by inhibiting tumor angiogenesis factors (VEGF and HIF-1α) or regulating adhesion factors (Integrin β5, ESM-1, and ICAM-1), although further investigation is required.

One of the key cell signaling pathways involved in cancer tumorigenesis and metastasis is the hypoxic pathway. As we know, HIF-1α binds to HREs and induces subsequent expression of genes encoding angiogenic factors, such as VEGF and MMPs, leading to angiogenesis [31]. TET could reduce the expression of HIF-1α and then decrease VEGF level and MMPs activity. It is generally believed that VEGF could activate ECs; activated ECs produce many types of enzymes such as matrix metalloproteinases (MMPs) that break down the stroma and ECM proteins [32]. This is

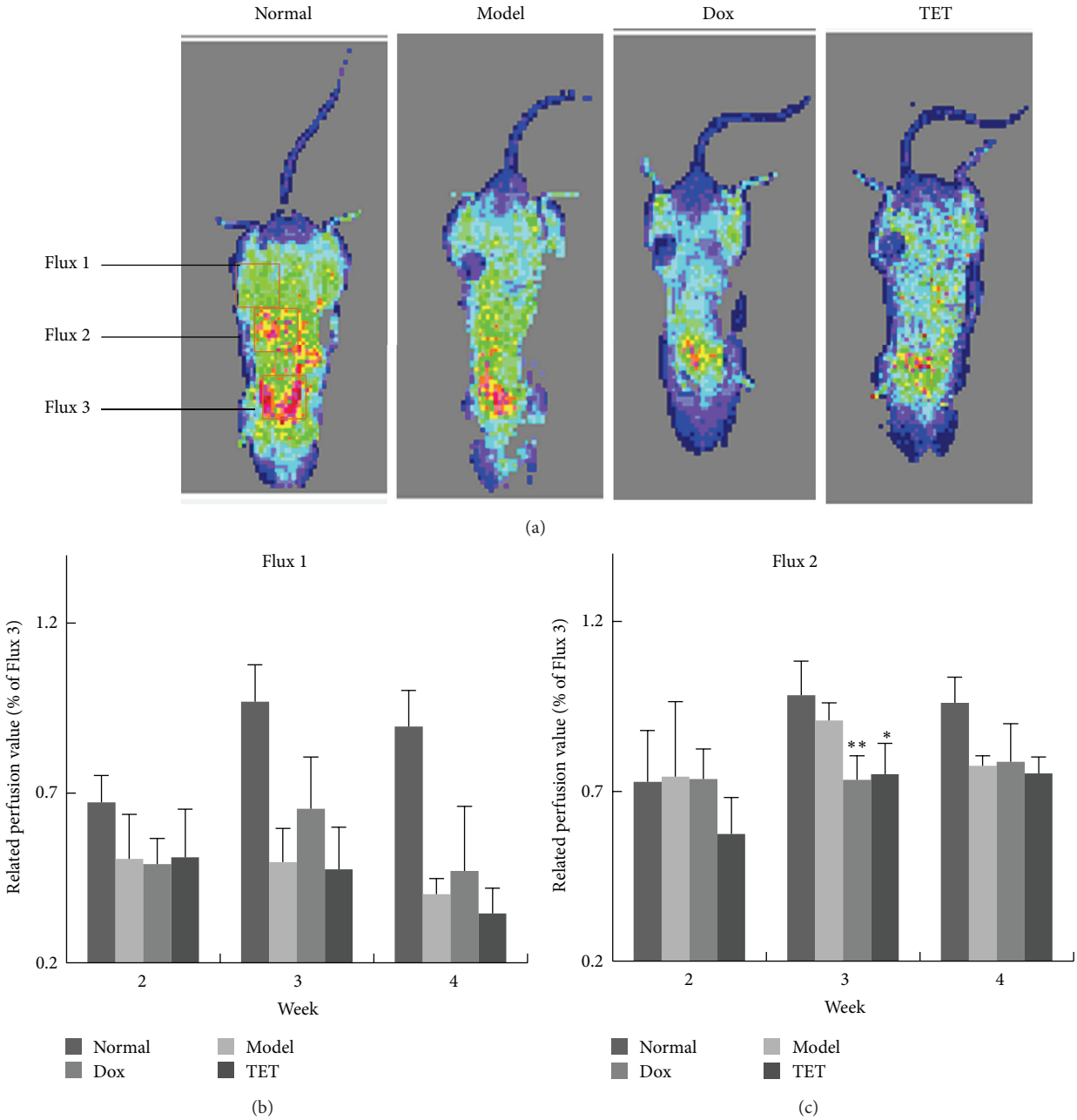

FIGURE 6: TET inhibits *in vivo* blood perfusion in 4T1 tumor bearing mice. (a) *In vivo* LDPI imaging of mice. TET was orally administered (10 mg/kg, once/2 days). With a distance of 10 cm between the scanner and the skin surface, three examined areas (1.4 ∗ 1.4 cm) covered the tumor (Flux 1), adjacent healthy skin around tumor (Flux 2), and the heart of mice (Flux 3). (b and c) Quantitative analysis of microvascular blood perfusion in mice. Related perfusion (Flux 1) = $F1/F3 * 100\%$; related perfusion (Flux 2) = $F2/F3 * 100\%$. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control.

the critical step in angiogenesis as well as metastasis. The invasion assay involving the migration of HUVEC through ECM (matrigel) demonstrated that TET could reduce the chemoinvasive ability of EC by reducing the gelatinases activities of the cell culture medium. These noteworthy results indicated that the angiosuppressive effect of TET could be possibly due to the reduction of HIF-1α and/or VEGF expression, as well as MMPs activities.

Recently, several research groups have tried to identify cell adhesion suppressors which could inhibit cancer metastasis by blocking the lodging in blood vessels in the distant organs of disseminated cancer cells or cell clusters [33]. Integrins are important mediators of the malignant phenotype during oncogenic transformation [34]. Breast carcinoma cells express high levels of integrin β5. Bianchi-Smiraglia et al. indicated that cells deficient in integrin β5 have lower migration and proliferative capacities, and ERK signaling pathway plays an important role in the function of integrin β5 in cancer [35]. Our results show that the expression of integrin β5 was decreased by TET treatment

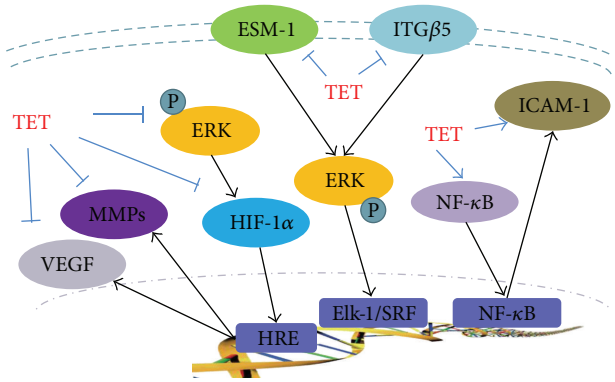

FIGURE 7: The influence of TET on the angiogenesis and metastasis related factors.

and accompanied by a reduced phosphorylated-ERK level. On the other hand, the adhesion factor ESM-1, which could inhibit leukocyte adhesion and migration through the endothelium, was increased in tissue and serum from colorectal cancer patients and ESM-1 silencing decreased cell survival, migration, and invasion and modulated cell cycle progression in hepatocellular carcinoma [36, 37]. We notified that TET also decreases ESM-1 expression in breast cancer cells, which leads to an inhibition effect of tumor metastasis. Taken together, TET suppressed the integrin $\beta 5$ and ESM-1 expressions, then depressed the activation of ERK, and regulated cellular proliferation, adhesion, and survival in ECs.

Another important adhesive factor involved in TET's angiosuppressive effect is ICAM-1; several researches showed that ICAM-1 synthesis in ECs is regulated by activation of p38 and NF-κB [38, 39], and ICAM-1 could induce cell adhesion by active ERK, JNK, and p38 pathways [40, 41]. Our IHC results present an upregulation of ICAM-1 in tumor tissue, along with the increasing of NF-κB, and suggested that the promotion effect of TET on NF-κB and ICAM-1 expressions is closely related to its antiangiogenesis effects. Taken together, as shown in Figure 7, our studies indicate that TET is a potential inhibitor of tumor angiogenesis and metastasis by targeting the angiogenesis and metastasis related factors.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

J.-L. Gao and X. Ji contributed equally to the work.

Acknowledgments

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Research Article

Efficacy of Contact Needle Therapy for Chemotherapy-Induced Peripheral Neuropathy

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Cancer chemotherapy-induced peripheral neuropathy (CIPN) often results in discontinuation of treatment with potentially useful anticancer drugs and may deteriorate the patient's quality of life. This study investigated the effect of contact needle therapy (CNT) on CIPN caused by responsible chemotherapeutic agents as taxanes and oxaliplatin. Six patients with CIPN were treated with CNT. The severity of CIPN was evaluated using the Common Terminology Criteria for Adverse Events (CTCAE) version 4 and FACT/GOG-Ntx before and after CNT. After the treatment, all of the patients showed some improvement. Four patients showed apparent improvement in breakthrough pain. One of the cases had difficulty in walking because of CIPN in lower extremities, but after 2 times of CNT, he could walk without pain and could continue the chemotherapy. Although its putative mechanisms remain elusive, CNT has strong potential as an adjunctive therapy in CIPN. Well-designed clinical trials with adequate sample size and power are necessary to confirm the findings of this study.

1. Introduction

With the increasing numbers of patients with cancer and cancer survivors and the development of multidisciplinary cancer therapy, treatment that considers the quality of life (QOL) of patients together with prognostic improvement is demanded. Multidisciplinary cancer therapy consists of surgical treatment, radiotherapy, and chemotherapy. Chemotherapy often causes side effects such as myelosuppression, digestive symptoms, renal failure, or peripheral neuropathy. Cancer chemotherapy-induced peripheral neuropathy (CIPN) is one of the most serious problems in clinical practice, and it sometimes results in the discontinuation of subsequent treatment [1, 2]. CIPN is well known in taxanes, platinum analogues, vinca alkaloids, and molecular target drugs such as bortezomib [3]. Neuropathy by taxanes stems from damage to microtubules of the neuraxis, mainly developing from gloves-and-socks type sensory disturbance [4]. Platinum analogues such as cisplatin and oxaliplatin damage nerve cells directly, followed by damage to the neuraxis [5]. Moreover, with oxaliplatin, acute accumulation-related disorder is detected, and acute peripheral neuropathy is induced by a low-temperature stimulus that is known to be reinforced [6]. To prevent CIPN, calcium and magnesium infusions, glutathione, and anticonvulsants (carbamazepine) are used, but their effects are limited. Also, when CIPN appears, anticonvulsants, tricyclic antidepressants, alpha-lipoic acid, and opioids are often used [2, 7], but their effects may not be enough. Abrogation of chemotherapy is required to prevent the exacerbation of symptoms. Specific and effective curative treatments are lacking.

Acupuncture is a frequently used alternative and complementary medicine in Japan. Contact needle therapy (CNT) is one of the traditional Japanese methods of acupuncture, which was thought up by Bunkei Ono. We use disposable needle and do not insert at all but only settle the needle on

the acupuncture point to perform the least but effective stimulus to unblock the meridian. The aim of CNT is to improve the patients' condition no matter what their diseases are by regulating the flow of Qi. This method has many advantages such that it is safe, painless, easy to perform, and it decreases the risk of infection.

From ancient times, it has been said that the larger the needle is and the deeper the needle is inserted, the stronger the stimulation will be. If the stimulation is too strong, the patients' condition becomes worse, especially when their constitution is weak. CNT is known as a method of weak stimulation. In this aspect, CNT is effective and appropriate for treating cancer patients. It is also expected to relieve symptoms such as the side effects of chemotherapy in several clinical reports in Japanese [8].

Recently, clinical trials of acupuncture for prevention or treatment of CIPN have been conducted [9]. Publications in English language journals on acupuncture as a symptomatic treatment for CIPN have been limited to only a few case studies, all of which report an improvement in symptoms [10–13]. But there is no report of CNT and it has never been clinically evaluated in English publications, because CNT may have been considered as placebo acupuncture.

In this study, we investigated whether CNT had beneficial effects in patients with CIPN after or during chemotherapy.

2. Patients and Methods

2.1. Preliminary Study

2.1.1. Patients. Between July 2012 and January 2013, acupuncture treatment was offered to all the patients who were diagnosed to have CIPN by surgeons in Japanese Red Cross Kanazawa Hospital. Seven patients agreed to receive acupuncture treatment, CNT. We excluded 1 patient whose CIPN had been partially caused by uncontrolled diabetes mellitus from this study. Therefore, we evaluated six patients with CIPN. We checked the patients' background (age, sex, and cancer types) and contents of chemotherapy. Six patients (four men and two women) of mean age 64.3 years received the best medical care and additionally were treated with CNT for CIPN. The types of cancer were one case of breast cancer and 5 cases of colorectal cancer.

2.1.2. Contact Needle Therapy (CNT). The specific acupuncture protocols employed in this study are described later, point location were as described in standard textbooks [14], and disposable sterile silver needles of 0.16×24 mm were used and left in place for 30 second to 1 min without insertion. Each patient received standard 4–6 treatments in 3 months.

