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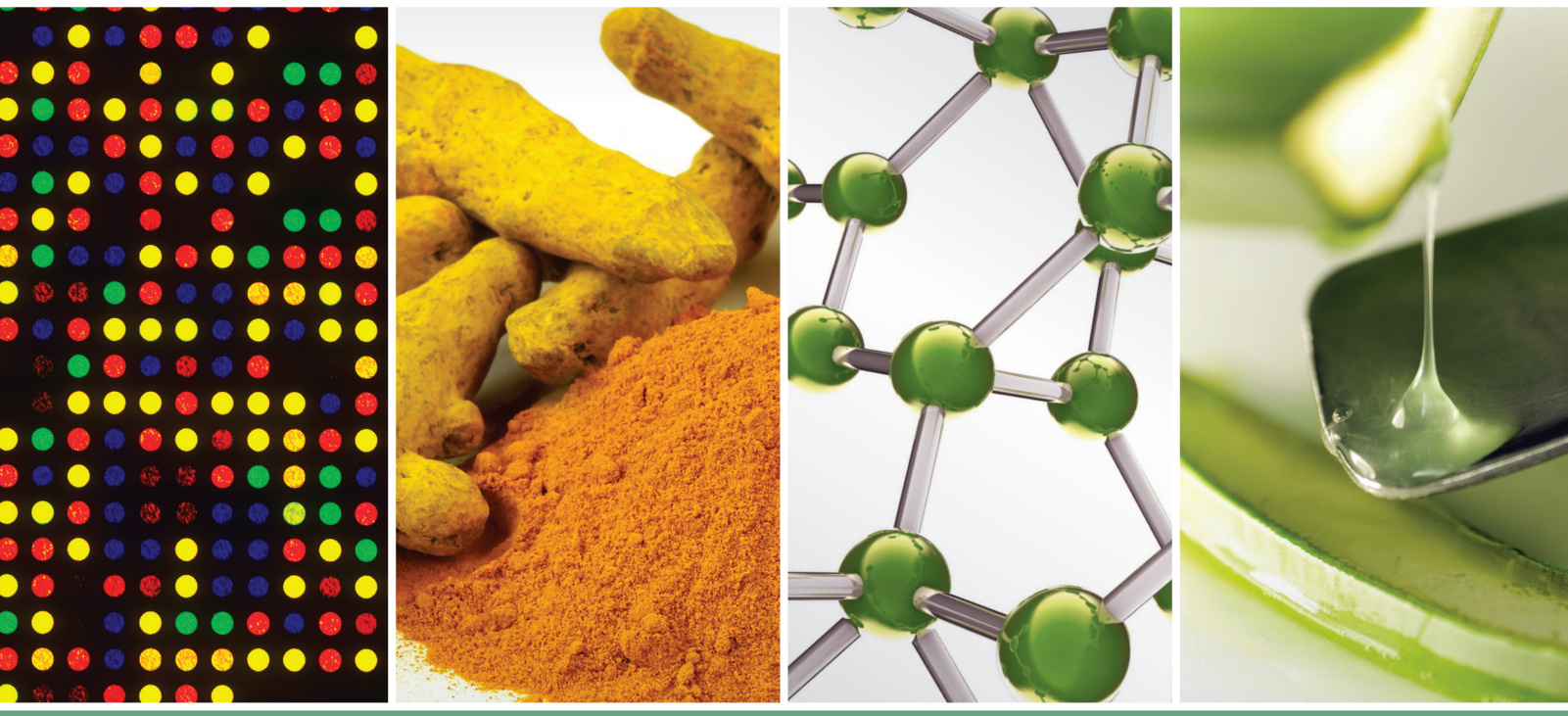
Mechanism Study of Traditional Medicine Using Proteomics Alone or Integrated with Other Systems Biology Technologies

Guest Editors: Xuan Liu, M. S. Kanthimathi, and Klaus Heese



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Editorial Board

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Editorial

Mechanism Study of Traditional Medicine Using Proteomics Alone or Integrated with Other Systems Biology Technologies

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The efficacy of traditional medicine such as traditional Chinese medicine (TCM) had been confirmed by many years of clinical use. However, the mechanisms of traditional medicine remained obscure. Since the development of systems biology in 2000, systems biology technologies have been popularly used in the mechanism study of traditional medicine. In this special issue, we particularly picked reviews and research papers related to mechanism study of TCM, TCM diagnosis, or TCM therapy methods using proteomics as well as other systems biology technologies. There are 10 papers collected in this special issue, in which 3 papers are reviews and 7 papers are original research papers.

The 3 reviews in the present issue introduce the advancements in applications of proteomics technologies in the study of TCM and the achievements in the mechanism study of TCM. Q. Ji et al. summarized the applications of proteomics technologies in TCM syndrome research as well as the mechanistic study of TCM treatments, including Chinese herbal medicine, Chinese herbal formula, and acupuncture. Furthermore, they introduced the combined analyses of proteomics with other "-omics" technologies in the study of TCM. Y. A. Sulistio and K. Heese discussed the utility of comparative proteomics for a better understanding of the mechanisms involved in TCM activities and its potential application as complementary therapy for the treatment of Alzheimer's disease (AD). Additionally, they reviewed the data from comparative proteomics studies of AD patients and established the relevance of the data with available AD hypotheses and potential TCM-based treatments, most notably regarding the ubiquitin proteasome system. Y. Yang et al. focused on discussing various results of proteomics studies of mechanisms of anticancer TCM, including terpenes, flavonoids, and glycosides. And they suggested future research strategies such as integrating translating mRNA analysis with proteomics in the study of the mechanisms of anticancer TCM.

Four of the research papers reported results of mechanism studies of TCM. J.-Y. Shiau et al. studied the mechanism of 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne, an active component of *Bidens pilosa*, in human T-cell acute lymphocytic leukemia cells by using combined differential proteomics and bioinformatics approaches. M.-N. Shi et al. reported the protective effects of scutellarin, a flavone isolated from *Erigeron breviscapus* (Vant.) Hand.-Mazz, on human cardiac microvascular endothelial cells against hypoxia-reoxygenation injury. Possible target-related proteins of scutellarin were searched using proteomics analysis and a possible interaction network was predicted using bioinformatics analysis. C.-C. Guo et al. studied the antiosteoporotic effects and possible target-related proteins of Huangqi Sanxian decoction, a traditional Chinese formula composed of *Radix astragali*, *Epimedii folium*, *Cistanche herba*, *Radix notoginseng*, *Radix Salviae Miltiorrhiae*, *Corydalis rhizoma*, *Radix Angelicae sinensis*, and *Radix Clematidis*, in cultured rat osteoblasts. Z. Bo et al. investigated the molecular mechanism of fibroblast regulation and the treatment of recurrent oral ulcer by Shuizhongcao granule-containing serum. Shuizhongcao granule is a TCM formula composed

of buffalo horn, urine sediment, callicarpa, and so forth, and it exhibited great efficacy in the clinical treatment of recurrent oral ulcer.

In the present issue, there are also 3 research papers reporting about the mechanisms of TCM used for the diagnosis of diseases or specialized TCM therapy methods such as moxibustion. In the TCM system, tongue diagnosis has been an important diagnostic method. X. Liu et al. used the GC/MS technology to determine the potential changes of metabolites and identify special metabolic biomarkers in the tongue coating of *H. pylori* infected chronic gastritis patients. W.-T. Dang et al. studied the expression of caspase-1 gene transcript variant mRNA in peripheral blood mononuclear cells of patients with primary gout in different TCM syndromes. X.-L. Shi et al. investigated the effects of moxibustion, a traditional Chinese practice that involves heated *Artemisia vulgaris* (mugwort) stimulation, on hormonal imbalance and ovarian granulosa cell apoptosis in a rat model of perimenopause.

In summary, this special issue provides a snapshot of the current status of mechanism studies of traditional medicine, especially TCM, using proteomics and other systems biology technologies. Authors of the present issue highlight both the achievements and challenges faced in the field of the mechanism study of traditional medicine. Hopefully, this publication will help readers to follow mechanism studies of traditional medicine and will contribute to improved applications of proteomics or other systems biology technologies in research of traditional medicine.

Acknowledgments

We would like to thank the Editorial Board of this journal for the approval of this concept and continuous help that made this special issue possible. With great respect we extend our thanks to all authors and reviewers for their contributions to the successful publication of this special issue.

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Research Article

The Metabonomic Studies of Tongue Coating in *H. pylori* Positive Chronic Gastritis Patients

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In Traditional Chinese Medicine (TCM), tongue diagnosis (TD) has been an important diagnostic method for the last 3000 years. Tongue coating can be used as a very sensitive marker to determine the progress of chronic gastritis. Therefore, the scientific, qualitative, and quantitative study for the pathophysiologic basis of tongue coating (TC) emerged as a major direction for the objective research of TD. In our current report, we used GC/MS technology to determine the potential changes of metabolites and identify special metabolic biomarkers in the TC of *H. pylori* infected chronic gastritis patients. Four discriminative metabolites were identified by GC/MS between the TC of *H. pylori* infection (G + H) and without *H. pylori* infection (G − H) patients: ethylene, cephaloridine, γ-aminobutyric acid, and 5-pyroglutamic acid, indicating that changes in amino acid metabolism are possibly involved in the formation of TC, and the amino acid metabolites are part of the material components of TC in G + H patients.

1. Introduction

Helicobacter pylori (*H. pylori*, *Hp*) infection is one of the most important causes of chronic gastritis and gastric cancer [1, 2]. *Hp*, a Gram-negative bacterium found in the stomach, is listed as Class I carcinogen by WHO. In 1984, *Hp* was first isolated from the gastric mucosa and epithelial surface by Marshall and Warren [3]. *Hp* infection can lead to chronic gastritis, gastric and duodenal ulcers, and increased risk of gastric cancer [1, 4–7]. Correa delineated the whole pathological process from *Hp* infection induced inflammation of gastric mucosa, to intestinal metaplasia, aplasia, and carcinoma [8]. In 1998, Watanabe et al. established the first animal model with Mongolian gerbils to demonstrate that *Hp* infection directly causes gastric cancer [9]. Our previous studies illustrated that *Hp* can grow in the stomach mucosa of C57BL/6 mice following oral gavage of the bacteria. Seventy-two weeks later, pathological examinations clearly revealed a 22.2% rate of gastric cancer incidence in the mice [10]. This piece of evidence again reaffirms the role of *Hp* in inducing gastric cancer; however, the mechanism remains elusive, is thought to be very complex, and involved numerous metabolic pathways in the body. This poses a huge challenge for the prevention and treatment of *Hp* induced chronic gastritis and gastric cancer. Among the many current studies of metabolic pathways and the metabolites in *Hp* induced chronic gastritis and cancer, Shi revealed that, in the serum samples of *Hp* infected patients, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) is significantly lower while the malonaldehyde (MDA) level is higher relative to *Hp* negative patients [11].

Tongue diagnosis is a noninvasive, simple, and valuable diagnostic tool, the use of which has been repeatedly affirmed by clinical practitioners of traditional Chinese medicine (TCM) for 3,000 years. Tongue appearance is closely associated with the physiology as well as pathophysiology of

the digestive system [12–14]. TCM theories state that the tongue coating (TC) is condensed stomach "Qi" and the essence "Qi" of food; tongue appearance is a very sensitive index of the physiological and pathological status of the organs, especially the stomach and spleen. Tongue appearance reflects the amount of bad "Qi" and the dynamic process of illness of the stomach, that is, the *Hp* infection status. Clinical research has reported that the tongue appearance changes provide essential information for the diagnosis, treatment, and prognosis of chronic gastritis, peptic ulcers, and gastric and colorectal cancers [15–18]. Huang et al. discovered that, in the patients with *Hp* induced chronic superficial gastritis, the colors of TC were mainly light white and yellow [19]. This finding was corroborated by another report by Wang et al., which suggests that, in 518 chronic gastritic cases, 81.6% of *Hp*-infection positive patients had yellow CT, significantly higher than the *Hp* negative group [20]. Mao reported that the tongue color of the majority of *Hp* positive patients was light red while the TC appeared greasy, thick, and yellow [21]. Together, these and other studies have demonstrated that tongue appearance correlates with *Hp* infection status: positive patients have red or purple tongues with yellow TC, and the more severe the *Hp* infection gets, the thicker and greaser the TC appears. Tongue appearance also reflects the degrees of gastric inflammation and prognosis: when TC turns to be thinner, it indicates a better function status of stomach and spleen, less inflammation, and less *Hp* infection [22].

Metabonomics is an important part of the system biology. Metabolites are the ultimate products of gene expression, closely related to the physiology and pathophysiology of the body. Metabonomics considers the human body as a whole system, which is consistent with the TCM concept, and therefore has wide application prospects in TCM research [23–25]. The analysis of syndrome ("zheng" in TCM) associated metabolites may help comprehend the changes of metabolic pathways and conditions when a disease progresses and understand the material basis of the disease. Chen et al. reported a specific metabolite, 1-methyladenosine, as biomarker in hepatocellular carcinoma patients using metabonomics [26]; Leichtle et al. investigated the levels of 26 amino acids in the blood of colorectal cancer patients and found that the cancer patients had lower concentration for 11 amino acids and proposed a carcinoembryonic antigen-(CEA-) glycine-tyrosine tri-biomarker, the best model for the diagnosis of the disease [27]. Chronic gastritis is associated with *Hp* infection and TC is a reliable status indicator of *Hp* infection, gastric inflammation, and prognosis. Hence, in the current study, we used GC/MS technology to investigate the spectrum of material composition in TC of *Hp* infected patients, determine the changes of TC metabolites, and identify microorganism biomarkers for the *Hp* positive, chronic gastritis patients.

2. Materials and Methods

2.1. Ethical Statement. All samples were obtained as part of diagnostic criteria after patients gave written informed consent. The study was approved by the local ethics committee of Shanghai University of Traditional Chinese Medicine Shuguang Hospital (SUTCMSH), Shanghai, China.

Table 1: General information of the chronic gastric patients.

Group	Tongue coating White/yellow W/Y	Gender		Age ($\overline{x} \pm$ SD)
		Male	Female	
Hp positive	2 13 8	12	11	51.71 ± 13.42
Hp negative	9 1 9	7	12	58.57 ± 10.69

2.2. Participant Selection Criteria and TC Samples Details. The participants of this study were mainly patients from SUTCMSH, from October 2012 to July 2013. All patients underwent a gastroscopy examination for diagnosis of chronic gastritis and a gastric mucosa biopsy and Giemsa staining to confirm *Hp* infection. Twenty-nine patients had both chronic gastritis and *Hp* infection while 13 patients had only chronic gastritis. Our study included 42 chronic gastritis patients at Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, between October 2012 and July 2013. Of the 42 cases, 23 were positive of *Hp* infection (12 male, 11 female), with the mean age of 51.71 ± 13.42 years; 19 were negative of *Hp* infection (7 males, 12 females), with the mean age of 58.57 ± 10.69 years. The TC color in the *Hp* group was mainly yellow while mainly white or white/yellow in the non-*Hp* group (Table 1). No significant differences in gender and age were observed between the two groups ($P > 0.05$).

2.3. Tongue Coating (TC) Samples Collection. The TC samples were collected as previously described [22]. All participants were required to gargle saline 2~3 times before sampling to rinse possible food contamination that might influence the TC. Small spoons were used to scrape the TC at the thickest area and samples were placed into sanitized Eppendorf tubes that had been filled with 2 mL of sterile saline. All samples were stored at −80°C until analysis (Figure 1).

2.4. Gas Chromatography-Mass Spectrometry (GC/MS) Analysis

2.4.1. GC/MS Measurement. TC samples were prepared by sonication and centrifugation at 4°C 3500 rpm for 10 min. The 100 μL supernatant was transferred to a new tube and after adding 200 μL methanol, it was vortexed for 30 s, incubated at −20°C for 10 min, and centrifuged at 4°C 10000 rpm for 10 min; then 200 μL supernatant was transferred to a sample tube. Sample was then freeze-dried, added 10 μL of chlorophenylalanine (0.3 mg/mL) and 30 μL of methoxamine pyridine (15 mg/mL), sealed, vortexed for 30 s, incubated at 37°C for 90 min, added 40 μL BSFTA (containing 1% TMCS), incubated at 80°C for 2 h, cooled at room temperature for 1 h, before being analyzed by GC/MS. The GC/MS system was from Agilent Technology (California, USA), Model# DB5MS, column: 30 m × 0.25 mm × 0.25 μm. GC/MS condition is as follows: the column temperature was held at 80°C for 3 min, then 10°C/min increased to 140°C, 4°C/min increased

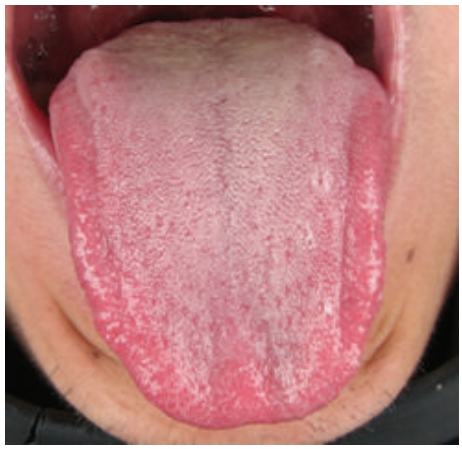

Figure 1: Sampling images of tongue coating from the center of the tongue, an area regarded as tongue coating in the traditional tongue diagnosis.

to 240°C, 10°C/min increased to 280°C, and it was held for 10 min. Injection inlet temperature was 280°C and sensor temperature was 300°C. The carrier gas is Helium and flow rate was 1 mL/min. MS condition is as follows: EI ionization, electron energy 70 eV, ion source temperature 250°C, interface temperature 250°C, solvent delay 5 min, full-spectral scan, and scan scope M/Z 40–600.

2.4.2. GS/MS Data Analysis. Raw data was processed through multiple stages including noise reduction, feature detection, alignment of peaks, and normalization. GC/MS data were analyzed by Agilent Mass Profiler Professional (MPP) software. We analyzed the previously processed data by SIMCA-P^+ software (V13.0, Umetrics AB, Umea, Sweden), principal component analysis (PCA) is used to analyze the data by Centered Scaling method, and the data is automatically modeled and analyzed; partial least squares-discriminate analysis (PLS-DA) is used to analyze the data by Centered Scaling method, and the data is automatically modeled, modeling analysis of the first and two principal components; and orthogonal partial least squares-discriminate analysis (OPLS–DA) showed the maximum differences between different groups within the model.

2.4.3. Identification of Metabolite Markers. Differential metabolites markers were selected according to the PLS-DA Variable Importance in the Projection (VIP), considering only variables with VIP values higher than 1, indicative of significant differences among groups. These potential markers were identified by retention time correction of peaks and mass-to-charge ratio (m/z) using the Mass Spectral Library (National Institute of Standards and Technology, NIST).

3. Results

3.1. Chromatographic Analysis and Comparison between the Hp-Infection Positive and Negative Chronic Gastritis Patients. The total ion chromatograms obtained by GC/MS from the TC samples of *Hp* positive and *Hp* negative chronic gastritis patients demonstrated a clear difference between the two groups (Figure 2). In order to determine the detailed metabolomic profiles, multivariate statistical analysis was performed for the samples, that is, the principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), and orthogonal partial least squares-discriminate analysis (OPLS-DA).

3.2. The Metabonomics of TC Samples from the Hp Positive and Hp Negative Chronic Gastritis Patients. PCA scores of the TC samples from the two groups showed that all the sample points fell in the 95% confidence intervals but appeared partially overlapped, indicating that this method was not able to discriminate between the groups (Figure 3(a)). Further research by PLS-DA showed that the sample points were clearly separated (obtaining good class separation value and predictive power, with $R_2Y = 0.82$), which indicated that the two groups' metabolic pathways were different: all the sample points of *Hp* positive patients were mainly in the left lower quadrant, while the *Hp* negative patients' sample points are in the right upper quadrant (Figure 3(b)). To improve the accuracy of the PLS discriminated model, OPLS–DA by SIMCA-P^+ software was used to analyze the results to better highlight the difference between the groups. The analysis result showed that the sample points from the two groups were completely separated in different quadrants: the *Hp* positive sample points were in the left quadrant and *Hp* negative in the right (Figure 3(c)).

3.3. The Different Metabolite Markers of TC Samples from the Hp Positive and Hp Negative Chronic Gastritis Patients. We used OPLS-DA to block out irrelevant signals, to acquire reliable metabolite marker peaks. The metabolites responsible for discrimination were selected according to the Variable Importance in the Projection (VIP) considering only variables with VIP values higher than 1.0, indicative of significant differences among groups (Table 2). These potential metabolite markers, identified using the NIST Mass Spectral Library and KEGG bioinformatics database, were ϒ-aminobutyric acid, 5-hydroxyproline, ethylene, and some amino acids (Table 3).

4. Discussion

As a unique method, the tongue diagnosis contributed a great deal for the formation and development of TCM theory system [28]. "Huang Di Nei Jing," an ancient TCM book written in Qin and Han era (~2000 years ago), recorded the uses of tongue diagnosis. A chapter in that book called "Ling Shu, Shi Zhuan" stated the following: "By observing lip and tongue, one can determine the stages of a disease." Tongue coating (TC), as the main part of the tongue appearance, is the moss or fur like material on the tongue surface. The TCM believes that the changes of TC reflect human body's physiology and pathophysiology status. As described by "Xing Se Jian Mo": "the TC is formed by stomach ("stomach-Qi" in Chinese) and the five organs ("Wu-Zang" in Chinese) are all supplied by

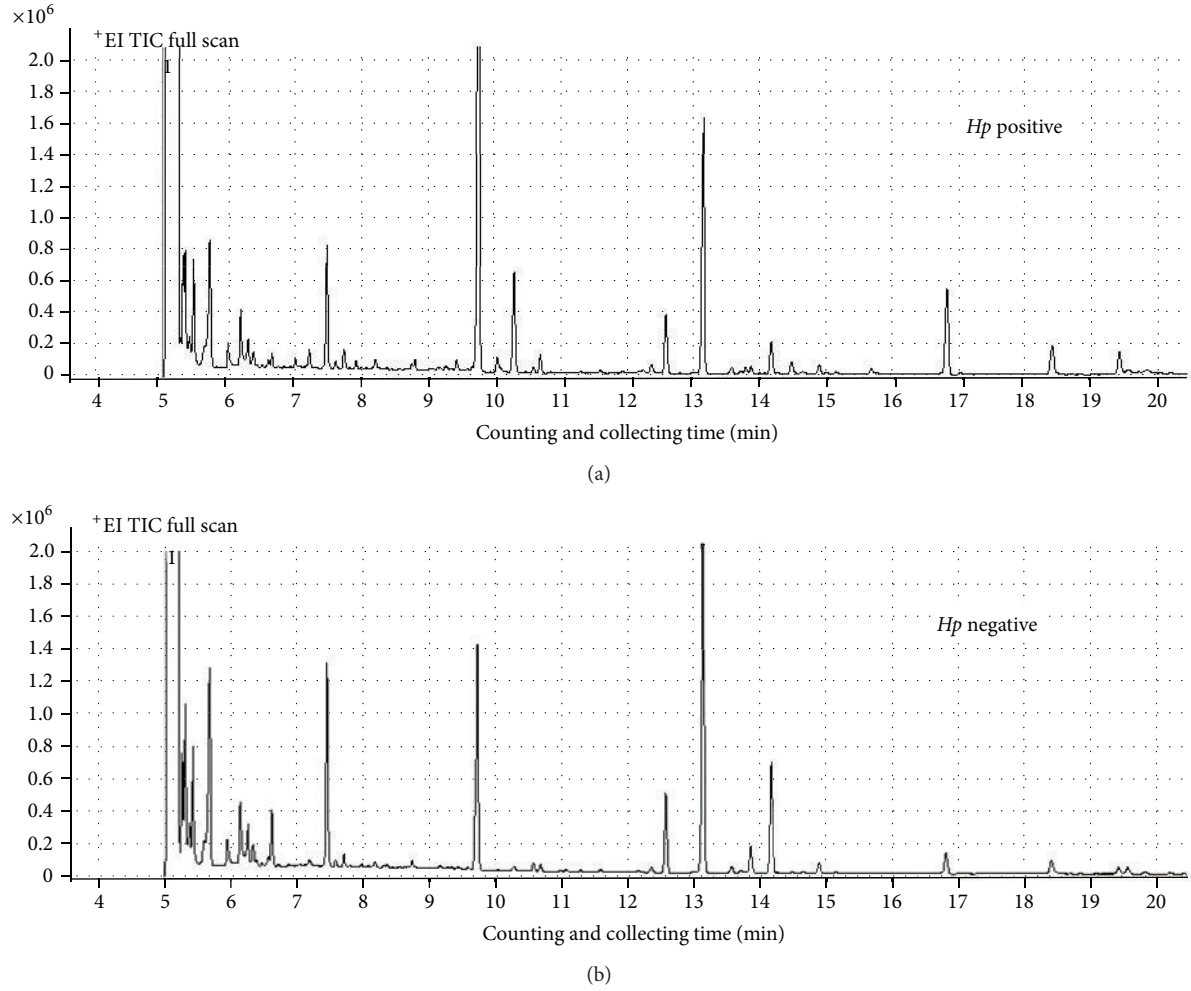

Figure 2: GC/MS metabolic fingerprinting total ion chromatogram of TC samples from *Hp* positive and *Hp* negative chronic gastritis patients.

the stomach, so the TC is the index of body status." "New Ling Shu" explained: "Tongue is closely related to the digestive system, whenever the digestive organs have problem, TC will show it." Therefore, not only can TC be an indicator of the pathophysiological status of the five essential organs, but also a "window" for the development stages of the gastric illnesses, a sensitive index for the progression of chronic gastritis [29]. In summary, to investigate the underlying mechanisms of TC formation, we can explore the nature of chronic gastritis TCM syndromes and obtain new clues and novel ideas for the objective studies of TCM syndromes.

Hp infection, one of the most causative factors of chronic gastritis and gastric cancer, has been listed as Class I carcinogen by WHO cancer institutions. Wang et al. reported that, in 518 chronic gastritis patients, 440 cases were *Hp*-infection positive (85%) and mainly had yellow TC (81.16%), significantly higher than the *Hp* negative group [20]. The reason for the yellow appearance of the TC was probably due to *Hp* infection increased gastric inflammation, which led to the malfunctioning of digestive system, lowered saliva secretion, and decreased oral cavity self-cleaning. This resulted in tongue surface dysbacteriosis that caused inflammation, exudate, and yellow-color change of the tongue. This is just a hypothesis, which apparently needs to be studied further and supported by experimental evidence. Therefore, how the *Hp* infection causes TC changes still remains an unsolved problem.

Metabolomics, based on the analysis of the entire set of metabolites in a sample, provides a comprehensive overview of the status of organisms, more directly and accurately reflecting the pathophysiology of the organisms. Biomarkers discovery is the current research "hotspot," but most of the metabolite biomarkers are identified in blood, urine, and tissue samples, rarely in TC samples. TC metabolomics, the study of the metabolites of TC samples to determine the pathophysiology status of the human body, has recently emerged. Li et al. established the methodology to process TC sample for metabolomics analysis [30]. Sun et al. discovered 10 discriminative metabolite biomarkers between TC samples of normal and chronic gastritis groups, using LC/MS technology [25]. Zhao et al. utilized GC/MS technology to uncover 17 metabolite biomarkers between normal and chronic hepatitis groups [31]. TC, as biological sample, is convenient and noninvasive to collect and is unique to TCM,

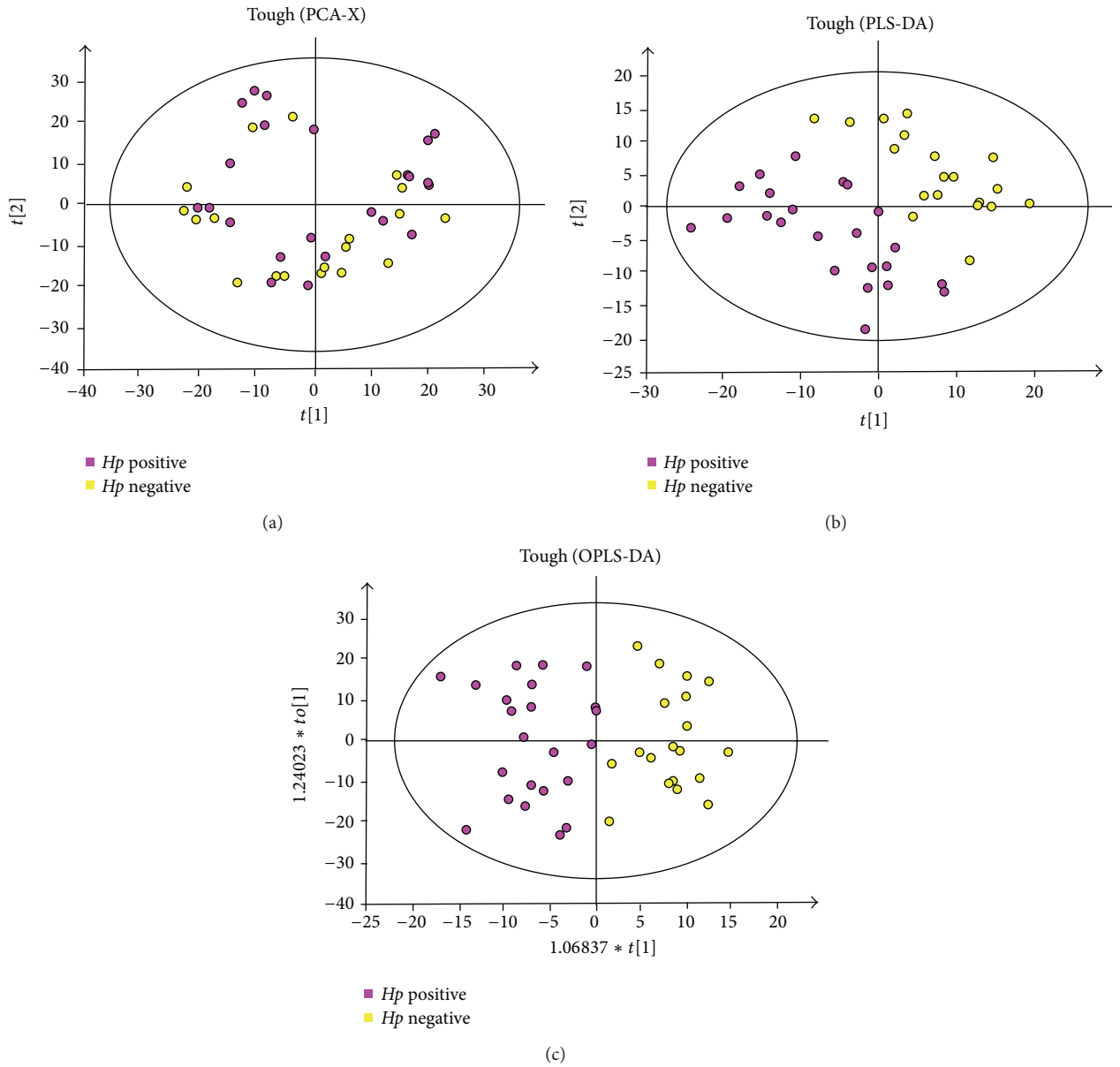

Figure 3: The metabonomics of tongue coating samples from *Hp* positive and *Hp* negative chronic gastritis patients. (a) PCA analysis, (b) PLS-DA analysis, and (c) OPLS-DA analysis.

which believes that TC is condensed Qi and liquid ("Jin" in Chinese) evaporated from the spleen and stomach on the tongue surface, so TC reflects the physiological and pathological status of the human body. Our current report researched TC samples to determine metabolite biomarkers in the TC of the *Hp* infection induced chronic gastritis patients.

We used PLS-DA and OPLS-DA statistical methods to analyze the GC/MS data of TC samples from *Hp* positive and *Hp* negative chronic gastritis patients and found a difference between the metabolites of each group. Using the NIST Mass Spectral Library and KEGG bioinformatics database, we identified these discriminative metabolite biomarkers as Υ-aminobutyric acid, 5-hydroxyproline, ethylene, and pyroglutamic acid which is derived from glutamine through dehydration and cyclization. Glutamine is one of the 20 common amino acids of the human body. It can form glutathione (GSH) by synthetically reacting with cysteine and glycine [32]. GSH plays a role in the biodefense system of the human body, that is, proimmunity, antiaging, and detoxicating. The TC samples of the *Hp* positive chronic gastritis patients had higher amount of pyroglutamic acid (VIP > 1), which indicates that the synthesis pathway of GSH was blocked, as the glutamine was not used to make GSH but directed toward the dehydration/cyclization reaction, to form pyroglutamic acid. The imbalance of GSH metabolism will disrupt normal physiology, causing a decrease of immune and detoxicating functions of the human body. McNulty and Dent uncovered that highly homogeneous groups of

Table 2: Comparison of chromatogram peaks from the TC samples of *Hp* positive and *Hp* negative chronic gastritis patients.

Var ID	m/z	Mean peak area (*Hp* positive)	Mean peak area (*Hp* negative)	P value	VIP
593	73	0.000124052	0.000290037	0.04129	2.0532
64	73	0.000231336	$8.16838E-05$	0.04928	2.05431
512	73	0.000162672	$2.74211E-05$	0.03997	2.09457
623	73	$7.71475E-05$	0.000303819	0.04789	2.09683
42	43	0.001224892	0.000927157	0.05121	2.17178
11	73	0.022093242	0.037576638	0.04812	1.06903
668	73	0.000341666	$5.39923E-05$	0.05087	2.29584
799	73	0.000312782	0.004340828	0.04879	2.62218
680	73	0.000954827	0.000677735	0.04762	2.75425
595	73	0.000208521	$5.79851E-5$	0.03584	2.79777
523	73	$5.86622E-05$	0.000264136	0.04978	2.87228
321	57	0.000112378	$9.7488E-05$	0.05041	1.16753

Note: we selected the different materials between *Hp* positive and *Hp* negative patients with $P < 0.05$, VIP > 1.0.

Table 3: The potential metabolite biomarkers and related metabolic pathways in the TC of *Hp* positive and *Hp* negative chronic gastritis groups.

Var ID	CAS1/NIST	CAS2/NIST	Name	KEGG ID
593	7381-30-8	—	Ethylene	C06547
64	1126-58-5	—	—	—
512	50-59-9	—	Cephaloridine	C11754
623	39508-23-1	—	γ-Aminobutyric acid GABA	C00334
42	1126-58-5	—	—	—
668	30274-77-2	—	Pyroglutamic acid	C01879, C02237
799	54477-01-09	55521-23-8	—	—
615	1126-58-5	50-59-9	Cephaloridine	C11754
680	1126-58-5	—	—	—
595	55521-23-8	50-59-9	Cephaloridine	C11754
523	7381-30-8	1126-58-5	Ethylene	C06547
321	39508-23-1	—	γ-Aminobutyric acid	C00334

Note: CAS1/NIST is the potential metabolite biomarkers number in NIST Mass Spectral Library, and KEGG ID is the potential metabolite biomarkers number in KEGG bioinformatics database.

C. pylori produce a similar panel of enzymes, including oxidase, DNase, oxidase, catalase, urease, alkaline phosphatase, leucine aminopeptidase, and Υ -glutamyl aminopeptidase [33]; therefore, our future research projects will be focused on interrogating whether the *Hp* produced Υ -glutamyl aminopeptidase affects the metabolism of glutamine.

5. Conclusions

We used GC/MS technology to determine the metabolic components of tongue coating samples in chronic gastritis patients with or without *Hp* infection. We found distinct metabonomic differences between the 2 patient groups and identified 4 discriminative metabolite biomarkers in the tongue coating of *Hp* positive chronic gastritis patients: ethylene, cephaloridine, γ-aminobutyric acid, and 5-pyroglutamic acid. The discovery of these metabonomic biomarkers in the tongue coating not only can help the diagnosis and treatment of *Hp* infection induced chronic gastritis, but also provide a theoretical basis for the utilization of tongue coating aided clinical diagnosis of diseases.

Disclosure

Xuan Liu and Zhu-Mei Sun are co-first authors.

Conflict of Interests

The authors report no conflict of interests.

Authors' Contribution

Xuan Liu and Zhu-Mei contributed equally to this work.

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Research Article

Expression of Caspase-1 Gene Transcript Variant mRNA in Peripheral Blood Mononuclear Cells of Patients with Primary Gout in Different TCM Syndromes

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A large number of studies have shown that cysteinyl aspartate specific protease-1 (CASP1) played an important role in the inflammatory response of primary gout, but the decreased expression of different CASP1 transcript variant could inhibit the activation of IL-1β. Our study mainly analyzed the expression level and function of CASP1 gene transcript variant mRNA in peripheral blood mononuclear cells of patients with gout in different TCM syndromes. The expression of CASP1 gene transcript variant and IL-1β mRNA in PBMCs were detected in patients with PG [acute phase (AP: 44 cases); nonacute phase (NAP: 52 cases)] and healthy controls (HC: 30 cases) by reverse transcription-polymerase chain reaction and/or real-time quantitative polymerase chain reaction. The expressions of plasma IL-1β in patients with PG and HC were detected by enzyme-linked immunosorbent assay. Dysregulated expression of the CASP1 gene and its transcript variant, plasma proinflammatory cytokines in all patients with primary gout in different TCM syndromes, correlation analysis showed that there was negative correlation between the expression of CASP1-gamma gene transcript variant mRNA and IL-1β protein in APPG group. The study suggested that CASP1 gene and its transcript variant may play a critical role in the inflammatory response of patients with PG in different phases and TCM syndromes.