Acupuncture was performed in all cases by the same senior acupuncturist who had used acupuncture for 20 years. CNT was performed to the patients according to the medical diagnosis of meridian therapy. Acupuncture points used in this study are as follows.

Points for all patients: CV12, CV4, ST25, KI2, well points of extremities.

Selected points: LR8, LR14, SP3, LR13, LU9, LU1, KI7, GB25, PC7, CV17, CV6, CV4, ST36, LU1, BL20, BL13, BL18, BL23.

2.1.3. Endpoints and Evaluation. The severity of CIPN was graded using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 and also FACT/GOG-Ntx [15]. Clinical evaluation was performed before and after CNT because of the limitation of CTCAE for precise evaluation of symptoms. Patients' objective evaluations were also obtained.

3. Results

3.1. Preliminary Results in 6 Patients. Table 1 shows the patient characteristics. The mean age of the 6 patients was 64.3 years old (range: 54–73). Each patient had undergone an operation with chemotherapy. Four of 6 received concurrent chemotherapy, but all of them excluded responsible chemotherapeutic agents from regimens for 1–26 months before the start of CNT. Five of 6 are tumor-bearing status, and case 2 is receiving chemotherapy as adjuvant setting. As chemotherapeutic agents responsible for peripheral neuropathy, taxanes were used in Patient 2, and in others platinum analogue oxaliplatins were used.

Prior to CNT, the patients had grades 1-2 CIPN according to the CTCAE and had symptoms scored as 4–13 according to the FACT/GOG-Ntx. All six patients had hypoesthesia in a globes and/or socks distribution; four had additional neuropathic breakthrough pain, and three had clinical motor involvement.

As for the start of CNT in relation to the duration of chemotherapy, 4 cases were during chemotherapy, and 2 cases were within half a year after last chemotherapy.

Some improvement of CIPN was found in 2 of the cases after CNT in CTCAE grading, but all of the patients showed improvement in the FACT/GOG-Ntx score. Hypoesthesia in a globe and/or stocking distribution was improved in all six patients. All four patients who complained of breakthrough pain showed apparent improvement (Table 2). Especially 2 of 6 patients with CIPN treated with CNT after they stopped chemotherapy improved after only 1 or 2 times of CNT.

In all of the cases, multiple drugs were used for CIPN. Also, Japanese traditional (Kampo) medicines were prescribed in two cases.

As well as peripheral neuropathy, improvement in some symptoms other cancer treatment-related symptoms was obtained after initiating CNT. Improvement of edema was obtained in 5 cases, fatigue and constipation in 4 cases (67%), and other symptoms in 6 cases.

3.2. Case Report. A 66-year-old man (Patient 5) presented with occult blood from the rectum and visited a hospital in May 2007. He was referred to Kanazawa University Hospital and was diagnosed with rectal cancer (RaT2N1M0, stage IIIa). He was initially treated by operation without adjuvant chemotherapy because of the earthquake. Three years after the operation, metastasis was found in S8 and caudal lobe of the liver. He was treated by chemotherapy

Table 1: Patient characteristics.

	Case					
	1	2	3	4	5	6
Age/sex	70/F	54/F	66/M	57/M	66/M	73/F
Performance status	0	0	0	0	0	0
Primary lesion	Colon	Breast	Colon	Colon	Colon	Colon
Chemotherapeutic agents	Oxaliplatin	Docetaxel paclitaxel	Oxaliplatin	Oxaliplatin	Oxaliplatin	Oxaliplatin
Regimen of chemotherapy	XELOX + Bev	DOC PAC	mFOLFOX	mFOLFOX + Bev	SOX + Bev FOLFIRI + Bev	XEROX + Bev
Past operations	Yes	Yes	Yes	Yes	Yes	Yes
Tumor-baring	Yes	No	Yes	Yes	Yes	Yes

XELOX: oxaliplatin, capesitabine.
Bev: bevacizumab.
SOX: oxaliplatin, S-1.
FOLFIRI: folinic acid, fluorouracil, irinotecan.
FOLFOX: folinic acid, acid, fluorouracil, oxaliplatin.
DOC: fluorouracil, epirubicin, cyclophosphamide, docetaxel.
PAC: paclitaxel, cyclophosphamide, doxorubicin.

Table 2: Results of symptoms scores pre- and post-CNT and clinical evolutions of patients.

	Case					
	1	2	3	4	5	6
CTCAE						
Before	2	1	2	1	2	1
After	2	1	1	1	2	0
FACT/GOG-NTX						
Before	11	6	10	4	13	9
After	5	2	5	4	2	4
Breakthrough pain						
Before	4	0	2	3	3	0
After	0	0	1	1	1	0
Patients' evaluation	Improved	Improved	Improved	Improved	Improved	Improved
Last responsible chemotherapy (month ago)	Concurrent 12	26	16	Concurrent 16	Concurrent 12	Concurrent 1
Adverse effect of CNT	None	None	None	None	None	None

Breakthrough pain: 0 (None)~4 (Very severe).

with SOX + Bev (oxaliplatin, S-1, bevacizumab) regimen for recurrent liver metastases. After 3 courses of chemotherapy, he developed grade 1 CIPN and grade 2 pigmentation of extremities in CTCAE. After 5 courses of SOX + Bev regimen, chemotherapy regimen was changed to FOLFIRI + Bev (folinic acid, fluorouracil, irinotecan, bevacizumab) because of the severe CIPN. Although he stopped oxaliplatin for 12 months, symptoms of CIPN were not improved and were even exacerbated with FOLFIRI + Bev. He decided to stop all chemotherapy, in July 2010, which led to the growth of liver metastasis. Chemotherapy with FOLFIRI + Bev was restarted in November 2011, but symptoms of CIPN were the same. CNT was started in August 2012. After 2 times of CNT, he demonstrated a dramatic improvement in pain, numbness, and discomfort of lower extremities and he could easily walk. After 6 times of treatment, FACT/GOG-Ntx score was improved to 2 from 13. The amelioration of his symptoms enabled chemotherapy to be continued.

4. Discussion

CIPN is one of the most serious problems in clinical practice, and it sometimes results in the discontinuation of subsequent treatment [1, 2]. CIPN is well known in taxanes, platinum analogues, vinca alkaloids, and molecular target drugs such as bortezomib [3]. Neuropathy by taxanes stems from damage to microtubules of the neuraxis, mainly developing from gloves-and-socks type sensory disturbance. It is commonly associated with a decrease in reflection, vibratory sensation, muscle ache, and muscle weakness [4]. Platinum analogues such as cisplatin and oxaliplatin damage nerve cells directly, followed by damage to the neuraxis. With oxaliplatin, acute accumulation-related disorder is detected, and acute peripheral neuropathy is induced by a low-temperature stimulus that is known to be reinforced [6].

In this investigation, some improvement of CIPN was found in all of the cases after CNT. CIPN of 2 patients

in concurrent chemotherapy was not exacerbated, which may indicate the effectiveness of its prophylactic role. In 4 patients who stopped chemotherapy, CNT was effective on peripheral neuropathy even 2 years after last chemotherapy. CIPN may spontaneously disappear over time in some cases [16] and in some patients in our study as well, but it will be difficult while undergoing the responsible chemotherapy, as well as after more than one year. Patient 5 demonstrated remarkable improvement in pain, numbness, breakout pain, and discomfort of his legs.

Although several prospective clinical trials have indicated acupuncture to be effective in painful diabetic neuropathy (PDN) and human-immunodeficiency-virus- (HIV-) related painful neuropathy [17–20], no known clinical trials have investigated the intriguing potential of acupuncture for the relief of breakout pain in CIPN, which has a dominant position among side effects. Various treatments have been tried, but few of them have demonstrated sufficient effects so far. In the present study, the usefulness of CNT for CIPN by experienced specialist, including the advantage of making adjustments to acupuncture point according to the patients' sho and safety of CNT compared with inserted acupuncture methods, is suggested. Several studies [17–20] have demonstrated that acupuncture is effective in the treatment of CIPN, with fewer adverse effects than analgesic drugs.

From a perspective of Japanese traditional medicine, acupuncture is based on the premise that there are patterns of qi flowing throughout the body, and blockage of qi leads to illness. Qi is defined as a kind of vital energy that is immaterial or invisible in narrow definition and is simultaneously both immaterial and material in wide definition. From the scientific point of view, the proposed putative mechanisms of acupuncture involve regulation of the nervous system, stimulation of the immune system and alteration of brain chemistry causing the release of various neurotransmitters and hormones. Some evidence has shown that acupuncture is a safe pain-control method with minimal side effects, and interest in acupuncture as an adjunctive therapy has grown rapidly in recent years among the general public as well as the scientific and academic communities.

Several human and animal studies have examined the neurochemical basis of acupuncture for pain control, and although no single theory is sufficient to completely explain all the effects of acupuncture, the mechanisms can be partly explained in terms of endogenous pain inhibitory systems. Acupuncture excites receptors or produces rhythmic discharges in nerve fibers activating both the peripheral and central nervous systems, resulting in the release of various neurotransmitters [21–24]. The exact effects of acupuncture depend on point selection and type of stimulation [25].

Serotonergic pathways have been implicated in pain relief and have been useful in relieving discomfort in neuropathy [26]. Thus, it has been hypothesized that acupuncture might work synergistically with serotonergic therapy to relieve neuropathic pain.

According to the research of Litscher et al. [27], acupuncture may increase the blood flow in the extremities. Increased blood flow to the vasa nervorum and dependent capillary beds supplying the neurons may contribute to nerve repair with measurable improvement of axons or myelin sheaths. In addition, the symptomatic effect of acupuncture may reflect morphological changes in the anatomy of peripheral nerves and also complex derangements of central and peripheral regulation [28]. It is not certain whether CNT has the same effect as inserted acupuncture, but those findings in acupuncture give us some suggestion on the mechanism of effect of CNT.

The reason that quite a few randomised controlled trials of acupuncture failed to show the significant effects is partially because of the real effect of no-insertion acupuncture. For example, a randomised controlled trial compared acupuncture and amitriptyline with their respective placebo controls showing that the sham device had greater effects than the placebo pill on self-reported pain and severity of symptoms over the entire course of treatment [29]. In this study, we showed the effect of "placebo" acupuncture, CNT. It might be better to reconsider the method of "placebo" acupuncture in the design of randomised controlled trials.

In this investigation, it is difficult to evaluate everything because of the small study population. We did not evaluate the efficacy after cessation of CNT. It is said that the effect of CNT continues about a week and the accumulative effect is reported in some other researches on acupuncture, so as in this study group. It should be investigated in our next research. However, we think that the potential of CNT for CIPN was clearly indicated. Another shortcoming of this study is the remarkable effect of CNT for pain breakthrough. The benefit of the application of CNT by specialists for CIPN was suggested for the first time in this study.

5. Conclusion

There are few standard therapies for CIPN, a condition that often leads to discontinuation of chemotherapy and to deterioration of the subsequent QOL. CNT may improve the symptoms of CIPN and associated side effects during the course of chemotherapy and even after a long interval since the last chemotherapy. CNT might be considered as one of the safe and effective alternative methods for CIPN.

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Research Article

Prenylated Flavonoids from *Morus alba* L. Cause Inhibition of G1/S Transition in THP-1 Human Leukemia Cells and Prevent the Lipopolysaccharide-Induced Inflammatory Response

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Morus alba L. (MA) is a natural source of many compounds with different biological effects. It has been described to possess anti-inflammatory, antioxidant, and hepatoprotective activities. The aim of this study was to evaluate cytotoxicity of three flavonoids isolated from MA (kuwanon E, cudraflavone B, and 4′-*O*-methylkuwanon E) and to determine their effects on proliferation of THP-1 cells, and on cell cycle progression of cancer cells. Anti-inflammatory effects were also determined for all three given flavonoids. Methods used in the study included quantification of cells by hemocytometer and WST-1 assays, flow cytometry, western blotting, ELISA, and zymography. From the three compounds tested, cudraflavone B showed the strongest effects on cell cycle progression and viability of tumor and/or immortalized cells and also on inflammatory response of macrophage-like cells. Kuwanon E and 4′-*O*-methylkuwanon E exerted more sophisticated rather than direct toxic effect on used cell types. Our data indicate that mechanisms different from stress-related or apoptotic signaling pathways are involved in the action of these compounds. Although further studies are required to precisely define the mechanisms of MA flavonoid action in human cancer and macrophage-like cells, here we demonstrate their effects combining antiproliferative and anti-inflammatory activities, respectively.