1. Introduction

Gout is a clinical syndrome which is attributed to precipitation and deposition of monosodium urate (MSU) crystals on the tissue or organ caused by purine dysbolism and/or excretion reduction and continuous elevation of uric acid, and it belongs to metabolic rheumatism [1]. Gout is similar to Lijei or severe and migratory arthralgia in traditional Chinese medicine; its early elaboration is reported in *Ge Zhi Yu Lun* written by Zhu DanXi in which the pathogenesis of gout was regarded as phlegm, wind-heat, wind-wet and blood deficiency. Then, some doctors classified migratory Bi syndrome or painful Bi syndrome of Bi syndrome as gout. Recent researches have showed that inflammation and immunity are also involved in the pathogenesis of gout besides metabolism factors [2]. We know that the MSU released by aging and death cells in the body is endogenous danger-associated molecular patterns (DAMPs) caused by inflammation and apoptosis through innate immunity [3]. Cysteinyl aspartate specific protease-1 (CASP1) is also called IL-1β invertase, is involved mainly in regulation of inflammation, and plays an important role in the inflammatory response [4]. Luksch et al. [5] found that the decreased expression of different CASP1 gene transcript variant could reduce the activation of the IL-1β. Recent study showed that CASP1 played a key role in the course of gout [6, 7]. However, there was no study reporting the role of CASP1 gene transcript variant in different traditional Chinese medicine (TCM) syndromes of primary gout yet. In our study, the expression level of CASP1 gene transcript variant mRNA in peripheral blood mononuclear cells (PBMCs) of patients with primary gout (PG) in different TCM syndromes was measured by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) and/or real-time quantitative polymerase chain reaction (qRT-PCR);

in the meantime, the interleukin 1β was measured to explore the role of CASP1 gene and its transcript variant in the pathogenesis of gout.

2. Methods

2.1. The Clinical Data. All the research objects conformed to the 1977 American Rheumatism Association (ACR) diagnostic criteria [8], excluding the objects which have secondary gout caused by diseases of kidney, cardiovascular and blood system, or drugs and so on and also excluding coinfection, autoimmune diseases, long-term use of hormone therapy or serious condition that may affect the efficacy and safety of our study [9, 10]. 96 male patients with PG who visited the Department of Rheumatology of the Affiliated Hospital of North Sichuan Medical College from December 2012 to October 2013 were admitted; among them 44 patients were in acute phase and 52 patients were in nonacute phase. The age of patients ranged from 23 to 79 years old, and the mean age was 40 ± 11 years with their disease course being 9 ± 3 years. 30 healthy people set as healthy controls (HC) were admitted from the Department of Physical Examination in the same hospital during the same period whose age ranged from 22 to 70 years, their mean age was 44 ± 7 years, and their laboratory indexes were normal, excluding the people who had diseases or family history of cardiovascular disease, diabetes, liver, gout, and so on [10]. The age between the two groups showed no statistical significance. The study has gained approval and agreement of the local ethics committee and all participants signed informed consent.

2.2. The TCM Syndromes. Based on the gout TCM syndrome differentiation in *Clinical Diagnosis and Treatment Terminology of Traditional Chinese Medicine-Syndrome Type Part* [11] and *Guideline of the Study on New Traditional Chinese Medicine* [12] and the TCM differentiation according to comprehensive analysis by the "*four examination methods,*" "*Eight Principle Pattern Identification,*" and "*Zang-fu pattern identification,*" the TCM syndromes differentiation of PG patients was divided into four types: obstruction of dampness and heat syndrome (ODHS), intermingled phlegm-stasis blood syndrome (IPSBS), Pi-deficiency induced dampness syndrome (PDIDS), and Qi-blood deficiency syndrome (QBDS) [13].

2.3. Main Reagents and Instruments. Human lymphocytes separation liquid (Batch number LTS10771) was product of Jingyang Tech Co., China; RNAiso Plus Reagent (Batch number A9701-1), PrimeScript RT reagent kit with gDNA eraser (Perfect qRT-PCR) kit (Batch number AK1801), SYBR Premix Ex Taq II (Batch number AK5004), and TaKaRa LA Taq kit (CKA4501A) were products of Takara BIO Inc., Japan; ELISA kits specific for human interleukin-1β (IL-1β) (Batch number 20131014) was product of Beijing 4A Biotech Co., Ltd., China. [7].

The 7900 real-time fluorescence quantitative PCR instrument was a product of ABI Company, USA. The hypothermic high-speed centrifugal machine 5417R was a product of Eppendorf Company, USA. The FlexCycler PCR instrument was a product of Analytik Jena AG, Germany. The FUSION-Fx5 lithography machine was a product of Oriental Science & Technology Development Co., Ltd., China [7].

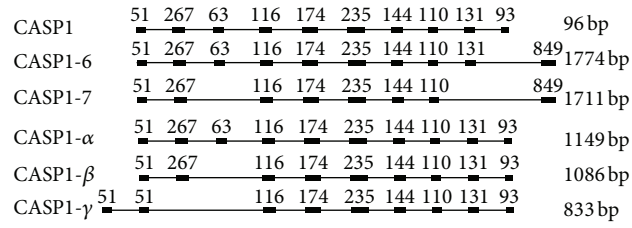


Figure 1: The exon of CASP1 gene and its transcript variant in human. Notes: rectangle: exon; straight line: intron; the number above the rectangle: length of exon; bp: fragment size.

2.4. Primer Design. Located in 11q23, the ID of CASP1 gene is 834, with ten exons, nine introns, seven transcript variants, and two functional domains (Figure 1). According to human β-actin and CASP1 gene and its transcript variant in GenBank, the primer was designed to be used in the measurement of RT-PCR or qRT-PCR and synthesized by polyacrylamide gel electrophoresis method in Genscript Biotechnology Co. Ltd., Designed primer sequences (Table 1).

2.5. Total RNA Extraction and cDNA Synthesis. 2.5 mL peripheral blood was taken and anticoagulated with heparin. PBMCs were separated by human lymphocytes separation liquid under sterile condition. According to instruction strictly, total RNA was extracted with Trizol reagent and the RNA was dissolved with 30 μL no RNA enzymes water. 5 μL RNA samples were taken and measured by agarose gel electrophoresis and three bands were showed in 1.5% agarose gel map: 28S, 18S, and 5S (Figures 2(c) and 3(c)). The absorbance value (A value) of RNA was detected by UV spectrophotometer detection at 260 and 280 nm wavelength and the A_{260}/A_{280} ratio was calculated (adopted 1.8 to 2.0) [9]. According to instruction strictly, cDNA was synthesized with reverse transcription kits on condition of 37°C for 15 min and 85°C for 5 sec, and then the reaction was terminated. 60 μL of the RT system was recorded as follows: 6 μL 5 ×g DNA eraser buffer, 3 μL gDNA eraser, 5 μL total RNA, 12 μL 5× PrimeScript buffer 2 (qRT-PCR), 3 μL prime script RT enzyme mix I, 3 μL RT primer mix, and 28 μL RNase free dH_2O [10]. The cDNA product was stored at −20°C.

2.6. Measurement of CASP1 Gene, Transcript Variant, and IL-1β by RT-PCR. PCR amplification was made in the 25 μL reaction system, which was created according to cDNA of HC and patients with PG: 0.25 μL TaKaRa LA Taq (5 U/μL), 2.5 μL 10× LA PCR buffer II (Mg^{2+} Free), 2.5 μL $MgCl_2$ (25 mM), 4 μL dNTP mixture (each 2.5 mM), 1 μL template DNA (cDNA), 0.5 μL primer 1 (upstream 20 μM), 0.5 μL primer 2 (downstream 20 μM), and 13.75 μL sterilized and distilled water. The reaction condition was initial denaturation in 95°C for 5 min, 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min and repeated 35 cycles, and extension in 72°C for 5 min [9]. Amplification products were measured by 1%

Table 1: Primer sequences of CASP1 gene and its transcript variant.

Gene (transcript variant) name	Upstream	Downstream	Genetic fragment size
CASP1	5′-CGCAGATGCCCACCACT-3′	5′-TGCCCACAGACATTCATACAG-3′	96 bp
CASP1-6 (NM_001257118.2)	5′-TACAGTTATGGATAAGACCCGAGC-3′	5′-GCAGACATAATTCCAAAAACCTTTA-3′	1774 bp
CASP1-7 (NM_001257119.2)	5′-TACAGTTATGGATAAGACCCGAGC-3′	5′-GCAGACATAATTCCAAAAACCTTTA-3′	1711 bp
CASP1-alpha (NM_033292.3)	5′-TACAGTTATGGATAAGACCCGAGC-3′	5′-GCAGACATAATTCCAAAAACCTTTA-3′	1149 bp
CASP1-beta (NM_001223.4)	5′-TACAGTTATGGATAAGACCCGAGC-3′	5′-GCAGACATAATTCCAAAAACCTTTA-3′	1086 bp
CASP1-gamma (NM_033293.3)	5′-TACAGTTATGGATAAGACCCGAGC-3′	5′-GCAGACATAATTCCAAAAACCTTTA-3′	833 bp
IL-1β	5′-ACAGATGAAGTGCTCCTTCCA-3′	5′-GTCGGAGATTCGTAGCTGGAT-3′	73 bp
β-actin	5′-GAGCTACGAGCTGCCTGACG-3′	5′-GTAGTTTCGTGGATGCCACAG-3′	120 bp

agarose gel electrophoresis and agarose gel was shot with exposure by FUSION-Fx5 lithography machine. The gray value of the exposed PCR strip images was measured by BIO-RAD Quantity-One software. The ratio of gray value of target gene to internal parameters was to reveal the expression level of target gene and its transcript variant mRNA.

2.7. Measurement of CASP1 and IL-1β Gene by qRT-PCR. cDNA of HC and patients with PG was measured by qRT-PCR instrument to create 20 μL reaction system: 10 μL SYBR Premix Ex Taq II, 0.4 μL ROX Reference Dye II, 0.8 μL upstream primer (10 μmol/L), 0.8 μL downstream primer (10 μmol/L), and 8 μL sterilized and distilled water. The reaction condition was 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min and repeated 40 cycles. Each specimen was done with multiple pores and the C_t value difference between the multiple pores was controlled within 0.5. All amplifications were performed on the ABI 7900 real-time PCR instrument. The melting curve was made after the amplification. ΔC_t derived from C_t value minus internal parameters of the target gene and $2^{-\Delta C_t}$ value represented the expression level of the target gene mRNA (the amplification of specific primer of target gene and its transcript variant).

2.8. The Purification and Sequencing of the PCR Products. The PCR products were purified and recycled by agarose gel purification. The recycled gene fragments were remeasured by agarose gel electrophoresis. After bands were confirmed, the nucleic acid sequence of purified target gene fragments was sequenced in Genscript Biotechnology Co. Ltd.

2.9. Measurement of the Level of Plasma IL-1β by ELISA Kit. According to instructions strictly, ELISA kit was operated, and OD values of each hole was measured with a microplate reader at 450 nm. The standard curve was made with standard sample of kit. The corresponding concentration was identified according to the absorbance value of the sample, and the final concentration of the sample was calculated by multiply the measured concentration by dilution factor.

2.10. Correlation Analysis. We analyzed the correlation between mRNA expression of CASP1 gene and its transcript variant and IL-1β in patients with PG in different phases and TCM syndromes and also analyzed the correlation between expression of CASP1 gene and its transcript variant mRNA and IL-1β protein in patients with PG in different phases and TCM syndromes.

2.11. Statistical Analysis. SPSS 16.0 software package was used for statistical analysis and all data were presented as mean ± standard deviation ($\overline{x} \pm s$). Comparison of mean values among multiple groups was done with ANOVA and comparison between two groups was done with the LSD test. The correlation of each group was done with spearman analysis. A $P < 0.05$ was considered as significant difference among groups.

3. Results

3.1. The Comparison of the Results between Different Phases and TCM Syndromes of Patients with PG. See Table 2.

3.2. The Primers Amplified Results of CASP1 Gene and Its Transcript Variant in PBMCs of Patients with PG in Different Phases and TCM Syndromes. See Figures 2 and 3.

3.3. The Expression of CASP1 Gene and Its Transcript Variant mRNA in PBMCs of PG Patients with PG in Different Phases and TCM Syndromes. The expression of CASP1 mRNA in APPG group was significantly higher than that in HC group ($P < 0.01$), the expression of CASP1-6 mRNA in APPG group and CASP1-6 and CASP1-7 mRNA in NAPPG group was significantly lower than in HC group ($P < 0.01$ or $P < 0.05$); the expression of CASP1 and CASP1-gamma mRNA in

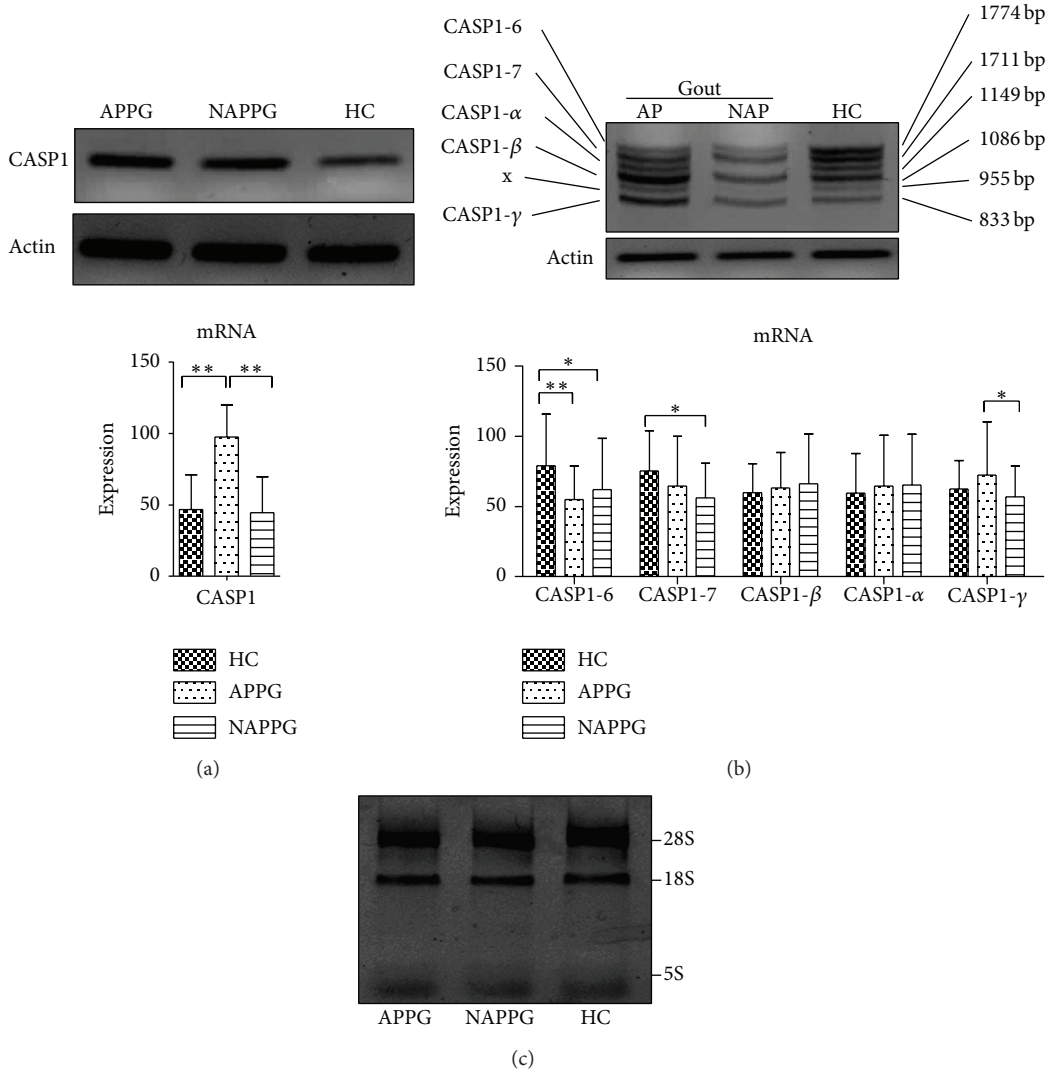


Figure 2: The mRNA expression of CASP1 gene and its transcript variant in PBMCs of patients with PG in different phases. Notes: HC: health control, AP: acute phase of primary gout, NAP: nonacute phase of primary gout; (a) CASP1 gene primers were amplified to one fragment (96 bp); (b) common primers of CASP1 gene transcript variants 6, 7, beta, alpha, and gamma were amplified to six fragments: 1774 bp was transcript variant 6, 1711 bp was transcript variant 7, 1086 bp was transcript variant beta, 1149 bp was transcript variant alpha, 833 bp was transcript variant gamma, and x was an unknown stripe. Delta and epsilon stripe were not observed in designed primers of our research, and we will carry on the design and experiment through different methods. (c) The RNA quality electropherogram of PBMCs. $^{*}P < 0.05$; $^{**}P < 0.01$.

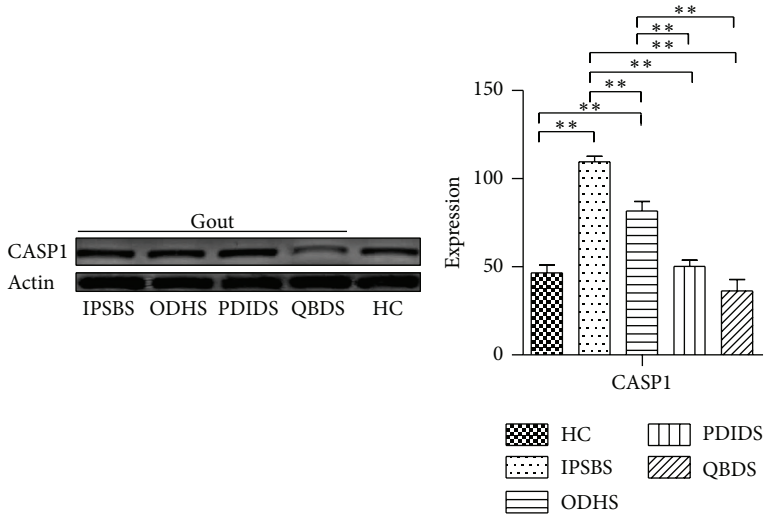
Table 2: The comparison of the results between the different phases and TCM syndromes of patients with PG.

Phases	APPG ($n = 44$)				NAPPG ($n = 52$)			
TCM syndromes	ODHS	IPSBS	PDIDS	QBDS	PDIDS	QBDS	IPSBS	ODHS
n	16	12	10	6	21	14	10	7
Percent (%)	36.36	27.27	22.73	13.64	40.38	26.92	19.23	13.46

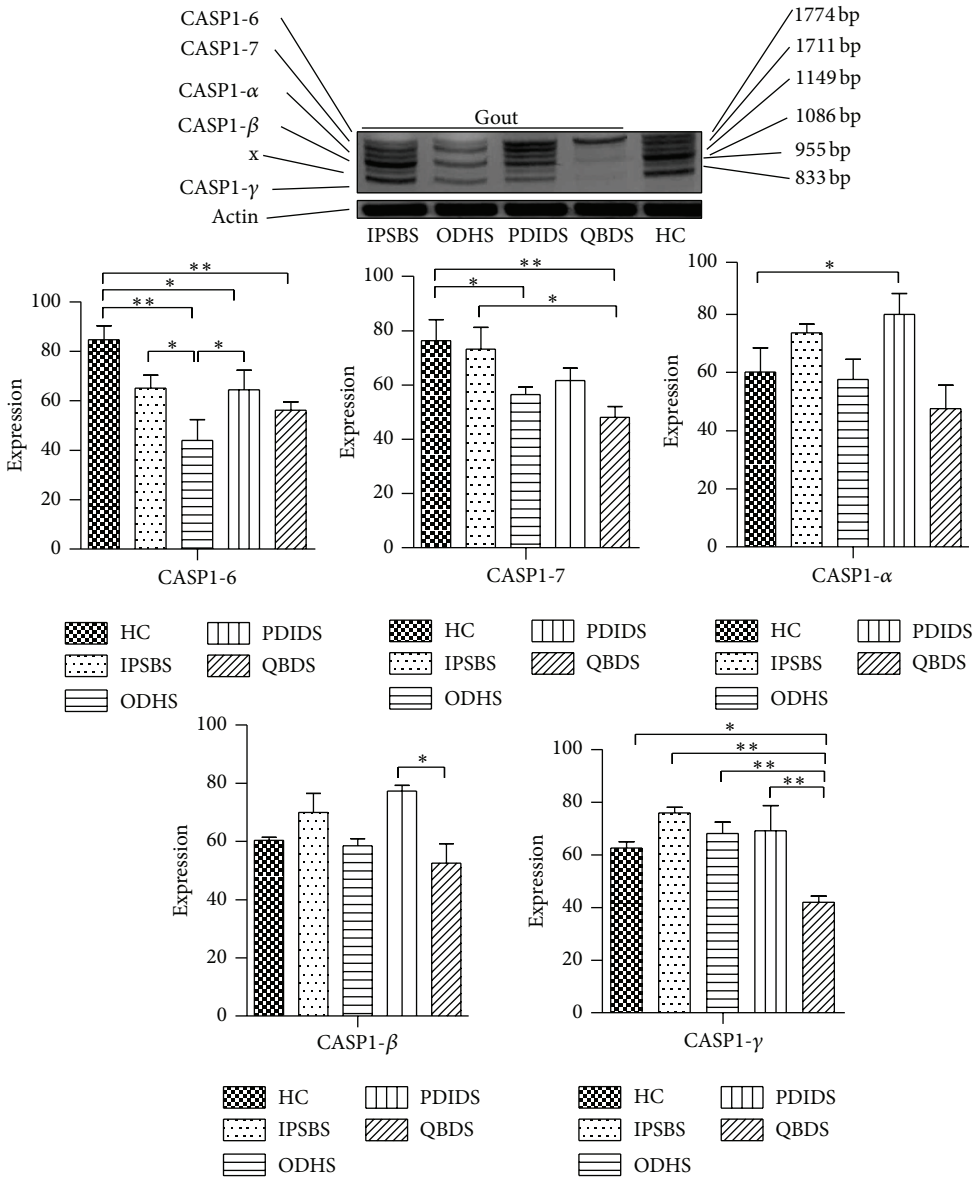
Notes: APPG: acute phase primary gout; NAPPG: nonacute phase primary gout; ODHS: obstruction of dampness and heat syndrome; IPSBS: intermingled phlegm-stasis blood syndrome; PDIDS: Pi-deficiency induced dampness syndrome; QBDS: Qi-blood deficiency syndrome.

NAPPG group was significantly lower than in APPG group ($P < 0.01$ or $P < 0.05$, Figure 2).

The expression of CASP1 gene mRNA in IPSBS and ODHS group was significantly higher than in HC group ($P < 0.01$); the expression of CASP1-6 and CASP1-7 mRNA in ODHS and QBDS group and CASP1-6 mRNA in PDIDS group all was significantly lower than in HC group ($P < 0.05$ or $P < 0.01$); the expression of CASP1-6 and CASP1-7 mRNA in ODHS group, CASP1 mRNA in PDIDS group, and CASP1, CASP1-7, and CASP1-gamma mRNA in QBDS group

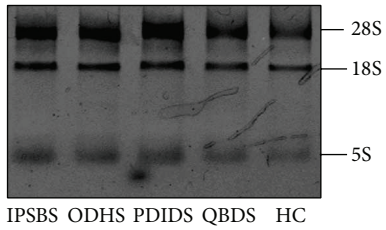


(a)



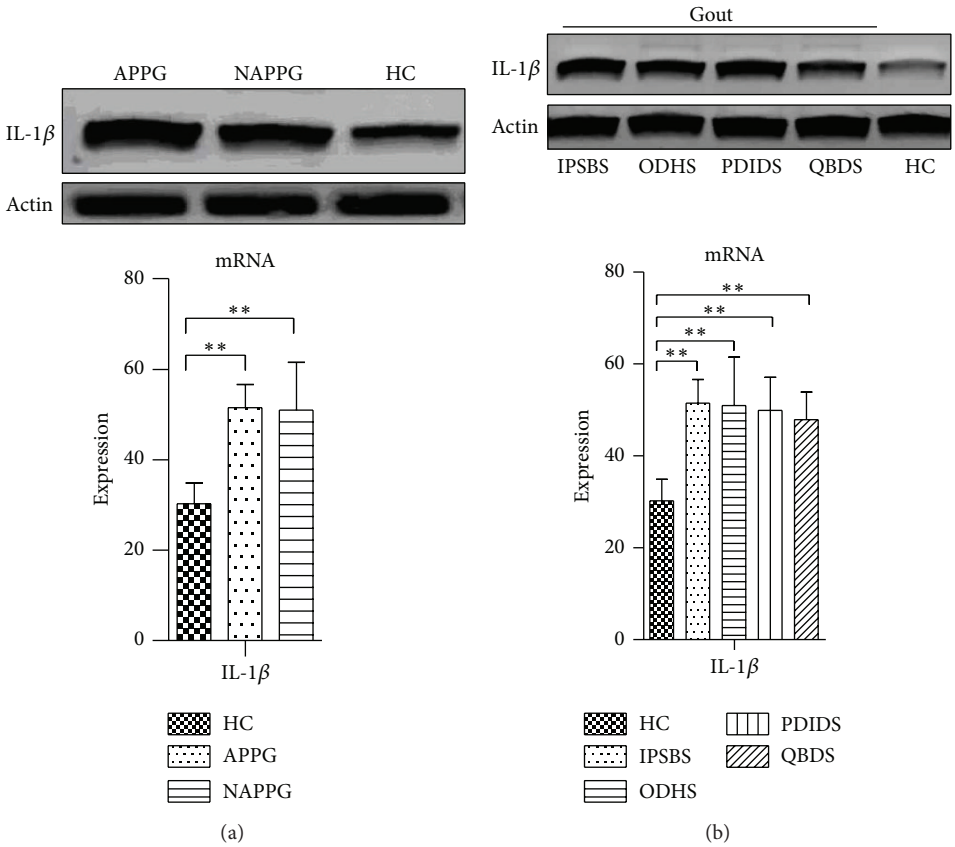
(b)

Figure 3: Continued.



(c)

Figure 3: The mRNA expression of CASP1 gene and its transcript variant in PBMCs of PG patients with different TCM syndromes. Notes: $^{*}P < 0.05$; $^{**}P < 0.01$.



(a) (b)

Figure 4: The mRNA expression of IL-1β in PBMCs of patients with PG in different phases and TCM syndromes. Notes: (a) and (b) IL-1β gene primers were amplified to one fragment (73 bp); (a) the mRNA expression of IL-1β in PBMCs of patients with PG in different phases; (b) The mRNA expression of IL-1β in PBMCs of patients with PG in different TCM syndromes. $^{**}P < 0.01$.

all was significantly lower than in IPSBS group ($P < 0.05$ or $P < 0.01$); the expression of CASP1 mRNA in PDIDS and CASP1-7 and CASP1-gamma mRNA in QBDS group all was significantly lower than in ODHS group ($P < 0.05$ or $P < 0.01$); the expression of CASP1-6 mRNA in PDIDS group was significantly higher than in ODHS group ($P < 0.05$); the expression of CASP1-beta and CASP1-gamma mRNA in QBDS group was significantly lower than in PDIDS group ($P < 0.05$ or $P < 0.01$, Figure 3).

3.4. The Expression of IL-1β mRNA in PBMCs of Patients with PG in Different Phases and TCM Syndromes. The expression of IL-1β mRNA in APPG and NAPPG group was significantly higher than in HC group ($P < 0.01$, Figure 4(a)).

The expression of IL-1β mRNA in IPSBS, ODHS, PDIDS, and QBDS group was significantly higher than in HC group ($P < 0.01$, Figure 4(b)).

3.5. The Expression of Plasma IL-1β Protein of Patients with PG in Different Phases and TCM Syndromes. The expression of plasma IL-1β protein in APPG and NAPPG group was significantly higher than in HC group ($P < 0.01$), and the expression of plasma IL-1β protein in APPG group

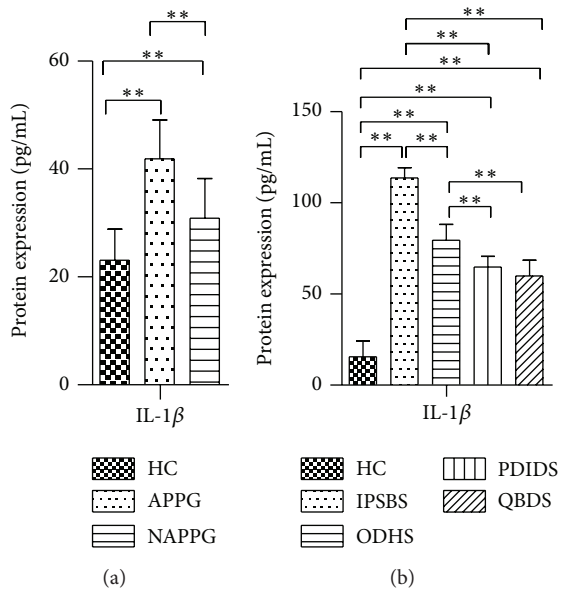


Figure 5: The expression of plasma IL-1β protein in patients with PG in different phases and TCM syndromes. Notes: (a) the expression of plasma IL-1β protein in patients with PG in different phases; (b) the expression of plasma IL-1β protein in patients with PG in different TCM syndromes. $^{**}P < 0.01$.

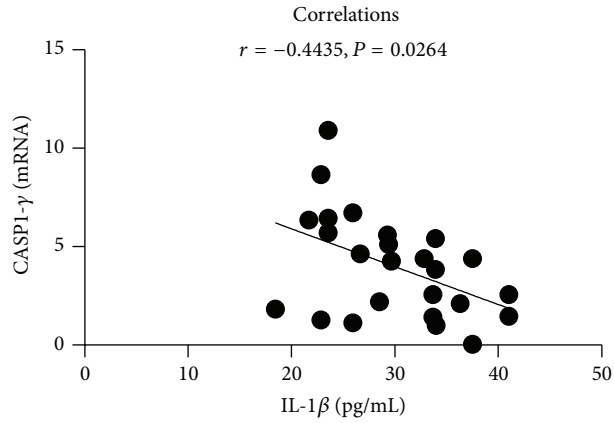


Figure 6: Correlation analysis of the expression level between CASP1-gamma gene transcript variant mRNA and IL-1β protein in APPG group.

was significantly higher than in NAPPG group ($P < 0.01$) (Figure 5(a)).

The expression of IL-1β protein in IPSBS, ODHS, PDIDS, and QBDS group was significantly higher than in HC group ($P < 0.01$); the expression of IL-1β protein in IPSBS group was significantly higher than in ODHS group ($P < 0.01$); the expression of IL-1β protein in IPSBS and ODHS group was significantly higher than in PDIDS and QBDS group ($P < 0.01$) (Figure 5(b)).

3.6. The Results of Correlation Analysis. Correlation analysis showed that there was negative correlation between the expression of CASP1-gamma gene transcript variant mRNA and IL-1β protein in APPG group ($r = -0.4435$, $P = 0.0264$; Figure 6), and no significant correlation was observed between the mRNA expression of CASP1 gene and its transcript variant and IL-1β in other groups ($P > 0.05$).

4. Discussion

Gout has a strong influence on people's health. The primary gout, with certain familial predisposition, was caused by both genetic and environmental factors, and the etiology was unknown except for about 1% due to congenital defects in purine metabolism enzymes [14]. In recent years, the incidence of gout in adults in China increases year by year and also increases with age [14]. Chinese medicine believes that the causes of gout were congenital deficiency, being worn out with age, dysfunction of spleen in transportation and with no ability to ascend lucidity or descend turbidity, or deficiency of kidney for activation of Qi and not distinguishing lucidity and turbidity resulted in cereal essence not being reformed along with noxious dampness which stranded and accumulated in the body, related to the dysfunction of middle jiao and lower jiao. By classifying gout patients into Western medicine phases and TCM syndrome, we found that the main TCM syndrome in the acute and nonacute phase of gout patients was IPSBS, ODHS, PDIDS, and QBDS, among which ODHS and IPSBS were mainly in the acute phase of gout while PDIDS and QBDS were in the nonacute phase. The results showed that the main syndromes in the acute phase of gout were obstruction of dampness and heat syndrome and intermingled phlegm-stasis blood syndrome, while, in the nonacute phase of gout, the main syndromes were Pi-deficiency induced dampness syndrome and Qi-blood deficiency syndrome.

Some researches had shown that inflammation and immunity played a certain role in the pathogenesis of gout [2], and the IL-1β level in peripheral venous blood of gout patients had significantly increased [15]. The body's innate immunity uses Toll-like Receptors (TLRs) to recognize the "naked" MSU crystals which can activate myeloid differentiation factor (MyD88) dependent NF-κB pathway and lead the gene transcription to produce prointerleukin 1β (Pro-IL-1β) precursor. Pro-IL-1β is cut into mature IL-1β through CASP1. By binding to IL-1 receptor, IL-1β can activate the IL-1 and NF-κB signal pathway, which can cause a large expression of proinflammatory factor like IL-1β, tumor necrosis factor-α (TNF-α), and so on, producing inflammatory cascade amplification effect [16].

It was known that an mRNA precursor (pre-mRNA) could produce different mRNA splice variant by selecting different splicing sites, and different splice variant plays an important role in the occurrence and development of diseases [17–19]. Studies showed that the immature CASP1 mRNA could be translated into six different subtypes through variable shear and transcription: alpha, beta, gamma, delta, epsilon, and zeta, all of which can not only mediate inflammatory response but also play different roles in cell death [20, 21]. Our study retrieved the already known seven gene transcript variants of CASP1 gene from Genbank. After CASP1 gene and

its transcript variant primers were designed and measured by RT-PCR, we found that the expression of CASP1 gene mRNA in IPSBS and ODHS group was significantly higher than in HC group ($P < 0.01$); the expressions of CASP1-6 and CASP1-7 mRNA in ODHS group, CASP1-6 mRNA in PDIDS group, and CASP1-6 and CASP1-7 mRNA in QBDS group were all significantly lower than in HC group ($P < 0.05$ or $P < 0.01$); the expression of CASP1 mRNA in APPG group was significantly higher than in HC group ($P < 0.01$), the expression of CASP1-6 mRNA in APPG group and CASP1-6 and CASP1-7 mRNA of NAPPG group was significantly lower than in HC group ($P < 0.05$ or $P < 0.01$); in the meantime, the expression of IL-1β mRNA in patients with PG in different phases and TCM syndromes was significantly higher than in HC group ($P < 0.01$). These results showed that CASP1 gene and its transcript variant are expressed abnormally in patients with PG in different phases and TCM syndromes, and these results also suggested that CASP1 gene and its transcript variant might play an important role in the regulation of inflammatory responses in patients with PG. The study also found that there are differences of the expression of CASP1 gene and its transcript variant between APPG and NAPPG groups. The results showed that the change of phases and TCM syndromes of patients with PG may relate to the change of the expression of CASP1 gene and its transcript variant.

Our study also found that the protein expression of plasma IL-1β of patients with PG in different phases and TCM syndromes was significantly higher than in HC group ($P < 0.01$); there were differences between the expression of plasma IL-1β protein in patients with PG in different phases and TCM syndromes, and the correlation analysis showed that there was negative correlation between the expression of CASP1-gamma gene transcript variant mRNA and IL-1β protein in APPG group ($r = -0.4435$; $P = 0.0264$). The results suggested indirectly that the CASP1 gene transcript variant may play an important role in the inflammatory responses of patients with PG, and the mechanism needs further and in-depth study [22, 23].

In conclusion, obstruction of dampness and heat syndrome and intermingled phlegm-stasis blood syndrome tend to occur in the acute phase of gout, while Pi-deficiency induced dampness syndrome and Qi-blood deficiency syndrome tend to occur in the nonacute phase of gout, and the mechanism may relate to the dysregulated expression of CASP1 gene and its transcript variant; the expression change of CASP1 gene and its transcript variant may be associated with the onset of gout. Therefore, further study for the mechanism of CASP1 gene transcript variant in PG is expected to provide new method for the effective prevention and treatment of PG.

5. Limitations

A small sample size may be a limitation for the present study. CASP1 transcript variant primers are not specific and cannot be detected by qRT-PCR; there may have been an error in the results detected by RT-PCR. Hence, every transcript variant specific primer of CASP1 should be redesigned later, and CASP1 gene transcript variant should be detected by qRT-PCR.

6. Conclusions

In summary, through our research, we initially demonstrated the existence of CASP1-6, CASP1-7, CASP1-alpha, CASP1-beta, and CASP1-gamma transcript variant in PBMCs of gout patients, and the expression of each transcript variant mRNA showed difference between patients with gout in different TCM syndromes and health controls, combined with relevant laboratory index; the results preliminarily indicated that CASP1 gene and its transcript variant might play an important regulating role in the pathogenesis of gout.

Abbreviations

ACR:	American Rheumatism Association
AP:	Acute phase
APPG:	Acute phase primary gout
CASP1:	Caspase-1
DAMPs:	Danger-associated molecular patterns
HC:	Healthy control
IPSBS:	Intermingled phlegm-stasis blood syndrome
LPS:	Lipopolysaccharides
LSD:	Least significant difference
MSU:	Monosodium urate
NAP:	Nonacute phase
NAPPG:	Non-acute phase primary gout
ODHS:	Obstruction of dampness and heat syndrome
PBMCs:	Peripheral blood mononuclear cells
PDIDS:	Pi-deficiency induced dampness syndrome
PG:	Primary gout
Pro-IL-1β:	Prointerleukin 1β
QBDS:	Qi-blood deficiency syndrome
TCM:	Traditional Chinese medicine
TLRs:	Toll-like receptor-specific.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

The authors contributed equally to this work.

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Review Article

Recent Advance in Applications of Proteomics Technologies on Traditional Chinese Medicine Research

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Proteomics technology, a major component of system biology, has gained comprehensive attention in the area of medical diagnosis, drug development, and mechanism research. On the holistic and systemic theory, proteomics has a convergence with traditional Chinese medicine (TCM). In this review, we discussed the applications of proteomic technologies in diseases-TCM syndrome combination researches. We also introduced the proteomic studies on the *in vivo* and *in vitro* effects and underlying mechanisms of TCM treatments using Chinese herbal medicine (CHM), Chinese herbal formula (CHF), and acupuncture. Furthermore, the combined studies of proteomics with other "-omics" technologies in TCM were also discussed. In summary, this report presents an overview of the recent advances in the application of proteomic technologies in TCM studies and sheds a light on the future global and further research on TCM.

1. Introduction

Traditional Chinese medicine (TCM), emphasizing most importantly the holistic concept, has been applied in the diagnosis, treatment, and prevention of illnesses in China and other Asian countries for more than 3000 years. Because of the complexity of the concept, the technology limitations, and the current investigation methodology, TCM diagnosis and treatment lack objective evaluation, and the essence and the mechanisms of TCM theory remain unclear.

Since proteins are the major executers of biological information, proteomic analysis provides a direct reflection of gene expression. Generally, proteomics is defined as the genome-scale analysis of protein abundance, structure, localization, modification, and activity. Currently, as a major component of systems biology, proteomics has gained comprehensive attention in the field of medical diagnosis, drug development, and mechanism studies [1, 2]. Proteomics technology is an important research tool for elucidating the differential expressions of proteins in peripheral body fluids, cells, tissues, blood, and urine samples [3]. Blood and urine are the most widely used specimens because their molecular compositions fluctuate in response to the dynamic physiological and pathological conditions of the body. Technically, proteomic analysis requires the combination of several technologies, that is, protein processing and separation such as two-dimensional polyacrylamide gel electrophoresis (2DE), high-performance liquid chromatography (HPLC), mass spectrography (MS) such as MALDI-TOF-MS, SELDI-TOF-MS, and MS/MS, isobaric tags for relative and absolute quantification- (iTRAQ-) based quantitative proteomic analysis, and bioinformatics [4].

TCM diagnosis and therapy depend on the intuition and experience of the TCM theory trained physicians. Compared with biomolecular science and western medicine, TCM appears to be nonobjective and lacking accuracy and reproducibility. In accordance with the holistic and systemic theory, proteomics has a convergence with TCM and can overcome biases in TCM research. Proteomics can be helpful in exploring the scientific connotation of TCM and the modernization of Chinese herbal medicine (CHM). First, proteomics could be used to characterize the differential

expression profiles between healthy individuals and patients with different TCM syndromes. For example, apolipoprotein A1 and apolipoprotein A4 expression levels analyzed by plasma proteomics were found to be the potential diagnostic and prognostic markers for chronic viral hepatitis B (CHB) with damp-heat retention in Middle-Jiao syndrome (DRMS) [5]. Similar findings [6–10] can discover other molecular markers of TCM syndromes in clinical applications.

Secondly, proteomics can help discover molecular targets, develop new bioactive compounds, and elucidate the underlying mechanisms of TCM treatment. For instance, a recent proteomic study showed that Tianma promoted neuroregenerative processes by inhibiting stress-related proteins and mobilizing neuroprotective genes such as Nucleoredoxin (Nxn), Drebrin-like protein (Dbnl), Ki67 protein, and Baxin mouse N2a cells [11]. This and other similar proteomic studies provide important insights into the molecular mechanisms underlining the beneficial effects of TCM treatments [12–15].

In this report, we reviewed the current proteomic approaches in TCM research including clinical TCM diagnosis and treatment and *in vitro* and *in vivo* mechanistic studies and shed light on future utilization of proteomics for TCM research (Figure 1).

2. Proteomics Studies on Disease-TCM Syndrome Combination

TCM syndrome, also called ZHENG in Chinese, is a profile of clinical symptoms and signs, which reflect the essence of pathological changes in the occurrence and development of diseases, provide great insights in understanding the human homeostasis, and guide specific TCM treatments. TCM syndrome differentiation, that is, the diagnosis of TCM syndrome, is to differentiate diseases by analyzing the information of each patient, for example, patients' symptoms and physical status, which were collected by four diagnostic methods: inspection, auscultation and olfaction, inquiry, and palpation [16]. Currently, although the applications of proteomic technologies in TCM are much less common than those in western medicine, clinical TCM studies using proteomic technologies have already achieved some great successes, with several biomarkers and the mechanisms of TCM syndrome differentiation being discovered in various diseases, such as hepatorenal, cardiocerebrovascular, and lung diseases.

2.1. Hepatorenal Diseases and TCM Syndromes. In a chronic hepatorenal diseases-TCM syndrome study, Wei et al. [5] investigated the plasma proteomics of chronic viral hepatitis B (CHB) of damp-heat retention in the Middle-Jiao syndrome (DRMS) using 2DE and MS technologies; they found apolipoproteins A1 and A4 as the diagnostic and prognostic markers or treatment targets. Liu et al. demonstrated that immunoglobulin J-chains protein could be a new biomarker for the diagnosis of different TCM syndromes in CHB [6]. Song et al. [7] established diagnosis models of excess syndrome and deficiency syndrome in CHB by SELDI-based protein chip analysis. Using MALDI-TOF-MS technology, Zhou et al. [8] set up diagnosis models of Spleen-Qi asthenia syndrome, Liver-Kidney Yin deficiency syndrome, and blood stasis syndrome in hepatitis B cirrhosis (HBC).

In addition, Hao et al. [9, 10] established a predictive model for clinical typing of chronic renal failure (CRF), screened for protein markers in urine samples of CRF patients with TCM damp syndrome (CMDS), and illustrated that urine protein biomarkers reflected different biological features of CRF with different TCM syndromes. For example, the levels of m/z 1674.53 and m/z 1952.7, two differentially expressed proteins, were elevated in Liver-Kidney Yin deficiency but lowered in Spleen-Kidney Qi deficiency, Spleen-Kidney Qi-Yin deficiency, Spleen-Kidney Yang deficiency, and Yin-Yang deficiency. m/z 2305.78 and m/z 4262.02, another two differentially expressed proteins, were expressed less in Liver-Kidney Yin but more in Spleen-Kidney Qi deficiency, Spleen-Kidney Qi-Yin deficiency, Spleen-Kidney Yang deficiency, and Yin-Yang deficiency.

2.2. Cardiocerebrovascular Diseases and TCM Syndromes. In a cardiocerebrovascular diseases-TCM syndrome research, Chu et al. [17, 18] demonstrated that the differentially expressed proteins, such as 9334.958 m/z (increased), 9280.191 m/z (decreased), 8030.794 m/z (increased), and 2941.551 m/z (increased), might be potential biomarkers of abundant phlegm-dampness syndrome (PDS) and liver-gallbladder dampness-heat syndrome (LGDHS) in hypertension patients.

Song et al. [19] studied correlation between the states of Zang-Fu organs and the levels of plasma biomarker proteins and found differential plasma protein profiles in hyperlipidemia and atherosclerosis of different patterns of phlegm stasis syndrome and blood stagnation syndrome. For example, the levels of albumin, adrenomedullin binding protein precursor, and haptoglobin precursor in patients with phlegm syndrome were different from those in the patients with blood stagnation syndrome and also correlated with kidney-Qi deficiency and heart-Qi deficiency, while the complement component C4 is independent of the deficient Zang-Fu organs. Zhao et al. [20] uncovered common proteomic characteristics in unstable angina with Qi deficiency and blood stasis syndrome (QBS) and phlegm stasis cross-blocking syndromes (PSS), indicating a correlation of these proteins with inflammatory reaction and metabolic disturbance. For instance, actin was found only expressed in Qi deficiency blood stasis syndrome (QDBS), while FN, ApoH, and ANXA6 are highly expressed in QDBS. Wang et al. [21] found that energy metabolism and myocardial structural injury associated proteins, isocitrate dehydrogenase 3 (NAD+) alpha, NADH dehydrogenase (NAD) Fe-S protein 1, chain A, heat shock protein 27 (HSP27), and oxidoreductase (NAD-binding protein), may be biomarkers for the diagnosis of chronic myocardial ischemia with QBS.

2.3. Lung Diseases and TCM Syndromes. Using proteomic technology, Liu et al. [22] established a diagnostic serum proteomic model for the three TCM syndromes in tuberculosis (TB), and ApoC-III was identified as a potential biomarker

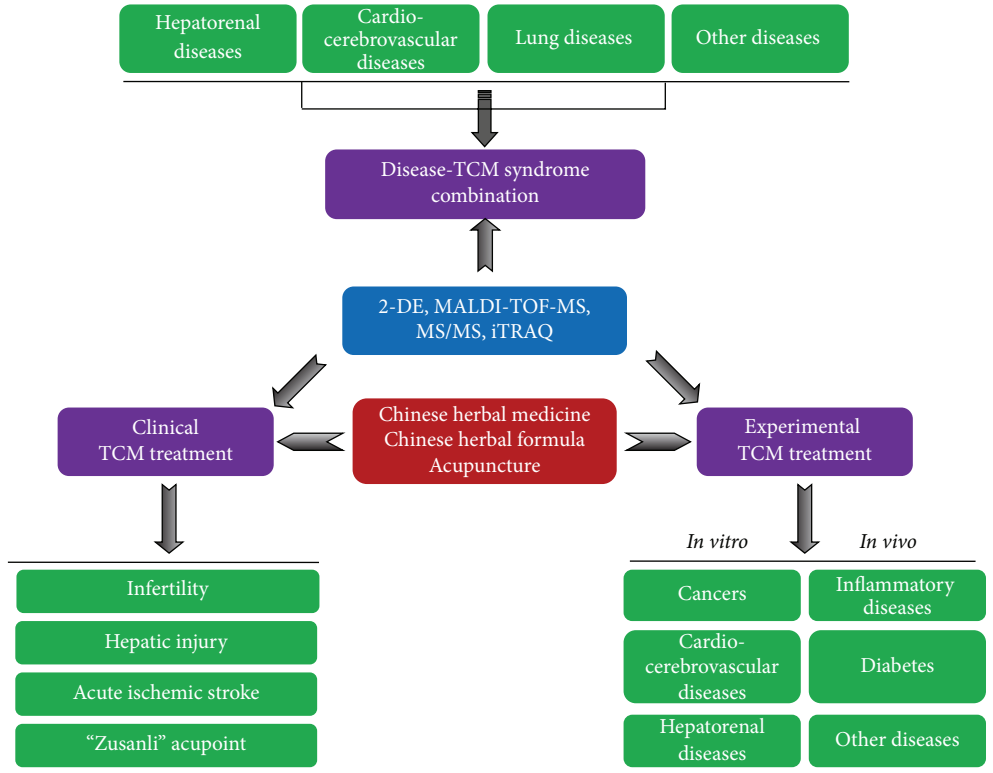

Figure 1: Scheme of the overview of the recent advance in the applications of proteomics technologies on traditional Chinese medicine research.

for TCM syndrome differentiation in TB. By combining SELDI-TOF-MS techniques with a decision tree model, nine upregulated and six downregulated proteins were identified in lung cancer patients with Qi deficiency syndrome and phlegm and blood stasis syndrome. Two candidate protein peaks, 2284.97 *m/z*, were selected to establish a predictive model, which can be applied in the TCM syndrome differentiation in lung cancer [23].

Other studies have also provided the evidence of syndrome differentiation using proteomic technologies in chronic stomach disease [24], myasthenia gravis [25], and systemic lupus erythematosus [26]. All these studies suggested that the rapid growth of proteomics has made it possible for the integration of diseases-TCM syndrome with modern technology, thereby supplying the diagnostic or prognostic markers for TCM syndrome differentiation, as well as TCM therapy targets.

3. Proteomic Studies of Clinical TCM Treatments Using Chinese Herbal Medicine (CHM), Chinese Herbal Formula (CHF), and Acupuncture

TCM treatment is based on the holistic characterization of patients' disease status, which is diagnosed to certain TCM syndrome type. Most of the TCM therapeutic methods, CHM, CHF, or acupuncture, provide modern medicine with a collection of complementary remedies for disease treatment and health maintenance. Although some of CHM, CHF, or acupuncture methods are known to have beneficial effects on the diseases, their therapeutic efficacy could not be well evaluated. Recently, proteomic technologies have been utilized to measure the therapeutic effect of TCM treatments [74].

Lian et al. [75] demonstrated that the favorable effects of Liuwei Dihuang granule (LDG) on infertility of women with Kidney-yin deficiency syndrome might be through regulating the expression levels of retinol binding protein 4, transthyretin, apolipoprotein, and complement C4-B, all being associated with HPT axis, lipid metabolism, estrogen level, and cellular immunity, and activation function of complement system-pathways might be the actionable targets for the treatment of infertility with LDG. To identify the drug targets of TCM formulae Yin-Chen-Hao-Tang (YCHT), which was used to treat hepatic injury, Sun et al. [76] performed 2DE and MALDI-TOF/TOF-MS analysis and found that YCHT modulated the expression levels of several proteins, that is, zinc finger protein 407, haptoglobin, transthyretin, and vitamin D-binding protein, all being involved in metabolism, energy generation, chaperone, antioxidation, signal transduction, protein folding, and apoptosis. Pan et al. [77] investigated the effects of acupuncture on serum protein levels in a total of 35 acute ischemic stroke (IS) patients, with the acupuncture treatment being performed on eight acupuncture points once a day for 10 consecutive days. After acupuncture, SerpinG1 protein expression in patients' serum was downregulated while those of gelsolin, complement

component I, C3, C4B, and beta-2-glycoprotein I proteins were upregulated. iTRAQ-based quantitative proteomics was performed to identify key proteins in the blood sample for acupuncture at "Zusanli" acupoint (ST-36) in patients, and a total of seven related proteins were identified. These proteins, aldolase A protein, hCG2008184, ATP synthase, ATP5A1 protein, and hexokinase type 1, were involved in the regulation of multiple metabolism pathways, which may help elucidate the action mechanism of ST-36 acupuncture [78].

Although proteomic studies in clinical TCM medicine have been successful, such studies in clinical TCM treatment still remain few. Ideally, proteomic studies should be conducted in both the diagnosis of a TCM syndrome and its corresponding treatment including CHM, CHF, and acupuncture. A large number of experimental proteomic studies are currently under way to uncover the molecular mechanisms of TCM treatment.

4. Proteomic Studies on Mechanisms of TCM Treatments *In Vitro* and *In Vivo*

The complex nature of TCM determines that a thorough investigation on the mechanisms and physical basis of TCM will not be so easy. Luckily, development of modern biotechnologies is constantly providing novel and powerful tools. Proteomic technologies can reveal statistically significant changes in the levels of proteins, identify novel target molecules, and provide clues for the underlying mechanisms of TCM treatments. Herein, we will focus mainly on the application of proteomics in the research of TCM treatment using *in vitro* and *in vivo* models over the past years [79].

4.1. Evaluation of TCM Treatment In Vitro. Proteomic technologies can be applied to screen target molecules and explore the effective mechanisms of TCM treatments in various cell lines originated from various diseases, such as cancers and cardiocerebrovascular and inflammatory diseases. TCM treatment includes CHM, CHM compounds, and CHF. As shown in Table 1, proteomics is often applied to investigate the change of proteins and of various related signaling pathways in TCM treatments *in vitro*.

4.1.1. Cancers. In cervical carcinoma, Cui et al. [27] tested the cytotoxicity of 9,11-dehydroergosterol peroxide (DHEP) isolated from *Ganoderma lucidum* on HeLa cells and revealed that Stathmin 1 might be a target of DHEP. Yue et al. [28] found that triterpenes from *Ganoderma lucidum* extract targets interleukin-17E, eukaryotic translation initiation factor 5A, peroxiredoxin-2, and ubiquilin-2, which are involved in cell proliferation, carcinogenesis, and oxidative stress. In addition, Pan et al. [29, 30] found by proteomics that Tanshinone IIA had cytotoxic activity against HeLa cells via regulating the expression of proteins involved in apoptotic processes, spindle assembly, and p53 activation.

In hepatocellular carcinoma, through proteomic analysis, Fu et al. [31] discovered that 1,3,6,7-tetrahydroxyxanthone (TTA) effectively induced apoptosis of HepG2 cells through upregulating the expression levels of P16 and 14-3-3σ protein while downregulating that of β-tubulin. Fu et al. [32] demonstrated that 1,3,5-trihydroxy-13,13-dimethyl-2H-pyran [7,6-b] xanthone promoted mitochondrial apoptosis of HepG2 cells via mediating the heat shock protein 27.

In colorectal adenocarcinoma, Huang et al. [33] found that Baicalein inhibited colorectal cancer DLD1 cell proliferation and reduced reactive oxygen species (ROS) by upregulating the levels of peroxiredoxin-6 (PRDX6). Liu et al. [34] discovered that 14-3-3 epsilon, a cell cycle- and apoptosis-related protein, was affected (including cleavage and perinuclear translocation) in colon cancer SW480 cells treated with Triptolide.

In gastric adenocarcinoma, Lin et al. [35] revealed that Tanshinone IIA suppressed gastric cancer AGS cell growth by blocking glucose metabolism via the downregulation of the levels of intracellular ATP, glucose-6-phosphate isomerase, and L-lactate dehydrogenase B chains, as well as altering the p53 and AKT expression. Zhu et al. [15] found in human gastric adenocarcinoma SGC-7901 cells that *Celastrus orbiculatus* suppressed TGF-β1-induced epithelial-mesenchymal transition by inhibiting HSP27 expression, and further investigation showed that the downregulation of HSP27 was associated with TNF-α-induced NF-κB/Snail signaling pathway.

In breast cancer, proteomic analysis by Fang et al. [14] identified 12 differentially expressed proteins, of which the downregulated proteins TDP-43, SF2/ASF, and eIF3i, as well as upregulated proteins including 3-PGDH, ERP29, and platelet-activating factor acetylhydrolase IB subunit beta, positively contributed to the anticancer activity of Curcumin in human breast cancer MCF-7 cells. In addition, a study by Chou et al. [36] illustrated that berberine induced apoptosis of MCF-7 cells, and a link between ROS generation and cell death was identified using lysine- and cysteine-labeling 2D-DIGE combined with MS.

In leukemia K562 cells, Wei et al. [37] demonstrated that, after treatment with triterpenes from *Patrinia heterophylla*, 4 proteins were upregulated (aldolase A, glyceraldehyde-3-phosphate dehydrogenase, flavin reductase, and hemoglobin subunit) and 4 downregulated (heat shock protein 90 Alpha, eukaryotic translation initiation factor 5A, moesin, and tubulin). These proteins were associated with energy metabolism, oxidative stress, apoptosis, signal transduction, differential induction, and protein biosynthesis.

4.1.2. Cardiocerebrovascular Diseases. In heart disease, Fan et al. [38] revealed that Shuanglong formula (SLF) induced autologous mesenchymal stem cells (MSCs) into cardiomyocyte-like cells, and 36 proteins, which functioned in cytoskeleton, cell tissue energy metabolism, and signal transduction, showed distinct differential expression patterns before and after SLF treatment. Feng et al. [39] clarified the signaling impact of salvianolic acid B (SB) in H9C2 cells using proteomic assay and bioinformatic analysis and found the signal cascade from EGFR to heat shock protein 27 (HSP27) and mitofilin might be the most important cascade that was affected by SB.

In Alzheimer's disease, Tao et al. [40] found that Huperzine A, from *Huperzia serrata*, protected N2a cells from

Table 1: Proteomics applied in the effective mechanisms of TCM treatments *in vitro*.

Diseases	CHF, CHM, and CHM compound	Targets or signaling pathways	Proteomics methods	References
Cancers				
Cervical carcinoma	9,11-Dehydroergosterol peroxide	Stathmin 1	MALDI-TOF MS/MS	[27]
Cervical carcinoma	Triterpenes	IL-17E, eIF5A, peroxiredoxin-2, and ubiquilin-2	2DE, MALDI-TOF MS/MS	[28]
Cervical carcinoma	Tanshinone IIA	Vimentin, Maspin, a- and b-tubulin, and GRP75	2DE, MALDI-TOF MS	[29]
Cervical carcinoma	Tanshinone IIA	Endoplasmic reticulum stress pathways	2DE, MALDI-TOF-TOF MS	[30]
Hepatocellular carcinoma	1,3,6,7-Tetrahydroxyxanthone	β-tubulin, 14-3-3σ, and P16	2DE, MALDI-TOF-MS, MS/MS	[31]
Hepatocellular carcinoma	1,3,5-Trihydroxyxanthone	HSP27	2DE, MALDI-TOF-TOF MS	[32]
Colorectal carcinoma	Baicalein	Peroxiredoxin-6	2DE, MALDI-TOF-TOF MS	[33]
Colon carcinoma	Triptolide	14-3-3ξ	2DE, MALDI-TOF-TOF MS	[34]
Gastric adenocarcinoma	*Celastrus orbiculatus*	HSP27, NF-κB/Snail signal pathways	2DE, MALDI-TOF-TOF MS	[15]
Gastric adenocarcinoma	Tanshinone IIA	p53, AKT, G6PI, and LDHB	iTRAQ	[35]
Breast carcinoma	Curcumin	TDP-43, SF2/ASF, eIF3i, 3-PGDH, and ERP29	2DE, MALDI-TOF MS	[14]
Breast carcinoma	*Coptis chinensis* Franch.	ROS generation	2D-DIGE, MS	[36]
Leukemia	Triterpenes	Aldolase A, GAPDH, and HSP90-Alpha	2DE, MALDI-TOF-MS	[37]
Cardiocerebrovascular diseases				
Heart disease	Shuanglong formula	Energy metabolism	2DE	[38]
Cardiovascular disease	Salvianolic acid B	HSP27 and mitofilin	2DE, MALDI-TOF-MS/MS	[39]
Alzheimer's disease	Huperzine A	Trp53	LC-MS/MS	[40]
Parkinson's disease	*Acanthopanax senticosus* Harms	Lewy body, mitochondrial energy metabolism	iTRAQ	[41]
Neurodegenerative disorders	Tianma	CALR, FKBP3/4, HSP70/90, and AIP5	iTRAQ	[42]
Neurodegenerative disorders	Tianma	Nxn, Dbnl, Mobkl3, Clic4, Mki67, and Bax	iTRAQ	[43]
Inflammatory diseases				
Inflammatory diseases	Bi-qi capsule	iNOS, COX-2, TNF-α, IL-6, and IL-1β	Proteome profiler array	[44]
Inflammatory diseases	Zuojin pill	iNOS, COX-2, IL-6, IL-1β, TNF-α, and NF-κB	Proteome profiler array	[45]
Neuroinflammation	*Acanthopanax senticosus* extract	Nitrosative stress pathway	2D-DIGE, LC-ESI-MS/MS	[12]
Other diseases				
Wound healing	Lithospermi radix	Antioxidant activity, antiapoptosis activity	2DE, LC-MS/MS	[46]
Oxidative damage	Isopsoralen	Proteins (m/z 6532 and m/z 6809)	SELDI-TOF-MS	[47]
Ischemia reperfusion injury	Tao Hong Si Wu decoction	Nrf2-mediated phase II enzymes	2DE, MALDI-TOF MS	[48]
Postcataracts	Curcumin	Proteins (m/z of 8093 and m/z 13767)	MS	[49]

amyloid β-induced cell death by decreasing the p53 protein levels.

In Parkinson's disease (PD), iTRAQ-based quantitative proteomics study [41] uncovered that, after treatment with extract of *Acanthopanax senticosus* Harms (EAS) in A53T-α-Syn transgenic SH-SY5Y cells, 16 out of 84 abnormally expressed proteins were altered. These proteins play roles mainly in formation of Lewy body, mitochondrial energy metabolism, protein synthesis, and apoptosis. Also in SH-SY5Y cells, Ramachandran et al. [42] discovered that Tianma promoted neuroregenerative signaling cascades by controlling chaperone proteins such as CALR, FKBP3/4, and HSP70/90, mobilizing a neuroprotective gene AIP5, and modulating RTN1/4, NCAM, PACSIN2, and PDLIM1/5 with various regenerative modalities and capacities related to neurosynaptic plasticity. In another proteomic research by Manavalan et al. [11], they proposed that Tianma promoted neuroregenerative processes in N2a cells by inhibiting stress-related proteins and mobilizing neuroprotective genes such as Nxn, Dbnl, Mobkl3, Clic4, Mki67, and Bax.

4.1.3. Inflammatory Diseases. In inflammatory diseases, the proteome profiler array analyses [44] displayed that 8 cytokines were downregulated while 6 were upregulated by Bi-Qi capsule in lipopolysaccharide-stimulated RAW 264.7 macrophages. The same research group [45] also found that Zuojin pill inhibited the levels of inflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-6, and interleukin 1β (IL-1β) in lipopolysaccharide-stimulated RAW 264.7 mouse macrophages. Additionally, Jiang et al. [12] established that *Acanthopanax senticosus* extract (ASE) suppressed LPS-induced nitrosative stress in BV-2 cells, and proteomic quantitative analyses revealed that the levels of 17 proteins significantly changed in response to ASE.

In other diseases, by proteomic analysis, several important molecular mechanisms of TCM treatment have been found, such as Lithospermi radix on wound healing [46], isopsoralen on oxidative damage [47], Tao Hong Si Wu decoction (THSWD) on cerebral ischemia reperfusion injury [48], and Curcumin on postcataracts [49].

One may know that *in vitro* studies may not reflect the actual effects of TCM treatments, as *in vivo* body environment and the influencing factors are very complex and hard to control. To better understand the effective mechanisms of TCM treatments, *in vivo* animal models and tests are highly desired.

4.2. Evaluation of TCM Treatments In Vivo. Presently, the *in vivo* proteomic research involves mainly the rat and mouse models. Recent studies focused mostly on the following diseases: cardiocerebrovascular diseases, hepatorenal diseases, diabetes, and so on. In addition, proteomic technologies were also applied to study the effective mechanisms of acupuncture on diseases such as asthma. As shown in Table 2, the proteomics was applied to investigate the mechanisms of TCM treatments including CHF, CHM, and CHM compound *in vivo*.

4.2.1. Cardiocerebrovascular Diseases. By proteomic analysis of heart tissues in rat ischemia/reperfusion (I/R) models, Jia et al. [50] confirmed that Dingxin recipe prevented ischemia/reperfusion-induced arrhythmias via upregulating prohibitin and suppressing inflammatory responses. In a rat model of myocardial infarction, Zhou et al. [51] established that Buyang Huanwu decoction (BYHWD) alleviated ventricular remodeling, which increased Bcl-2/Bax ratio and decreased caspase 3 activity via downregulating atrial natriuretic factor (ANF) while upregulating heat shock protein beta-6 (HSPB6) and peroxiredoxin-6 (PRDX6).

By the differential proteomic analysis in platelet samples of SD rats, Ma et al. [52] proposed that salvianolic acid B (SB) caused regulation of 20 proteins such as heat shock-related 70 kDa protein 2 (HSP70), LIM domain protein CLP-36, copine I, peroxiredoxin-2, coronin-1B, and cytoplasmic dynein intermediate chain 2C. Furthermore, SB bound with integrin $\alpha 2\beta 1$ to regulate intracellular Ca^{2+} level and the levels of cytoskeleton-related protein coronin-1B and to affect cytoskeleton structure of platelets. In addition, Yue et al. [53] from the same studying group demonstrated that salvianolic acids (SA) and notoginsenoside (NG) showed both similarity and difference in their protein targets involved in cardioprotective effects.

Lo et al. [54] investigated the effect of *Uncaria rhynchophylla* (UR) on the differentially expressed proteins in SD rats with kainic acid- (KA-) induced epileptic seizures using a proteomic analysis and found that macrophage migration inhibitory factor (MIF) and cyclophilin A were underexpressed in frontal cortex by an average of 0.19- and 0.23-fold, respectively, suggesting that both MIF and cyclophilin A at least partially participated in the anticonvulsive effect of UR. Zhang et al. [55] explored the effective mechanisms of Yizhijiannao granule (YZJN) in treating Alzheimer's disease (AD) with proteomic tools, and the results indicated that YZJN regulated multiple protein expressions in entorhinal cortex tissues of SAMP8, suggesting that it had multitarget therapeutic action and the mechanism in treating AD is possibly via improving mitochondria function, antagonizing oxidation stress, preventing nerve cell apoptosis, and protecting neurons. Koh [56] has identified the proteins differentially expressed in cerebral cortexes of *Ginkgo biloba* extract- (EGb761-) treated rats in a middle cerebral artery occlusion model, and the results showed that EGb761 protected neuronal cells against ischemic brain injury through the specific up- and downmodulation of various proteins.

Manavalan et al. [43] found that *Gastrodia elata* (Tianma) affected synaptic plasticity and neurorestorative processes and thus might be a novel candidate agent for the treatment of neurodegenerative diseases by regulating the brain proteome. In detail, the long-term treatment with Tianma modulated the brain protein metabolism at the proteome level by downregulating the expressions of various proteins, such as Gnao1 and Dctn2, which are related to neuronal growth cone control and synaptic activities. Tianma treatment also induced the upregulation of molecular chaperons and proteins related to the misfolded protein response, such as Pacsin1 and Arf3 involved in Huntington's disease (HD).

Table 2: Proteomics applied in the effective mechanism of TCM treatment *in vivo*.

Diseases	CHF, CHM, CHM compound, and acupuncture	Targets or signaling pathways	Proteomics methods	References
Cardiocerebrovascular diseases				
Arrhythmias	Dingxin recipe	Prohibitin	2DE, MALDI-TOF MS	[50]
Ischemic myocardial injury	Buyang Huanwu decoction	Atrial natriuretic factor	2DE, MALDI-TOF MS	[51]
Cardiovascular disorders	Salvianolic acid B	Integrin $\alpha 2\beta$	2DE, MALDI-TOF MS/MS	[52]
Cardiovascular disorders	Salvianolic acids	Energy metabolism, lipid metabolism	2DE, MALDI-TOF MS/MS	[53]
Epileptic seizures	*Uncaria rhynchophylla*	MIF and cyclophilin A	2DE	[54]
Alzheimer's disease	Yizhijiannao granule	NADH dehydrogenase iron-sulfur protein 6	2DE, peptide mass fingerprint	[55]
Neurodegenerative diseases	*Ginkgo biloba* L. extracts	PPAP subunit Band CRMP2	2DE, MALDI-TOF MS	[56]
Cerebrovascular diseases	Tianma	Gnao1, Dctn2, Anxa5, Pacsin1, and Arf3	iTRAQ	[43]
Hepatorenal diseases				
Liver cirrhosis	Yiguanjian decoction	Cu/Zn SOD, DJ-1	2DE, MALDI-TOF-TOF MS	[57]
Liver fibrosis	Fuzheng Huayu	Vimentin	2DE/MS	[58]
Liver injury	Yin-Chen-Hao-Tang	Zinc finger protein 407, haptoglobin	RP-HPLC	[59]
Immunological liver injury	*Salvia miltiorrhiza*	PRDX6	2D-DIGE, MALDI-TOF MS	[60]
Nephropathy	Tanshinone IIA	Oxidative stress	2DE	[61]
Diabetes				
Diabetes	Granules	Apolipoprotein E (apoE) and C3	2DE, MALDI-TOF MS/MS	[62]
Type 2 diabetes mellitus	ZiBuPiYin recipe	DRP-2 and PDHE1α	Fluorescence-based DIGE, MS	[63]
Type 2 diabetes mellitus	Tianqijiangtang capsule	Haptoglobin, transthyretin, and prothrombin	2DE, MALDI-TOF-TOF/MS	[64]
Other diseases				
Gan stagnancy syndrome	—	TTR, aryl sulfotransferase	2DE, MALDI-TOF MS	[65]
Functional dyspepsia	Wei Kangning	Glutathione S-transferase, pi2	2DE, MALDI-TOF MS	[66]
Spontaneously hypertensive	Formula	HSP-27, annexin-A1, MFN-2, and Rho	2DE, MALDI-TOF MS	[67]
Allergic airway	Xiao-Qing-Long-Tang	Spectrin $\alpha 2$	2DE, MS, MS/MS	[68]
Silicosis	*Gymnadenia conopsea*	Cathepsin D precursor, peroxiredoxin-1	2DE, MALDI-TOF MS	[69]
Anxiety disorders	Polysaccharides	Beta-synuclein, DJ-1, and peroxiredoxin-2	2DE/MS	[70]
Normal	Acupuncture	NAD-isocitrate dehydrogenase	2DE, MALDI-TOF MS	[71]
Normal	Acupuncture	Local stimulus response, energy metabolism	2DE/MS	[72]
Asthma	Acupuncture	S100A8, RAGE, S100A11, and CC10	2DE, LC-MS/MS	[73]

4.2.2. Hepatorenal Diseases. Using proteomics technologies and *in vivo* model, Shen et al. [57] investigated the effects of Yiguanjian decoction on rats with cirrhosis and found that increasing expression of proteins that were related to antioxidative stress such as Cu/Zn SOD and DJ-1 is probably the mechanism of Yiguanjian decoction in treating CCl4 induced cirrhosis. Xie et al. [58] proposed that the action mechanism of anti-liver fibrosis effect of Fuzheng Huayu (FZHY) may be due to modulation of proteins associated with metabolism and stress response, as well as myofibroblast activation, including aldehyde dehydrogenase, vimentin isoform (CRA_b), gamma-actin, vimentin, fructose-bisphosphate aldolase B, aldo-keto reductase, S-adenosyl homocysteine hydrolase isoform, and HSP90. Wang et al. [80] studied the effects of Isoline on mouse liver protein profile and showed that the liver samples from mice of Isoline group had about 13 differentially expressed proteins compared with the normal. These proteins may be involved in Isoline-induced liver injury, as 9 of them were involved in the process of oxidative stress or cellular energy metabolism.

Sun et al. [60] investigated mechanisms of the protective effects of *Salvia miltiorrhiza* polysaccharide (SMPS) against lipopolysaccharide- (LPS-) induced immunological liver injury (ILI) in Bacille Calmette-Guérin- (BCG-) primed mice, and the results showed that SMPS antagonized liver injury by upregulating the enzymes of the citric acid cycle, namely, malate dehydrogenase (MDH) and 2-oxoglutarate dehydrogenase complex, as well as inhibiting the NF-κB activation by upregulation of PRDX6 and the subsequent attenuation of lipid peroxidation, iNOS expression, and inflammation.

Liu et al. [61] observed the effect of Tanshinone II A sodium sulfonate (TSNIIA-SS) on oxidative stress in mice and used two-dimensional electrophoresis (2DE) to find that TSNIIA-SS treatment not only improved DXR lesion but also regulated the expression of several proteins associated with the cytoskeleton, oxidative stress, and protein synthesis or degradation.

4.2.3. Diabetes. In diabetes research, Guo and Xiong [62] observed changes of serum proteome in model rats treated with Granules of Eliminating Phlegm and Removing Blood Stasis (GEPRB). It suggested that 13 proteins changed in response to GEPRB *in vivo*, and these proteins may play key roles in the GEPRB treatment of diabetes deafness. Among them, 2 highly differentially expressed proteins apolipoprotein E (apoE) and C3 may be potential drug targets of GEPRB.

Shi et al. [63] investigated the effects of the Chinese medicine ZiBuPiYin recipe (ZBPYR) on the hippocampus in a rat model of diabetes-associated cognitive decline and found that 13 protein spots were altered between control and diabetes groups and 12 spots were changed between diabetes and DM/ZBPYR groups. Nine proteins were involved in energy metabolism, cytoskeleton regulation, and oxidative stress. The protein alterations observed in the diabetes group were ameliorated to varying degrees following ZBPYR treatment.

Another proteomic study of serum proteins in a type 2 diabetes mellitus (T2DM) rat model by Chinese traditional medicine Tianqijiangtang capsule was performed by Zhang et al. [64], and the distinct effect of T2DM on rat serum protein patterns included the downregulation of apolipoprotein E, apolipoprotein A-I, and Ig gamma-2A chain C region and upregulation of transthyretin (TTR), haptoglobin (Hp), serum amyloid P-component (SAP), and prothrombin. The majority of those protein levels were restored to those of healthy rats after Tianqijiangtang capsule treatment.

In other diseases, the proteomics was also applied to uncover the effective mechanisms of TCM treatments in *in vivo* models, such as chronic restraint stress-induced liver stagnancy syndrome rats [65], stressed rats treated with Wei Kangning [66], spontaneously hypertensive rats treated with CHM compound PingganQianyang [67], airway inflammatory mice treated with Xiao-Qing-Long-Tang [81], silica exposing rats treated with *Gymnadenia conopsea* alcohol extract [69], and anxious rats treated with polysaccharides extracted from Shudihuang [70]. In addition, the proteomics was also performed to study the effect of acupuncture on different diseases, such as the effects of acupuncture at Taixi acupoint (KI3) on kidney proteome [71], the nonspecific physiological background effects of acupuncture revealed by proteomic analysis in normal rats [72], and the proteomic analysis in acupuncture-treated rats with asthma onset [73].

In summary, from the *in vivo* models, using proteomics method, the researchers can successfully identify novel candidate proteins involved in the development of disease and define potential targets for TCM treatment. Further studies are required to investigate the exact role of these selected proteins and validate their potential as therapeutic targets.

5. Combination of Proteomics and Other "-Omics" Technologies in TCM Researches

Because of the complexity of TCM and the differential expression profiles of various biomolecules, that is, DNAs, RNAs, proteins, and metabolites, there are indeed numerous regulatory mechanisms and signaling pathways awaiting to be investigated for TCM research. Systematically integrating collected information from genomics, transcriptomics, proteomics, and metabolomics will greatly help explore the molecular mechanisms of TCM, as well as finding biomarkers or targets for future research and clinical practice.