1. Introduction

The root bark of *Morus alba* L. (Moraceae; white mulberry) is used for its diuretic, antitussive, antidiabetic, and antipyretic effects in world traditional medicine [1]. Therefore, *Morus* species plants have been intensively studied from phytochemical point of view, and bioactive compounds of flavonoid character have been isolated. Within the flavonoid class of natural products, the prenylated subclass is quite rich in structural variety and pharmacological activities. Compounds obtained from *M. alba* L. possess anti-inflammatory, antibacterial, antiviral, antioxidant, and hepatoprotective activities [2–6]. Extracts obtained from *M. alba* L. were evaluated for their cytotoxicity against various tumor cells, such as K-562, B380 human leukemia cells, and B16 mouse melanoma cells [7]. Several studies have been published in

which bioactive compounds isolated from white mulberry exerted potent effect on human cancer cell lines. Morusin, one of the most efficient substances, showed strong activity against cervical carcinoma HeLa, breast carcinoma MCF-7, and hepatocarcinoma Hep3B cells [8]. Anticancer mechanism of morusin action in colorectal HT-29 cells is proposed to be mediated by induction of apoptosis and by suppression of NF-κB activity [9]. Another mulberry constituent, albanol A, induces apoptotic cell death in HL60 leukemia cell line via both the cell death receptor pathway by stimulation of death receptor and the mitochondrial pathway by topoisomerase II inhibition through caspase 2 activation [10].

The connection between inflammation and cancer can be thought of as consisting of two pathways: an extrinsic mechanism, where a constant inflammatory state contributes to increased cancer risk (such as in an inflammatory bowel disease), and an intrinsic mechanism, where acquired genetic alterations (such as activation of oncogenes) trigger tumor development [11]. The NF-κB signaling plays crucial roles in both precancerous chronic inflammation as well as cancer induced inflammation. An activation of this pathway induces expression of inflammatory cytokines, adhesion molecules, enzymes involved in the prostaglandin-synthesis pathway (such as COX-2), inducible nitric oxide synthase (iNOS), angiogenic factors, and antiapoptotic genes (such as Bcl-2) [12]. Proinflammatory cytokines implicated in carcinogenesis include, for instance, IL-1, IL-6, IL-15, colony stimulating factors (CSF), or TNF-α [13].

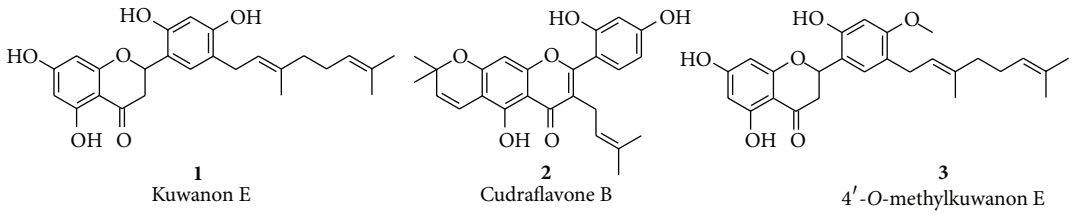
We have previously identified prenylated and geranylated flavanone compounds from plants of Moraceae and Paulowniaceae families with cytostatic activity in normal human fibroblasts and five human cancer cell lines [14]. Furthermore, we clarified the underlying molecular mechanisms mediating the effects of geranylated flavanone tomentodiplacone B on cell growth [15]. We showed that tomentodiplacone B induced accumulation of human monocytic leukaemia (THP-1) cells in G1 phase of cell cycle, which was in concert with downregulation of the cyclin E1 isoform and cyclin A2 levels, reduced CDK2 activity, and reduced pRb phosphorylation [15]. Our most recent work focusing on prenyl flavonoid cudraflavone B, which is contained in large amounts in the roots of white mulberry, showed unusually pronounced anti-inflammatory properties of this compound [2]. Moreover, throughout the course of experiments, we found that cudraflavone B had a strong effect on proliferation of human macrophage-like cells. It was therefore interesting to evaluate its effect on cell cycle progression and to elucidate the mechanisms of its cell proliferation inhibitory action. However, besides cudraflavone B (given designation **2**) we also isolated and characterized two other prenylated flavanones from *M. alba* L., which we have identified as kuwanon E (**1**), and 4′-*O*-methylkuwanon E (**3**), a new compound detected and described in our laboratory. Structures of all three tested *M. alba* L. prenylated (geranylated) flavonoids are shown in Figure 1(a). Based on our preliminary pilot data and the literature search (structure-effect relationship) we expected cytotoxic effect via targeting the cell cycle kinetics and viability.

The aim of our work was to evaluate effect of prenylated and geranylated flavonoids isolated from *M. alba* L. on proliferation of THP-1 cells and also to determine cell cycle profiles in several human cancer cells treated with *M. alba* L. flavonoids. As the role of inflammation in cancer is recently intensively discussed, we have also assessed anti-inflammatory effects of the previously mentioned flavonoids.

2. Methods

2.1. Test Compounds and Reagents. All three tested compounds (**1**, **2**, and **3**) were isolated and supplied by the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The identification of substances was carried out using HRMS, ^{1}H, and ^{13}C NMR analyses,and their purity exceeded 95% according to the HPLC analysis [2, 14]. These compounds are poorly soluble in water; therefore, fresh 10 mM stock solutions in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were prepared 1 day prior to experiments and stored at −20°C. These solutions were further diluted in the culture media to the desired final concentrations. RPMI 1640, DMEM, and IMDM culture media, phosphate buffered saline (PBS), and antibiotics (penicillin and streptomycin) were purchased from Lonza (Verviers, Belgium). Foetal bovine serum (FBS), phorbol myristate acetate (PMA), prednisone (purity ≥ 98%), and the lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich. Instant ELISA Kits (eBioscience, Vienna, Austria) were used to evaluate the production of TNFα and IL-1β. Cytoscreen Kit (BioSource Europe S.A., Nivelles, Belgium) was used to detect IL-1RA cytokine by ELISA method. Mouse monoclonal antibody against cyclin B1 (MS-868) was purchased from Neomarkers (Fremont, CA, USA). Mouse monoclonal antibody against cyclin A (sc-53228) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against poly(adenosine diphosphate (ADP) ribose) polymerase (PARP), caspase 3, and phospho histone H3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibody against γ-H2AX [pS139] (05-636) was purchased from Millipore (Billerica, MA, USA). Mouse monoclonal antibodies against pRb (554136) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal antibody against pRb [pT821] (44-582G) was purchased from BioSource (Carlsbad, CA, USA). Parthenolide (PTL), oxaliplatin, cisplatin and camptothecin, and all other reagents were purchased from Sigma-Aldrich.

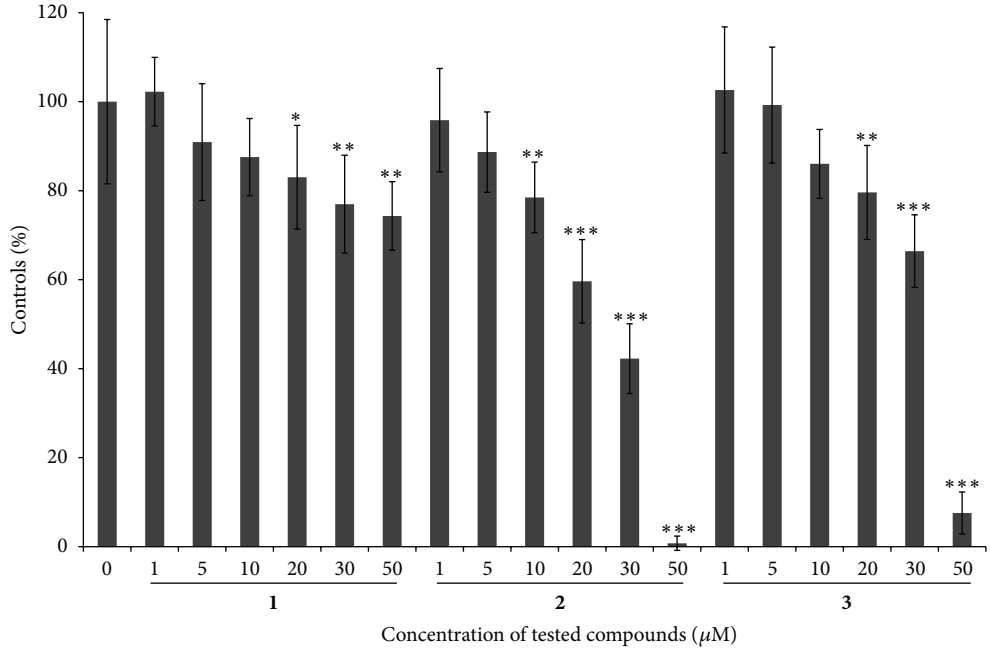
2.2. Cell Culture. The human monocytic leukemia THP-1 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK; methods of characterization: DNA fingerprinting (multilocus probes) and isoenzyme analysis). Cells were cultured in RPMI 1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), 10% FBS, and 2 mM L-glutamine. Cultures were kept in an incubator at 37°C in a water-saturated 5% CO_2 atmosphere in air. Cells were passaged at approximately 1-week intervals. Cells were routinely tested for the absence of mycoplasma infection (Hoechst 33258 staining method). Mouse mammary epithelial cell line, SCp2 cells



(a)

Tested substance	LD_{50} (μM ± SD)
1	>50
2	24.3 ± 2.41
3	45.7 ± 3.72
Oxaliplatin	1.7 ± 0.64
Camptothecin	0.2 ± 0.07

(b)



(c)

Figure 1: Toxicity and inhibitory effects of tested MA compounds on THP-1 leukemia cell proliferation. (a) Structure of *M. alba* L. prenylated flavonoids. (b) THP-1 cells were seeded (2×10^5 cells/mL), treated with the indicated concentrations of **1**, **2**, and **3** for 24 h, cell numbers were counted, and viability was determined by erythrosin B exclusion. Toxicity was expressed as the LD_{50} values. (c) THP-1 cells were seeded (5×10^4 cells/well) in 96-well plates. Proliferation of cells was determined using WST-1 assays. Bars represent the proliferation of cells cultured in the presence of increasing concentrations of MA compounds as a percentage of controls at 24 h. The results shown are expressed as the means ± S.D. of three independent experiments, with each condition tested in triplicate ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

(kindly provided by P. Y. Desprez, Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, CA, USA), was cultured in DMEM supplemented with insulin 5 μg/mL (Sigma, St. Louis), 1% penicillin/streptomycin mixture (Lonza Walkersville, Inc., USA), and 5% heat inactivated FBS (Sigma-Aldrich), in a humidified incubator (95% air, 5% CO_2) at 37°C [16]. THP-1 cells were split into 24-well plates to achieve concentration of 100 000 cells/mL and were differentiated to macrophages by a phorbol myristate acetate (PMA) as described previously [17]. PC3 and DU-145 cells were obtained from the American Type Culture Collection (ATCC). PC3 and DU-145 were cultured in RPMI-1640, Ham's F12, or McCoy's media, respectively (Gibco Invitrogen Corporation, Carlsbad, CA, USA) with 2 mM L-glutamine, streptomycin (0.1 mg/mL), and penicillin (100 U/mL), and supplemented with 10% fetal bovine serum. LAPC-4 cells [18], a generous gift of Dr. R. Reiter (UCLA, Los Angeles, CA, USA), were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with $NaHCO_3$, penicillin/streptomycin, 10% FBS, and 1 nM R1881 (PerkinElmer). Benign prostatic hyperplasia (BPH) epithelial cells BPH-1 [19] were obtained from the German Collection

of Microorganisms and Cell Cultures. The cells are androgen unresponsive and were cultured in RPMI 1640 (Invitrogen), supplemented with 20% bovine fetal serum (PAA Laboratories, Pasching, Austria), 5 μg/mL transferrin, 5 ng/mL sodium selenite, 5 μg/mL insulin (Invitrogen), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (PAA). Cells were cultured at 37°C in a humidified 5% CO_2 incubator.

2.3. In Vitro Analysis of Cytotoxicity and Cell Proliferation. THP-1 cells were seeded (2×10^5 cells/mL) and incubated for 24 h at 37°C with 5% CO_2 with tested compounds dissolved in DMSO (Sigma-Aldrich) in concentrations ranging from 1 to 50 μM in RPMI 1640 medium. The maximum concentration of DMSO in the assays never exceeded 0.1%. Numbers and viabilities of the cells were determined by counting with a hemocytometer as we previously described [15]. Cell proliferation was determined using a WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For WST-1 assays, cells were seeded into 96-well plates (5×10^4 cells/well in 100 μL culture medium) in triplicates in complete RPMI 1640 medium, and measurements were taken 24 h after adding the tested MA compounds. All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com/).

2.4. Cell Cycle Analysis. Cancer THP-1, LAPC-4, DU-145, PC3 cells, and human nontumorigenic benign prostatic hyperplasia BPH-1 cells were incubated with increasing concentrations of tested MA compounds for 24 h, washed in PBS (pH 7.4), and fixed for 30 min in an ice-cold 70% ethanol. Fixed cells were washed three times in PBS (pH 7.4) and incubated with RNase A (0.02 mg/mL) (Boehringer, Ingelheim, Germany) for 30 min at 37°C. Nuclei were stained with propidium iodide (40 μg/mL) and analysed by flow cytometry using a Beckman Coulter Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). Cell cycle distribution was analysed using FlowJo software (http://www.flowjo.com/).

2.5. Western Blotting. Cells were washed three times with PBS (pH 7.4) and lysed in 100 mM Tris-HCl (pH 6.8) containing 20% glycerol and 1% SDS. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Lysates were supplemented with bromophenol blue (0.01%) and β-mercaptoethanol (1%). Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA), immunodetected using the appropriate primary and secondary antibodies, and visualised with ECL Plus reagent (Amersham, Aylesbury, UK) according to the manufacturer's instructions.