Over the past few years, several researchers have combined the use of transcriptomics and proteomics in TCM researches. Using next-generation RNA sequencing and iTRAQ, in gastric cancer cell line AGS treated with TIIA, Lin et al. [35] characterized 16,603 unique transcripts and 102 proteins, which were involved in carbohydrate metabolism, cell cycle, apoptosis, DNA damage, and cytoskeleton reorganization. Intracellular ATP levels, the levels of glucose-6-phosphate isomerase, L-lactate dehydrogenase B chains, p53, and AKT were characterized to be associated with these changes. Moreover, proteomics and transcriptomic analysis coupled with pharmacological tests were employed to reveal the diversity of antithrombosis proteins from the medicinal

insect, *Eupolyphaga sinensis*. By this approach, Wang et al. [82] found that serine proteases contained both plasmin- and plasminogen-activating-like activities, the excellent candidates for antithrombosis medicines.

Recently, an integrated proteomic and metabolomic study found that three principal components of Yin-Chen-Hao-Tang, *Artemisia annua* L., *Gardenia jasminoides* Ellis, and *Rheum palmatum* L., contained major active ingredients 6,7-dimethylesculetin (D), geniposide (G), and rhein (R), respectively. Wang et al. [83] found that the DGR combination had a better therapeutic effect through intensifying dynamic changes in metabolic biomarkers, regulating target proteins, and activating both intrinsic and extrinsic pathways.

However, what needs to be emphasized is that, because of the high cost of "-omics" technologies, the studies combining proteomics with other "-omics" still remain of a small number, especially in TCM research, but it is a very promising approach since the combined "-omics" can provide much more information.

6. Prospects and Challenges

Recent technological advances in "-omics" including genomics, transcriptomics, proteomics, and metabolomics have helped cast light on the essence and molecular basis of TCM syndrome. High-throughput proteomics technologies assist the researchers to identify candidate proteins, which play key roles in TCM treatment response and toxicity. Subsequently, pharmacoproteomics emerges, seeking to characterize the molecules affecting the response to drugs in individual patients and helping target-based therapy. Furthermore, disease susceptibility proteins representing potential new drug targets could also be identified by pharmacoproteomics methods. All these novel approaches provide very useful tools for TCM drug discovery and individualized application of TCM therapy.

Proteomics can combine tightly with other technologies to better understand the essences of TCM [84]. For example, combining proteomics and bioinformatics to study protein signaling pathways and protein-drug interactions would be favorable to molecular evidence-based TCM research. Posttranslational modifications by small compounds, lipids, or even a group of chemicals can regulate the protein activity and its function. Proteomics could be applied to detect the posttranslational modifications and protein-protein interactions after TCM treatment. With the development of the latest proteomics techniques in TCM research, more and more researchers begin focusing on how proteomics can determine the mechanism of TCM treatment for various diseases including cancers, cardiocerebrovascular diseases, hepatorenal diseases, lung diseases, and diabetes. Proteomics could also be applied to determine the anticancer mechanisms of CHM compounds or CHM and CHF extracts. In addition, many researchers have paid more attention to proteomics studies based on treatment-TCM syndrome animal models. For example, Xie et al. focused on change of serum proteome in noxious Heat Blood Stasis syndrome treated by Radix [85]; Liao et al. performed an experimental study on proteomic analysis of gastric mucosa in chronic gastritis rats of Spleen-Stomach Damp-Heat syndrome treated by Sanren decoction [86]. These studies greatly help understand the molecular basis of TCM treatment based on syndrome differentiation.

However, every coin has two sides; proteomics technology has its limitation or disadvantage. Presently, the identified proteins from the proteomics technology are relatively fewer in comparison with the data from genomics and transcriptomics. The proteomics results are frequently instable and variable, which means that the reproducibility of proteomics data is poor. To obtain the ideal results, it is necessary to integrate all the proteomic technology such as 2DE, HPLC, MALDI-TOF-MS, SELDI-TOF-MS, MS/MS, iTRAQ, and the bioinformatics.

Furthermore, there are still many limitations for the application of proteomics in TCM research. For example, most studies have identified differentially expressed proteins among different TCM syndromes or TCM treatments. Few identified molecules were investigated in depth from the aspects of function and mechanism. Moreover, although many *in vivo* animal models associated with TCM syndromes have been established based on TCM theory, it remains unknown whether these models accurately simulate the real human environment and reflect human body conditions [87]. In addition, many CHM or CHF compounds are very complex and not stable because of the origin and production process; the proteomics results could not keep consistent, which affect greatly the further mechanism investigation of CHMs and CHFs.

Nowadays, proteomics bridges TCM and modern life sciences, greatly facilitates the quality evaluation and standardization of TCM, and promotes the modernization and internationalization of TCM. Although the application of proteomics in TCM research is full of challenges, it also provides very good opportunity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Proteomic Analysis of Anticancer TCMs Targeted at Mitochondria

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Traditional Chinese medicine (TCM) is a rich resource of anticancer drugs. Increasing bioactive natural compounds extracted from TCMs are known to exert significant antitumor effects, but the action mechanisms of TCMs are far from clear. Proteomics, a powerful platform to comprehensively profile drug-regulated proteins, has been widely applied to the mechanistic investigation of TCMs and the identification of drug targets. In this paper, we discuss several bioactive TCM products including terpenoids, flavonoids, and glycosides that were extensively investigated by proteomics to illustrate their antitumor mechanisms in various cancers. Interestingly, many of these natural compounds isolated from TCMs mostly exert their tumor-suppressing functions by specifically targeting mitochondria in cancer cells. These TCM components induce the loss of mitochondrial membrane potential, the release of cytochrome c, and the accumulation of ROS, initiating apoptosis cascade signaling. Proteomics provides systematic views that help to understand the molecular mechanisms of the TCM in tumor cells; it bears the inherent limitations in uncovering the drug-protein interactions, however. Subcellular fractionation may be coupled with proteomics to capture and identify target proteins in mitochondria-enriched lysates. Furthermore, translating mRNA analysis, a new technology profiling the drug-regulated genes in translatome level, may be integrated into the systematic investigation, revealing global information valuable for understanding the action mechanism of TCMs.

1. Introduction

Traditional Chinese medicine (TCM) has been used for thousand years in China. There is a well-established theoretical approach in TCM treatment based on Chinese philosophy. According to Chinese medicine, diseases resulted from a disturbance of the balance that maintains health (yin-yang balance). Physicians adopt different combinational formulas of TCM to regulate the harmony of the body-mind-environment network of patients according to the syndromes, age, gender, and physique [1], and therefore patients in different backgrounds receive specific treatments, equivalent to modern medical conception-personalized therapy.

However, for a long time TCM had been treated with skepticism in academic medicine because of the lack of herbal standardization and quality control [2] and the ambiguity of functional molecules and their action mechanisms. Beginning from past decades, increasing studies with modern chemical, biochemical, and molecular biological methods showed that TCMs are rich with various functional compounds active in cancer therapy [3–6]. There is a revival of interest in TCMs and many scientists turn to explore the action mechanisms of the bioactive natural products in cellular and molecular levels.

The mainstream strategy to study TCMs is to isolate and purify bioactive components from herbs or animals, observe their biological and medical effects in cellular and animal models, and then investigate the signaling pathways involved by the compounds in molecular level [7]. Up to now, thousands of active components have been isolated from TCMs and their potentials for the treatment of cancer, cardiovascular disease, and diabetes have been explored. However, the technologies for holistically investigating and understanding the mechanisms of TCMs are limited. Systems biology is regarded as the possible method that can bring breakthroughs in the study of TCM [8], because its advantage

is in accord with the holistic philosophy of Chinese medicine. Based on the systems theory, multiomics strategies [9] and multiple-target approaches must be the good choices for molecular screening, providing global views for elucidating the essence and molecular basis of TCMs. Meeting the urgent need for the high-throughput technologies, proteomics, as a powerful tool of systems biology, can be used to profile the differential expression of proteins in response to the biological action by TCM compounds, summarizing the top molecular pathways induced by the compounds and then the complex mechanisms can be further investigated in detail [10].

2. TCMs Induce Cancer Cell Death in Mitochondrial-Dependent Pathway

Mitochondrion is the key regulator in cellular energy homeostasis and plays a central role in determining cell apoptotic process [11, 12]; it is therefore regarded as a vital target for cancer chemotherapy [13]. Many investigations revealed that bioactive compounds can act on mitochondria to trigger the permeabilization of the mitochondrial outer membrane and lead to the impairment of the mitochondria, including the alteration of electron transport, the loss of mitochondrial transmembrane potential, and the cytosolic release of apoptotic proteins such as cytochrome c (Figure 1). Our previous studies based on proteomics also demonstrated that many natural active molecules, including *isodeoxyelephantopin* [14], *andrographolide analogue* [15, 16], *tubeimoside-1* [17], and *dioscin* [18] extracted from TCMs, induce cancer cell apoptosis mainly in mitochondria-dependent pathway. Mitochondria are likely the primary and common targets for TCM compounds as suggested by proteomic profiling, showing the substantial TCM-induced alterations of mitochondrial proteins among others. In this paper, we attempt to discuss the functional roles of several TCM compounds with anticancer properties, with special emphasis on the involved molecular mechanism *via* mitochondria as cellular targets using proteomics as a primary screening technology.

2.1. Terpenoids. Terpenoids are the largest and diverse class of natural products, which can be found in all classes of living things. These compounds feature five-carbon isoprene units assembled and modified in thousands of ways. Figures 2(a) and 2(b) display several structures of terpenoids extracted from TCM herbs. Accumulating reports demonstrated that many terpenoids exhibit strong effects on preventing carcinomas, as shown in Table 1.

2.1.1. Effects of Sesquiterpene on Mitochondria. Elemene is a sesquiterpene extracted from the TCM herb *Curcuma wenyujin* and *Curcuma zedoaria* Roscoe [19], including β-elemene, γ-elemene, and δ-elemene (Figure 2(a)). Among them, β-elemene has been widely used to inhibit cancer. A study demonstrated that β-elemene is able to reverse the drug resistance of A549 cells by decreasing the mitochondrial membrane potential, in which the membrane damage initiates apoptosis process *via* cytochrome c release,

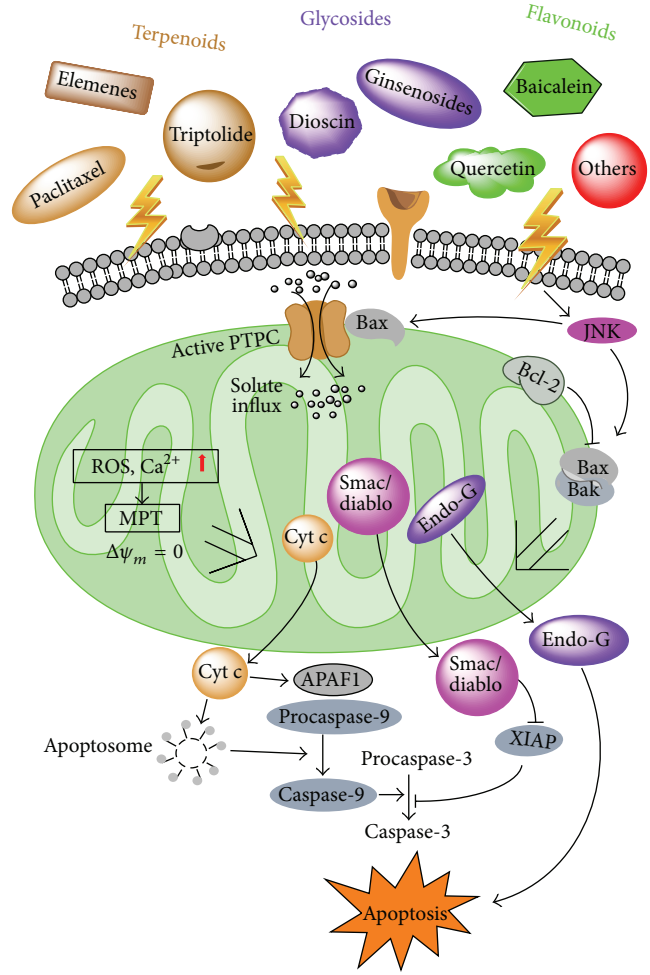

Figure 1: Modulation of mitochondrial-dependent apoptosis pathways by natural compounds. Mitochondrial transmembrane potential ($\Delta\psi_m$) is maintained by permeability transition pore complex (PTPC). PTPC interacts with Bcl-2, contributing to the exchange of small metabolites between the cytosol and the mitochondrial matrix. When the cell suffers stimuli from natural compounds, the accumulation of reactive oxygen species (ROS) and Ca^{2+} activates the PTPC, favored by its interaction with Bax, allowing small solutes to get into the mitochondrial matrix, resulting in decreased $\Delta\psi_m$. This eventually leads to mitochondrial outer membrane permeabilization, where many cytotoxic proteins such as cytochrome c, diablo, and endonuclease G are released to cytoplasm, initiating apoptotic signaling.

caspase activation, and the modulation of the expression of Bcl-2 family proteins [20]. Moreover, β-elemene can augment the cisplatin activity and carry out a synergistic effect on disrupting the mitochondrial transmembrane potential, inducing apoptosis in ovarian carcinoma cells [21]. Through targeting mitochondria, the antitumor effect of β-elemene was also observed in prostate, brain, breast, cervical, and colon cancers [22]. Research with iTRAQ-based proteomics revealed that several pathways in gastric cancer (SGC7901) may be involved by β-elemene, including ribosome signaling, peroxisome proliferator-activated

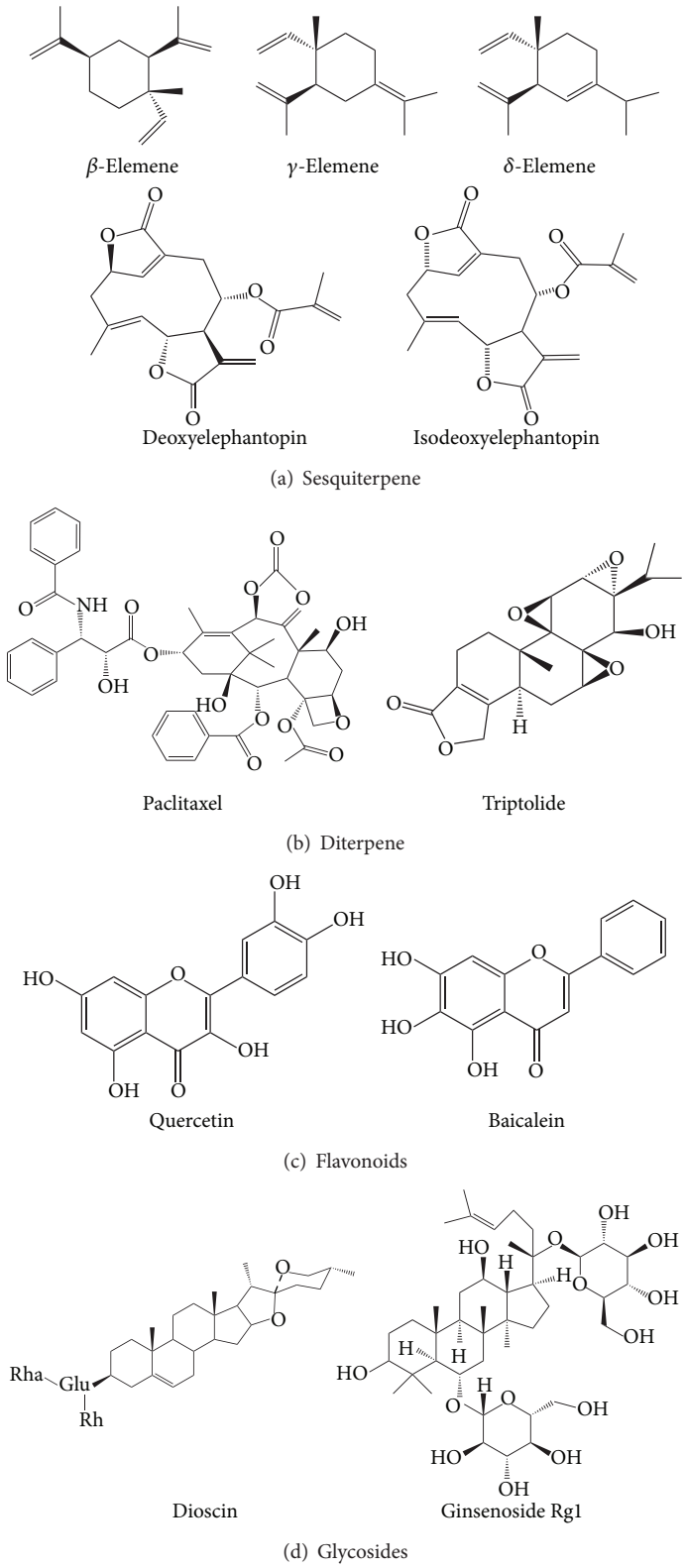

Figure 2: Chemical structures of compounds isolated from TCMs.

Table 1: Summary of the application of proteomics to determine the mechanism of action of terpenoids.

Compounds	Proteomics method	$\Delta\psi_m$	ROS	Effects	Mechanism of action	Cell lines	References
Sesquiterpene							
β-Elemene	iTRAQ	N/A	N/A	N/A	Ribosome signaling, PPARs signaling, actin cytoskeleton, phagosome, biosynthesis, and amino acids metabolism	SGC7901	[23]
β-Elemene	N/A	↓	↑	Apoptosis↑	Release of cytochrome c/caspase-3	A549/DDP	[20]
δ-Elemene	N/A	↓	N/A	Apoptosis↑	p38 MAPK	NCI-H292	[97]
β-Elemene	N/A	↓	↑	Apoptosis↑	Release of cytochrome c/caspase-3/caspase-8/caspase-9	A2780/CP MCAS	[21]
β-Elemene	N/A	N/A	N/A	Apoptosis↑	Release of cytochrome c/caspase-3/caspase-8/caspase-9, p53	H23, H358, H460, and A549	[19]
Deoxyelephantopin	2-DE DIGE	N/A	N/A	Apoptosis↑	ER stress-independent apoptotic pathway	TS/A	[27]
Deoxyelephantopin	N/A	↓	↑	Apoptosis↑	Release of cytochrome c/caspase-3/caspase-7/caspase-8/caspase-10, Akt, ERK, and JNK pathways	CNE	[25]
Isodeoxyelephantopin	SILAC	↓	↑	Apoptosis↑	ROS-dependent DNA damage, mitochondrial-mediated apoptosis, and antitumor inflammation factor pathway	CNE1	[14]
Isodeoxyelephantopin	N/A	N/A	N/A	Apoptosis↑	NF-κB	HL60, H1299, MM.1S, U266, and KBM-5	[82]
Diterpene							
Celastrol	SILAC	↓	↑	Apoptosis↑ Proliferation↓	Stress response, oxidative stress, and effects	Lymphoblastoid cells	[98]
Triptolide	2-DE	N/A	N/A	Proliferation↓	Perinuclear translocation of 14-3-3ε	SW480 and Lovo	[36]
Triptolide	N/A	↓	↑	Apoptosis↑	Release of cytochrome c/Smac/diablo, activation of caspase-3/caspase-9	HS-sultan, IM9, RPMI8226, and U266	[38]
Triptolide	N/A	↓	↑	Apoptosis↑	Release of cytochrome c, caspase-3/caspase-9, Bcl-2↓, Bax, and p53↑	L-02	[40]
Tanshinone IIA	2-DE	↓	↑	Apoptosis↑	p38, JNK/IRE1/PERK pathways, and ROS/ER stress pathways	CaSki	[99]

N/A: not applicable; ↑: upregulation; ↓: downregulation.

receptors (PPARs) signaling, regulation of actin cytoskeleton, phagosome, biosynthesis, and metabolism of amino acids [23]. In particular, as observed by the proteomic study, the expression of p21-activated protein kinase-interacting protein 1 (PAK1IP1) and Bcl-2-associated transcription factor 1 (BTF) is significantly regulated by β-elemene, indicating the antitumor effect probably by targeting mitochondria, though the binding proteins remain to be explored.

Deoxyelephantopin (ESD) and isodeoxyelephantopin (ESI) are two germacranolide sesquiterpene lactones (Figure 2(a)) isolated from TCM herb *Elephantopus scaber* [24–26]. This herb is a folk medicine usually used for preventing and treating respiratory diseases in China. However, accumulating evidences demonstrated that both ESD and ESI are able to induce cell apoptosis in TS/A cells (lung cancer) and CNE1 (nasopharyngeal carcinoma). Their underlying molecular mechanisms were further characterized with the advanced proteomic technologies. Lee et al. used 2DE DIGE and LC-ESI-MS/MS to profile proteins that are significantly regulated by ESD treatment in TS/A cells [27]. As revealed in this experiment, protein alternations regulated by ESD are involved in proteolysis and calcium ion transport, indicating that ESD may target proteasome and endoplasmic reticulum in TS/A cells. At the same time, Su and colleagues found that ESD is able to inhibit the cell proliferation, induce cell cycle arrest, and trigger apoptosis in CNE cells by decreasing the mitochondrial membrane potential ($\Delta\psi_m$) [25]. SILAC (stable isotope labeling with amino acids in cell culture) quantitative proteomics coupled with bioinformatics was also made full use of to characterize the molecular mechanism of ESI in nasopharyngeal carcinoma [14]. ESI was found to provoke G2/M arrest and apoptosis by inducing ROS generation, in which the accumulated ROS promote DNA breakage and mitochondrial-mediated apoptosis. Obviously, mitochondria are likely a key target, among others, for sesquiterpene.

2.1.2. Effects of Diterpene on Mitochondria. Paclitaxel (taxol), a famous diterpene plant product extracted from the *Taxus brevifolia* in 1971, has been currently employed as an antimitotic agent in chemotherapy for the treatment of various human cancers. The structure is showed in Figure 2(b). It is well known that taxol can induce cell apoptosis by preventing tubulin depolymerization during mitosis [28, 29], particularly in lung cancer and ovarian cancer [30, 31]. Mounting clinical evidences proved that many cancers acquire paclitaxel resistance during chemotherapy because of heterogeneity. Thereby growing researches, based on proteomics, aimed to reveal the complex molecular mechanisms of paclitaxel resistance in cancers [31–35]. Table 3 lists several paclitaxel resistance studies determined by proteomics in different cancers.

In lung cancer, a prevalent research strategy is to establish paclitaxel-resistant tumor subline and parental-sensitive cell line *via* stepwise selection by paclitaxel and then compare the differentially expressed proteins in two cell lines by proteomics. With such a method, Sun's group used 2DE DIGE to identify 30 altered proteins, which mainly belong to signal transduction, cytoskeleton, redox reaction, energy, and metabolism [35]. Another proteomic study found that the treatment of paclitaxel combined with MEK inhibitor significantly alters the level of RS/DJ-1 (RNA-binding regulatory subunit/DJ-1 PARK7) and RhoGDIα (Rho GDP-dissociation inhibitor α) in NSCLC H157 cell line, suggesting an important role that RS/DJ-1 and RhoGDIα are involved in drug resistance [30]. Furthermore, Tian and his colleagues employed multiple quantitative proteomic methods (iTRAQ labeling and label-free) to analyze paclitaxel resistance associated proteins in ovarian serous carcinoma cell line (SKOV-3) [31]. This in-depth proteomic screening identified 1371 differential proteins, including mitochondrial trifunctional enzyme, mitochondrial ATP synthase, complement component 1 Q subcomponent binding protein, cytochrome c, GrpE protein homolog 1, mitochondrial inner membrane protein, and mitochondrial malate dehydrogenase, suggesting that mitochondria play a core role in responding to paclitaxel treatment. These observations indicate that advanced proteomic techniques should be applied to obtain comprehensive views on protein alterations, in which mitochondria associated pathways can be fairly evaluated.

Triptolide, also called *Tripterygium wilfordii lactone alcohol*, is an oxygenated diterpene isolated from the Chinese herb *Tripterygium wilfordii* HOOK F, which contains three epoxy groups of diterpene lactone compounds (Figure 2(b)). Present studies showed that triptolide possesses high toxicity and thus can exert proapoptotic and antiproliferative effects on multiple tumor cell lines *in vitro* [36–38]. Comprehensive proteomics was employed to determine the triptolide-regulated proteins, which are related to oxidative stress, mitochondria, and signal transduction, confirming that triptolide inhibits the activation of JNK and p38 by decreasing ROS level [39]. The toxic effect of triptolide in normal liver cell (L-O2) was also investigated; the observed loss of mitochondrial membrane potential and cytochrome c releasing suggests that triptolide can also work on the mitochondria in normal cells to induce apoptosis [40].

2.2. Flavonoids. Flavonoids are kinds of natural products with abundant contents in TCM herbs, fruits, and vegetables. They are a group of polyphenolic compounds containing more than 6000 flavonoids, which are divided into 6 subclasses, including flavonols, flavanols, isoflavones, anthocyanidins, flavanones, and flavones [41]. Some epidemiological studies reported that intake of flavonoids may be uncertain or even harmful to cancer therapy [42, 43]; nevertheless, the majority of evidences supported their potential cancer protective properties [44–46]. Regarding this controversial role of flavonoids in cancer, proteomics may provide a global picture with comprehensive information for assessment. As shown in Table 2, the action mechanisms of some well-known flavonoids have been characterized by proteomics.

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a major flavonoid compound in fruits, which possesses a wide range of biological activities, including antioxidant [47], antitumor [48], and metabolic regulation [49] (Figure 2(c)). A proteomic study using SILAC method found that quercetin inhibits HepG2 proliferation and migration by regulating IQGAP1 and β-tubulin expression [50]. Experimental results

showed that quercetin-regulated proteins are involved in multipathways, including antioxidation-relating pathway and mitochondria-dependent apoptosis pathway. To understand the relationship between high fruit intake and the risk of colon cancer, Mouat et al. used 2DE-based proteomics to determine the influence of quercetin on human colon adenocarcinoma cell line (SW480), revealing that type II cytoskeletal 8 keratin and NADH dehydrogenase (ubiquinone) Fe-S protein 3 are downregulated, while the annexin family related that proteins are upregulated [51]. These data suggest that quercetin may decrease tumorigenicity through impairing the transfer of electrons from NADH to ubiquinone in the respiratory chain; that is, mitochondria may be the potential target of quercetin. With regard to metabolic regulation, a study applied transcriptome and proteome profiling to investigate the effect of quercetin on colon mucosa in rats model, indicating that mitogen-activated protein kinase (MAPK) pathway is downregulated while phases I and II metabolism pathway, PPARα, and mitochondrial fatty acid degradation pathway are enhanced [52]. This observation implicates that quercetin may induce a shift in energy production pathways from decreased cytoplasmic glycolysis to increased mitochondrial fatty acid degradation during cancer development.

Baicalein, the main active component isolated from *Scutellaria baicalensis*, is a flavonoid that shows cytotoxic effect on various human cancer cell lines [53, 54] and also plays a vital role in protecting cell against surrounding stress [55] (Figure 2(c)). To better illustrate the antitumor effect of baicalein on colorectal cancer, Huang et al. used 2DE-proteomic approach to identify 11 differentially expressed proteins as the potential targets of baicalein [56]. Peroxiredoxin-6 (PRDX6), an upregulated protein after baicalein treatment, was found to decrease the generation of ROS and inhibit the growth of colorectal cancer cells. Similarly, antiproliferation effect of baicalein was also reported in bladder cancer T24 cells, showing that baicalein inhibits Akt signaling and downregulates Bcl-2 expression [57]. At the same time, loss of mitochondrial membrane potential and activation of caspase-9 and caspase-3 were observed, implicating that baicalein induces apoptosis *via* mitochondrial-dependent caspase activation pathway. Nevertheless, an opposing observation was also reported: a baicalein isolated from *Scutellaria baicalensis* extract (SbE) increases colorectal cancer cell growth, whereas SbE without baicalein significantly induces mitochondrial apoptotic pathway [58]. This discrepancy remains to be reassessed by using advanced systems biological technologies including proteomics.

2.3. Glycosides. Glycosides are another class of natural products widely stored in living organisms. They can transform into active status through enzyme hydrolysis with their sugar groups broken off. These sugar groups of glycosides usually and susceptibly interact with toxic compounds from surroundings and are easily eliminated from the body. Recently, with the application of proteomics, increasing studies focused on the anticancer effects of glycosides. Here are some examples as shown in Table 2.

Dioscin (Figure 2(d)), a typical glucoside saponin derived from TCM plants *Polygonatum zanlanscianense* pamp, shows multiple pharmacological activities such as apoptosis induction in various carcinoma cell lines [59, 60] and liver injury protection [61]. Regarding the target of dioscin, accumulating evidences pointed to mitochondria. Earlier work in our laboratory used 2DE-based proteomics to profile the proteomic changes in response to dioscin treatment in human myeloblast leukemia HL-60 cells, revealing that dioscin exerts cytotoxicity *via* mitochondrial apoptotic pathway [18]. Our further investigation demonstrated that dioscin is capable of inducing mitochondria dysfunction and reactive oxygen species (ROS) generation with decreased $\Delta\psi_m$, leading to the initiation of the death receptor signaling pathway.

In parallel, ROS generation was observed as well in human colon cancer cells (HCT-116) after dioscin stimulation. Peng's laboratory employed iTRAQ-based proteomics to analyze the cytotoxic mechanism of dioscin in HCT-116 cells and identified 288 differentially expressed proteins, which are involved in oxidative phosphorylation, Wnt, p53, and calcium signaling pathways [62]. By regulating mitochondria, dioscin exerts not only proapoptotic effect but also hepatoprotective function in acetaminophen-induced liver injury [61]. As screened by 2DE-proteomics, 15 dioscin-regulated proteins probably associated with hepatoprotection are identified, including Suox, Krt18, Rgn, Prdx1, MDH, and PNP. In addition, dioscin is able to mediate Ca^{2+} balance *via* regulating Rgn and upregulating Ktr18 in cells that suffer from acetaminophen-induced mitochondrial damage.

Ginseng (*Panax ginseng* Meyer) is a medicinal herb of the family Araliaceae; its root has been commonly used for keeping healthy in China over 2000 years [63]. Ginsenosides, the major active compounds of ginseng, were reported to possess anticancer [64, 65], antimutagenic, anti-inflammatory, antidiabetes, and neurovascular effects [66]. Ginsenoside Rg1 is one of the ginsenosides that belong to triterpene glycosides (Figure 2(d)). It was reported to reverse TNF-α-attenuated nitric oxide production in human umbilical vein endothelial cells by a proteomic-based study [67]. TNF-α stimulation increases the expression of MEKK-3, reticulocalbin, phosphoglycerate, zinc finger protein, NSAP1 protein, and 6-phosphogluconolactonase, with reduced nitric oxide synthase. However, all these alterations can be restored by ginsenoside Rg1 pretreatment, suggesting a protective role of ginsenoside Rg1 in alleviating the injury of inflammatory factor on vascular disease.

The protective effect of ginsenoside Rg1 can be observed as well in cardiomyocytes [68]. Hypoxia condition induces neonatal rat cardiomyocytes death in mitochondrial apoptotic pathway, including ROS accumulation, loss of mitochondrial membrane potential, and cytochrome c release; nevertheless, ginsenoside Rb1 can markedly inhibit this process. Mitochondria are the arbiter in cardiomyocytes injuries by releasing apoptogenic proteins into the cytosol [69]; however, the exact targets of ginsenoside Rb1, probably associated with mitochondria, remained to be investigated.

2.4. Others. Honokiol (HNK) is a neolignan isolated from TCM herb *Magnolia officinalis* (also named *Houpu* in Chinese), exhibiting various pharmacological effects in

Table 2: Summary of the application of proteomics to determine the mechanism of action of flavonoids and glycosides.

Compounds	Proteomics methods	$\Delta\psi_m$	ROS	Effects	Mechanism of action	Cell lines	References
				Flavonoids			
Baicalein	2-DE	N/A	↓	Proliferation↓	PRDX6↑ and ROS↓	DLD-1	[56]
Baicalein	N/A	↓	↑	Apoptosis↑	Mitochondrial respiration↑ and cytochrome c oxidase activity↑	H2.35	[57]
Luteolin	2-DE	N/A	N/A	Apoptosis↑	p38↓, HSP27↓, intracellular ATP levels↑, and mitochondrial activity↑	CH27	[100]
Quercetin	SILAC	N/A	N/A	Migration↓ Proliferation↓	IQGAP1 and β-tubulin	HepG2	[50]
Quercetin	2-DE	N/A	N/A	N/A	NADH dehydrogenase↓	SW480	[51]
Rotenone	SILAC	↓	↑	N/A	Oxygen-sensing pathway	SH-SY5Y	[101]
Panduratin A	iTRAQ	N/A	N/A	Angiogenesis↓	mTOR signaling↓	HUVECs	[102]
Calycosin	2-DE	N/A	N/A	Proliferation↓	Cell-cycling pathway	BEL-7402	[103]
Tea polyphenols	N/A	↓	↑	Apoptosis↑	NF-κB	HeLa and SiHa	[104]
Tea polyphenols	N/A	↓	↑	Apoptosis↑	Bax and p53↑	Mouse skin cancer	[105]
				Glycosides			
Ginsenoside Rb1	N/A	M	M	Apoptosis↓	Mitochondrial apoptotic pathway↓	NRC	[68]
Ginsenoside Rg1	2-DE	M	M	Apoptosis↓	eNOS pathway	HUVEC	[67]
Dioscin	N/A	↓	↑	Apoptosis↑	Mitochondria-initiated apoptosis pathway	HL-60	[18]
Dioscin	iTRAQ	↓	↑	Apoptosis↑	Oxidative phosphorylation, Wnt, p53, and calcium signaling pathways	HCT-116	[62]

M: maintenance; NRC: neonatal rat cardiomyocytes; HUVEC: human umbilical vein endothelial cells; N/A: not applicable; ↑: upregulation; ↓: downregulation.

Table 3: Summary of proteomics-based studies of paclitaxel resistance in various cancers.

Treatments	Proteomics methods	Cancer types	Cellular models	References
Paclitaxel-sensitive A549 versus paclitaxel-resistant A549	2-DE DIGE	Non-small-cell lung carcinoma	A549	[35]
Untreated versus paclitaxel versus MEK inhibitor versus paclitaxel + MEK inhibitor	2-DE	Non-small-cell lung carcinoma	H157	[30]
Untreated versus paclitaxel treated	2-DE DIGE	Breast cancer	MDA-MB-435S	[106]
Untreated versus paclitaxel treated	2-DE	Cervical cancer	Hela	[107]
Untreated versus paclitaxel treated	2-DE DIGE	Promyeloid leukaemia	HL-60	[108]
Untreated versus paclitaxel treated	2-DE	Dermal papilla	Dermal papilla	[109]
Paclitaxel-sensitive CaOV3 versus paclitaxel-resistant variant CaOV3	N/A	Ovarian cancer	CaOV3 and OV90	[32]
SK-BR-3 versus paclitaxel-resistant SK-BR-3	2-DE	Breast cancer	SK-BR-3	[34]

N/A: not applicable.

preclinical experimental models [70]. A large volume of evidences demonstrated that HNK is able to induce apoptosis and antiproliferation in several cancers [71] and thus is regarded as a promising chemotherapy candidate in cancer therapy. In acute myeloid leukemia, HNK shows antileukemia effect by inhibiting enzyme activity of histone deacetylases, followed by the upregulation of p21/waf1 and Bax, leading to apoptosis [72]. HNK was also reported to promote cytoprotective autophagy mediated by ROS signaling in prostate cancer cells [73]. Using quantitative proteomic method (SILAC), Liang and colleagues found that HNK treatment in HepG2 is able to modulate cell migration by the downregulation of Ras GTPase-activating-like protein (IQGAP1), which interacts with Cdc42/Rac1 [74]. The interaction links to VEGFR-2/3 pathway to reduce cancer metastasis and proliferation. Another proteomic-based study profiled HNK-regulated proteins in Hela cells, showing 8 proteins with upregulation and 77 proteins with downregulation [75]. GO analysis revealed that 10% of these proteins are located in mitochondria, melanosome, and lysosome and over 17% are associated with metabolism, suggesting that HNK induces cell apoptosis *via* mitochondria signaling pathway, further confirming the previous result from Yang's group [76].

3. The Role of TCM-Regulated Proteins in Cancer Therapy

With the progresses in TCM investigation by proteomics approach, increasing TCM-regulated proteins were determined. Many TCMs were observed to suppress tumor development by regulating oncogenes. Can these TCM-regulated proteins serve as biomarkers to monitor cancer progression and measure treatment effectiveness?

Heat shock proteins (HSPs) are stress-inducible proteins including HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs; they act as molecular chaperones to regulate protein folding and transport, allowing cells to survive in lethal environments. In cancer, cells with higher metabolic requirement need more chaperones to maintain survival; these proteins are strong antiapoptotic proteins [77] and thus are regarded as antitumor targets in cancer therapy. Increasing reports revealed that many TCMs induce cancer cells apoptosis by decreasing HSPs [78–80]. Tanshinone IIA, an active component extracted from the roots of *Salvia miltiorrhiza* Bunge, was shown to inhibit HSP27 and promote apoptosis in cervical cancer [79]. Bufalin, a primary active ingredient of TCM *Chan-Su*, has the capacity of downregulating p-AKT and HSP27 and activating procaspase-3, procaspase-9, finally leading to cell apoptosis in pancreatic cancer [78]. HSPs, especially HSP90, are now confirmed to be essential for malignant transformation and progression [81]. In clinic, HSPs inhibition by TCMs might make the cancer cells more sensitive to chemotherapy.

NF-κB signaling is the well-known pathway that mediates immune and inflammatory responses in cells. The family includes p65 (RelA), NF-κB1 (p105/p50), and NF-κB2 (p100/p52), able to dimerize in numerous combinations and determine the fate of cells. NF-κB is constitutively activated and correlated with increasing grades in many tumors; it is thus received as the essential target for cancer therapy. To our knowledge, a great number of TCMs are able to suppress NF-κB and induce apoptosis. Isodeoxyelephantopin isolated from *E. scaber* [82], ginsenosides isolated from *ginseng* [64, 65], and isorhamnetin isolated from pollen *Typha angustifolia* or *Hippophae rhamnoides* L. [83] have the capacity of inhibiting NF-κB and exerting anti-inflammatory effect in cancers. In clinic, the subcellular localization of NF-κBs determined by immunohistochemistry is the convictive biomarker for monitoring cancer progression.