2.6. Treatment of THP-1 Cells with Drugs, Induction of Inflammatory Response, and Determination of Cytokines Production. Macrophages differentiated from THP-1 cells were pretreated for 1 h with tested compounds dissolved in DMSO to obtain final concentrations of 10 μM (this concentration lacked cytotoxic effect). For comparison with conventional drugs, 1 μM prednisone dissolved in DMSO was used. Vehicle-treated cells contained a vehicle (DMSO) only. The concentration of DMSO was 0.1% in each well. The inflammatory response was triggered by adding LPS dissolved in water (1 μg/mL) to drug-pretreated macrophages, and cells were incubated for another 24 h. After this time period, medium was collected and the concentration of cytokines was measured by ELISA assay. The lowest detection limit was 7.8 pg/mL for TNF-α and 31.3 pg/mL for both IL-1β and IL-1RA. LPS-untreated cell served as controls.

2.7. Treatment of SCp2 Cells with Drugs, Induction of Inflammatory Response, and Zymography. SCp2 cells were plated in a 24-well plate in density of 4×10^4 cells/mL. After 24 h of incubation in medium containing 5% FBS, the medium was replaced, the cells were washed by PBS and fresh media supplemented with 1% FBS, and tested compounds were added. Final concentrations of tested compounds were 5 μM (this concentration lacked cytotoxic effect (data not shown)). Vehicle-treated and control cells were prepared using the same protocol as for THP-1 macrophages. For comparison with conventional drugs, parthenolide (5 μM dissolved in DMSO) was used, because of its usual use as a control for this type of cells and experiments and its well-known ability to inhibit the expression of matrix metalloprotease (MMP)-9 [20]. The inflammatory response was triggered by adding a nontoxic dose of LPS (10 μg/mL) to the drug-pretreated SCp2 cells, which were then incubated at 37°C for another 24 h [21]. After this time period, medium was collected and the pro-MMP-2 and MMP-2 activity was measured by zymography as described previously by Talhouk et al. [22]. Briefly, 20 μL of collected medium was loaded into polyacrylamide gel impregnated by 0.1% gelatin. After electrophoresis, SDS from gels was washed out by 2.5% Triton X-100, and gels were incubated for 30 min at room temperature (~23°C) and overnight (16–20 h) at 37°C in developing buffer (50 mM Tris (pH 8.8), 5 mM $CaCl_2$, 3 mM NaN_3, and 0.5% Triton X-100). Gels were then stained by Coomassie blue [22]. Intensity of digested regions was determined by densitometry followed by calculation using AlphaEaseFC 4.0.0 software (Alpha Innotech, USA). It should be noted that the conditioned medium contained active MMP-2, which represented 75.3% of measured activity. Therefore, this value was subtracted from all obtained results of MMP-2 activity.

2.8. Statistical Analysis. Statistical significance was tested using the one-way ANOVA with Dunnett's test and Tukey post test for comparisons between the means, and differences between two conditions were retained for $P < 0.05$. Statistical significance was determined at levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

*3.1. Cytotoxic and Growth Inhibitory Effects of **1**, **2**, and **3** on THP-1 Cells.* To determine the effects of all three tested

substances obtained from *M. alba* on the viability and growth of human leukemia cells, the THP-1 cells were exposed for 24 h to increasing concentrations (1, 2.5, 5, 10, 20, and 50 μM) of **1**, **2**, and **3**, respectively, stained for viability, and counted by hemocytometer. From this data, the LD_{50} values for each MA compound were calculated (Figure 1(b)). Toxicity expressed as LD_{50} increased as follows: **1** (>50 μM), **3** (45.7 ± 3.72), and **2** (24.3 ± 2.41). To compare toxicity of MA compounds with already known chemical or natural substances, we assessed LD_{50} of oxaliplatin (1.7 ± 0.64) and camptothecin (0.2 ± 0.07), and, in both, it showed much lower toxic concentration values. Subsequent WST-1 assay, determining cell number using metabolic activity as a readout following exposure to MA compounds for 24 h, revealed that proliferation of THP-1 cells was inhibited by all three tested substances. As shown in Figure 1(c), substance **2** exhibited the strongest effect, as 10 μM and higher doses caused dose-dependent inhibition of THP-1 cell growth. The significant reduction of metabolic activity ($P < 0.05$) was though observed in cells treated with each of the three flavonoids at concentrations of 20 μM or higher. Based on cytotoxicity and proliferation data, the concentration range of MA compounds from 5 to 30 μM was selected for all subsequent experiments.

*3.2. Effects of **1**, **2**, and **3** on Distribution of Cells in Cell Cycle Phases.* In order to investigate the effect of tested substances on the cell cycle progression, we performed cell cycle analysis based on DNA content using flow cytometry of THP-1 cells. The data shown in Figures 2(a)–2(c) demonstrate that all compounds tested (**1**, **2**, and **3**) accumulate human leukemia cells in G1 phase dose-dependently after 24 h treatment. While the percentage of S phase cells decreased, the percentage of cells with 4N DNA content, representing G2/M phase, was unchanged upon treatment with tested compounds. This effect was dominant in substance **3**, lasting even after 72 h (data not shown).

Since compound **2** exerted the strongest impact on viability and proliferation, together with impact on the cell cycle profile of THP-1 cells (observed already from 10 μM concentration), we have expanded our analysis with this substance to further 3 human cancer cell lines (Figure 2(e)). The inhibition of G1/S transition, accompanied by the decreased proliferation caused by **2**, was observed in all cancer cell lines used in this experiment (LAPC-4—metastatic prostate, established from lymph nodes in SCID mice; PC3—androgen receptor null, p53-null, metastatic (bone) prostate cancer; and DU145—androgen receptor, p53-mutated, metastatic (brain) prostate cancer). To assess whether **2** affects also the cell cycle of human nontumorigenic cell line, we exposed the prostate epithelial BPH-1 cells, derived from the benign prostatic hyperplasia, to this compound. Interestingly, the distribution of BPH-1 cells in all three cell cycle phases remained unchanged even after the treatment with high concentrations of **2** used in the study (20 and 30 μM) (Figure 2(e)).

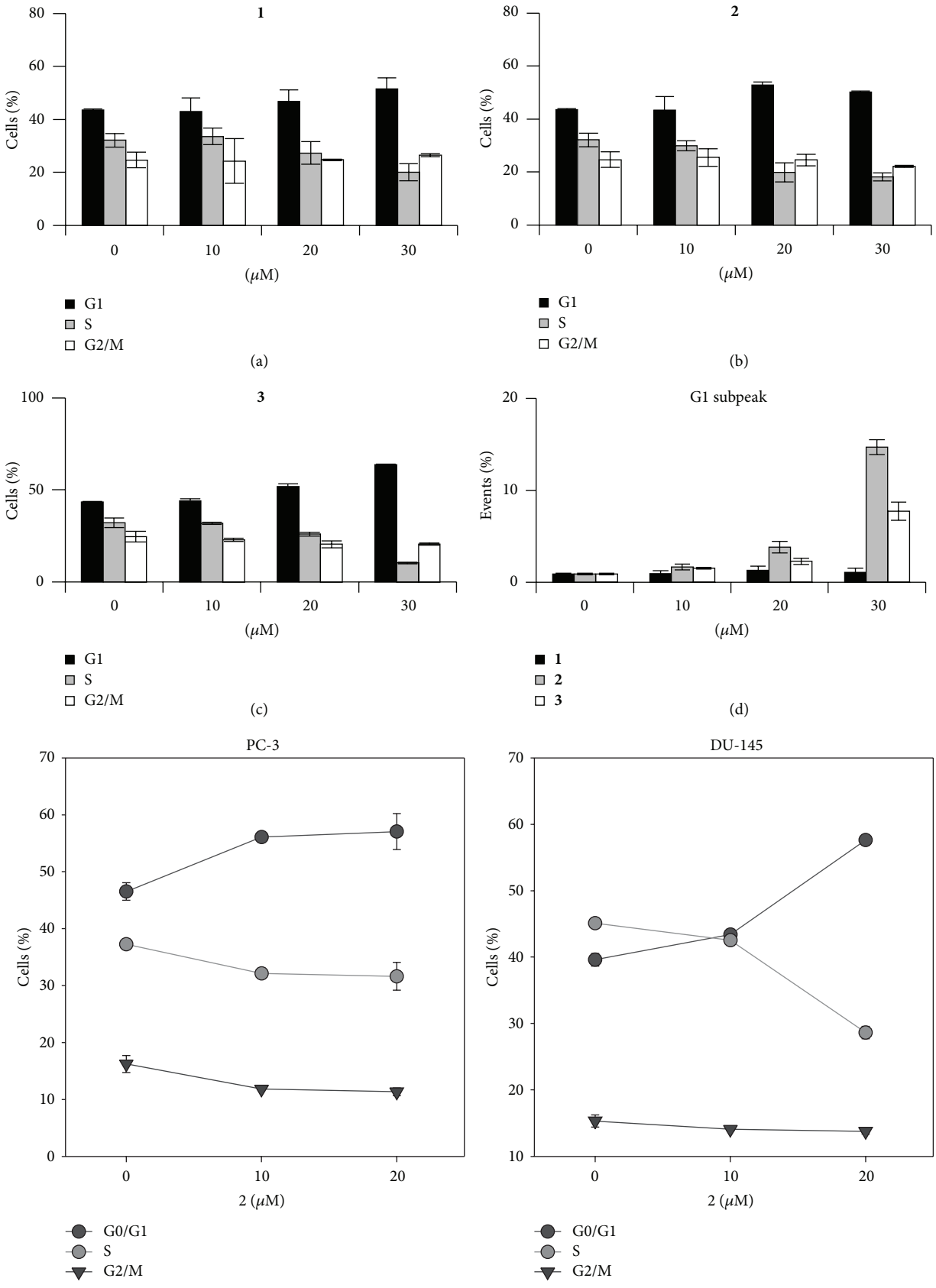
Although a G1 subpeak in a DNA histogram detected by flow cytometry cannot be considered as specific hallmark of apoptosis, it represents besides cellular debris also the apoptotic population of cells [23, 24]. The appearance of the G1 subpeak was increased at 24 h after beginning the treatment with MA compounds, although with a different intensity of this effect (Figure 2(d)). While **1** exerted no G1 subpeak increase, the strongest induction of apoptosis was found in 30 μM **2**-treated THP-1 cells (~15-fold higher compared to control). Significant increase of G1 subpeak (~8-fold higher compared to control) was caused also by 30 μM **3** compound. Nevertheless, even the highest concentration of **2** used did not cause such massive apoptosis that we found in 5 or 10 μg/mL cisplatin, included as a model compound (~36- and ~60-fold higher, resp., compared to control).

3.3. Expression of Cell Cycle Regulators in MA-Treated Cells. Based on the fact that all tested compounds cause accumulation of cells in G1 phase, we determined the expression and phosphorylation status of key cell cycle and stress-related proteins. Phosphorylated Rb protein is the key regulatory molecule, which coordinates processes critical for G1/S progression. We therefore examined whether pRb phosphorylation is suppressed in THP-1 cells treated by MA compounds. As shown in Figures 3(a) and 3(c), 24 h exposure to 20 μM **1** or **3** results in reduced phosphorylation of Rb protein on serine 780. For **2** this effect was even more pronounced (Figure 3(b)). Phosphorylation on serines 807/811 was also decreased in THP-1 cells exposed to MA compounds, in clearly dose- dependent manner (Figures 3(a)–3(c)). It is highly probable that MA-induced Rb dephosphorylation corresponds to the accumulation of cells in G1 phase.

Another protein involved in cell cycle machinery, which we analyzed in MA-treated cells, was proliferating cell nuclear antigen (PCNA). This protein is well known as a DNA sliding clamp for DNA polymerase delta and as an essential component for eukaryotic chromosomal DNA replication and repair [25]. All flavonoids tested downregulated the expression of PCNA in THP-1 cells (Figures 3(a)–3(c)), again correspondingly to the observed decrease of cells in S phase of the cell cycle.

Cyclins A and B are members of the cyclin family, expression of which fluctuates during cell cycle progression peaking in S and G2 phases, respectively. We found that none of the tested MA compounds affects the quantity of these cyclins, when measured in asynchronously growing cells. Moreover, phosphorylation of histone H3 at threonine 11, which normally peaks at M phase, remained unaffected even after 24 h treatment with MA compounds. Unchanged phosphorylation of histone H3 with normal expression of cyclins A and B suggests that MA compounds do not influence progression through G2 and M phases of cell cycle.

Caspase 3-mediated PARP cleavage has been considered as a hallmark of apoptosis. It is also known that PARP activation is induced by DNA strand breaks [26]. Neither **1** nor **3** did cause PARP cleavage, and so its activation in THP-1 cells. However, increased histone γ-H2AX phosphorylation together with cleavage of both caspase 3 and PARP in **2**-treated THP-1 cells indicates the activation of the stress signaling apoptotic pathways caused by the highest concentration used (20 μM).



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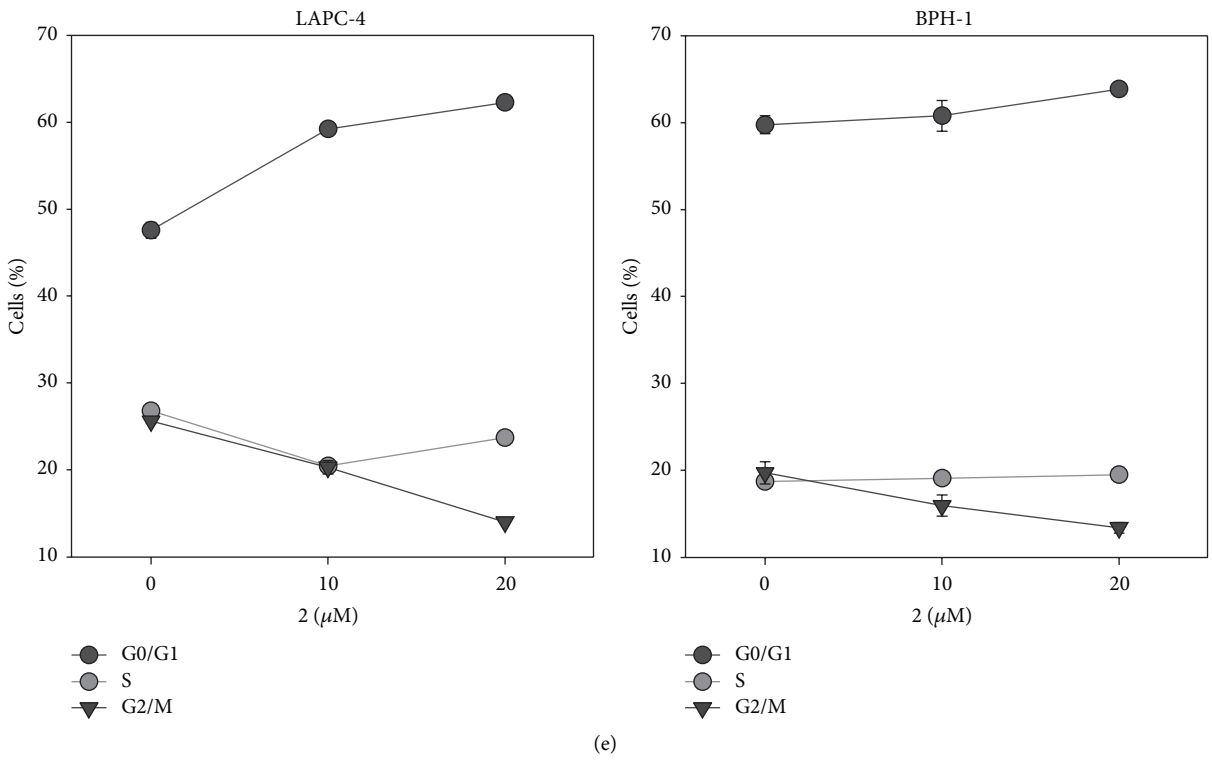

Figure 2: Treatment with *M. alba* L. prenylated flavonoids causes accumulation of several cancer cells in G1 phase. (a) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **1** as determined by flow cytometry. (b) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **2** as determined by flow cytometry. (c) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **3** as determined by flow cytometry. (d) Quantification of G1 subpeak in MA flavonoids treated THP-1 cells. (e) Cell cycle distribution at 24 h upon treatment of cancer PC3, DU-145, LAPC-4, and immortalized BPH-1 cells with **2** as determined by flow cytometry.

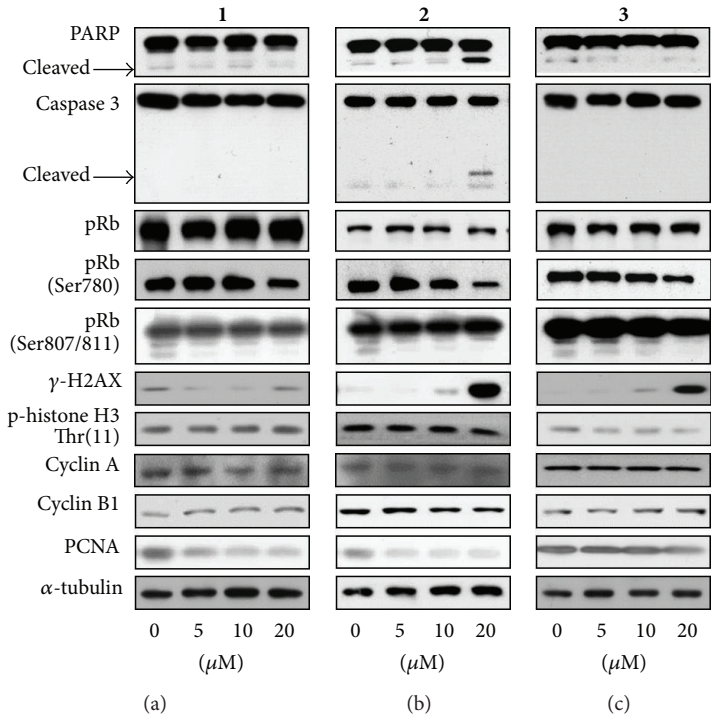

Figure 3: Expression of cell cycle regulators and stress response proteins after 24 h of (a) **1**, (b) **2**, and (c) **3** treatment.

3.4. Behaviour of Inflammatory Response Markers in MA-Treated Cells. Protein TNF-α together with other cytokines, such as interleukins, not only plays crucial role in the inflammatory response but also is involved in carcinogenesis [13]. To investigate whether antiproliferative effects of MA compounds are accompanied by anti-inflammatory activity, we assessed levels of selected inflammatory response markers secreted into the culture medium by LPS-activated macrophages derived from THP-1 cell line. As evident from Figure 4(a), LPS-induced TNF-α secretion by macrophages was reduced upon the treatment with MA compounds, similarly to prednisone used as the reference anti-inflammatory drug. Notably, all three compounds tested were significantly more effective than prednisone. Levels of IL-1β, the most studied member of the IL-1 family [27], produced by THP-1-derived macrophages were slightly decreased by tested substances, except for **3** (Figure 4(b)). Treatment with this compound (10 μM) led to significant ($P < 0.01$) increase of IL-1β secreted to cell culture medium. The natural antagonist of IL-1β is IL-1RA, and their mutual ratio is crucial for a progression of inflammation and maintaining a homeostasis. All tested flavonoids, similarly to prednisone, significantly decreased the secretion of IL-1RA (Figure 4(c)). This secretion attenuation affected the IL-1β/IL-1RA ratio (Figure 4(d)). This increase was nonsignificant for compounds **1**, **2**, and prednisone. On the other hand, 4′-*O*-methylkuwanon E (**3**) increased the IL-1β/IL-1RA ratio by the factor of 5.33. It is caused by enormously elevated secretion of IL-1β. Matrix metalloproteinase 2 (MMP2) is involved in the tissue development and remodelling, but it also contributes to inflammation progression. It is secreted as inactive pro-MMP2 form, which is extracellularly cleaved to its active form. The amount of (pro-)MMP2 was significantly decreased only by **2** and the control drug parthenolide (PTL) (Figure 5(a)) in SCp2 cell line. Whereas PTL inhibits proteinase activity to the level typical for unstimulated cells, **2** was able to reduce the (pro-)MMP2 activity below these control cells. **2** uniquely and significantly decreased the pro-MMP2/MMP2 ratio (Figure 5(b)).

4. Discussion and Conclusions

Relevance of the crosstalk between components of the immune system and cancer cells is widely discussed. During the last decade the clear evidence that inflammation plays a critical role in tumorigenesis has been obtained, and some of underlying molecular mechanisms have been elucidated [28]. A role of inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven [11].

In the present study, we assessed cytotoxicity and the effects of three prenylated (geranylated) flavonoids from *M. alba* L., kuwanon E (**1**), cudraflavone B (**2**), and 4′-*O*-methylkuwanon E (**3**) on cell cycle progression and selected cell cycle regulatory proteins. We have also extended our study with the aim of evaluating the effect of these substances on proinflammatory markers, because we recently reported that **2** has potent anti-inflammatory properties in human macrophages [2]. Compounds are poorly soluble in water; therefore, we used DMSO as a solvent. The final DMSO concentration of 0.5–1% is frequently employed in *in vitro* studies to solubilize/deliver bioactive compounds to cells. However, it has been shown that DMSO exhibits a myriad of biological actions, such as reported effects on cell cycle, differentiation, inflammatory response, and apoptosis studies [29–31]. Since our intention was focused on evaluation of these types of effects, it was necessary to take into account the effects of DMSO in arrangement of all conducted experiments. In particular, the concentration of DMSO in experiments never exceeded 0.1%. Moreover, to minimalize misinterpretations of our results due to biological effects of DMSO, we employed DMSO-only-treated THP-1 cells as controls in each experiment setting. Based on our previously published results [14, 15, 17] we used human monocytic leukaemia cells THP-1 as a model system to detect cytotoxic and cytostatic effects of newly isolated natural compounds and THP-1-derived macrophages for studies on inflammatory response. We found strong antiproliferative effects of all three tested MA compounds in concentrations ranging from 10 to 50 μM. When comparing these data with the LD_{50} values, we may conclude that unlike **2**, both **1** and **3** at concentrations of 20 μM and 30 μM had significant growth inhibitory effect without being cytotoxic to the cells. As regards substance **2**, we speculate that the observed reduction of metabolic activity is more likely a sign of cell dying rather than growth inhibition.

To reveal whether antiproliferative effects seen in THP-1 cells after 24 h treatment with MA flavonoids reflect inhibition of cell transition between specific cycle phases, we conducted the cell cycle analysis. Our results showed that all tested compounds caused accumulation of THP-1 human leukemia cells in G1 phase of cell cycle (and inhibited their entry into the S phase) in a dose-dependent manner. Taking into account the strength of **2** effect on viability, proliferation, and the cell cycle profile (showed from the concentration of 10 μM, in contrast to other MA substances), we exposed three other human cancer as well as nontumorigenic cell lines to **2**. While in all tumor cells **2** exhibited inhibitory effect on the G1/S transition, in nontumor line (prostate epithelial BPH-1 cells) such activity was not observed. This might indicate a partially selective effect of this substance on tumor versus nontumor cells. Nevertheless, such selectivity of **2** would have to be verified by more detailed analysis.

The cell cycle analysis allowed us to study the percentage of THP-1 cells in specific phase, including determining sub-G1 peak, which covers also cells undergoing the process of apoptosis. One of the characteristic events of apoptosis is the proteolytic cleavage of poly(ADP-ribose)polymerase (PARP), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. Caspases, in particular caspases 3 and 7, cleave the 116-kDa form of PARP at the DEVD site to generate an 85- and a 24-kDa fragment [26]. PARP is inactivated by caspase 3 cleavage (in a specific domain of the enzyme) during programmed cell death. One-day treatment with **1** had no effect on induction of apoptosis as determined