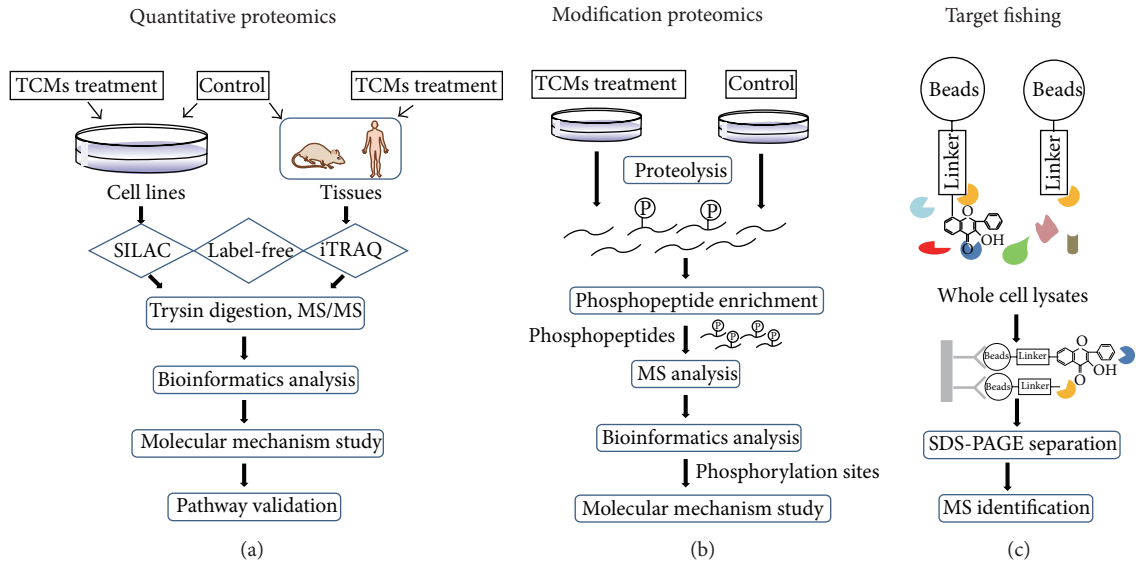

FIGURE 3: Scheme of approaches for the application of proteomics in the mechanistic study of TCMs. (a) Overview of conventional quantitative proteomics on TCMs including SILAC, label-free, and iTRAQ. Among them, SILAC is suitable for cell models, while label-free and iTRAQ can be applied to samples from various sources. Samples can be analyzed by MS after trypsin digestion. After comparison, the differently expressed proteins are sorted out by bioinformatics and selected for further mechanism studies. (b) The procedure of modification proteomics on TCMs investigation, with phosphoproteomics as a widely used approach. Samples are digested into peptides, followed by enrichment and MS analysis. (c) Abridged general view of target fishing. Beads linked with certain components of TCMs are used to capture "preys" in whole cell lysates; the target proteins can then be identified by mass spectrometry.

Drug resistance is emerging as a big challenge in cancer therapy and mitochondria play a vital role in this progression. Bcl-2 family is a group of mitochondrial proteins that regulate a diverse range of death signals. Among them, Bcl-2 is a strong antiapoptotic protein; its overexpression results in a more aggressive and treatment resistant phenotype [84]. A meta-analysis showed that Bcl-2 expression is a poor prognostic marker in lung cancer [85] and can be used as drug resistant marker as well. Some natural products, β-elemene extracted from TCM herb *Curcuma wenyujin* and triptolide isolated from *Tripterygium wilfordii* HOOK F, are able to inhibit Bcl-2 expression and thus sensitize cancer cells to chemotherapy [20, 86]. Besides, mitochondrial malate dehydrogenase also plays an essential role in docetaxel resistance in clinic [31, 87]; knockdown of malate dehydrogenase 2 (MDH2) increases docetaxel sensitivity by inducing metabolic inefficiency. Taken together, mitochondrial proteins are the arbitrator on drug resistance; therefore, the combination of mitochondrial protein inhibitors with chemotherapeutics may be the efficient method in cancer therapy.

4. The Application of Proteomics in TCMs in Cancer

Proteomics is a multifunctional tool in investigating the mechanisms of TCMs, not only providing global views of molecular alterations induced by TCMs but also identifying protein-drug interactions. Quantitative proteomics such as SILAC and iTRAQ provides conventional ways to study the mechanism of TCMs. As shown in Figure 3(a), samples including cell lines or tissues with and without TCM treatments are labeled with various tags; the cell lysates are then digested by trypsin, followed by MS analysis. The proteins with expression alterations can be further analyzed by bioinformatics to uncover the signaling pathways regulated by TCMs.

In addition, posttranslational modifications are the most common phenomenon in eukaryotic cells; many proteins are activated or inactivated after posttranslational modifications including phosphorylation, ubiquitination, and glycosylation. The rising modification proteomics provides efficient methods to investigate the action of TCMs. Figure 3(b) shows the whole procedure of phosphoproteomics: proteins from different TCM treatments are digested by trypsin, and the phosphopeptides are enriched by TiO_2 and then analyzed by LC/MS. The global identification of modification sites offers us new insights into the action mechanism of TCMs.

For the target identification, magnetic "Fishing" assay [88] coupled with proteomics is an effective approach to screen protein-drug interactions, as shown in Figure 3(c). Single agents of TCMs are linked to magnetic beads, with affinity-based isolation; the target proteins that interact with the agents of interest can be identified by mass spectrometry; bioinformatics analysis such as molecular docking can further calculate the binding sites of TCMs.

It must be noted that although TCMs are purified as single agents, they may have more than one or multiple targets in tumor cells and their actions may be involved in more than one or several pathways. Proteomics coupled with bioinformatics is able to identify and integrate various signaling

pathways of each agent thus figuring out which component is toxic to human body. Removing the useless components and optimizing the prescription of TCMs by proteomics can provide directive effects for developing therapeutic regimens.

5. The Development of Proteomics in Studying TCMs

With the rapid development of technology, proteomics progresses from the early stage of 2DE, label-free, to SILAC and iTRAQ, which highly promotes our investigation on TCMs. Back to 1975, 2DE was first used to separate proteins [89] and it was generally applied in TCM research until 2004 [90]. As can be seen from Tables 1–3, the majority of proteomic approach for TCM studies is 2DE, which can only detect and quantify over 1000 proteins by comparing a couple of paired gels at one time [91]. For the inherited limitation of 2DE in protein separation [92], 2DE is gradually replaced by SILAC and iTRAQ, which can identify approximately 5000–8000 proteins in one experiment. In SILAC, an *in vivo* labeling approach that feeds cells with isotopically labeled "light" or "heavy" nutrients for different treatments, the altered proteins can be sensitively identified by MS/MS [90]. As an *in vitro* labeling method, iTRAQ can analyze up to eight different samples and thus is widely used in profiling animal tissues. According to PubMed as of April 2015, a total of 186 papers including 34 reviews related to the application of proteomics on TCMs are published. Among them, 17 recent reports involved TCMs characterization with advanced quantitative proteomics, revealing many more identifications of altered proteins for further mechanistic investigation. Obviously, advancements in systematic screening technology are required for profiling TCMs-induced protein alterations in depth, so that follow-up specific mechanistic researches become possible.

6. A New Dawn in TCM Investigation

As the rapid development in mass spectrometry, sophistic proteomics now has a capacity to identify over 10000 proteins at one time [93]. However, there still exist many limitations of mass spectrometry in protein identification, such as low identification rates for low-abundance proteins and poor-soluble proteins [94]. To deal with this problem, we recently introduced a new systematic profiling technique, translating mRNA analysis, or translatomic sequencing [95]. This method isolates ribosome-nascent chain-mRNA complex (RNC-mRNA) from cells/tissues and sequences the mRNAs released from the complex, and then the proteins corresponding to the translating mRNAs can be identified.

Under a steady state, the abundance of translating mRNAs corresponds to protein expressions [95], and thus phenotype-related protein alterations can be derived by comparing the RNC-mRNAs from two cellular conditions. For its independence from physical and chemical properties of proteins, translatomic sequencing has its inherent advantage in detecting translating mRNAs corresponding to the low-abundance and low-solubility proteins, thus offsetting the shortcoming of mass spectrometry. Translatomic sequencing has been successfully applied to identify "missing proteins" in Chromosome-Centric Human Proteome Project (C-HPP) [96]. We believe that, by integrating translatome sequencing with proteomics, systematic screening will bring TCM research to a new stage, where more comprehensive views about the TCM-induced biological alterations can be obtained and thus global investigations on the action mechanism of TCMs can be proceeded.

In view of the fact that mitochondria are the common target for most TCM compounds, a feasible strategy is to isolate mitochondria from the cells with and without treatments by TCMs, and then translatomics coupled with proteomics can be carried out to differentiate the mitochondrial mRNAs and proteins. This combination using both subcellular enrichment and in-depth transomics must provide comprehensive information for the identification of specific targets of TCMs in mitochondria. Similar ways can be applied to other subcellular fractionations; all together, the molecular mechanism of bioactive TCM components in cancer can be uncovered in a holistic view.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Investigation on Molecular Mechanism of Fibroblast Regulation and the Treatment of Recurrent Oral Ulcer by Shuizhongcao Granule-Containing Serum

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The purpose is to study the intervention, proliferation, and differentiation on fibroblast by Shuizhongcao Granule during the treatment of ROU and investigate the action mechanism in inflammatory microenvironment. Proliferation of rat fibroblasts was detected using CCK8. Western blot was used to detect the effect of drug-containing serum on the expression of protein associated with NF-κB and ERK pathway in rat fibroblasts. Expression of IL-10 and IL-12 was detected by PCR. Shuizhongcao Granule group successfully inhibited proliferation of rat fibroblast. Western blot results revealed that p65 and IKKB were significantly less expressed in Chinese medicine group, while pIκBα and pIKK$\alpha\beta$ expression were significantly increased. We have also found that in this group the expression of pAKT was evidently suppressed and expression of pERK significantly decreased. PCR results showed significantly decreased expression levels of IL-10 and 1IL-12b in Chinese medicine group, while the expression of IL-12a was increased. Our results suggest that Shuizhongcao Granule can suppress the proliferation of fibroblast and inhibit the activation of NF-κB and thus suppress inflammatory reactions, possibly involving the inhibited expression of phosphorylated AKT, rather than the canonical pathway. Furthermore, it can inhibit ERK pathway and reduce IL-10 and IL-12b gene expression while enhancing IL-12a expression.

1. Introduction

Recurrent oral ulcer (ROU) is the most frequent oral mucosal disease with an incidence rate up to 25–30% [1]. The main type, minor ROU, accounts for 70–80% of all cases [2]. ROU is also one of the most common complications of chemotherapy. The current treatments mainly include antibiotic therapy [3], hormonal therapy [4], medicine mouthwash [5], and laser therapy [6], but none of them proves to be very effective. Finding a safe and effective medication to suppress inflammation is a hot and difficult research topic in this field. Suppression of NF-κB pathway to reduce the incidence gives a novel resolution to treat ROU [7]. From the perspective of traditional Chinese medicine, ROU is a type of "aphthae," which is usually considered to be associated with the phase of "fire." A proven formula in our hospital, Shuizhongcao Decoction (composed of buffalo horn, urine sediment, *Callicarpa*, etc.), exhibited great efficacy in the clinical treatment through a mechanism of heat reduction, blood cooling, and detoxification [8]. This study evaluated the intervention effects of serum containing Shuizhongcao on rat fibroblasts and tested the optimal dosage and intervention time. We also detected the effect of Shuizhongcao Granule on ERK and NF-κB pathways using Western blot and expression of IL-12 and IL-10 by RT-PCR. Therein, we evaluated the effect and the mechanism of Shuizhongcao Granule on

the inflammatory microenvironment and the rat fibroblasts. The results are reported as follows.

2. Materials

2.1. Experimental Cells. Rat ear-tip fibroblasts were purchased from Shanghai SiDanSai Biotechnology, Ltd. (lot number 1702-100).

2.2. Experimental Animals. Sixty Sprague-Dawley (SD) rats in healthy and hygiene grade (weighted 180 ± 20 g, 6–8 weeks, half male and half female) were provided by Laboratory Animal Center of Zhejiang Chinese Medical University.

2.3. Chemicals and Medicines. 5-FU was purchased from Zhejiang Chinese Medical Hospital, manufactured by Tianjin Jinyao Amino Acid, Ltd. (10 mL: 0.25 g, lot number 0909162, National Medicine Permit number H12020959). Chloral hydrate was purchased from Zhejiang Chinese Medical Hospital (specification 30 m: 3 g, 30 mL/bottle, Zhejiang Medicine Permit H20050357). NaOH crystal was purchased from Eastern China Medicine Ltd., manufactured by Hangzhou Xiaoshan Chemical Reagent Manufacturing (lot number 201003020). Shuizhongcao Granule (buffalo horn tablet 30 g, burned urine sediment 15 g, and *Callicarpa* leaf 15 g), 2.0 g crude drug per milliliter, was purchased from Chinese Medicine Pharmacy of Zhejiang Chinese Medical Hospital (buffalo horn, produced in Zhejiang, lot number 130112; burned urine sediment, produced in Anhui, lot number 121103; *Callicarpa* leaf, produced in Zhejiang, lot number 120922).

Preparation of medicine is as follows: buffalo horn tablets, burned urine sediment, and *Callicarpa* leaf (2 : 1 : 1 in weight) were soaked in pure water of 8–10 times in volume for 30 min. Buffalo horn tablets were boiled alone for 30 min and then added with burned urine sediment and *Callicarpa* leaf. After boiling with high heat, the medicines were then boiled with low heat for 30 min. For the second time of boiling, the herb residues were added with pure water of 1.5 times in volume and boiled for another 30 min. The decoctions from the two boiling medcine juice were then condensed to a concentration with containing crude drug 2.0 g/mL. The medicines were sterilized and packed for use.

2.4. Reagents. The reagents are trypsin (product number 1310S, Jinuo Biomedical Technology, Hangzhou, Zhejiang, China), fibroblast complete culture media (product number 0810-500, Si Dan Sai Biotechnology, Shanghai, China), CCK8 (catalog number CK04, Do jin do Molecular Technologies, Tokyo, Japan), NF-κB signaling pathway kit (product number 9936S, Cell Signaling Technology, Santa Cruz, CA, USA), MAPK signaling pathway kit (product number 8922S, Cell Signaling Technology, Santa Cruz, CA, USA), SIRT1 antibody (product number 9475S, Cell Signaling Technology, Santa Cruz, CA, USA), Immobilon-P membrane (PVDF, Shanghai Bai wei Biotechnology, Shanghai, China), RIPA lysis buffer (RIPA, Beyotime Institute of Biotechnology, Hangzhou, Zhejiang, China), BCA standard protein (product number 10735108001, Roche, Danvers, MA, USA), phosphatase inhibitor (number P5726-1 mL, Sigma-Aldrich, WI, USA), protease inhibitor (product number WBP1265-1 mL, Sigma-Aldrich, WI, USA), 5x-loading-buffer (number WB11236-1 mL, Sigma-Aldrich, WI, USA), anti-beta-actin (Actb, 1 : 3000, Sigma-Aldrich, WI, USA), total RNA extraction kit (product number: 15596-026, Invitrogen, New York, USA), Trizol (product number 1226-210, Invitrogen, New York, USA), PCR reverse transcription kit (product number DRR037A, TaKaRa Bio Inc., Palo Alto, CA, USA), and SYBR Premix Ex TaqTM (product number RR420A, Palo Alto, CA, USA).

2.5. Laboratory Apparatus. 5% CO_2 incubator HEPA Class 100 was purchased from Thermo Fisher. Water bath thermostat oscylator was bought from Taicang Laboratory Instrument Manufacture. CO_2 incubator (5410-220) was made by Precision Scientific. Laminar flow hood was made by Suzhou Antai Air Technology Co., Ltd. (BCM-1000A). Counter top centrifuge was purchased from Beijing Jingli Centrifuge Co., Ltd. (LDZ 5-2). Electric Thermostat water bath (DK-450 B type) was purchased from Shanghai Senxin Experimental Instrument Co., Ltd. Liquid nitrogen tank was MVE CRYOSYSTEM750. Microplate reader was made by Thermo Fisher (3001-1249). UV spectrophotometer was made by Eppendorf. PCR gene amplifier was made by Bio-Rad Laboratories, Inc. (US). iQTM5 real-time quantitative PCR was made by Bio-Rad Laboratories, Inc. (US).

2.6. Preparation of Reagents. Cell lysis buffer (0.25% pancreatic enzyme +EDTA) was purchased freshly and stored in freezer at −20°C. PBS buffer (pH 7.2–7.4) was purchased freshly and stored in refrigerator at 4°C. 30% acrylamide/bis solution (propylene thalidomide 0.8 g and N-methylene bisacrylamide thalidomide 29.2 g in dd-water to a final volume of 100 mL) was made in a fume hood, stored at room temperature, and protected from light. 10% SDS (sodium dodecyl sulfate) was made by dissolving 10 g SDS in dd-water to a final volume of 100 mL. 10% ammonium persulfate (0.1 g ammonium persulfate in 1.0 mL dd-water) was made freshly and stored at 4°C less than a week. 15 mL 10% separating gel was prepared in the fume hood with 6 mL dd-water, 5 mL 30% acrylamide/bis solution, 3.75 mL pH 8.8 Tris-HCL, 150 μL 10% SDS, 150 μL 10% ammonium persulfate, and 7.5 μL TEMED. The gel was mixed and loaded immediately after TEMED was added. 5 mL 5% stacking gel was prepared in the fume hood with 3.75 mL dd-water, 0.67 mL 30% acrylamide/bis solution, 0.62 mL pH 6.8 Tris-HCL, 50 μL 10% SDS, 50 μL 10% ammonium persulfate, and 5 μL TEMED. The gel was mixed and loaded immediately after TEMED was added. 1x electrophoresis buffer was made by diluting 100 mL 10x electrophoresis buffer in dd-water to a final volume of 1000 mL. 1x transfer buffer was made by mixing 3.03 g Tris, 14.4 g glycine, and 200 mL methanol in dd-water to a final volume of 1000 mL. 10x TBS was made by dissolving 24.2 g Tris and 80 g NaCl in dd-water to a final volume of 800 mL, adjusting pH to 7.5 using appropriate amount of HCl, and bringing up to 1000 mL dd-water. 1x TBST was prepared

by mixing 10x TBS 100 mL and Tween 20 1 mL in dd-water to a final volume of 1000 mL. Blocking buffer was made by dissolving 5 g fat-free milk powder or bovine serum albumin (BSA) in 100 mL 1x TBST.

3. Methods

3.1. Experimental Animal and Group Assignment. Sixty healthy rats (180 ± 20 g, 6–8 weeks, half male and half female) were fed for an acclimated period of a week and then randomized into three groups: blank control group, saline group, and Shuizhongcao treatment group.

3.2. Establishment of Experimental Animal Model. The rats were intraperitoneally administrated with 5-fluorouracil (5-Fu) with a dose of 5 mg/100 g for chemotherapy. On the fourth day after chemotherapy, rats were anesthetized with 10% chloral hydrate intraperitoneal injection (0.3 mL/100 mL). A piece of NaOH of appropriate size (1.5 mm × 1.5 mm) was placed by a straight head tweezer on the rat's right side cheek mucosa for 5–10 seconds until the naked appearance of redness and ulcer. Afterward all three groups were fed with normal diet. From the second day after surgery, saline group and Chinese medicine group were intragastrically administrated with standard saline and Shuizhongcao (1 mL/100 g) twice a day, respectively, for a total of 10 days. No additional treatment was applied to the blank control group.

3.3. Preparation of Rat Serum. At day 11 after surgery, the rats were anesthetized with 10% chloral hydrate intraperitoneal injection (0.3 mL/100 mL) and euthanized by abdominal aorta exsanguination. The peripheral blood obtained from rats was pipetted into a centrifuge tube containing 10% EDTA and pancreatic enzymes. After 10 min centrifugation at 200 g, the supernatant, which was the serum, was collected and stored in an ultralow freezer at −80°C.

3.4. Culture of Rat Fibroblast. Rat fibroblasts were cultured in fibroblast complete media and passaged when they reached confluence. After being subcultured for 6-7 passages, the cells reached the log phase of growth and were ready for experiment.

3.5. Detection of Drug-Containing Serum on Fibroblast by CCK8 Assay. The fibroblasts in log growth phase were counted and seeded in three 96-well plates, labeled with 12 h, 24 h, and 36 h, respectively. Each plate was divided into ten groups and each group contained 7 subgroups: 5%, 10%, and 15% Chinese medicine group and 5%, 10%, and 15% saline group and blank group. After 12 h, 24 h, and 36 h incubation, the corresponding 96-well plates were taken out, and CCK8 solution was added to each well with 10 μL and continued incubation for 3h. The absorbance was detected and growth curve was plotted.

3.6. NF-κB and ERK Pathway Protein Expression Detected by Western Blot. Cells from Chinese medicine group, saline group, and blank group were collected, washed in PBS solution, and lysed with lysis buffer. Total protein was extracted and quantified using BCA assay. 40 μL protein sample was loaded in each well for SDS-PAGE. The protein was transferred from polyacrylamide gel to PVDF membrane by electrotransfer. The PVDF membrane with transferred protein was blocked with TBST containing 5% BSA (bovine serum albumin) overnight. The membrane was incubated with primary antibody (1 : 1000) for 2 h at 37°C and washed with TBS 3 times, 10 min each time. The membrane was then incubated with secondary antibody (1 : 5000) for 2 h at 37°C and washed with TBS 3 times, 10 min each time. The membrane was developed using ECL solutions and imaged.

3.7. Expression of IL-10, IL-12a, and IL-12b in Rat Fibroblasts in Each Group Detected by Real-Time Quantitative PCR. Total mRNA of cells from each group was extracted at 48 h after serum treatment using absorption column centrifugal method. After concentration, purity, and integrity assessment, mRNA was reversely transcribed into cDNA following the instruction of reverse transcription kit. Levels of IL-10, IL-12a, and IL-12b were detected by fluorescence-based quantitative RT-PCR. Primers were designed using software

Table 1: Real-time PCR primers.

Gene	Primer sequence
GAPDH	GGAGCGAGATCCCTCCAAAAT
	GGCTGTTGTCATACTTCTCATGG
IL-10	GCTATGTTGCCTGCTCTTACTG
	TCTGGCTGACTGGGAAGTG
IL-12a	AGACATCACACGGGACAAAAC
	CACAGGGTCATCATCAAAGAAG
IL-12b	AGCACTCCCCATTCCTACTTCT
	AACGCACCTTTCTGGTTAC

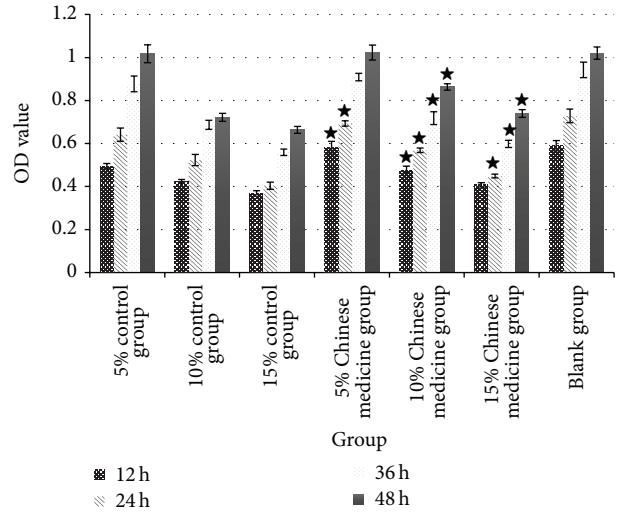

Figure 1: Cell growth and time relationship following serum intervention in control group and Chinese medicine group. Note: $^{\star}P < 0.05$ between Chinese medicine group and control group at the same time point.

Table 2: OD values of rat fibroblast following intervention by Chinese medicine containing serum and control serum ($\overline{X} \pm S$, $n = 6$).

Group	Time				Zero pores
	12 h	24 h	36 h	48 h	
5% control group	0.4944 ± 0.01294	0.6412 ± 0.03167	0.8769 ± 0.03631	1.0179 ± 0.04123	0.0834 ± 0.01123
10% control group	0.4240 ± 0.00884	0.5231 ± 0.02622	0.6874 ± 0.02147	0.7213 ± 0.01843	0.0782 ± 0.00873
15% control group	0.3713 ± 0.00968	0.4035 ± 0.01732	0.5589 ± 0.01483	0.6633 ± 0.01627	0.0761 ± 0.01374
5% Chinese medicine group	0.5833 ± 0.02643*	0.6934 ± 0.01216*	0.9088 ± 0.01736	1.0235 ± 0.03513	0.0851 ± 0.01165
10% Chinese medicine group	0.4780 ± 0.01637*	0.5687 ± 0.01072*	0.7189 ± 0.02987*	0.8636 ± 0.01532*	0.0801 ± 0.02198
15% Chinese medicine group	0.4083 ± 0.00996	0.4488 ± 0.00832*	0.5986 ± 0.01653*	0.7399 ± 0.01843*	0.0719 ± 0.01682
Blank group	0.5901 ± 0.02398	0.7287 ± 0.03128	0.9432 ± 0.03612	1.0213 ± 0.02871	0.0912 ± 0.01572

Note: $^\star P < 0.05$ between Chinese medicine group and control group at the same time point.

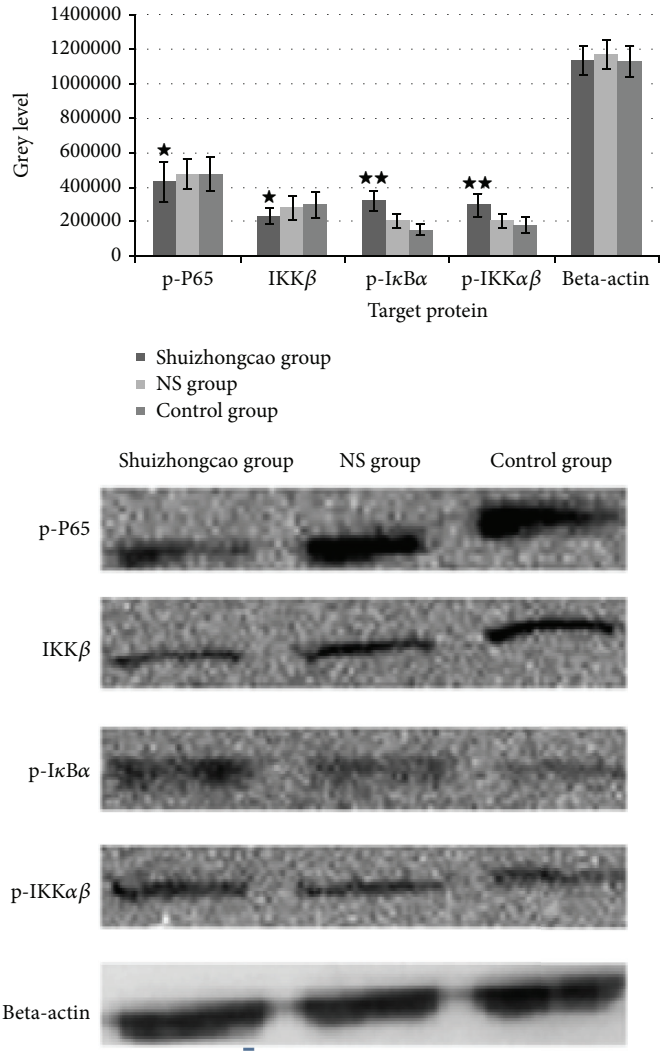

Figure 2: Protein expression of NF-κB pathway.

Primer Premier 6.0 and Beacon Designer and synthesized by Shenggong Bioengineering Technology Co., Ltd. (Shanghai). Primer sequences are listed in Table 1.

Reaction conditions were as follows: reverse transcription, 37°C for 15 min and 85°C for 5 sec; PCR reaction was as follows: 95°C, for 5 sec and 60°C for 30 sec (annealing), 40 cycles (fluorescence collection); melting curve analysis was 50°C to 95°C.

3.8. Statistical Analysis. Quantitative data were expressed as mean ± standard deviation ($\overline{X} \pm S$). SPSS 17.0 statistical software was used for statistical and variance analysis.

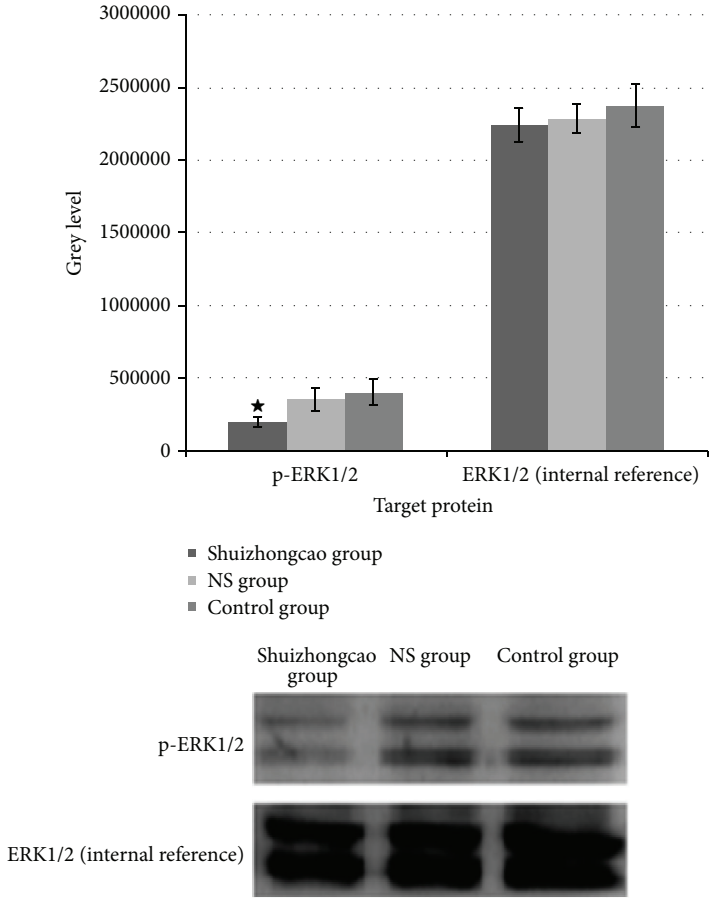

Figure 3: Expression of phosphorylated ERK1/2.

4. Results

4.1. Rat Model Establishment. 12 h after rat model establishment, buccal mucosal vasodilatation, increased capillary network, and tissue edema were observed. At 24 h, edema became serious. The mucosal epithelial layer slightly exfoliated and the surface was covered with exudates. At 48 h, the oral ulcer formed, and the mucosa turned red extensively, which was covered by large pieces of pseudomembranes, appearing yellowish white in color and easily exfoliated. A specialist in pathology confirmed the diagnosis and the success of model establishment.

4.2. Culture of Rat Fibroblasts. The rat fibroblast culture grew well after subculturing.

4.3. CCK8 Results of Fibroblast Intervention by Control Serum and Drug-Containing Serum. As shown in Table 2 and Figure 1, serum in both control group and Chinese medicine group was able to suppress rat fibroblast proliferation, significantly different from that in blank group ($P < 0.05$). The suppressive effect was positively, but not proportionally, correlated with dosage and treatment time. The serum containing Chinese medicine reduced the suppressive effect of inflammation on rat fibroblasts. The difference between each group was statistically significant ($P < 0.05$).

4.4. Western Blot Results following Serum Intervention of Rat Fibroblasts

4.4.1. Expression of Protein Associated with NF-κB Pathway. The results showed that serum containing Shuizhongcao Granule significantly downregulated the expression of phosphorylated P65, demonstrating its ability to suppress the activation and relevant protein expression of NF-κB pathway. In the meantime, Shuizhongcao increased phosphorylated IκBα and phosphorylated IKK$\alpha\beta$ expression, facilitating the activation of canonical NF-κB pathway. The differences between each parameter from Shuizhongcao group (except for internal standard) and that of two other groups were statistically significant ($P < 0.05$). Differences between NS group (NS group is short for "normal saline group") and control group were not significant. See Figure 2.

4.4.2. Expression of Phosphorylated ERK1/2. The results demonstrated that Shuizhongcao-containing serum led to suppressed expression of phosphorylated ERK1/2 and thus inhibited ERK mediated proliferation pathway. The difference of phosphorylated ERK1/2 expression level between

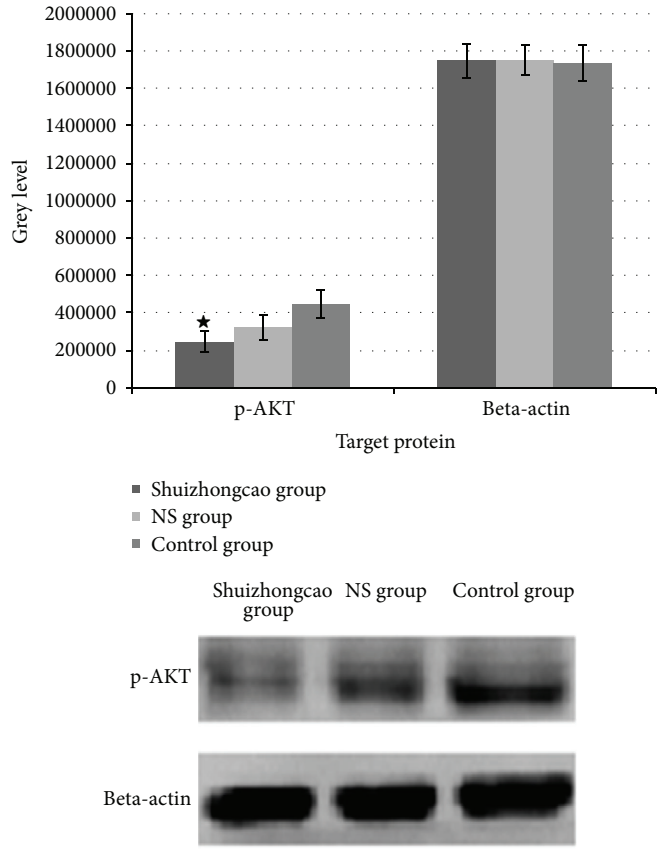

Figure 4: Expression of phosphorylated AKT.

Shuizhongcao group and control group, as well as NS group and control group, was statistically significant ($P < 0.05$). See Figure 3.

4.4.3. Expression of Phosphorylated AKT. The results demonstrated that Shuizhongcao-containing serum suppressed the expression of phosphorylated AKT. Differences between the phosphorylated AKT expression level of Shuizhongcao group and that of two other groups were statistically significant ($P < 0.05$), respectively. The difference between NS group and control group was not significant. See Figure 4.

4.5. PCR Results of IL-10, IL-12a, and IL-12b. Considering the large variations in PCR measurements, a normal group was set up, that is, using drug-containing rat serum without any treatment as reference. The measurements in the control group and Chinese medicine group were, respectively, divided by the values in the normal group to calculate relative expression amounts to more objectively reflect the expression difference.

The results showed that levels of IL-10 expression in both Chinese medicine group and control group were significantly increased ($P < 0.01$), compared with normal control group. The expression levels of IL-10 and IL-12a in Chinese medicine group were significantly decreased compared with those in control group ($P < 0.01$). When compared with normal group, IL-12a expression level was increased in Chinese medicine group while it decreased in control group, with significant differences ($P < 0.01$). When compared with normal group, the IL-12b expression level was decreased in Chinese medicine group while it increased in control group, with significant differences. See Figure 5.

5. Discussion

Fibroblast proliferation is crucial for the healing of oral ulcers. CCK8 assay indicated that Shuizhongcao Granule did enhance fibroblast proliferation. Western blot showed that the serum containing Shuizhongcao Granule inhibited AKT expression in fibroblasts. We supposed that fibroblast proliferation was related to inflammation inhibition caused by AKT inhibition. However, this needs to be confirmed by controlled trial of gene silencing. We aimed to identify the effect of Shuizhongcao Granule on oral ulcers in animal and cell models using CCK8 assay, and the results confirmed that fibroblast proliferation was enhanced. Subsequent Western blot and PCR were intended to find out the reasons for this and to collect data for analysis.