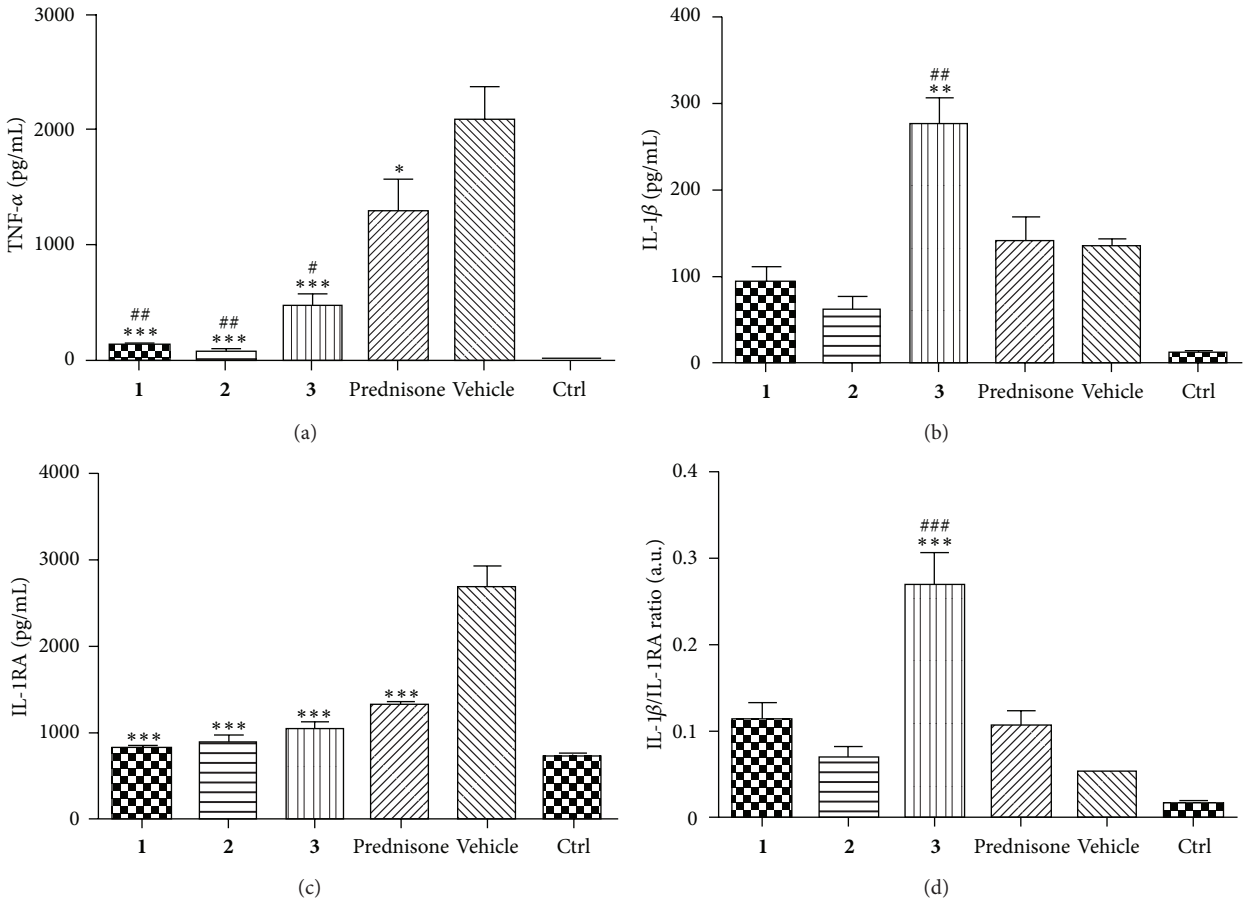

Figure 4: (a) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced TNF-α secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. *Significant difference in comparison to vehicle-treated cells ($P < 0.05$), ***significant difference in comparison to vehicle-treated cells ($P < 0.001$), $^{\#}$significant difference in comparison to prednisone-treated cells ($P < 0.05$), and $^{\#\#}$significant difference in comparison to prednisone-treated cells ($P < 0.01$). (b) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced IL-1β secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. **Significant difference in comparison to vehicle-treated cells ($P < 0.01$); $^{\#\#}$significant difference in comparison to prednisone-treated cells ($P < 0.01$). (c) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced IL-1RA secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$). (d) Ratio IL-1β/IL-1RA production calculated for macrophages derived from THP-1 cell line. Values were obtained from ELISA measurements of individual cytokines as it is described in Figures 2 and 3. Results are expressed as means ± S.E. for three independent experiments. A.U. = arbitrary unit. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$); $^{\#\#\#}$significant difference in comparison to prednisone-treated cells ($P < 0.001$).

by flow cytometry assessment of G1 subpeak and western blot analysis of PARP and caspase 3 cleavage. Significant increase of G1 subpeak (~8-fold higher compared to control) was caused by 30 μM **3** compound; however, no cleavage of PARP and caspase 3 was observed (Figure 3(c)). Conversely, massive increase in G1 subpeak (~15-fold higher compared to control), together with occurrence of both apoptotic markers (cleaved PARP and subsequently caspase 3), was observed in cells exposed to **2** for 24 h. However, effects of **2** on THP-1 cells are not comparable with those of cisplatin, added as a model anticancer drug. Cisplatin caused considerably more substantial changes in both G1 subpeak accumulation and caspase 3 cleavage (see Figure S1 in Supplementary Material Available online at http://dx.doi.org/10.1155/2013/350519), suggesting that **2** mechanism of action is not similar to that of platinum derivatives. These results prompted us to experimentally address molecular mechanisms underlying the effects of MA compounds on cell growth.

Cyclins A and B are members of the cyclin family, with the maximum of their expression during S and G2 phases of

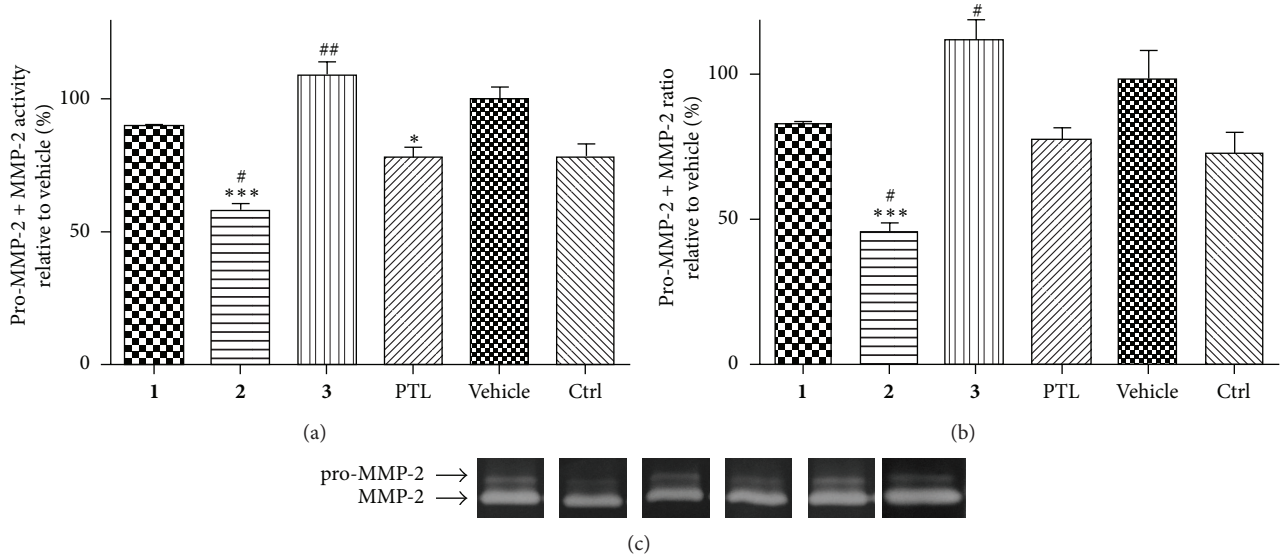

Figure 5: Effects of *M. alba* L. prenylated flavonoids and the reference drug parthenolide (PTL) on LPS-induced (pro-)MMP-2 activity at SCp2 cells. Cells were pretreated with given compounds (5 μM), parthenolide (5 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Activity of (pro-)MMP-2 was detected by zymography (a). Intensity of digested bands was analyzed by densitometry. (b) shows pro-MMP-2/MMP-2 ratio. Showed gels represent results of three independent experiments (c). Results are expressed as means ± S.E. for three independent experiments. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$), $^{\#}$significant difference in comparison to parthenolide-treated cells ($P < 0.05$), and $^{\#\#}$significant difference in comparison to parthenolide-treated cells ($P < 0.01$).

a cell cycle. Cyclin A is required for cell to progress through the S phase, and cyclin B is necessary for cells to enter mitosis and divide into two daughter cells [32]. It is also known that activation of tumor suppressor retinoblastoma protein (pRb) permits transcription of key S-phase-promoting genes, including some that are required for DNA replication. In contrast, dephosphorylation of pRb slows the progression of cells into S phase [33]. None of the MA compounds tested were found to reduce, after 24 h exposure, the expression of cyclins A and B. This fact, together with the reduced pRb phosphorylation caused by all MA compounds, possibly indicates that these substances affect rather the G1/S than G2/M transition. Flow cytometry data further support this hypothesis, since significant accumulation of cells in G1 at the expense of S phase was observed upon the treatment with MA compounds. Since such cell cycle distortion could be mediated by stress response signaling pathways, their activation was evaluated in THP-1 cells treated by all three MA flavonoids. In THP-1 cells stress-associated regulators such p21, p27, and p53 proteins are not detectable [15]. Therefore, we focused on histones γ-H2AX (becomes phosphorylated on damaged DNA) and H3 (its phosphorylation on Thr11 correlates with mitotic/meiotic chromosome condensation). Cells treated with any MA compound displayed no changes in phosphorylation of histone H3 on the given residue. For γH2AX we observed increased phosphorylation only in cells treated with **2** at concentration of 20 μM, which is the same as such causing cleavage of caspase 3 and PARP. Collectively, we speculate that **2** exerts mode of action that is different from that of **1** and **3**. Compound **2** seems to inhibit proliferation via triggering the stress-related pathway leading to Rb dephosphorylation and apoptosis with typical cleavage of PARP and caspase 3. On the other hand, no induction of the stress-related proteins occurs in **1**- and **3**-treated cells, and **1** in all tested concentrations clearly affects PCNA, which facilitates and controls DNA replication, and is at the very heart of cell-cycle progression.

As mentioned at the beginning, our previous study on effects of cudraflavone B (**2**) in human macrophages showed an interesting anti-inflammatory activity of this flavonoid. Since newly characterized compounds **1** and **3** were also isolated from *M. alba* L., and chemically belong to the same category, we expected similar effects. Yet, except secretion of TNF-α and IL-1RA, we found different results after application of MA substances to macrophages. Therefore, only relatively little structural differences between compounds tested (presence of 2,3 double bond at **2**, presence and position of prenyl or geranyl side chains, or substitution of flavonoid *B*-ring) strongly affect the mechanism of action and play a role in final effect of compound.

Importantly, our results pointed to huge differences among the tested compounds. **1** and **2** showed similar inhibition effect on TNF-α, IL-1β, and IL-1RA expression. On the other hand, **3**, which differs from **1** by substitution of one hydroxyl group on the *C*-ring for methoxy group, attenuated only TNF-α and IL-1RA expression, but less effectively than **1** or **2**, and secretion of IL-1β was strongly elevated. It is obvious that all three compounds are able to downregulate expression of genes that are under transcriptional control of NF-κB. In comparison with other two cytokines, IL-1β is synthetized as proprotein, and it is cleaved into active form by caspase-1-containing inflammasome [34]. Increased IL-1β

production in the presence of LPS in cells was observed following incubation with doxorubicin and daunorubicin [35] or Cu(II) complexes [36]. We cannot exclude the possibility that **3** activates an inflammasome, and, thus, augments IL-1β secretion. It should be noted that although **1** and **2** inhibited IL-1β secretion, the effect is much smaller than in the case of TNF-α. This may indicate that all three compounds are able to positively regulate inflammasome action. The low ability of tested compounds to downregulate proinflammatory IL-1β and significant downregulation of anti-inflammatory IL-1RA are showed in higher IL-1β/IL-1RA ratio. The MMP-2 activity is in agreement with TNF-α and IL-1β expression—**2** significantly decreased its level, **1** inhibited its activity only slightly, and **3** moderately raised its level. According to these results, the highest antiphlogistic potential has **2** followed by **1**. Depending on conditions, flavonoids can act both as prooxidants and antioxidants. The ability to cause dysfunction of mitochondria by prooxidant effect is connected with possible mechanisms of anticancer action, which may lead to apoptosis of tumor cells. Their antioxidant activity is connected with direct scavenging effect of excessive reactive oxygen/nitrogen species or with interaction with enzymes involved in their production or elimination. Interaction with enzymes responsible for carcinogen activation can lead to prevention of tumor formation [37]. Only a few reports on anti/prooxidative activity of compounds analysed in this study have been published. In general, these compounds do not fulfil the structural requirements needed for direct scavenging effect *in vitro* [38], which was confirmed for compound **2** [6, 39]. Park et al. [40] showed only weak activity of **2** in protecting LDL particles against oxidation (TBARS assay), but the inhibition of NO formation mediated via inhibition of iNOS was proved using RAW 264.7 cells. Protective effects of prenylated compounds (**1** and **3**) against oxidative stress-induced damage of human neuroblastoma SH-SY5Y cells were observed, showing their potential antioxidant activity [41]. Compound **1** showed inhibitory activity on NO production in RAW 264.7 cells [42]. Possible pro/antioxidant activity of tested compounds and its interconnection with their anticancer effects should be clarified in further experiments.

In conclusion, the reported active agents isolated from *M. alba* L. have an interesting impact on human cells, which are involved in both tumor and inflammation. Of the three compounds tested, **2** showed the strongest effects on cell cycle progression and viability of tumor cells and on inflammatory response of macrophage-like cells. In addition, substances **1** and **3** exerted more sophisticated rather than direct toxic effect on used cell types. Our data indicate that mechanisms different from stress-related or apoptotic signaling pathways are involved in the action of these compounds. Although further studies are required to precisely define the mechanisms of MA flavonoid actions, here we clearly demonstrate their effects combing antiproliferative and anti-inflammatory activities in human cancer and macrophage-like cells, respectively. Confirmed dual activity of tested prenylated flavonoids could be an inspiration for chemical modifications of their structures or isolation of similar substances in order to get more potent agents usable for clinical practice in future.

Abbreviations

MA: *Morus alba* L.
1: Kuwanon E
2: Cudraflavone B
3: 4′-*O*-methylkuwanon E
PBS: Phosphate buffered saline
FBS: Fetal bovine serum
DMSO: Dimethylsulfoxide
PMA: Phorbol myristate acetate
LPS: Lipopolysaccharide
PTL: Parthenolide.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Impact of Standardized Allergen-Removed *Rhus verniciflua* Stokes Extract on Advanced Adenocarcinoma of the Ampulla of Vater: A Case Series

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Background. Adenocarcinoma of the ampulla of Vater (AAV) is a rare malignancy that has a better prognosis than other periampullary cancers. However, the standard treatment for patients with relapsed or metastatic AAV has not been established. We investigated the clinical feasibility of standardized allergen-removed *Rhus verniciflua* stokes (aRVS) extract for advanced or metastatic AAV. *Patients and Methods*. From July 2006 to April 2011, we retrospectively reviewed all patients with advanced AAV treated with aRVS extract alone. After applying inclusion/exclusion criteria, 12 patients were eligible for the final analysis. We assessed the progression-free survival (PFS) and overall survival (OS) of these patients during the follow-up period. *Results*. The median aRVS administration period was 147.0 days (range: 72–601 days). The best tumor responses according to Response Evaluation Criteria in Solid Tumors were as follows: two with complete response, two with stable disease, and eight with progressive disease. The median OS was 15.1 months (range: 4.9–25.1 months), and the median PFS was 3.0 months (range: 1.6–11.4 months). Adverse reactions to the aRVS treatment were mostly mild and self-limiting. *Conclusions*. Prolonged survival was observed in patients with advanced AAV under the treatment of standardized aRVS extract without significant adverse effects.

1. Background

Adenocarcinoma of the ampulla of Vater (AAV) is a rare cancer representing about 6% of all periampullary tumors and only 0.2% of all gastrointestinal cancers [1, 2]. From 1973 to 2005, 5,625 cases of AAV were reported in the USA, and the frequency of the disease has been increasing [3]. Furthermore, Asian-Pacific people have a higher incidence of this cancer, whereas African American men have a slightly lower incidence in the USA [3, 4].

The prognosis of AAV is better with a higher resectability rate compared to other periampullary malignancies including the pancreatic or the distal bile duct cancers because even small tumors of the ampulla generally produce obstructive jaundice earlier than tumors in other locations [4–6]. On the other hand, patients who relapse or present with metastatic AAV have a poor prognosis with a two-year overall survival rate of about 10% [3]. There is no standard chemotherapeutic regimen for advanced AAV patients and no standard adjuvant therapy after curative pancreaticoduodenectomy. Further, a considerable number of patients could not undergo aggressive chemotherapy or radiation therapy because of poor performance status or adverse effect [4].

Rhus verniciflua Stokes (RVS) of the Anacardiaceae family, commonly known as the lacquer tree, was traditionally used for treating diseases of the digestive system, including tumors, in Korea and China in the 15th century [7, 8]. Several reports have been published on the clinical efficacy of RVS in stabilizing cancer progression or inducing tumor regression [9–14]. Many experimental studies have recently

shown that the flavonoids from RVS or the whole extract have antiproliferative and apoptotic activities in human cancer cell lines [7, 8, 15–17]. Here, we report on the outcomes of using a standardized extract from RVS (aRVS) without conventional therapy regimens in patients with advanced AAV.

2. Patients and Methods

2.1. Patients. All patients had advanced AAV and were treated with the standardized aRVS extract at the Integrative Cancer Center, Kyung Hee University Hospital at Gangdong (Seoul, Republic of Korea) between June 1, 2006 and April 30, 2011. We selected patients based on the following eligibility criteria: (1) histologically confirmed AAV, (2) radiological findings showing nonresectability of the tumor that included evidence of distant metastasis, and (3) more than a day of standardized aRVS extract administration. Exclusion criteria included response evaluation not available after aRVS treatment and concurrent conventional treatment including chemotherapy or radiotherapy during aRVS treatment. All patients signed a written informed consent form. Medical records were retrospectively reviewed with particular attention to the initial history and physical examination, histopathologic findings, operative and postoperative treatments, and followup. This study was approved by the institutional review board of the Kyung Hee University Hospital at Gangdong (KHNMC-OH-IRB 2011-12).

2.2. Standardized Extract of aRVS and Treatment Course. The clinical application of RVS has been limited because of an allergenic component, urushiol, which causes severe contact dermatitis in sensitive individuals [18–20]. Therefore, urushiol, a mixture of several derivatives of catechol, must be removed from RVS prior to its pharmaceutical use. A standardized extract of allergen-removed RVS (aRVS) was manufactured based on thorough historical research (fustin > 13.0%, fisetin > 2.0%, urushiol not detected). The daily oral administration of 1350 mg (one 450-mg capsule, three times a day) of aRVS extract was prescribed.

2.3. Evaluation of Efficacy and Safety. We identified 15 patients with advanced AAV who were consecutively treated with aRVS. Three patients were excluded because of a lack of response evaluation ($N = 2$) and concurrent radiotherapy ($N = 1$).

We assessed the treatment outcomes of progression-free survival (PFS), overall survival (OS), and toxicities. Progression of radiological findings was determined according to Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. Disease status was radiologically checked every two to three months after aRVS treatment. OS was defined as the period from the date of the start of aRVS treatment until death from any cause. We verified the time of death using official Korean National Health Insurance records on March 15, 2012. Both PFS and OS were estimated using the Kaplan-Meier method. Safety was assessed in terms of toxicity and assigned a severity grade ranging from 1 to 4 based on the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

3. Results

3.1. Clinical Characteristics. The baseline characteristics of the patients are presented in Table 1. The median age was 52 years (range: 36–73 years), with a low BMI (median BMI: 20.3; range: 14.6–25.2). Ten (83.3%) patients had undergone surgical resection of their primary tumor, and, of those, two (20.0%) had received adjuvant treatment.

Only three patients (25.0%) were chemotherapy naïve because of advanced age, poor performance status, anxiety about the toxicity of chemotherapy, or preference for herbal medicine. Before starting aRVS treatment, nine patients (75.0%) had received prior palliative chemotherapy, and three of those patients (33.3%) had received second chemotherapy regimens. All patients had progressive disease during or after prior chemotherapy except for one patient (ID 9).

3.2. Safety and Treatment Outcomes of the aRVS Extract. On March 15, 2012, nine patients had expired, and the remaining three patients were living. The median aRVS administration period was 147.0 days (range: 72–601 days). Overall, treatment was well tolerated, even in patients with a worse performance status. Although hematologic toxicity related to aRVS treatment was not observed, nonhematologic adverse effects were reported. One case of gastritis (Gr 2) and two cases of pruritus (Gr 1) were each observed in three patients. The reported gastritis developed after surgery, and the symptoms waxed and waned. The symptom of pruritis spontaneously diminished without reducing the dosage of aRVS. Patients discontinued aRVS treatment because of disease progression, not because of adverse effects of aRVS.

The best tumor responses based on RECIST, PFS, and OS are summarized in Table 2. The best responses among the 12 patients were complete response in 2 cases (ID 8 and 11), stable disease in 2 cases (ID 3 and 6), and progressive disease in 8 cases. The median OS was 15.1 months (range: 4.9–25.1 months). The median PFS was 3.0 months (range: 1.6–11.4 months).

Patient 11 is 73 years old and was diagnosed with adenocarcinoma with invasive pancreas (pT3N0 M0; IIA) in June 2010, following an abnormal presentation during a routine health examination. She underwent pylorus preserving pancreatoduodenectomy (PPPD). Follow-up CT scans in February 2011 revealed newly developed peritoneal seeding and local tumor recurrence around the PPPD site. PET-CT whole body scans confirmed multiple peritoneal metastases (Figure 1(A)). The patient and her family refused palliative chemotherapy because of old age and poor performance status. For these reasons, the patient visited our hospital to receive alternative therapy. The treatment plan did not include any orthodox therapies such as surgical operation, chemotherapy, or radiation therapy. Only aRVS was administrated since March 2011. Follow-up CT scans in July 2011 showed a decrease (12 mm ← 20 mm) in the recurrent mass in the superior mesenteric artery lesion and disappearance

Table 1: Demographic and clinical characteristics of all patients ($N = 12$).

Patient number	Gender	Age	BMI	Diagnosis day	Initial stage*	Prior surgery	Differentiation	Prior adjuvant treatment	Day of advanced stage diagnosis	Metastasis region	Chemotherapy regimen	Response
1	Female	65	23.4	2005-07-15	I	PPPD	NA		2006-07-15	Liver	(1) Clinical trial 4 cycles (2) Chemotherapy 2 cycles not reported	PD
2	Female	61	20.6	2005-04-25	IIB	PPPD	Well		2005-12-30	Metastatic LN	(1) Oral FU/CDDP 15 cycles (2) Gemcitabine 4 cycles	PD
3	Female	58	25.2	2006-08-07	IV	PPPD (R2)	Moderate			Lung, LN	(1) Oral FU with concurrent RT 1 cycles	PD
4	Female	36	18.5	2008-05-20	IIB	PPPD	Moderate		2008-12-15	Lung, bone, LN	(1) Gemcitabine/CDDP 8 cycles (2) FOLFOX 2 cycles	PD
5	Male	66	24.7	2006-12-18	IB	PPPD	NA	IV FU with concurrent RT	2007-08-21	Liver	(1) Oral FU/CDDP 21 cycles	PD
6	Female	45	20.1	2010-03-31	IV		Poor			Liver	(1) IV FU/CDDP/EPS 3 cycles	PD
7	Male	68	17.9	2009-08-04	IIB	PPPD	Moderate		2009-12-02	Lung, LN	(1) Oral FU/CDDP 8 cycles	PD
8	Male	41	20.8	2010-03-17	IV		Well			Liver	(1) Oral FU/Oxaliplatin 6 cycles	SD
9	Male	46	19.7	2009-08-05	IIB	PPPD	NA		2010-12-01	Peritoneal seeding	Chemotherapy refused because of adverse effects	
10	Female	41	14.6	2009-09-22	IIB	PPPD	Moderate	Oral FU 2 cycles	2010-02-06	Lung, liver	Chemotherapy refused because of adverse effects	
11	Female	73	22.6	2010-06-14	IIA	PPPD	Well		2011-02-09	Peritoneal seeding	Chemotherapy refused because of old age	
12	Male	37	19.3	2008-11-15	IIB	PPPD	Moderate		2009-10-15	Mesenteric LN	(1) Oral FU with concurrent RT 1 cycles	PD

*Staging is based on the seventh edition of the *TNM Classification of Malignant Tumors*.

BMI: body mass index, LN: lymph node, NA: not available, PD: progressive disease, PPPD: pylorus preserving pancreaticoduodenectomy, RT: radiotherapy, and SD: stable disease.

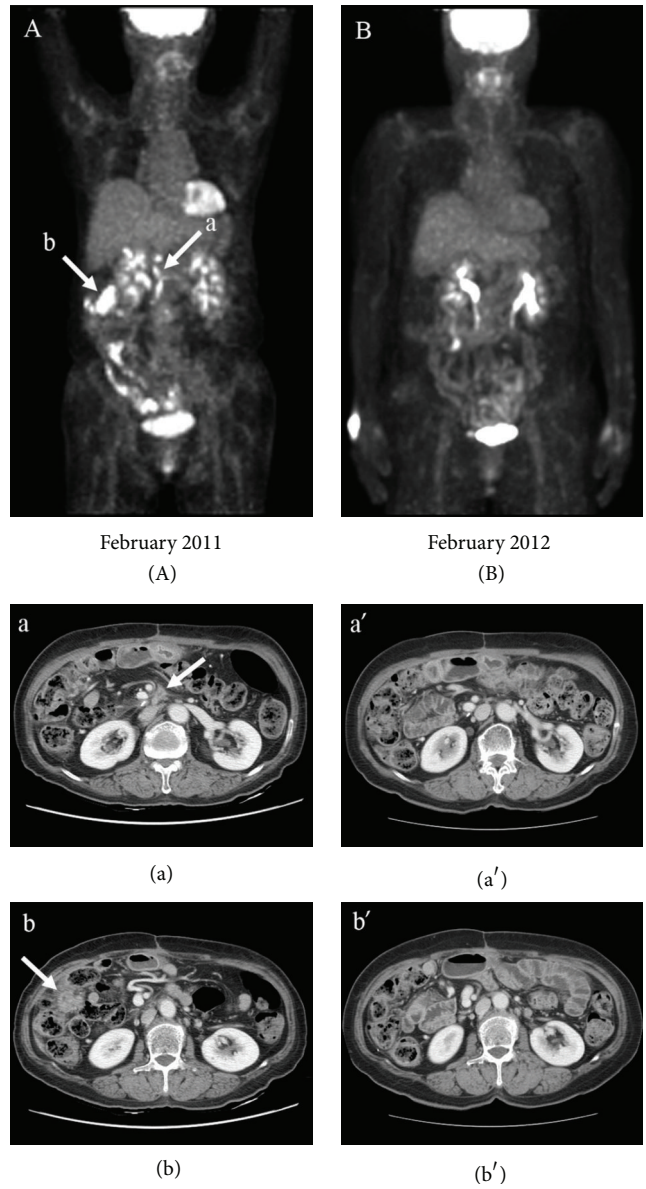

Figure 1: Multiple peritoneal metastases after surgery were confirmed by a PET-CT whole-body scan in February 2011 (A). The irregular speculated soft tissue mass in the proximal superior mesenteric artery lesion (20 mm; (a)) and the right side mesentery mass (32 mm; (b)) disappeared in the recent CT scans ((a′) and (b′)) after only aRVS treatment. All the metastases disappeared in the recent PET-CT scans in February 2012 after only aRVS treatment (B).

of the right side mesenteric mass (32 mm) and peritoneal nodules. Recent PET-CT scans in February 2012 revealed complete disappearance of her AAV (Figure 1(B)). She is currently doing well.