According to our preliminary research, Shuizhongcao Granule was able to increase the red blood cell Cab receptor rosette formation rate but decrease immune complex rosette formation rate in red blood cells [9]. We also demonstrated that, in ROU, Shuizhongcao Granule was capable of

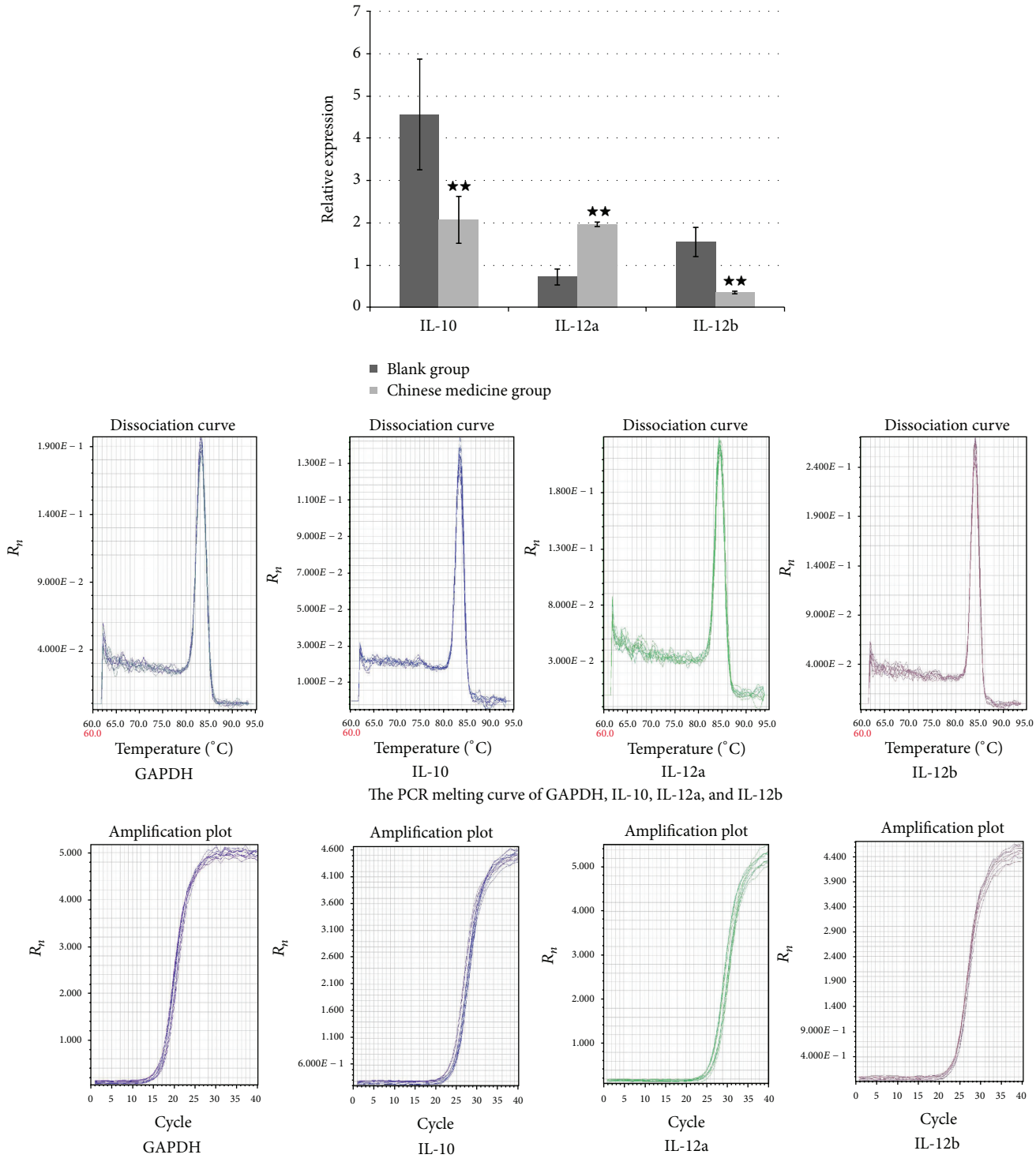

FIGURE 5: Relative expression of IL-10, IL-12a, and IL-12. Note: ★★ compared with control group, $P < 0.01$.

increasing the percentage of CD3 and CD4 positive cells, CD4/CD8 ratio, and serum level of IL-12 and decreasing serum level of IL-10 [10]. Through the rat ROU model, we compared the recovery time of ulcers in Shuizhongcao group and model group and demonstrated the efficacy of Shuizhongcao Granule in treating ROU. The *in vivo* effect of inflammation inhibition of Shuizhongcao was demonstrated by levels of IL-4, IL-8, and GM-CSF in rat serum.

Based on the preliminary research, this study focused on exploring the pathways associated with inflammation.

NF-κB is a canonical pathway associated, among others, with inflammation. It has also been reported that NF-κB regulates several hundred genes involved in cell growth, differentiation, and apoptosis [11]. Here we reported that the level of phosphorylated P65 in Chinese medicine group was significantly decreased compared with two other groups. Since the expression of NF-κB pathway mainly relies on the binding of phosphorylated P65 on the particular gene target in the nucleus, NF-κB pathway might be inhibited in the Chinese medicine group. However, further experiments showed that, after the treatment of Chinese medicine, expression levels of phosphorylated IκBα and phosphorylated IKK$\alpha\beta$ increased, while IKKβ expression was decreased, which was contrary to what should be presented by the canonical pathway. Therefore, we speculate that Shuizhongcao Granule suppresses the activation of NF-κB pathway through inhibiting an alternative unknown pathway, rather than inhibiting the canonical pathway.

Our preliminary data showed that serum containing Shuizhongcao facilitated fibroblast proliferation (or inhibited inflammation lesions). Thus we speculated that serum containing Shuizhongcao Granule would be able to activate ERK pathway, promote cell proliferation, and suppress apoptosis. However, the results demonstrated that drug-containing serum inhibited the expression of phosphorylated ERK1/2, which are the key players of ERK pathway. Therefore, we inferred that the ability of Shuizhongcao Granule to promote cell proliferation is through the inhibition of p-AKT expression and thus to regulate the NF-κB pathway.

As a major player on antiapoptotic pathway, AKT can exert a dominant negative effect and inhibit insulin-like growth factor 1 (IGF1) mediated cell growth; thus persistent activation of AKT inhibits PTEN mediated apoptosis. AKT also affects cell survival by an indirect effect on Pi3k-AKT and P53 [12]. It has been shown that [13–15] AKT indirectly affects NF-κB and P53 and thus influences cell survival. By phosphorylating and activating kB kinase (IKK), AKT leads to the degradation of IκB, an inhibitor of NF-κB, and thus results in cytoplasm release and nuclear translocation of NF-κB, which in turn activates its target genes and promotes cell survival. Our experiments showed that Chinese medicine containing serum significantly inhibited the level of phosphorylated AKT. Therefore, we inferred that it was the AKT-NF-κB signaling pathway that Shuizhongcao Granule acted through on fibroblasts.

Our study confirmed the ability of Shuizhongcao to accelerate the recovery of ROU rat and further demonstrated that the mode of action was associated with inflammatory reaction inhibition. The mechanism underlying facilitation of fibroblast proliferation by Shuizhongcao is yet to be discovered. Our research provides an experimental rationale for the application of Shuizhongcao in clinical therapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Zhang Bo, Ruan Shan-ming, Wang Bei, and Li Qing-lin contributed equally to the project and are considered co-first authors.

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Research Article

Mechanistic Study of the Phytocompound, 2-β-D-Glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne in Human T-Cell Acute Lymphocytic Leukemia Cells by Using Combined Differential Proteomics and Bioinformatics Approaches

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Bidens pilosa, a medicinal herb worldwide, is rich in bioactive polyynes. In this study, by using high resolution 2-dimensional gel electrophoresis coupled with mass spectrometry analysis, as many as 2000 protein spots could be detected and those whose expression was specifically up- or downregulated in Jurkat T cells responsive to the treatment with 2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (GHTT) can be identified. GHTT treatment can upregulate thirteen proteins involved in signal transduction, detoxification, metabolism, energy pathways, and channel transport in Jurkat cells. Nine proteins, that is, thioredoxin-like proteins, BH3 interacting domain death agonist (BID protein involving apoptosis), methylcrotonoyl-CoA carboxylase beta chain, and NADH-ubiquinone oxidoreductase, were downregulated in GHTT-treated Jurkat cells. Further, bioinformatics tool, Ingenuity software, was used to predict signaling pathways based on the data obtained from the differential proteomics approach. Two matched pathways, relevant to mitochondrial dysfunction and apoptosis, in Jurkat cells were inferred from the proteomics data. Biochemical analysis further verified both pathways involving GHTT in Jurkat cells. These findings do not merely prove the feasibility of combining proteomics and bioinformatics methods to identify cellular proteins as key players in response to the phytocompound in Jurkat cells but also establish the pathways of the proteins as the potential therapeutic targets of leukemia.

1. Introduction

The omics technologies are being increasingly utilized as the systems biology approach to study diseases and drug discovery [1]. For instance, proteomics research using a combination of techniques, including high-resolution 2-dimensional electrophoresis analysis and mass spectrometry (2DE/MS) for protein identification, has become a powerful approach in the rapid identification and validation of protein expression profiles, posttranslation modifications, and new protein targets. The proteomics approach can also lead to identifying potential biomarkers and elucidate cross-talk molecules among signal pathways in human cancer diseases [2]. Bioinformatics is emerging as an indispensable tool to process and analyze the big data generated from omics technologies. The development of bioinformatics can assist

to gain comprehensive insight into a complexity of signal network of cellular proteins, leading to the identification of drug targets. Now it is a golden opportunity to employ the systems biology approach to unveil the global signal network of traditional medicine, which can explain its mode of action and pharmacology.

Acute lymphocytic leukemia (ALL) is the most common cancer of childhood. Its incidence rate is 1.6 per 100,000 men and women per year in United States. Around 15% of acute lymphocytic leukemia cases are T-cell acute lymphocytic leukemia (T-ALL), a disease caused by malignant transformation of T cells [3]. T-ALL pathogenesis is related to genetic alterations or aberrant expression of oncogenes or tumor suppressor genes. Though treatment outcomes for T-ALL have been much improved, novel lead compounds for T-ALL are necessary. Jurkat cells, which were established from a T-ALL patient, are a physiologically relevant tumor model of T-ALL [4].

Plant extracts or compounds are considered to be of great potential as therapeutic agents that can prevent or treat human cancers, immune disorders, and others. *Bidens pilosa* (Asteraceae), an edible herb, has been claimed to treat 41 diseases worldwide [5]. *B. pilosa* has a complex phytochemistry. Naturally occurring polyynes have been shown to have a broad range of medicinal and biological properties such as antitumor activities [6, 7]. Of note, a group of polyynes were identified from *B. pilosa* [5]. Our group first demonstrated that some of them could promote apoptosis of endothelial cells and angiogenesis [8, 9]. Nevertheless, their antitumor effect and mechanism are poorly understood.

To gain a comprehensive picture of anticancer action of the polyynes present in *B. pilosa*, an integrative approach to use proteomics and bioinformatics methods was simultaneously applied in this study. We used the Jurkat cells as a model system to investigate the signal network of cellular proteins in responses to GHTT treatment. Biochemical methods were applied to verify the putative pathways of the responsive proteins of the phytocompound.

2. Material and Methods

2.1. Chemicals and Reagents. Most of the chemical reagents were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Immobilized pH gradient (IPG) strips, buffers, and electrophoresis apparatus (Multiphor II and Protean IEF cell) were from Bio-Rad Laboratories (Hercules, CA, USA) and Amersham Biosciences (Denmark). SYPRO Ruby Protein Gel Stain was from Molecular Probes (Eugene, OR, USA). Trypsin (sequencing grade, modified) was from Promega (Madison, WI, USA). All other chemicals and solvents used in this study were of reagent grade or HPLC grade. GHTT was isolated from *B. pilosa* and structurally elucidated as published [10]. Briefly, the whole fresh plant was crushed and mixed with 10-fold volumes (L/kg) of 70% ethanol at room temperature. This crude extract was subsequently partitioned with ethyl acetate (EA) and *n*-butanol (BuOH), each with the same volume of water for 3 repeats. GHTT was purified from the BuOH fraction and used for this study. Antibodies against PARK7, VDAC2, LMNB1, NDUA5, and PRDX3 were purchased from Cell Signaling and/or Proteintech.

2.2. Cell Culture. Human T-ALL cell line, Jurkat cells (E6-1 clone, TIB 152), was obtained from American Type Culture Collection and cultivated in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 10 mM HEPES, 1 mM pyruvate, 10 μM β-mercaptoethanol, and 1% (v/v) penicillin-streptomycin glutamate at 37°C in a humidified incubator at 5% CO_2. GHTT at 15 μg/mL was added to the culture at cell density of 3–10 $\times$ 10^5 cells/mL. The treatment was continued for 24 h before cell harvest by centrifugation at 624 g for 5 min. Cell pellets were washed twice in PBS and stored at −70°C until use for protein extraction.

2.3. Protein Extraction. Jurkat cells (~5 $\times$ 10^7 cells) were lysed in 200 μL of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), 10 mM spermine, and 0.5% pharmalyte 3–10) for 1 h at room temperature with gentle vortex [11]. The viscous lysate was centrifuged at 100,000 g for 1 h to pellet the nucleic acids. The supernatant was recovered and stored in a deep freezer (−70°C) before use. Protein concentration was determined using *RC DC* protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

2.4. Two-Dimensional Gel Electrophoresis (2DE). Protein lysates from DMSO- and GHTT-treated Jurkat cells were first separated on an isoelectric focusing gel with pH ranges from pH 4 to 7, 5 to 8, or 6 to 9 and then a SDS-PAGE (18 $\times$ 20 cm) as a 2-dimensional gel with 10–16% acrylamide gradient. Protein on those gels was visualized by silver or fluorescence staining and scanned for image analysis using PDQuest 2D gel analysis software. Isoelectric focusing (IEF) of IPG strips (18 cm, pH 4–7 and pH 6–9) on Multiphor II and IPG strips (17 cm, pH 5–8) on Protean IEF Cell was essentially followed by the protocol of Gorg et al. (Electrophoresis, 2000, 21, 1037). Briefly, IPG dry strips were rehydrated for 16 h with protein (0.6–1 mg) in a rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.005% (v/v) bromophenol blue, 2% (v/v) IPG buffer for IPG pH 4–7 and pH 5–8 strips on Multiphor II, or 0.13% (w/v) Biolyte 5–8 and 0.07% (w/v) Biolyte 8–10 for IPG pH 5–8 strips on PROTEAN IEF Cell. IEF on Multiphor II system was conducted at 20°C for 74.6 Vh with voltages setting as follows: 300 V for 3 h, 300–1400 V linear gradient for 6 h, 1400 V for 10 h, 1400–3500 V linear gradient for 3 h, and 3500 V for 14 h. IEF on PROTEAN IEF Cell was conducted at 20°C for 92.7 Vh with voltages setting as follows: 300 V for 3 h, 300–1500 V linear gradient for 4 h, 1500 V for 8 h, 1500–4000 V for 3 h, 4000 V for 4 h, 4000–8000 V for 2 h, and 8000 V for 5 h. The strips were then stored at −70°C or equilibrated in 10 mL of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, trace bromophenol blue, and 65 mM DTT for 20 min. Alkylation was followed in 10 mL of the same buffer containing 135 mM iodoacetamide (IAA) instead of DTT for 20 min. The IPG strips were embedded with 0.5% w/v melted agarose on the top of 10–16% T gradient SDS-PAGE slabs (18 cm $\times$ 20 cm $\times$ 1 mm). The SDS-PAGE was run at 15°C

with constant current setting at 12 mA/gel for 30 min and then at 24 mA/gel through the end of run. After electrophoresis, the gels were stained with SYPRO Ruby protein gel stain (Molecular Probes, Eugene, OR, USA) or silver staining with ammoniacal silver as described [11]. SYPRO Ruby stained gels were scanned at 100 dpi on a scanner (Typhoon 9200, Amersham Biosciences) and image of protein spots analysis was performed with the PDQuest 2D software (Bio-Rad).

2.5. Protein Identification by Mass Spectrometry (MS) and Database Searching. By gel-to-gel matching, the overestimating qualitative variations were removed and the spots constantly present in all gels from the same sample were further quantified and analyzed. Relative comparisons of protein spot volumes in fluorescence stained gels were analyzed using PDQuest 2D gel analysis software (Bio-Rad). Differentially expressed protein spots were then identified by in-gel trypsin digestion and analysis using MALDI-MS/MS or capillary LC ESI-MS/MS. Namely, the protein spots detected on 2DE gel were manually excised from the gel and cut into pieces for in-gel trypsin digestion. The gel pieces were dehydrated with acetonitrile for 10 min, vacuum dried, rehydrated with 55 mM DTT in 25 mM ammonium bicarbonate, pH 8.5, at 37°C for 1 h, and subsequently alkylated with 100 mM IAA in 25 mM ammonium bicarbonate, pH 8.5, at room temperature for 1 h. The pieces were then washed twice with 50% acetonitrile in 25 mM ammonium bicarbonate, pH 8.5 for 15 min each time, dehydrated with acetonitrile for 10 min, dried, and rehydrated with 25 ng trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8.5, at 37°C for 16 h. Following digestion, tryptic peptides were extracted twice with 50% acetonitrile containing 5% formic acid for 15 min each time with moderate sonication. The extracted solutions were pooled and evaporated to dryness under vacuum.

Trypsin-digested hydrolysates from protein spots were subjected to MALDI MS and CID MS/MS analyses for protein identification using Q-Tof Ultima MALDI mass spectrometer (Waters/Micromass, Manchester, UK). Selected spots were also submitted to nano-LC-MS/MS analysis on a separate Q-Tof Ultima MS instrument equipped with a capillary LC system and a nano-ESI source for complementary protein identification.

For MALDI MS and MS/MS analysis, samples were premixed 1 : 1 with matrix solution (5 mg/mL CHCA, 2% ammonium citrate, and 0.1% TFA in 50% acetonitrile) and spotted onto a 96-well MALDI sample stage. Data dependent acquisition on the Q-TOF Ultima MALDI instrument was operated with predefined probe motion pattern and peak intensity threshold for switching over from MS survey scan to MS/MS. Precursor ions meeting the predefined criteria (m/z 800–3000 range with intensity above 10 counts) were selected for CID MS/MS using argon as collision gas and a mass dependent ±5 V rolling collision energy starting from the most intense peak. The quadrupole selection window for a precursor ion was set at 4 Da wide. The instrument was externally calibrated to 5 ppm accuracy over the mass range of m/z 800–3000 using a sodium iodide and PEG 200, 600, 1000, and 2000 mixtures, and Glu-Fibrinopeptide B was used as the lock mass calibrant during data processing.

For LC-MS/MS analysis on the nano-LC-Q-Tof Ultima MS system, tryptic peptide samples were first injected into an autosampler, trapped, desalted on a precolumn (LC-Packings PepMap C18 μ-Precolumn Cartidge, 300 μm I.D. × 5 mm, 5 μm; Dionex), and separated on an analytical C18 capillary column (Zorbax 300 SB C18, 75 μm I.D. × 15 cm, 5 μm, Micro-Tech Scientific) connected online to the mass spectrometer at 300 nL/min flow rate using a 40 min gradient of 5% to 40% acetonitrile in 0.1% formic acid. For routine protein identification analysis, the 1 s survey scans were acquired over the mass range m/z 400–2000 and a maximum of 3 concurrent. Data dependent MS/MS acquisitions were triggered for 2+, 3+, and 4+ charged precursors.

After data acquisition, MS and MS/MS data acquired on MALDI- and LC-MS runs were processed into peak list files (.txt and .pkl) using the Micromass ProteinLynx Global Server (PGS) 2.0 data processing software. The peak list files were used for peptide/protein identification based on peptide mass fingerprinting and/or MS/MS ions searching using the online Mascot database search engine (http://www.matrixscience.com/) against the SwissProt *Homo sapiens* protein sequence database with the following parameters: peptide mass tolerance, 50 ppm; MS/MS ion mass tolerance, 0.25 Da; missed cleavage allowed, 1; variable modifications, methionine oxidation; and fixed modifications, cysteine carbamidomethylation. Only significant peptide hits as defined by Mascot significant threshold ($p < 0.05$) were considered initially. In addition, a minimum total score of 20 comprising at least one peptide match of ion score more than 20 was arbitrarily set as the threshold for acceptance.

2.6. Flow Cytometry. To measure the mitochondrial membrane potential, Jurkat cells were preloaded with tetramethylrhodamine methyl ester (TMRM, 10 nM) at 37°C in the dark for 30 min. After washing, the cells were incubated with DMSO vehicle or GHTT at the indicated doses for an additional 30 min. The cells underwent BD LSR II flow cytometry analysis. The data were processed using FCS express 3 software (De Novo Inc., CA, USA). For cell apoptosis, Jurkat cells were incubated with DMSO vehicle or GHTT at the indicated doses for 30 min. After washing, the cells were stained with annexin V plus propidium iodide (PI) and, in turn, underwent flow cytometry analysis and FCS express 3 analysis.

2.7. Ingenuity Pathways Analysis. Using a web-based entry tool developed by Ingenuity systems [12], in which specific molecular network of direct physical, transcriptional, and enzymatic interactions could be observed between mammalian orthologs. For better understanding the biological meaning of changes in protein expression in Jurkat cells treated with DMSO and GHTT, we constructed possible candidate signaling pathways of 22 of their responsive proteins, identified by 2DE/MS analyses, in Table 1 by using the Ingenuity software.

2.8. Statistics. Data from three or more independent experiments are presented as mean ± standard error (SE).

Table 1: Differentially expressed proteins in GHTT-treated Jurkat T cells identified using 2DE/MS analysis.

ID	Protein name	Accession number	Theor. Mr (kDa)	Theor. pI	Sequence coverage (%)	Number of peptides matched	Avg. of fold difference[a]	Functional categorization[b]	Identification method
GDIR2	Rho GDP-dissociation inhibitor 2	P52566	22974	5.10	41	5	+4.0	Signal transduction	MS, MS/MS
GSTO1	Glutathione S-transferase omega 1	P78417	27548	6.23	44	12	+2.0	Detoxification	MS, MS/MS, ESI-MS/MS
HBB	Hemoglobin beta chain	P68871	15871	7.26	15	4	+3.4	Transport	MS/MS
PDIA3	Protein disulfide isomerase A3 precursor	P30101	56747	5.98	28	15	++	Metabolism	MS/MS
ADA	Adenosine deaminase	P00813	40555	5.54	35	14	+3.2	Metabolism; energy pathways	MS, MS/MS
SERA	D-3-phosphoglycerate dehydrogenase	O43175	56483	6.31	23	10	+2.3	Metabolism; energy pathways	MS, MS/MS
ECHM	Enoyl-CoA hydratase, mitochondrial precursor	P30084	31379	8.34	33	7	+2.9	Metabolism; energy pathways	MS, MS/MS
PARK7	DJ-1 protein (oncogene DJ1)	Q99497	19878	6.33	42	7	+1.6	Nucleoside, nucleotide, and nucleic acid metabolism	MS, MS/MS
IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic	O75874	46630	6.53	28	9	+1.7	Metabolism; energy pathways	MS, MS/MS
LTOR3	Mitogen-activated protein kinase kinase 1 interacting protein 1	Q9UHA4	13614	6.73	85	8	+1.5	Signal transduction	MS, MS/MS
LMNB1	LMNB1 protein	AAH78178	38118	5.37	26	9	+5.1	Miscellaneous	MS, MS/MS
GSTP1	Glutathione S-transferase P	P09211	23210	5.44	40	6	+1.8	Metabolism; energy pathways	MS, MS/MS, ESI-MS/MS
VDAC2	Voltage-dependent anion-selective channel protein 2	P45880	38069	6.32	9	7	+1.5	Transport	MS, MS/MS
RM39	Mitochondrial 39S ribosomal protein L39	Q9NYK5	34204	6.47	26	7	—	Metabolism	MS, MS/MS
MCCB	Methylcrotonoyl-CoA carboxylase beta chain	Q9HCC0	61294	7.57	31	12	—	Metabolism; energy pathways	MS, MS/MS
TXNL1	Thioredoxin-like protein 1	O43396	32100	4.84	58	10	−4.4	Metabolism	MS, MS/MS
C3orf60	Chromosome 3 open reading fragment 60	AAH02873	20337	8.48	29	5	−2.1	Miscellaneous	MS, MS/MS
BID	BH3 interacting domain death agonist	P55957	21981	5.27	41	7	−3.8	Apoptosis	MS, MS/MS
PRDX3	Thioredoxin-dependent peroxide reductase, mitochondrial precursor	P30048	27675	7.67	14	3	−3.6	Metabolism; energy pathways	MS/MS
PPID	40 kDa peptidyl-prolyl cis-trans isomerase	Q08752	40607	6.76	36	12	−2.0	Metabolism; energy pathways	MS, MS/MS, ESI-MS/MS
GLRX3	Thioredoxin-like protein 2	O76003	37408	5.31	34	13	−2.7	Metabolism	MS, MS/MS, ESI-MS/MS
GLRX3	Thioredoxin-like protein 2	O76003	37408	5.31	31	10	−3.5	Metabolism	MS, MS/MS, ESI-MS/MS
NDUA5	NADH-ubiquinone oxidoreductase 13 kDa-B subunit	Q16718	13319	5.76	49	7	−2.1	Metabolism; energy pathways	MS/MS

[a]Data were obtained from the analysis of protein spots (GHTT versus control) using PDQuest software. Symbol — represents that the respective protein spot in the GHTT gel was not detected.

[b]Proteins were queried in Human Protein Reference Database (http://www.hprd.org/) for their functional categorization.

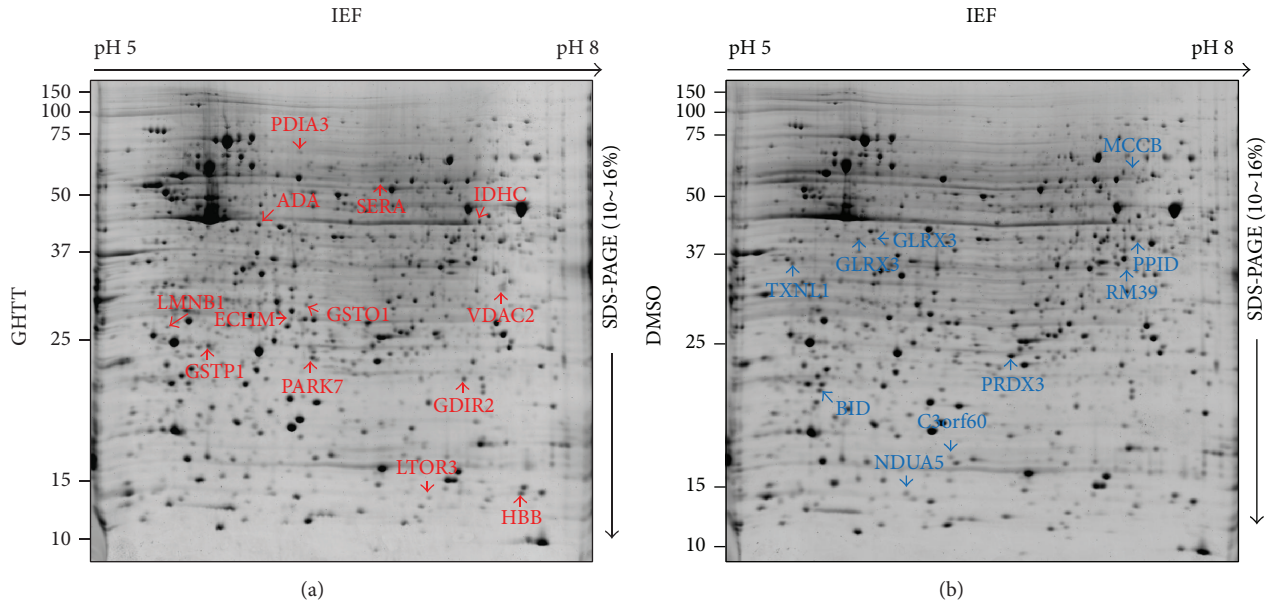

FIGURE 1: 2DE gels of cellular proteins from the GHTT-treated (a) and DMSO-treated Jurkat T cells (b). Upregulated proteins (red on gel (a)) and downregulated proteins (blue on gel (b)) were identified by MS or MS/MS analysis. Identity of these spots is listed in Table 1.

Comparisons between multiple groups were made with ANOVA. $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Identification of the Differentially Expressed Proteins in Jurkat Cells in Response to GHTT Using Two-Dimensional Gel Electrophoresis Coupled to Mass Spectroscopy (2DE/MS). To explore novel signal pathway network of the phytocompound, GHTT, on Jurkat cells, the 2DE/MS was engaged to compare the protein expression profiles of Jurkat cells treated with DMSO vehicle and GHTT. With this technology, we were able to routinely obtain representative, high resolution, and highly reproducible 2D protein profiles of Jurkat cells (Figure 1). Around 1,200–2,000 protein spots with a molecular mass range of 10–150 kDa in each gel were detected (Figure 1). In order to avoid erroneous and ambiguous evaluation of the experimental data, comparative difference of the protein spots was verified at least in three replicates from each batch of T cells collection. Red and blue circles denoted the up- and downregulation of the proteins in GHTT-treated Jurkat cells as opposed to those in control cells (Figure 1). Differentially expressed proteins of 22 spots showing >1.5-fold change were further identified. Table 1 lists proteins identified by MALDI-MS/MS or capillary LC-ESI-MS/MS and databases search based on peptide mass fingerprinting and MS/MS ions search using Mascot search program. Thirteen upregulated and nine downregulated proteins could be identified (Figure 2). In fact, some of the protein spots could not be identified due to low protein abundance or lack of peptides digestible with trypsin (data not shown).

Thirteen proteins, including Rho GDP-dissociation inhibitor 2 (GDIR2), glutathione transferase omega 1 (GSTO1), hemoglobin beta chain (HBB), protein disulfide isomerase A3 (PDIA3), adenosine deaminase (ADA), D-3-phosphoglycerate dehydrogenase (SERA), DJ-1 protein (PARK7), cytoplasmic NADP-dependent isocitrate dehydrogenase (IDHC), mitogen-activated protein kinase kinase 1 interacting protein 1 (LTOR3), LMNB1 protein (LMNB1), glutathione S-transferase P (GSTP1), and anion-selective channel protein 2 (VDAC2), were found to be upregulated in GHTT-treated Jurkat cells compared to control Jurkat cells (red, Figures 1 and 2). Nine proteins, mitochondrial 39S ribosomal protein L39 (RM39), methylcrotonoyl-CoA carboxylase beta chain (MCCB), thioredoxin-like protein 1 (TXNL1), chromosome 3 open reading frame 60 (C3orf60), BH3 interacting domain death agonist (BID), thioredoxin-dependent peroxide reductase, mitochondrial precursor (PRDX3), 40 kDa peptidyl-prolyl cis-trans isomerase (PPID), thioredoxin-like protein 2 (GLRX3), and NADH-ubiquinone oxidoreductase 13 kDa-B subunit (NDUA5), were observed to be downregulated in GHTT-treated Jurkat cells compared to control Jurkat cells (blue, Figures 1 and 2).

To identify specific signaling pathways in response to GHTT treatment in Jurkat cells, we analyzed the 22 up- and downregulated proteins using the Ingenuity software similar to the publications [12, 13]. Two likely pathways related to mitochondrial function (Figure 3(a)) and cell survival (Figure 3(b)) in Jurkat cells were postulated to elucidate the pharmacological action and mechanism of GHTT. The first pathway was relevant to mitochondrial function, including the participation of PRDX3, VDAC2, and PARK7 proteins, with an expression change over 1.5-fold, as shown in Figure 3(a). PRDX3 is multifunctional protein in mitochondria, involving the regulation of ROS accumulation and apoptosis [10]. PRDX3 acted as a mitochondrial ROS scavenger and, therefore, diminished ROS content under cellular oxidative conditions [14, 15]. However, PRDX3 was associated with

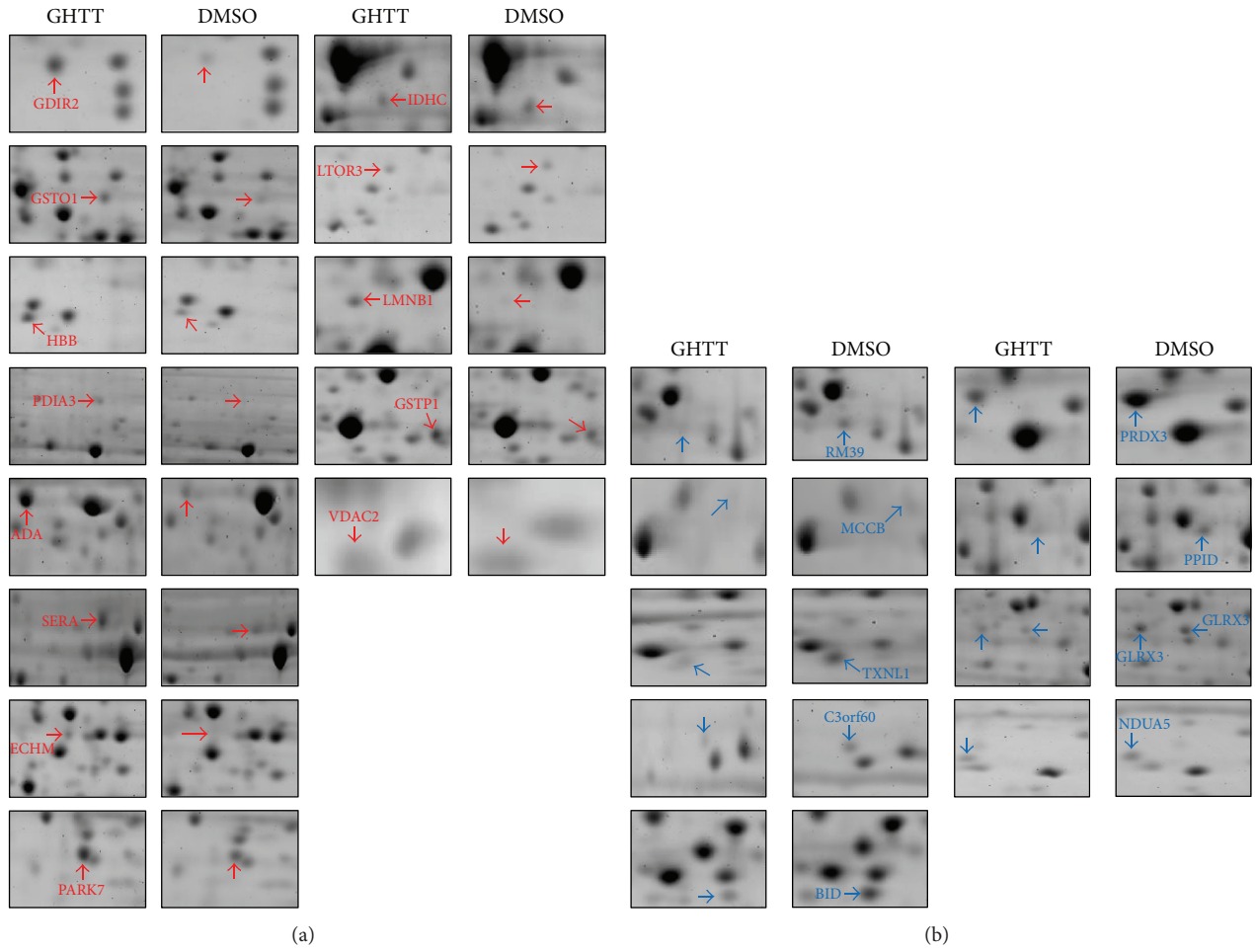

Figure 2: Enlarged view of the spots up- and downregulated by treatment with DMSO and GHTT. Red and blue circles indicate the increase fold and decrease fold of the proteins listed in Table 1, respectively.

carcinogenesis in Caucasian patients [16]. VDAC2 was also shown to regulate apoptosis. During apoptosis, the permeability of VDAC2 was increased and, in turn, led to the release of cytochrome c (CYTC) [17]. PARK7, a DJ-1 oncogene, was thought to maintain mitochondrial function during oxidative stress and thereby alter mitochondrial dynamics and autophagy indirectly [18]. In this work, the protein expression level of PRDX3 was decreased (−3.6-fold) in Jurkat cells by GHTT. In parallel, the protein level of VDAC2 (+1.5-fold) was increased by GHTT in the cells (Figure 3(a)). Both data suggest that GHTT treatment promotes the dysfunction of mitochondria and apoptotic activity in Jurkat cells. However, the protein level of PARK7 (+1.6-fold) was increased after GHTT treatment, suggesting that the ability of PARK7 to maintain mitochondrial function was induced by GHTT in an attempt to balance the mitochondria dysfunction.

The second likely pathway was related to cell death, involving the participation of BID and LMNB1. Granzyme B (GZB) was reported to promote apoptosis through two main pathways, either through BID-dependent CYTC release or through direct caspase processing and activation [19]. Further, GZB also directly cleaves several caspase substrates such as LMNB1 and others via BID-independent death pathway [20]. In this study, the protein expression level of BID was decreased (−3.8-fold). However, tBID was not detected in this study. In contrast, the protein expression level of LMNB1 was significantly increased (+5.1-fold) in Jurkat cells after GHTT treatment (Figure 3(b)). These data suggest that GZB-mediated cell death, which was implicated in LMNB1 but not BID, was activated by GHTT in Jurkat cells (Figure 3(b)). For the rest of GHTT-responsive proteins, the Ingenuity software failed to predict any matched biological pathways. However, PDIA3 [21], TXNL1 [22], and GLRX3 [23–25] were also proposed to be implicated in cancers. Upon the recognition of tumor antigen, GZB, released by cytotoxic T cells, can induce cell death in tumor. Since GHTT was involved in GZB-mediated tumor cell death, this raised the possibility that GHTT could potentiate GZB-mediated tumor cell death, which could be beneficial for tumor therapy.

Overall, the structured network knowledge-based strategy using the Ingenuity software pointed to two putative apoptotic pathways in Jurkat cells in response to the plant chemical, GHTT. This approach not only simplifies the processing of proteomics data (proteins and their expression

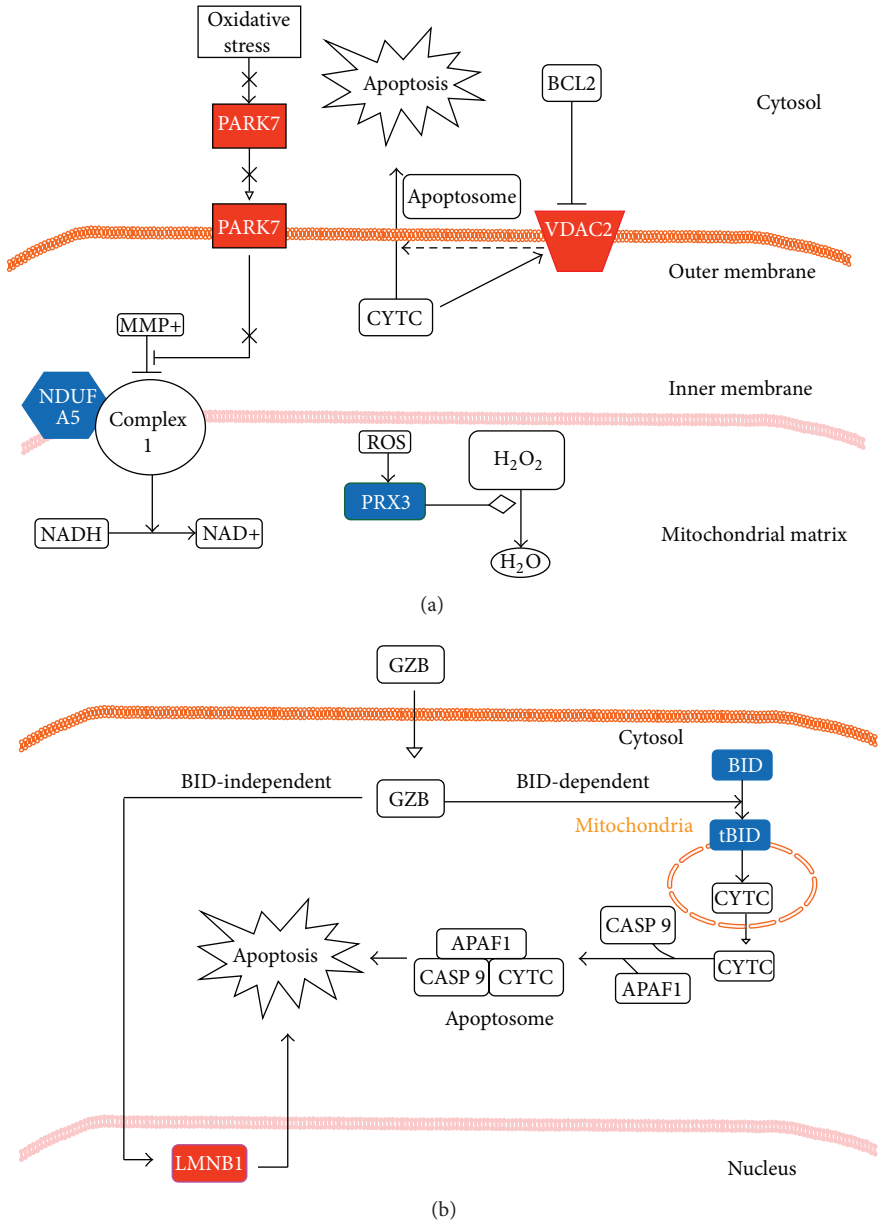

Figure 3: Pathways analysis of the proteins listed in Table 1 by means of the Ingenuity software. Mitochondrial dysfunction pathway (a) and granzyme B (GZB) signaling pathway (b) are proposed according to the structured network knowledge-based strategy. In (a), increase in the permeability of VDVC proteins in the outer mitochondrial membrane is assumed to allow for CYTC and, therefore, relevant to mitochondrial dysfunction and apoptosis. The VDVC-mediated apoptosis involves the formation of apoptosome and an activation of the caspase cascade. PRDX3 acts as an antioxidant protein to catalyze the degradation/reduction of hydrogen peroxide to water. Oxidative stress promotes the formation of ROS in mitochondrial complexes 1 to 4 and can cause mitochondrial damage. PARK7 acts as an antioxidant player and antagonizes the loss of mitochondrial function. In (b), GZB exerts its apoptotic function via the BID-dependent and BID-independent pathways. In BID-dependent route, GZB degrades BID to generate its active truncated BID (tBID), thereby inducing cell death via the formation of apoptosome and activation of caspases. In BID-independent route, GZB can activate different caspases and caspase substrates (e.g., LMNB1, etc.) independent of BID cleavage, leading to apoptosis. Red and blue indicate the proteins which are up- or downregulated by the plant compound, GHTT, in Jurkat cells. Translocation (—▷), activation (⟶), inhibition (⊣), and catalysis (—◇) are indicated.

change and signal network) but also provides constructive information about the signaling pathways and mechanism of GHTT. Of course, the putative signaling pathways need to be ascertained with further experiments.

3.2. Effect of GHTT on Mitochondrial Dysfunction and Apoptosis of Jurkat Cells. Next, we wanted to verify the effect of GHTT on mitochondrial function and cell survival of Jurkat cells. TMRM has been used as a fluorescent dye

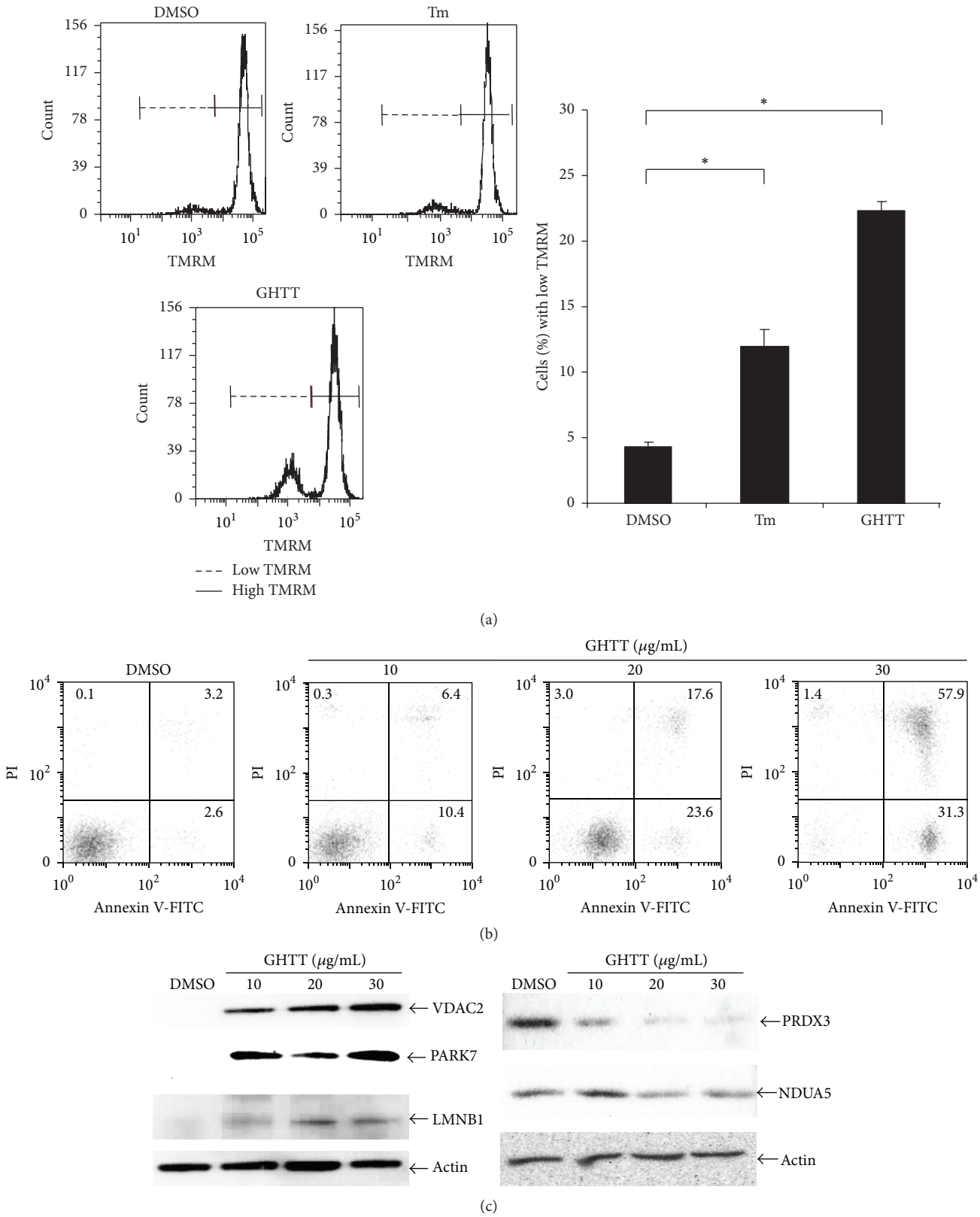

Figure 4: Effect of GHTT on mitochondrial membrane potential (a) and apoptosis (b) in Jurkat cells. (a) Jurkat cells were incubated with TMRM. After washing, the cells were treated with GHTT, DMSO, a negative control, and tunicamycin (Tm, 10 μg/mL), a positive control, followed by flow cytometry analysis. (b) Jurkat cells were incubated with GHTT and DMSO. After washing, the cells were stained with PI and annexin V and subjected to flow cytometry analysis. (c) Total lysates of Jurkat cells treated with GHTT and DMSO underwent SDS-PAGE. Following protein transfer, the membrane was blotted with the indicated antibodies.

to detect mitochondrial membrane potential, an indication of mitochondrial function [26]. As shown in Figure 4(a), less than 5% of Jurkat cells decreased their mitochondrial membrane potential in exposure to DMSO vehicle (DMSO, Figure 4(a)). In contrast, 12% of the cells decreased their mitochondrial membrane potential in exposure to tunicamycin, an ER stress inducer (Tm, Figure 4(a)). Further, 23% of the cells decreased their mitochondrial membrane potential in exposure to GHTT (GHTT, Figure 4(a)). The results corroborate the function of GHTT in causing mitochondrial dysfunction, akin to the predictive data deduced from proteomics and bioinformatics analyses. In parallel, we tested the effect of GHTT on life and death of Jurkat cells. DMSO vehicle increased apoptosis by 5.8% (DMSO, Figure 4(b)). In contrast, GHTT elevated apoptosis from 16.8% to 88.2% (GHTT, Figure 4(b)). This elevation was dose-dependent. Besides, we verified the expression level of key proteins involved in mitochondrial dysfunction and cell death. Consistent with the proteomics data (Figure 2), VDAC2, PARK7, and LMNB1 were upregulated in Jurkat cells in response to GHTT (Figure 4(c)). In contrast, NDUFA5 and PRX3 were downregulated in Jurkat cells in response to GHTT (Figure 4(c)). The regulation of the above proteins in Jurkat cells by GHTT appeared to be dose-dependent. The data confirmed that mitochondrial dysfunction and cell death are related to these proteins.

Overall, the biochemical studies confirm the omics approach-driven discovery of signaling pathways in Jurkat cells and reveal a novel molecular basis of mitochondrial dysfunction and cell death of the anticancer compound, GHTT, in T-ALL cells. This concept-of-proof study exemplifies the feasibility of the novel interdisciplinary approach to combine proteomics and bioinformatics methods in basic research and application of herbal medicine and its active components.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Protective Effects of Scutellarin on Human Cardiac Microvascular Endothelial Cells against Hypoxia-Reoxygenation Injury and Its Possible Target-Related Proteins

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Scutellarin (SCU) is one of the main components of traditional Chinese medicine plant *Erigeron breviscapus (Vant.)* Hand.-Mazz. In this paper, we studied the protective effects of SCU on human cardiac microvascular endothelial cells (HCMECs) against hypoxia-reoxygenation (HR) injury and its possible target-related proteins. Results of MTT assay showed that pretreatment of SCU at doses of 1, 5, and 10 μM for 2 h could significantly inhibit the decrease in cell viability of HCMECs induced by HR injury. Subcellular fractions of cells treated with vehicle control, 1 μM SCU, HR injury, or 1 μM SCU + HR injury were separated by ultracentrifugation. The protein expression profiles of cytoplasm and membrane/nuclei fractions were checked using protein two-dimensional electrophoresis (2-DE). Proteins differentially expressed between control and SCU-treated group, control and HR group, or HR and SCU + HR group were identified using mass spectrometry (MS/MS). Possible interaction network of these target-related proteins was predicted using bioinformatic analysis. The influence of SCU on the expression levels of these proteins was confirmed using Western blotting assay. The results indicated that proteins such as p27BBP protein (EIF6), heat shock 60 kDa protein 1 (HSPD1), and chaperonin containing TCP1 subunit 6A isoform (CCT6A) might play important roles in the effects of SCU.

1. Introduction

SCU (4,5,6-trihydroxyflavone-7-glucuronide) is a flavone isolated from *Erigeron breviscapus (Vant.)* Hand.-Mazz. Extract containing mainly SCU, called as breviscapine, has been successfully used in China in clinic for treatment of both cardiac ischemic and cerebral ischemic diseases [1–5]. Previous studies showed that purified SCU had protective effects on cardiovascular ischemia as well as cerebrovascular diseases and its effects were better than those of breviscapine [5]. SCU was found to have protective effects on cardiomyocytes against ischemic injury [6, 7]. Endothelium was an active early participant in ischemia-reperfusion (IR) injury and endothelial cells were particularly susceptible to and actively participate in IR injury [8–10]. In the present study, we checked whether SCU could also protect endothelial cells against simulated IR injury, 12 h hypoxia followed by 12 h reoxygenation. Firstly, we observed the effects of SCU on cell viability of HCMECs under normal condition. Then, protective effects of SCU on cell viability of HCMECs underwent HR treatment were studied.

For a comprehensive study of the mechanism of SCU on HCMECs, a proteomic approach (2-DE combined with MS/MS) was used for seeking possible target-related proteins of SCU in HCMECs, both under normal condition and under HR treatment. Proteins differentially expressed between

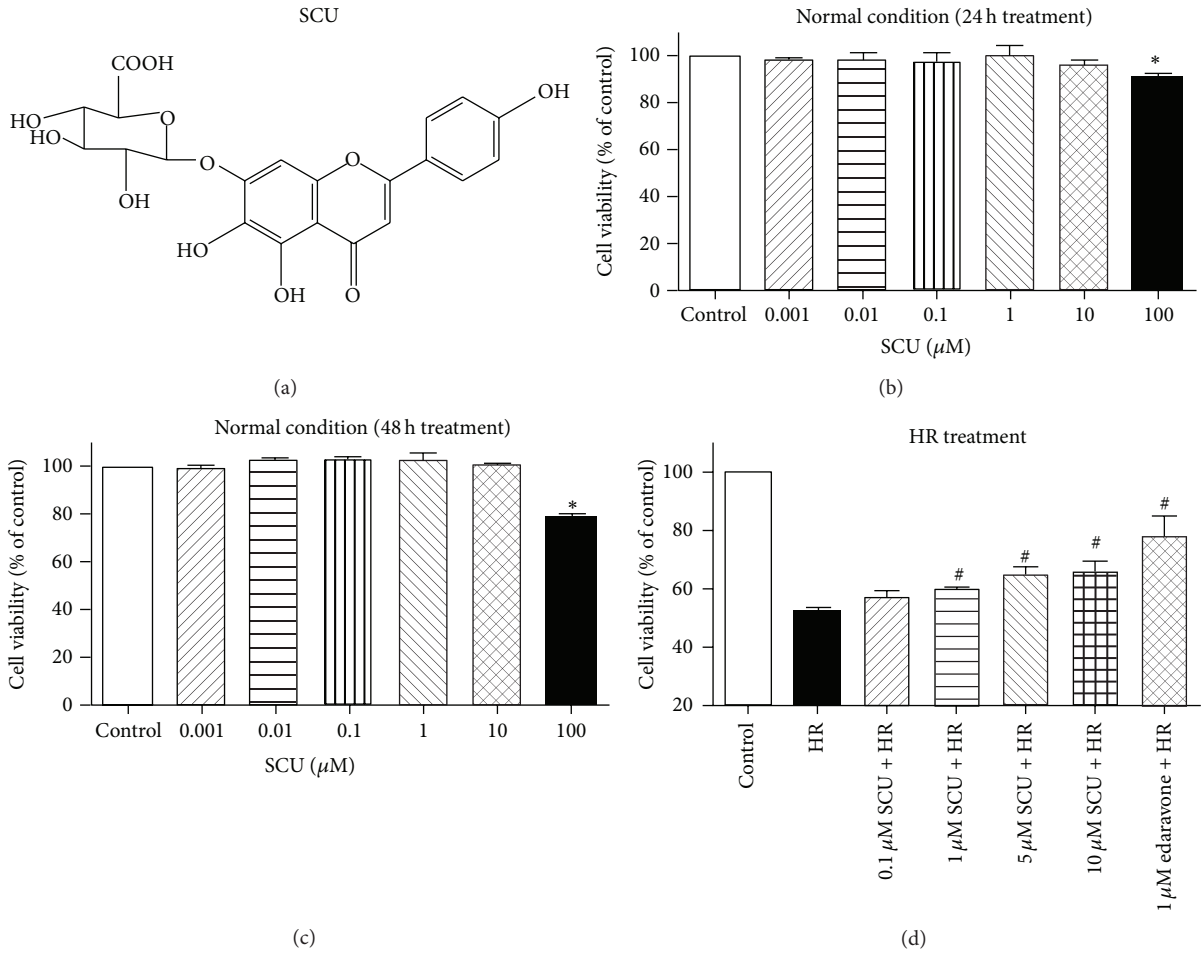

Figure 1: Effects of SCU on cell viability of HCMECs under normal condition or HR treatment. (a) Chemical structure of SCU. (b) Results of MTT assay of cell viability of cells treated with SCU at different concentrations or vehicle control for 24 h. $^{*}p < 0.05$, compared with control. (c) Results of MTT assay of cell viability of cells treated with SCU at different concentrations or vehicle control for 48 h. $^{*}p < 0.05$, compared with control. (d) Results of MTT assay of cell viability of cells treated with vehicle control, HR treatment, and HR treatment with 2 h pretreatment of SCU at different concentrations. $^{\#}p < 0.05$, compared to HR group. Data are presented as mean ± SD, $n = 3$ independent experiments.

control and SCU-treated group and proteins differentially expressed between HR and SCU + HR group were considered as possible target-related proteins of SCU. At the same time, proteins differentially expressed between control and HR group were also identified to provide information about possible proteins involved in HR injury. Using proteomic approach offers us the opportunity to search possible target-related proteins of SCU in an unbiased way. Furthermore, subcellular fractions of cells were separated and used in proteomic analysis to increase the coverage of analysis and study possible cross-talk between different cell organelles.

2. Materials and Methods

2.1. Reagents. Powdered SCU (purity 99%, formula weight 464.4) was obtained from Mr. Renwei Zhang of Kunming Longjin Pharmaceuticals Co. (Kunming, China). The structure of SCU was shown in Figure 1(a). SCU was dissolved in physiological saline solution. Cell culture reagents including modified RPMI-1640 medium and fetal bovine serum were obtained from HyClone (Thermo Fisher Scientific, Waltham, MA, USA). Edaravone with a purity of 99% was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Cell Culture. HCMECs were obtained from the Shanghai Yansheng Biochemical Technology Company (Shanghai, China) and grown in 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics. Tightly confluent monolayers of HCMECs from the 4th to the 15th passages were used in all experiments.

2.3. Effects of SCU on Cell Viability under Normal Condition or HR Treatment. Cell viability was determined using MTT assay. To check the influence of SCU on cell viability under normal condition, cells were plated in 96-well flat-bottomed plates at density of 3×10^3 cells/well in complete medium and incubated overnight. Then, cells were treated with vehicle

control (physiological saline solution) or SCU with final concentrations of 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM for 24 or 48 h. At the end of the incubation, 20 μL of the dye (3, [4,5-dimethylthiazol-2-yl-] diphenyltetrazolium bromide, 5 mg/mL), MTT, was added to each well and the plates were incubated for 3 h at 37°C. Then, 100 μL of lysis buffer (20% sodium dodecyl sulfate [SDS] in 50% N,N-dimethylformamide, containing 0.5% [v : v] 80% acetic acid and 0.4% [v : v] 1 N HCL), was added to each well and incubated overnight (16 h). Cell viability was evaluated by measuring the mitochondrial-dependent conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. The optical density (proportional to the number of live cells) was assessed with a Microplate Reader Bio-Rad 550 at 570 nm. Each experiment was performed in triplicate. Results of three independent experiments were used for statistical analysis.

To check the influence of SCU on cell viability under HR treatment, cells were plated in 96-well flat-bottomed plates at a density of 3×10^3 cells/well in complete medium and incubated overnight. HR injury was induced as described before with minor modifications [11]. Briefly, cells were placed in a humidified hypoxic chamber (HF100, Heal Force Biotech Co., Shanghai, China) for 12 h of hypoxia (5% CO_2 + 2% O_2 + 93% N_2) with medium free of glucose and serum at 37°C, followed by 12 h of reoxygenation (5% CO_2 + 95% air) in complete medium containing glucose and serum. Cells were classified into four groups, control, HR, SCU + HR, and edaravone + HR group. Cells of HR group were pretreated with vehicle control (physiological saline solution) for 2 h and then underwent HR treatment. Cells of SCU + HR group were treated with SCU at nontoxic doses, with final concentrations of 8 nM, 40 nM, 100 nM, 200 nM, 1 μM, 5 μM, 10 μM, and 25 μM, for 2 h and then underwent HR treatment. Cells of edaravone + HR group (positive control group) were treated with edaravone with final concentration of 100 nM for 2 h and then underwent HR treatment. Control cells were cultured in parallel and kept in normal culture condition for the entire time period (26 h). After treatments, cell viability of different groups was determined using MTT assay as described above.

2.4. Proteomic Analysis. To make sure correspondence between the reaction of cells in experiment of checking cell viability using MTT assay and the reaction of cells in proteomic study, cells were plated in 96-well plates and 75 cm^2 flasks at similar density and used for MTT assay and proteomic study, respectively. Only after MTT assay of cells in 96-well plates confirmed the protective effects of SCU on cells against HR injury, cells collected from 75 cm^2 flasks were further used for proteomic study. Cells were classified into 4 groups, control, HR, SCU (1 μM), and SCU + HR (SCU at 1 μM) groups. After treatments, cells in 75 cm^2 flasks were washed three times with ice-cold PBS and then scraped off with a cell scraper. After centrifugation at 2500 ×g for 3 min, the supernatants were discarded and cell pellets were resuspended for 5 min on ice in 500 μL CLB buffer containing 10 mM HEPES, 10 mM NaCL, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5 mM EDTA, 1 mM $CaCL_2$, and 0.5 mM $MgCL_2$. Homogenization of the cells was achieved by ultrasonication (30 strokes, 4 HZ) on ice. Thereafter, 50 μL of 2.5 M sucrose was added to restore isotonic conditions before differential centrifugation at 4°C. The first round of centrifugation was performed at 6500 g for 5 min. The pellet was supposed to contain nuclei. The supernatant was further ultracentrifugated at 107000 g for 30 min in a Hitachi CS150GXL ultracentrifuge. The resulting supernatant was supposed to contain cytoplasm and the pellet was supposed to contain membrane fraction. Then, the purity of the cytoplasm fraction, nuclei fraction, and membrane fraction was checked with Western blotting assay using antibodies against autophagy-related protein 7 (ATG7), histones (H3), and sodium-potassium adenosine triphosphatase (AT1A1) which were specific for cytoplasm, nuclei, and membrane, respectively. Since the results showed that the purity of cytoplasm fraction was acceptable while the membrane fraction contained part of nuclei fraction, the cytoplasm fraction was used without further treatment for proteomic analysis while the membrane fraction and nuclei fraction were merged together to be nuclei/membrane fraction. Protein samples from at least three independent experiments were collected and used in proteomic analysis.

Protein two-dimensional electrophoresis (2-DE) was conducted as reported in our previous studies [12–14]. The nuclei/membrane fraction were dissolved in 2-DE lysis buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and 0.8% pharmalyte and protease inhibitor (all from Bio-Rad) on ice for 30 min. After centrifugation at 15000 ×g for 30 min at 4°C, the supernatant containing solubilized nuclei/membrane proteins could be used for 2-DE analysis. The proteins in cytoplasm fraction were precipitated by adding 2X volume of precooled acetone. After centrifugation at 15000 ×g for 15 min at 4°C, the pellet was washed twice with 75% ethanol, dried down by evaporation in a vacuum concentrator/centrifugal evaporator, and then redissolved in 2-DE lysis buffer for 2-DE use. The protein concentrations of cytoplasm fraction or nuclei/membrane fraction were determined using the Bradford method.

For 2-DE analysis, 150 μg protein sample was applied for IEF using the ReadyStrip IPG Strips, 17 cm, and pH 4–7 (Bio-Rad). The strips were placed into a Protein IEF cell (Bio-Rad) and were rehydrated at 50 V for 12 h and then the proteins were separated based on their pI according to the following protocol: 250 V with linear climb for 30 min, 1000 V with rapid climb for 60 min, 10000 V with linear climb for 5 h, and 10000 V with rapid climb until 60 000 Vh was reached. After IEF, the IPG strips were equilibrated and then directly applied onto 12% homogeneous SDS-PAGE gels for electrophoresis using a PROTEIN II xi Cell system (Bio-Rad). The gels were then silver stained using Bio-Rad Silver Stain Plus kit reagents (Bio-Rad) according to the manufacturer's instructions. The silver-stained gels were scanned using a Densitometer GS-800 (Bio-Rad) and then analyzed using PDQuest software (Bio-Rad). Protein samples from 3 independent experiments, with each including 4 groups of control, SCU, HR, and SCU + HR, were analyzed by 2-DE. And, for each protein sample, triplicate electrophoreses were performed to ensure reproducibility. Comparisons were made between gel images of protein profiles obtained from control group versus HR

group, control group versus SCU group, and HR group versus SCU + HR group. Quantitative analysis was performed using the Student's t-test and significantly differentially expressed protein spots ($p < 0.05$) with 1.5-fold or more increased or decreased intensity between different groups were selected and subjected to further identification by MALDI-TOF MS/MS. MS/MS analysis was performed in Institutes of Biomedical Sciences, Fudan University, Shanghai, China, as described before [12–14]. The PMF and MS/MS data collected were submitted as a combined search to MASCOT (Matrix Science, London, UK) using the GSP Explorer software, V3.5 (Applied Biosystems, Foster City, CA) against the NCBInr databases. The probability-based score, assuming that the observed match is significant ($p < 0.05$), had to be more than 64 when submitting PMF data to the database and had to be be more than 30 for individual peptide ions when submitting peptide sequence spectra.

2.5. Bioinformatic Analysis. Bioinformatic analysis was conducted similar to previous reports [15, 16]. Protein-protein interactions were obtained from the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string.embl.de/) which include direct (physical) and indirect (functional) associations derived from four sources: genomic context, high throughput, coexpression, and previous knowledge. For the 18 proteins found in the proteomic analysis, we focused on the greatest possible correlations (i.e., interactions with a score larger than 0.15) without any other proteins added by custom limit. Only direct interactions among the proteins were included in the interaction network.

2.6. Western Blotting Analysis. Western blotting assay was conducted as reported before [12–14]. In assay of protein levels of EIF6, HSPD1, and CCT6A, cells of control group, HR group, SCU group, or SCU + HR group were washed three times with cold TBS and then harvested using a cell scraper. Cells were lysed in 10 volume of cold lysis buffer (50 mM Tris-HCl, pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol, 1 mM PMSF, 5 μg/mL aprotinin, and 5 μg/mL leupeptin) on ice. Lysates were centrifuged and then the supernatant protein was denatured by mixing with equal volume of 2 × sample loading buffer and then boiling at 100°C for 5 min. Then, an aliquot of 50 μg protein was loaded onto a 12% SDS gel, separated electrophoretically, and transferred to a PVDF membrane (Bio-Rad). After the PVDF membrane was incubated with 10 mM TBS with 1.0% Tween 20 and 10% dehydrated skim milk to block nonspecific protein binding, the membrane was incubated with primary antibodies overnight at 4°C. The primary antibodies for HSPD1 and EIF6 were rabbit polyclonal anti-HSPD1 antibody (number 15282-1-AP, 1 : 1500) and rabbit polyclonal anti-EIF6 antibody (number 10291-1-AP, 1 : 1500), all from Proteintech Group, Inc. (Chicago, USA). The primary antibody for CCT6A was rabbit polyclonal anti-CCT6 antibody (number ab155541, 1 : 800) bought from Abcam company (Cambridge, USA). Blots were then incubated with HRP-conjugated goat anti-rabbit IgG (#7074, 1 : 500, Cell signaling company) for 2 h at room temperature and then visualized using chemiluminescence (Pierce Biotechnology, Rockford, IL).

2.7. Statistical Analysis. Data are given as the mean ± SD. For each variable, three independent experiments were carried out. Significances of difference between groups were determined by a nonpaired Student's t-test.

3. Results

3.1. Effects of SCU on Cell Viability under Normal Condition or HR Treatment. As shown in Figure 1, SCU at concentrations from 0.001 to 10 μM exhibited no significant influence on cell viability after 24 h treatment (Figure 1(b)) or 48 h treatment (Figure 1(c)). SCU at 100 μM exhibited cytotoxicity on cells. As shown in Figure 1(d), HR treatment caused decrease in cell viability while pretreatment of SCU at 1, 5, and 10 μM could significantly inhibit decrease in cell viability induced by HR treatment. The results indicated the protective effects of SCU on cells against HR injury. Edaravone, used as positive control in the present experiment, also could inhibit the decrease in cell viability induced by HR treatment.

3.2. Proteomic Study of Protein Expression Profiles of Cells of Control Group, SCU Group, HR Group, and SCU + HR Group. To search possible target-related proteins of SCU related to its protective effects against HR injury, protein profiles of cells of control group, SCU group, HR group, and SCU + HR group were studied by comparative proteomic analysis. And, both cytoplasm fraction and nuclei/membrane fraction of the cells were analyzed. Representative 2D gel images of cytoplasm fraction and nuclei/membrane fraction were shown in Figures 2 and 3, respectively. Comparison was made between control group and HR group, control group and SCU group, and HR group and SCU + HR group. Significantly and differentially expressed protein spots ($p < 0.05$) with 1.5-fold or more increased or decreased intensity were accepted as differentially-expressed proteins. The protein spots were then cut from the gels, digested by trypsin, and identified using MS/MS. The result of MS/MS analysis of spot7 found in analysis of nuclei/membrane fraction was shown in Figure 4 as an example.

For cytoplasm fraction analysis (Figure 2), 11 differentially-expressed proteins were found totally, including 7 proteins found between control and HR, 2 proteins found between control and SCU, and another 2 proteins found between HR and SCU + HR group. Table 1 showed both the information of these 11 protein spots such as the average intensity values and their standard deviations of the proteins spots and the fold differences between different groups as well as the MS/MS identification results of these proteins such as the protein score, sequence coverage, and best ion score of each spot.

For nuclei/membrane fraction analysis (Figure 3), 7 differentially-expressed proteins were found totally, including 3 proteins found between control and HR, 4 proteins found between control and SCU, and 1 protein found between HR and SCU + HR group. Notably, the differentially-expressed

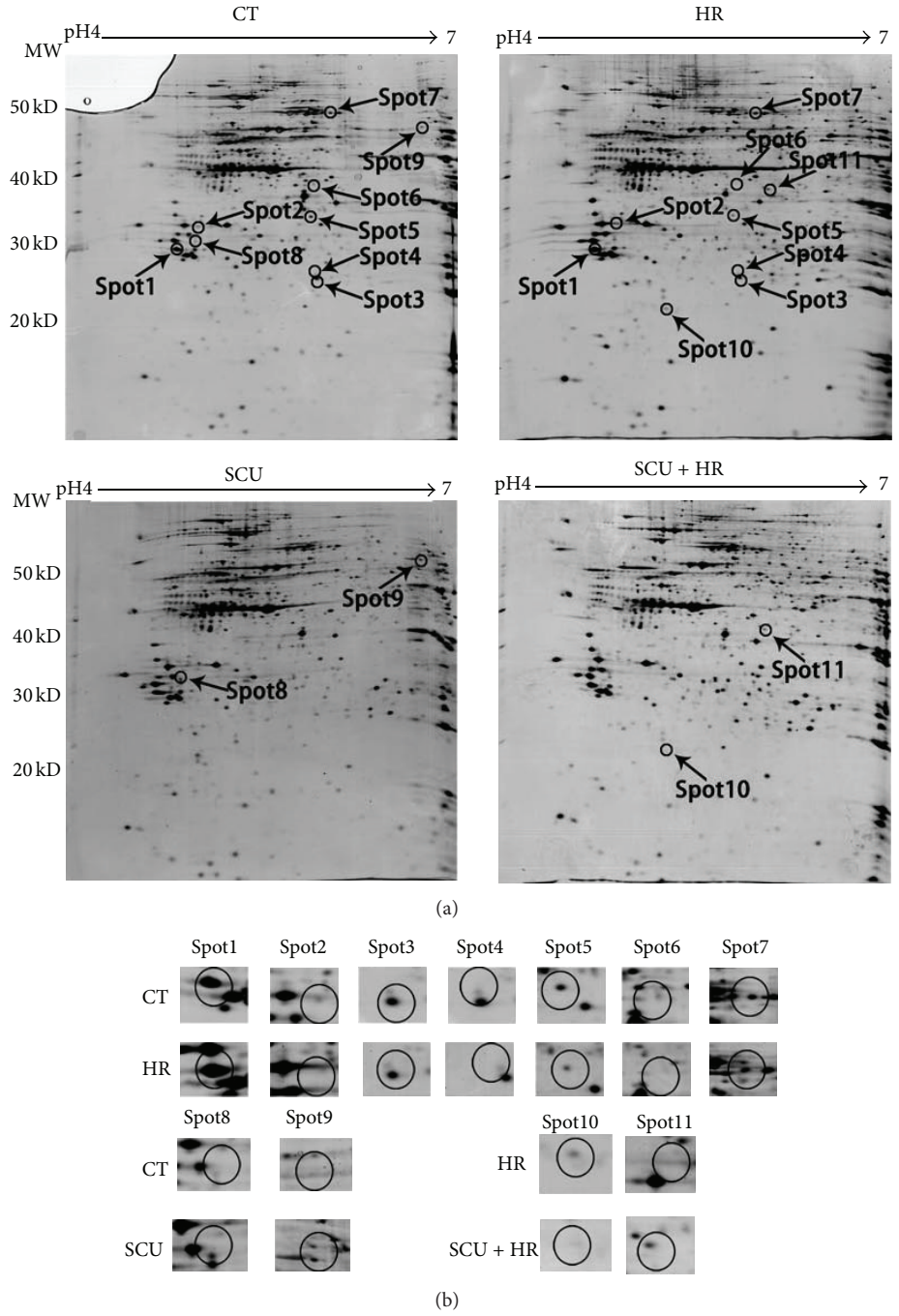

Figure 2: The proteome maps (2-DE images) of cytoplasm fraction of cells of control group, SCU group, HR group, and SCU + HR group. (a) Representative group of gels of nine replicate groups of gels, which were gotten by triplicate electrophoresis of samples from three independent experiments. Differentially expressed spots were shown by the arrows. (b) The expanded region of differentially expressed protein spots. The proteins within the circles were the differentially expressed proteins. Comparison was made between control group and HR group, control group and SCU group, and HR group and SCU + HR group.

protein between HR and SCU + HR group, identified as p27BBP protein, was also found to be one of the differentially proteins between control and HR group. Table 2 showed both the information of these 7 protein spots such as the average intensity values and their standard deviations of the proteins spots and the fold differences between different groups as well as the MS/MS identification results of these proteins such as the protein score, sequence coverage, and best ion score of each spot.

3.3. Possible Interaction Network of the Differentially-Expressed Proteins. The differentially-expressed proteins found

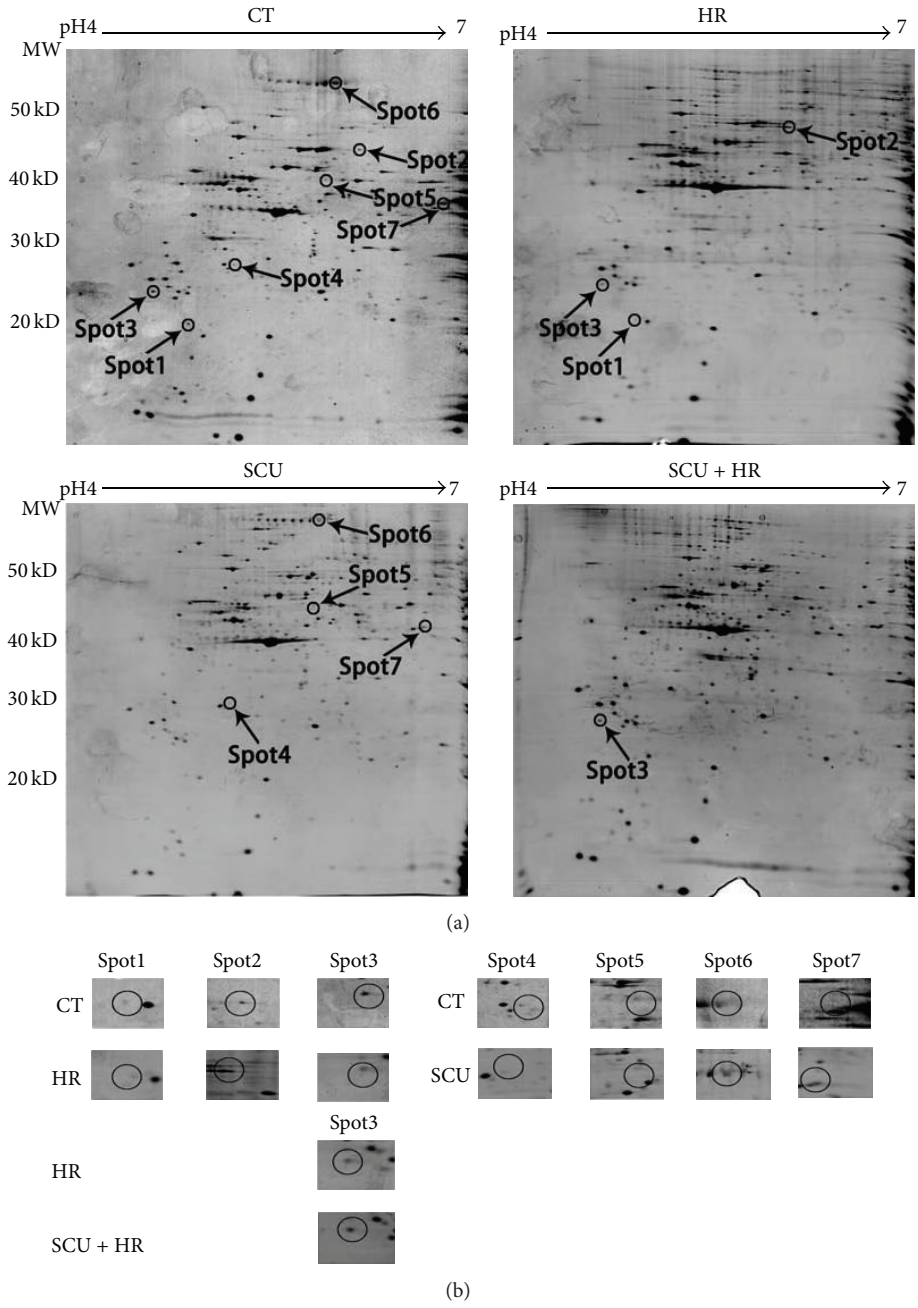

Figure 3: The proteome maps (2-DE images) of nuclei/membrane fraction of cells of control group, SCU group, HR group, and SCU + HR group. (a) Representative group of gels of nine replicate groups of gels, which were gotten by triplicate electrophoresis of samples from three independent experiments. Differentially expressed spots were shown by the arrows. (b) The expanded region of differentially expressed protein spots. The proteins within the circles were the differentially expressed proteins. Comparison was made between control group and HR group, control group and SCU group, and HR group and SCU + HR group.

in both comparison of control and SCU group and comparison of HR and SCU + HR group could be considered as possible target-related proteins of SCU. While the differentially-expressed proteins found in comparison of control and HR group might be important proteins involved in HR injury of cells. To elucidate possible mechanism of protective effects of SCU against HR injury, interaction network including both target-related proteins of SCU and proteins involved in HR injury was predicted using bioinformatic analysis. The information about abbreviated gene names and their corresponding protein full names was shown in Supplementary Table 1 available online at http://dx.doi.org/10.1155/2015/278014. As shown in Figure 5, target-related proteins of SCU (showed as red balls) and proteins involved in HR injury (showed as green balls) could link together into one net through direct interaction, that is, without adding intermediate partner.