Patient 8, a 35-year-old patient with liver metastasis from AAV, underwent six cycles of treatment with oxaliplatin/capecitabine and achieved complete response of the liver mass and stable disease in the ampullary mass. However, the patient stopped his treatment because of thrombocytopenia and neuropathy from chemotherapy. Instead, he has received aRVS treatment since August 2010. After three months of only aRVS treatment, the ampullary mass completely disappeared. Follow-up CT scans in July 2011 showed no evidence of tumor recurrence (Figure 2). The patient is currently doing well with refusing further radiological examination.

Patient 6, a 45-year-old patient was diagnosed with liver, pancreas, and peritoneal metastases from AAV in March 2010. She received three cycles of cisplatin/5FU/epirubicin, and follow-up CT scans in July 2010 unfortunately showed the progression of metastatic masses in liver. Continuous palliative regimen such as gemcitabine-based chemotherapy was recommended, but she strongly refused it because she experienced adverse effects and poor response from chemotherapy. She has received aRVS treatment as alternative therapy since July 2010 (Figures 3(A) and 3(B)). In spite of the

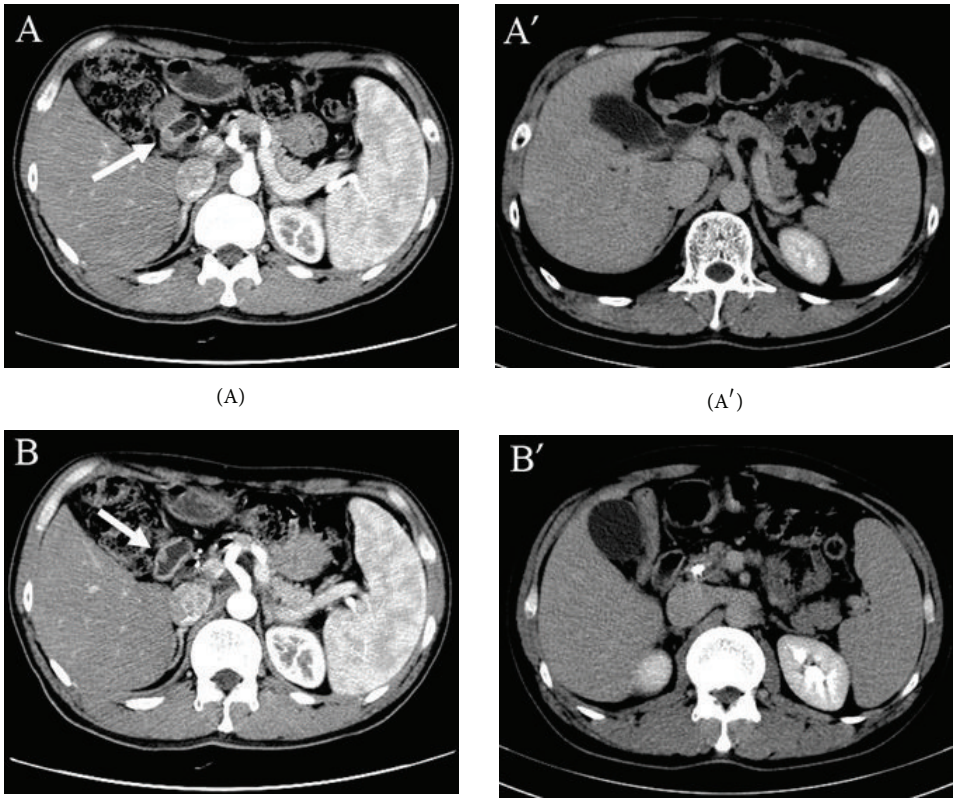

Figure 2: An abdominal CT scan in August 2010 revealed a mass in the ampulla of Vater legion ((A) & (B)). After only aRVS treatment, a recent abdominal CT scan in July 2011 showed that no definite mass is seen in the region of the ampulla of Vater nor is there any definite evidence for metastatic mass ((A′) and (B′)).

Table 2: Patient response to aRVS extract treatment.

Patient number	Initial day of aRVS treatment*	Time from advanced stage diagnosis to aRVS treatment (month)	aRVS treatment duration (month)	Best response RECIST	Progression-free survival (month)	Overall survival (month)
1	2007-02-28	7.6	6.0	PD	2.7	6.7
2	2007-06-01	17.3	4.6	PD	1.6	15.1
3	2007-06-18	10.5	4.7	SD	4.1	25.1
4	2009-09-09	8.9	15.5	PD	1.9	16.9
5	2010-07-14	35.3	5.1	PD	3.3	14.2
6	2010-07-23	3.8	20.0	SD	11.2	20.0 (alive)
7	2010-07-27	7.9	3.5	PD	1.7	10.3
8	2010-08-11	4.9	13.8	CR	11.3 (+)	19.4 (alive)
9	2011-01-14	0.5	2.4	PD	2.7	4.9
10	2011-01-26	11.8	4.0	PD	2.2	11.8
11	2011-03-16	1.2	10.7	CR	11.4 (+)	12.2 (alive)
12	2011-03-30	17.7	2.6	PD	3.3	6.7

*The standardized allergen-removed *Rhus verniciflua* Stokes (aRVS) extract (1350 mg) was orally administered daily.
CR: complete response, PD: progressive disease, PR: partial response, and SD: stable disease.

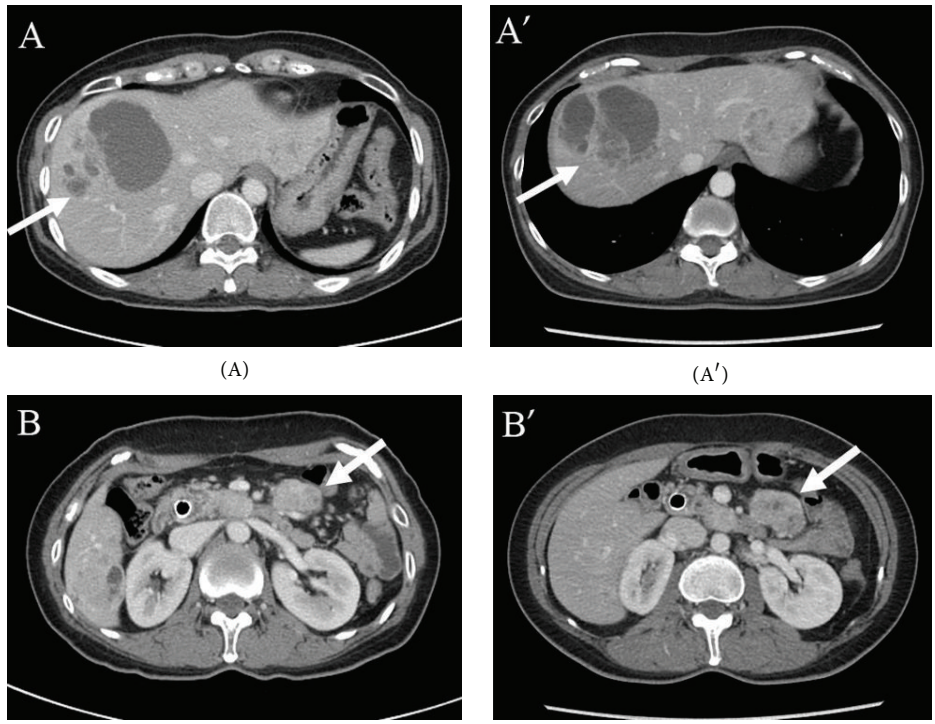

Figure 3: An abdominal CT scan in July 2010 revealed a necrotic metastatic lesion in the right lobe of liver and a metastatic mass in mesentery LUQ ((A) & (B)). After only aRVS treatment, a recent abdominal CT scan in March 2012 showed slight increase in size of metastatic masses over 20 months ((A′) and (B′)).

tumor progression in June 2011, she insisted aRVS treatment because her disease slowly progressed and there were no adverse effects from aRVS treatment. The recent CT scans in March 2012 showed no significant change, indicating stable disease compared to the CT scans in June 2011 (Figures 3(A′) and 3(B′)). The patient is fully active doing well without signs or symptoms of disease.

4. Discussion

AAV represents a rare disease, and only a few prospective studies on the palliative treatment of AAV have been published [21, 22]. Moreover, previous studies were small and focused on small bowel adenocarcinoma having significantly better OS than AAV. To the best of our knowledge, there is no prospective randomized controlled study evaluating the benefit of palliative chemotherapy against AAV. Therefore, patients with advanced AAV are obliged to receive chemotherapy based on their physician's preference [4].

Recently, two clinical reports were published on the efficacy of chemotherapy in patients with advanced relapsed /metastatic AAV. A phase II trial of capecitabine plus oxaliplatin in 25 patients with metastatic adenocarcinoma of the small bowel and ampullary origin reported a median time to progression (TTP) of 6.6 months and a median OS of 15.5 months [21]. However, the study included patients with small bowel origin rather than AAV. The response rate was actually 33% in AAV cases, which is poor, compared to a rate of 61% in cases with small bowel origin. The retrospective data from another Korean hospital showed that first-line cisplatin-based combination chemotherapy in AAV resulted in a median TTP of 4.9 months and a median OS of 12.5 months [23]. These results are consistent with the outcomes of the current study because it was a retrospective review of patients of the same ethnicity with only AAV, not small bowel adenocarcinoma.

The median OS of 15.1 months in our study is comparable to the findings based from the other Korean hospital, although our sample size was smaller (12 patients versus 29 patients). It should be noted that most patients (75.0%) in our study were refractory to the first or second regimen of chemotherapy and the median time was 8.4 months from the day of advanced or metastatic stage diagnosis to the initiation of aRVS treatment (Tables 1 and 2). The more conventional treatments were not available and recommended for them by oncologist because of disease progression or adverse effect. Nonetheless, there were two cases (ID 8 and 11) out of 12 patients showing complete response after only aRVS treatment, while the front-line cisplatin-based chemotherapy did not result in a single case of complete response out of 29 patients.

The aRVS treatment was safe with over one year of administration (ID 4, 6, and 8), and there were no aRVS discontinuations related to adverse effects. Hematologic toxicity was not reported, and only minor grade nonhematologic adverse effects were reported in three patients. The results

also suggest that aRVS treatment could improve OS, in spite of a median PFS of only 3.0 months, which is supported by the outcomes of patients in this study (ID 2, 3, 4, 5, and 6). Therefore, the aRVS treatment should not be evaluated as the cytotoxic agents focusing on tumor response.

Biological studies on AAV have been difficult because there are only a few established cell lines and histological subtypes are mixed with either pancreatobiliary or intestinal differentiation [24, 25]. Therefore, there is a need for a better understanding of the biology of AAV and the identification of potential prognostic factors and clinically relevant molecular targets for therapy. Up to now, several clinical reports have demonstrated that the expression of death-promoting proteins such as Bax is highly correlated with survival in patients with radically resected AAV, and cyclooxygenase-2 (COX-2) expression is highly elevated in patients with AAV and strongly associated with vascular endothelial growth factor (VEGF) expression [26–28].

The flavonoids are known to have significant anticancer properties as they induce apoptosis and inhibit COX-2 [29]. A purified flavonoid fraction prepared from RVS has been shown to induce apoptosis in some cancer cell lines, while sparing normal cells [7, 16]. A phenolic-rich fraction from RVS was reported to suppress inflammatory response via the NF-κB and JNK pathway [30]. Sulfuretin (3′,4′,6-trihydroxyaurone) isolated from RVS inhibits COX-2 as well as proinflammatory cytokine expression via the downregulation of NF-κB [31]. Fisetin (3,7,30,40-tetrahydroxyflavone) from RVS was also found to have apoptotic, antiproliferative, and anti-invasive effects by downregulation of the NF-κB signaling pathway in chemo-resistant human pancreatic cancer cells [32]. Butein (3,4,2′,4′-tetrahydroxychalone) has also been shown to downregulate COX-2 expression in cancer cells and suppress cancer cell micrometastasis by inhibiting fibroblast formation [33, 34]. It has been experimentally demonstrated that the standardized aRVS extract has antiangiogenic activities by inhibiting VEGF and an inhibitory effect on matrix metalloproteinase-2 (MMP-2) and MMP-9 activities in a human fibrosarcoma cell line [35, 36]. The single component such as fustin or fisetin shows poor results compared to the whole extract. Though the mechanism of action is not clear, the multifactorial synergistic interactions among unknown compounds in RVS are thought to block AAV progression with major biologically active components such as sulfuretin, fisetin, and/or butein.

Even though this study is limited by the rarity of the disease, our results indicate that aRVS extract is beneficial in tumor response to some patients (ID 6, 8, and 11) previously described and could be applied as a salvage regimen or adjuvant agent against AAV in the selected patients for whom chemotherapy is not feasible. Therefore, we have tried to find several markers in common among responders, based on traditional medicine and modern science in the view of personalized medicine. For example, the alterations of cytokines-related immune system or metabolites in urine and plasma are investigated before and after treatment. We also hope that this clinical outcome would stimulate different investigation in natural products from conventional drug discovery and development based on cytotoxicity [14].

Conflict of Interests

The authors declare that they have no conflict of interests.

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