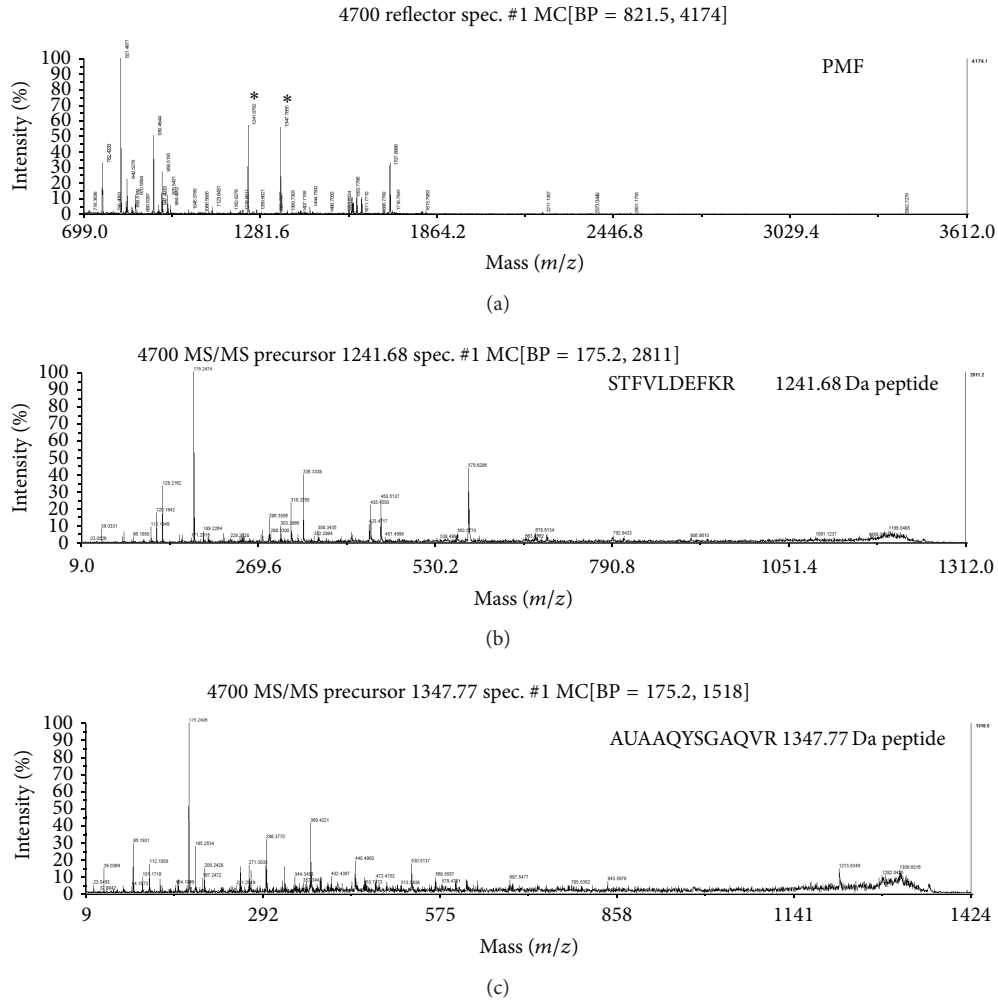

Figure 4: The result of the MALDI-TOF MS/MS analysis of spot7 cut from the 2-DE gels of nuclei/membrane fraction. It was identified to be human eukaryotic translation elongation factor 1 gamma by protein database search. (a) Peptide mass fingerprint of tryptic digest of the spot. ∗ unique peptides further identified by MS/MS. (b) MS/MS profile of the peptide with a mass of 11241.68 Da. (c) MS/MS profile of the peptide with a mass of 1347.77 Da.

Notably, HSPD1 (heat shock 60 kDa protein 1) was exhibited to be the protein that had the most numerous connections with other proteins in the network. The results suggested that HSPD1 might be a critical factor in the protective effects of SCU.

3.4. Confirmation of Differentially-Expressed Proteins by Western Blotting. Western blotting was employed to assess the expression of EIF6, HSPD1, and CCT6A in cells of control and HR, SCU, and SCU + HR groups. As shown in Figure 6, expression level of EIF6 was decreased in HR group while SCU pretreatment (SCU + HR group) inhibited the decrease of EIF6 induced by HR injury. Expression levels of HSPD1 and CCT6A were increased in SCU group compared with control group. These results were consistent with the proteomic results.

4. Discussion

The present study demonstrated that SCU, a flavonoid glycoside successfully used in clinic in China for treatment of ischemic diseases, exhibited protective effects on HCMECs against HR injury. Previous reports had shown that SCU had protective effects on cardiomyocytes [6] and neurons [17] against hypoxia-related injury. Our results suggested that, in treatment of cardiac ischemia or brain ischemia, SCU might also exert protective effects on endothelium besides direct protective effects on cardiomyocytes or neurons. It was well known that cardiac ischemia injury could result in endothelium dysfunction [18, 19]. Damaged endothelium would reduce perfusion to areas of ischemia and thus exacerbated organ damage [20]. Furthermore, abnormalities in endothelial cell structure and function occurred earlier in ischemia-reperfusion injury than those in parenchyma

Table 1: Differentially-expressed proteins found in proteomic analysis of cytoplasm protein expression profiles of different groups and results of MS/MS identification.

Spot	Target protein name	Accession number of NCBI database	Theoretical Mr (Da)/pI	Protein score	Sequence coverage (%)	Best ion score	Spot volume (ppm)		Fold change
							CT group	HR group	HR/CT
1	Chain A, 14-3-3 sigma in complex with Yap Ps127-peptide	306991738	26501/4.85	236	41	68	333.96 ± 223.4	172.3 ± 108.6	0.52
2	Chain B, crystal structure of human Nudt5 complexed with 8-oxo-Dgmp	317455160	21604/4.94	92	15	62	11.99 ± 14.9	1.36 ± 2.63	0.11
3	Glutathione S-transferase P	121746	23341/5.43	328	53	136	77 ± 71.7	27.6 ± 29.0	0.36
4	CGI-114 protein	5817183	26844/6.41	93	30	48	7.28 ± 6.2	2.4 ± 2.9	0.33
5	Inorganic pyrophosphatase	8247940	32639/5.54	179	34	66	36.88 ± 30.0	14.7 ± 8.3	0.40
6	COP9 complex subunit 4	5410300	46169/5.57	242	39	89	5.57 ± 4.79	2.26 ± 1.8	0.41
7	Heat shock protein	386785	69825/5.42	200	22%	59	52.9 ± 42.3	16.1 ± 17.9	0.30
							CT group	SCU group	SCU/CT
8	Heat shock 60 kDa protein 1 (chaperonin), isoform CRA_c	119590557	40896/5.09	73	11	58	7.17 ± 5.3	17.5 ± 11.6	2.44
9	Chaperonin containing TCP1, subunit 6A isoform variant	62089036	57725/6.25	224	22	56	1.76 ± 2.1	9.4 ± 8.4	5.34
							HR group	SCU + HR group	SCU + HR/HR
10	Sorcin isoform b	38679884	20332/5.11	98	31	64	5.3 ± 7.7	0.32 ± 0.58	0.06
11	Isocitrate dehydrogenase 3 (NAD+) alpha	18314368	39566/5.84	206	43	70	5.09 ± 4.26	10.13 ± 8.48	1.99

Table 2: Differentially-expressed proteins found in proteomic analysis of nuclei/membrane protein expression profiles of different groups and results of MS/MS identification.

Spot	Target protein name	Accession number of NCBI database	Theoretical Mr (Da)/pI	Protein score	Sequence coverage (%)	Best ion score	Spot volume (ppm)		Fold change
							CT group	HR group	HR/CT
1	Eukaryotic translation initiation factor 3, subunit 12, isoform CRA_b	119577208	17921/6.02	110	21	70	614.7 ± 423	182.1 ± 211.9	0.30
2	Albumin-like	763431	52048/5.69	138	9	86	831 ± 784	1997.7 ± 1131.4	2.41
3	p27BBP protein	13785574	26332/4.56	290	27	121	1868.7 ± 1220.8	726.1 ± 822.6	0.39
							CT group	SCU group	SCU/CT
4	Chain A, structural and electrophysiological analysis of annexin V mutants	157831406	35782/4.98	128	18	44	725.4 ± 314.8	241.7 ± 273.7	0.33
5	ATPase, H+ transporting, lysosomal 56/58 kDa, V1 subunit B2, isoform CRA_a	119584163	56465/5.57	87	15	31	584.1 ± 613.7	74.3 ± 126.8	0.13
6	VLA-3 alpha subunit	220141	113433/6.13	118	15	32	1812.1 ± 1377.5	536.9 ± 482.8	0.30
7	Eukaryotic translation elongation factor 1 gamma, isoform CRA_d	119594432	48407/6.24	187	28	47	4283.8 ± 2973.2	1790.3 ± 1335.2	0.42
							HR group	SCU + HR group	SCU + HR/HR
3	p27BBP protein	13785574	26332/4.56	290	27	121	726.1 ± 822.6	1797.3 ± 1085.5	2.48

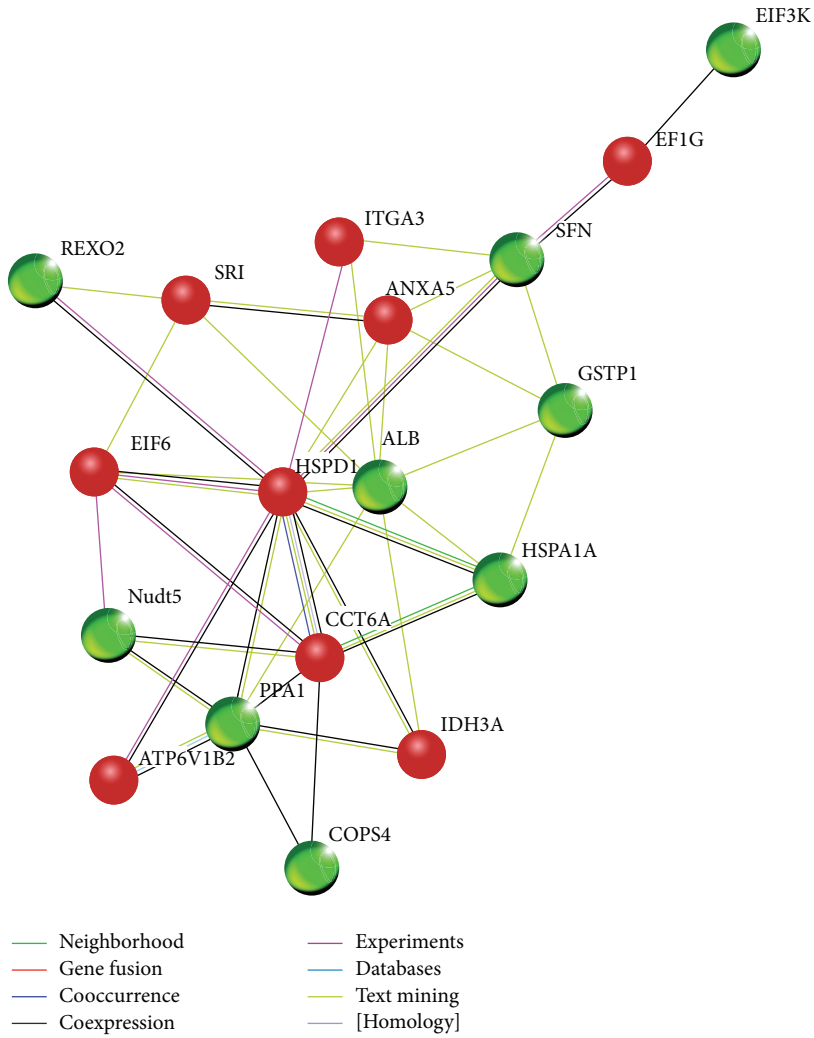

Figure 5: Protein-protein interaction network obtained from bioinformatic analysis based on STRING database. Possible target-related proteins of SCU were shown as round balls in red while possible proteins involved in HR injury were shown as round balls in green. Possible interactions between proteins were shown in line with different colors, as indicated in the figure.

cells, and the recovery of endothelial cells was later [21]. Since endothelial cells play a critical role in determining the extent of ischemia-reperfusion injury, protective effects of SCU on endothelial cells under injury might contribute to its efficiency in treatment of ischemic diseases.

Proteomic analysis of protein expression profiles of control cells, SCU-treated cells, HR-treated cells, and SCU + HR-treated cells in the present study provided information about both possible target-related proteins of SCU as well as proteins possibly involved in HR injury. The differentially-expressed proteins found in both comparison of control and SCU group and comparison of HR and SCU + HR group, with the total of 9 proteins including 4 found in cytoplasm fraction analysis and 5 found in nuclei/membrane fraction analysis, could be considered as possible target-related proteins of SCU. The differentially-expressed proteins found in comparison of control and HR group, with the total of 10 proteins including 7 found in cytoplasm fraction analysis and 3 found in nuclei/membrane fraction analysis, might be important proteins involved in HR injury of cells. Based on these results, possible interaction network including both target-related proteins of SCU and proteins involved in HR injury was predicted by bioinformatic analysis. Among these proteins, EIF6, HSPD1, and CCT6A might play important roles in the protective effects of SCU against HR injury.

EIF6, a monomeric protein of about 26 kDa, is an evolutionarily conserved protein involved in ribosomal subunit biosynthesis and assembly [22–24]. In the present study, EIF6 was found to be both a protein involved in HR injury and a possible target-related protein of SCU. In proteomic analysis, its expression level was downregulated in HR group compared with control while pretreatment of SCU could ameliorate the HR-induced decrease in EIF6 level. It was reported that EIF6 was a rate-limiting factor in translation, growth, and transformation [25, 26]. By binding to the large ribosomal subunit, EIF6 could prevent inappropriate interactions with the small subunit during initiation of protein synthesis [27]. Importantly, EIF6 might be part of a mechanism

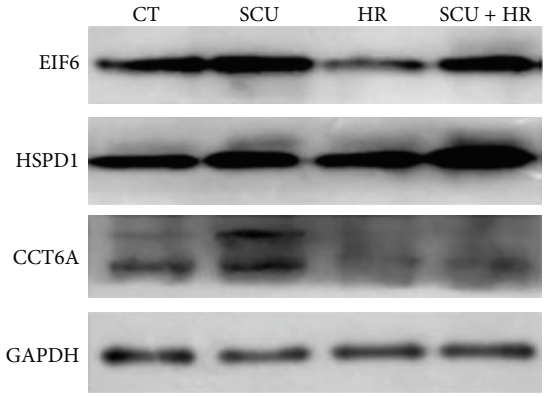

Figure 6: Results of Western blotting assay of protein levels of EIF6, HSPD1, and CCT6A in cells of control group, SCU group, HR group, and SCU + HR group shown were representative results of three independent experiments.

acting on the specific translation of messengers regulating cell survival such as regulating translation of factors upstream of Bcl2/Bax [28]. Previous reports showed that prolonged serum starvation could induce gradual decrease in EIF6 expression level [29]. Our finding that expression level of EIF6 was downregulated in HR-treated cells compared with control supported the role of EIF6 in cell survival. And, high level of EIF6 in SCU + HR-treated cells might protect cells against cell death induced by HR injury.

HSPD1 and CCT6A were found in the present study to be possible target-related protein of SCU whose expression levels were induced by SCU treatment. Interestingly, both proteins were chaperonin proteins. HSPD1 is a highly conserved protein which facilitates folding of nascent proteins into proper conformations thus plays a critical role in the assembly, folding, and transport of proteins in the mitochondria [30]. Mutations in HSPD1 were associated with diseases of nervous system [31, 32] and inactivation of HSPD1 in mice was embryonically lethal [33]. Furthermore, under heat stress, HSPD1 could interact with nascent cellular proteins to prevent their denaturation [30]. In the present study, HSPD1 was found to be located at the center of the interaction network and have the most numerous connections with other proteins in the network. Increase in HSPD1 in SCU-treated cells might protect proteins from HR injury thus increase viability of cells. Our results were consistent with previous reports that the overexpression of heat shock proteins could protect cells against apoptosis induced by ischemia-reperfusion injury [34–36]. CCT6A is the zeta subunit of the chaperonin containing TCP1 complex and is the only cytosolic chaperonin in eukaryotes. It was estimated to assist in the folding of multiple proteins such as actin, tubulin, cyclin E, myosin, and transducin [37]. Our finding about the increase levels of HSPD1 and CCT6A in SCU-treated cells suggested that induction of expression of chaperonin proteins might be involved in the protective effects of SCU against HR injury.

In the present study, HSPD1 and CCT6A were found to be a possible target-related protein of SCU in proteomic analysis of cytoplasm fraction and EIF6 was found to be a possible target-related protein of SCU in analysis of nuclei/membrane fraction. Our results were consistent with the reported subcellular location of HSPD1 in mitochondria [38, 39], CCT6A in cytoplasm [37], and nucleocytoplasmic shuttling of EIF6 [40, 41]. Interestingly, as shown in the network of bioinformatic analysis, EIF6 had direct interactions with HSPD1 and CCT6A. Therefore, it is possible that SCU might induce expression of chaperonin proteins, protect EIF6 in HR injury, keep translation of factors important in cell survival, and thus protect cells against HR injury. The roles of EIF6, HSPD1, and CCT6A in the protective effects of SCU against HR injury deserve further study.

In all, in the present study, the protective effects of SCU on HCMECs that underwent HR treatment were checked and possible target-related proteins of SCU were searched using proteomic analysis and analyzed by bioinformatic analysis. Results of the present study suggested possible important roles of EIF6, HSPD1, and CCT6A in the effects of SCU.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Meina Shi and Yingting Liu contributed equally to this study.

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Review Article

Proteomics in Traditional Chinese Medicine with an Emphasis on Alzheimer's Disease

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In recent years, there has been an increasing worldwide interest in traditional Chinese medicine (TCM). This increasing demand for TCM needs to be accompanied by a deeper understanding of the mechanisms of action of TCM-based therapy. However, TCM is often described as a concept of Chinese philosophy, which is incomprehensible for Western medical society, thereby creating a gap between TCM and Western medicine (WM). In order to meet this challenge, TCM research has applied proteomics technologies for exploring the mechanisms of action of TCM treatment. Proteomics enables TCM researchers to oversee various pathways that are affected by treatment, as well as the dynamics of their interactions with one another. This review discusses the utility of comparative proteomics to better understand how TCM treatment may be used as a complementary therapy for Alzheimer's disease (AD). Additionally, we review the data from comparative AD-related TCM proteomics studies and establish the relevance of the data with available AD hypotheses, most notably regarding the ubiquitin proteasome system (UPS).

1. Introduction

Traditional Chinese medicine (TCM) has promising potential as a complementary or alternative therapy for the treatment of neurodegenerative diseases (NDs). In recent years, TCM has become increasingly popular in Western countries [1]. The mechanisms of TCM on cellular levels, however, are poorly understood due to the complexity of the active components of TCM and the poor documentation available for mechanistic studies. TCM often utilizes several active ingredients, which may have either synergistic or antagonistic effects on cells. Accordingly, with the use of "omics" methods such as proteomics, the mechanisms of TCM treatment can be better explained and more fully understood [2, 3].

Alzheimer's disease (AD) is an ND that causes patients to exhibit high cognitive dysfunction, memory impairment, language deterioration, depression, and other debilitating conditions caused by the death of neurons in specific areas of the brain [4, 5]. Numerous TCM treatments have been reported to be effective therapies for AD [6–9]. The therapies, however, are still lacking sound scientific explanations. Comparative proteomics studies of TCM, which compare the relative quantity of proteomes between control and TCM-treated cells, may provide a holistic perspective on the mechanisms of active TCM phytochemicals [10, 11]. In this review, we focus on the proteomics approaches of existing TCM studies and on the relevance of these approaches in deciphering general TCM mechanisms for the treatment of NDs such as AD. Further, we emphasize AD as an example of how proteomics studies are able to explain the ways in which TCM treatments have positive effects at cellular and molecular levels.

2. TCM

TCM is a therapeutic system that has been practiced for more than 2,000 years, making it one of the oldest medical systems in the world [12, 13]. It is influenced by ancient Chinese philosophy and the idea that coherency between nature and human beings has a vital effect upon the health of people [12]. TCM is distinct from conventional Western medicine (WM) in many aspects. First of all, the primary difference

is the fundamental theory of medicine [14]. Unlike WM wherein diseases are explained theoretically using biology and physiology, TCM etiologies of diseases are described by theories from ancient Chinese philosophy [13]. Secondly, TCM formulas are often mixtures of several herbs, in which each component may have several active ingredients which can interact with each other in any number of ways, including mutual accentuation, mutual enhancement, mutual counteraction, mutual suppression, mutual antagonism, and mutual incompatibility [15]. In contrast, WM often only consists of a single active compound. Next, the emergence of TCM and WM is greatly divergent. TCM was developed over a couple of millennia and is practiced naturally by a massive population in East Asia as a folk remedy, while WM has been driven by scientific researches [1]. Nevertheless, since the 1950s, TCM has experienced a trend toward modernization and increased acceptance in Western countries [16]. This phenomenon demands more TCM research to establish scientific mechanistic studies, safety assessments, and standardized manufacturing practices.

3. Proteomics and TCM

The biggest challenge for the modernization of TCM involves unraveling the complexities of TCM mechanisms with sound scientific principles [17]. To achieve this, TCM research has applied "omics" methods in order to elucidate the complex network of TCM mechanisms [2]. "Omics" methods, such as proteomics, offer advantages for understanding the disease in the bigger picture and can reveal the dynamic interactions between the active components of TCM formulas [2]. Consequently, proteomics has become a crucial tool for deciphering the intricate mechanisms of TCM. To achieve this goal of broader empirical understanding, three proteomics strategies are available for TCM researchers. These strategies are syndrome proteomics, screening proteomics, and comparative proteomics.

3.1. Syndrome Proteomics Strategy. The first proteomics strategy is syndrome proteomics, which is used for translating a syndrome, as it is understood in TCM terminology, to biological principles [18]. This can be achieved by way of proteomics analysis of the organs or bodily fluids related to defined TCM syndromes. For example, the work of Sun et al. uses a stress-induced Gan-stagnancy syndrome model involving rats and 2D protein electrophoresis (2DE) proteomics to determine differentially regulated proteins in blood and tissues [19].

3.2. Screening Proteomics Strategy. The second proteomics strategy is screening proteomics. This strategy intends to elucidate the mechanisms of medicinal herbs used in TCM by identifying the binding partners of the active ingredients. This strategy is carried out by immobilizing target phytochemicals with an immobilized matrix. Subsequently, the whole proteome of a targeted tissue or plasma is screened through the immobilized phytochemical. Proteins that have the ability to bind to the phytochemicals will be immobilized in the matrix, while noninteracting proteins will be washed away. The immobilized proteins are then eluted and identified, in some cases, by using tandem mass spectrophotometry (MS/MS). This strategy is adopted in research to identify the molecular targets of curcumin, for example [20].

3.3. Comparative Proteomics Strategy. The last strategy is comparative proteomics, otherwise known as differential proteomics, which quantitatively determines the relative or absolute amount of proteins in TCM-treated and control groups and determines the key proteins altered between the groups. This strategy is widely adopted in the field of TCM research for determining the molecular actions of a TCM treatment. To date, there are several comparative proteomics techniques available for TCM researchers [21, 22]. The most prominent proteomics technique used in TCM studies is 2DE [2, 21, 22]. In this method, the whole proteome of a cell is separated based on the isoelectric point of the proteins on the first dimension, followed by a standard protein separation according to molecular weight on the second dimension (see Supplementary Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/393510). Consecutively, the proteomics profiles of the control or diseased cells and the TCM-treated cells are compared to find distinct protein expressions. Some of the proteins from the TCM-treated cells may be overexpressed or underexpressed in comparison to proteins in the control group. The differentially regulated proteins are the proteins of interest, and these are subsequently excised from the gel. The proteins of interest are then subjected to protein digestion, chromatography purification (e.g., by high performance liquid chromatography (HPLC)), and mass spectrophotometry (MS) fingerprinting or MS/MS sequencing to identify and quantify the proteins. An additional step involving a Western blot may be added to verify the differentially expressed proteins by comparing blots of control and TCM-treated cell protein extracts, as well as immunocytochemistry.

In addition to 2DE, there are several other methods to quantitatively assess the proteomics profiles of cell extracts. Stable-isotope labeling by amino acids in cell cultures (SILAC) *in vitro* is a proteomics method, which uses "light" or "heavy" isotope labeled amino acids to label respective (i.e., control and TCM-treated) samples (Supplementary Figure S2) [23]. These amino acids are introduced to the cell culture media and are then incorporated with the cellular proteins during cell growth and proliferation. The protein extracts for each sample are combined and then digested by protease (e.g., trypsin) and purified. Consequently, during MS/MS analysis, the peaks will appear as a pair: one with a lower mass-to-charge ratio (m/z), or the "lighter" peak (i.e., the control), and the other with a higher m/z, or the "heavier" peak (i.e., the TCM-treated sample). The relative amount of the respective proteins can be determined by comparing the respective peaks of the control and TCM-treated groups. An example of SILAC proteomics in TCM research is the identification of protein targets for celastrol, a phytochemical derived from *Tripterygium wilfordii*, in lymphoblastoid cells [24].

Alternatively, proteomics quantification can be achieved with protein-tagging methods, such as isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tags (TMT) [22]. These methods label the proteins after cell extraction according to the chemical reactions between the tag reagents and N-terminus of the peptides or the ε-amino group of lysine residue. The tags are composed of a reporter region, a balancer region, and a reactive region. The total mass of the tags is identical; hence the tags are isobaric, which is achieved by the inverse relationship of the mass in the reporter and balancer regions. A general overview of both the iTRAQ and TMT methods is as follows: the proteins are first extracted from the cells/tissue; then the proteins are digested to generate shorter peptides, which are then reacted with the tagging chemicals (e.g., 114 Da-reporter tag for the control group and 117 Da-reporter tag for the TCM-treated group) (Supplementary Figure S3). The peptides from both the control and TCM-treated groups are then combined and are subsequently purified to remove excess detergents that may interfere with the MS analysis. Following this step, the peptide solution may also be fractionated for preventing overcrowding of the peaks. The prepared peptides then undergo MS/MS analysis. In the first MS spectra, peptides from the control and treatment groups will appear as identical peaks, because the tags are still intact. During collision induced disassociation (CID), however, the tags will be fragmented, leaving only reporter ions, which appear as distinct peaks according to m/z. During MS/MS analysis, the peptide sequence is identified from the spectra, while the relative amounts of peptides in the control and treatment groups are deduced from the reporter ion peaks. For further discussion of the iTRAQ and TMT methods, readers are directed to other reviews [25, 26].

The advancement of proteomics technologies has made it possible for research to opt for the label-free method [27]. In this method, control and treated groups are prepared and analyzed in parallel (Supplementary Figure S4). In label-dependent proteomics, samples from control and TCM-treated groups are combined at some point after protein extraction. In label-free proteomics, however, the sample control and TCM-treated groups are processed separately. The relative quantification of proteins is achieved by comparing the spectra of the respective samples. This method offers a simple and streamlined protocol in comparison to other methods. However, this method may have higher variability due to the discrete processing of separate groups. A systemic comparison of the proteomics methods described above has been done in a previous study [28]. This comparison may provide insight for TCM researchers to design optimal proteomics experiments.

4. Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia, in which patients suffer from loss of higher cognitive functioning, memory impairment, language deterioration, depression, and other debilitating conditions caused by the death of neurons of specific areas of the brain [4, 29]. This disease is named after a German neuropathologist, Alois Alzheimer, who first presented a patient case for Auguste Deter in 1906. AD is characterized by hallmark pathological markers, which are aggregates of amyloid plaques (APs) and neurotoxic neurofibrillary tangles (NFTs). APs are composed of aggregated 40 or 42 amino acid amyloid beta peptides ($A\beta_{40/42}$), while NFTs are composed of aggregated hyperphosphorylated Tau proteins. These pathological markers become more pronounced as the disease progresses in the brain [30]. The first area of the brain to be impacted by AD is the transentorhinal region, followed by the hippocampus, amygdala, and frontal lobe areas [31, 32].

AD can be initiated by genetic or nongenetic causes. A small proportion of AD is caused by a genetic mutation of the $A\beta$ precursor protein (APP), microtubule-associated protein Tau (MAPT), and the presenilins-1 and presenilins-2 (PS1 and PS2, resp.) genes [4]. The mutations in APP, PS1, or PS2 genes shift the production of the $A\beta$ precursor protein toward the amyloidogenic pathway [33–35]. The genetic type of AD usually has an earlier onset; hence it is also known as early-onset AD. The other type of AD is caused by nongenetic factors, with onset at a later age. This type of AD is termed sporadic AD or late-onset AD [36].

There are several hypotheses about the pathogenesis of AD, leading to eventual neuronal death. One of the earliest hypotheses is the cholinergic hypothesis [37–39]. The cholinergic hypothesis is underlined by evidence of the loss of cholinergic neurons in the brain, which ultimately results in cognitive decline in AD patients [40]. According to this hypothesis, the proposed causes of neuronal death include reduced expression of acetylcholine receptors, decreased production of the acetylcholine neurotransmitters, and impaired axonal transport. This results in a failure to maintain synaptic connections between neurons, thus triggering neuronal death in AD patients.

The second hypothesis about the pathogenesis of AD is the amyloid cascade hypothesis, which is the most prominent AD hypothesis to date. This hypothesis reasons that accumulation of $A\beta$ causes the formation of NFTs in neurons and eventually induces apoptosis [41, 42]. $A\beta$ is a product of the APP protein when cleaved by β- and γ-secretases by a mechanism called regulated intramembrane proteolysis (RIP) [43, 44]. Cytotoxic $A\beta$ may cause cell death by inducing oxidative stress, calcium imbalance, and mitochondrial damage or by disturbing energy production, inducing Tau protein phosphorylation, and/or impairing the protein degradation system [45–52].

The next hypothesis is the Tau protein hypothesis [53, 54]. Tau proteins are a component of microtubules and are imperative for maintaining microtubule architecture in the axons [55, 56]. However, Tau proteins are easily hyperphosphorylated, which results in loss-of-function and in turn causes the loss of attachment to the microtubule [53, 57]. Detached Tau proteins may interfere with the axon's ability to maintain axonal transport, eventually resulting in synaptic dysfunction and neuronal death [54, 58, 59]. Tau protein hyperphosphorylation can be reversed with the application of protein phosphatases such as protein phosphatase 2A (PP2A) or protein phosphatase 5 (PP5), which restores the ability of Tau proteins to stabilize microtubules [60, 61]. Conversely, in

terms of AD, Tau protein hyperphosphorylation can be triggered by the reduced expression or activity of phosphatases [60, 62].

In recent years, a paradigm shift has occurred in AD research due to the failure of preexisting theories to provide a satisfactory explanation of AD pathophysiology and effective therapeutic strategies [63–66]. Researchers are now investigating the process of regulating misfolded proteins to gain alternative hypotheses for explaining AD and other NDs and ultimately for the development of new strategies for the treatment of AD. Misfolding-prone proteins are classified as intrinsically disordered proteins (IDPs), a group of proteins lacking defined tertiary structures, which are thus susceptible to aggregation [67]. Many representative proteins of NDs, such as Aβ, Tau proteins, α-synuclein, superoxide dismutase-1 (SOD-1), and TAR DNA-binding protein 43 (TDP-43), are included in this group [68–72]. In particular, this hypothesis emphasizes the importance of the ubiquitin proteasome system (UPS) and molecular chaperones for preventing the aggregation of IDPs [73, 74]. The UPS is responsible for degrading the majority of cellular proteins and maintaining protein homeostasis and is also able to degrade misfolded proteins [75, 76]. The ubiquitin proteasome system consists of the 26S proteasome as the proteolytic complex, ubiquitin ligases as the targeting mechanism, and ubiquitin recycling enzymes [76–78]. While the 26S proteasome is a protein complex comprised of a 20S proteasome catalytic core capped with a pair of 19S regulatory complexes, it has been numerously reported that inhibition of this proteasomal system leads to perturbed degradation of Aβ and hyperphosphorylated Tau protein, which eventually leads to an accumulation of these proteins and cytotoxicity [79]. The 20S proteasome core itself is composed of two α- and β-rings with seven subunits for each ring. Subunits β1, β2, and β5 are the active sites with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively [80, 81]. It has been demonstrated that the 20S proteasome is able to degrade APP or at least the C-terminus of APP [82]. This degradation is inhibited by the addition of MG132, a proteasome inhibitor that targets the β5 subunit, and to a lesser extent also the β1 subunit of the 20S proteasome [83]. Therefore, it appears that the β5 subunit is the key subunit for the degradation of targeted proteins, including APP. In addition, other studies found that Tau protein may be degraded independent of the 20S proteasome chymotrypsin-like activity [84].

Besides, the regulatory subunits of the 20S proteasome also play a decisive role in the proteasome activity. This fact is demonstrated by a study that showed that an upregulation of PA28 subunits improves the 20S proteasome ability to degrade proteotoxic substrates [85].

In AD, the specific pathways of the UPS are impaired, due possibly to inhibition on the catalytic core of the UPS by Aβ and aggregated Tau proteins, or due to decreased expression of ubiquitin-conjugating enzymes [48, 86–93]. Indeed, one study shows that ubiquitin overexpressing neurons have better survivability after ischemic stress in rodent brains [94]. The relationships among IDPs, the UPS, and molecular chaperones are also reviewed in other studies [73, 92, 95].

5. AD in TCM

The long history of dementia in TCM is documented in the books *Jingyue Quanshu* (1624 A.D.) and *Bian Zheng Lu* (1690 A.D.) [12]. The philosophy of TCM asserts that the brain is an outgrowth of and is nourished by kidney essence. It further explains that kidney essence produces the body's marrow, including cerebral marrow, spinal cord marrow, and bone marrow. When kidney essence is deficient, the production of cerebral marrow is reduced, which leads to various symptoms, including dementia. Therefore, according to TCM, AD is a deficiency of kidney essence [12]. Accordingly, AD treatment is achieved by tonifying kidney essence [96]. A more comprehensive TCM-based explanation states that AD, as well as other dementias, is caused by a deficiency of the vital energy of the kidneys (*Shen*), marrow (*Sui*), heart (*Xin*), or spleen (*Pi*), together with a stagnation of blood (*Xie*) and/or phlegm (*Tan*) [6]. Not surprisingly, TCM also perceives AD as the malfunctioning of multiple organs, including the kidneys, liver, heart, and spleen, in addition to the consequent accumulation of toxins and blood stasis [6, 9]. Hence, from a TCM point of view, effective treatment of AD must reverse the symptoms, as in a study that uses *Bushenhuatanyizhi* (an herbal mixture) to treat AD by bolstering kidney essence, removing phlegm, and promoting mental health [7].

Until recently, there has been no feasible treatment for AD. A great number of drugs have been developed based on existing AD theories, which have failed to be effective for treating or delaying AD [64, 65, 97, 98]. The drugs developed for AD thus far target only a single mechanism, while AD is often described as a multifaceted disease (meaning that the pathology cannot be described with only one theory) [64, 65, 97–101]. As described above, AD has several probable causes, which may interact with each other (Figure 1).

Under these circumstances, incorporating a TCM-based holistic perspective to the conventional AD treatment may be valuable.

In order to treat AD, TCM often uses a mixture of active ingredients with a diverse mechanistic action to target various molecular events. For instance, *Panax ginseng* extract has an array of active constituents that has been demonstrated *in vitro* to attack AD from different pathways. For example, ginsenoside Rg1 has been demonstrated to reduce apoptosis and to decrease the activity of β-secretase [102]. This activity is in synchrony with ginsenoside Rg3 that increases the expression of neprilysin, an Aβ-degrading enzyme, that further reduces the amyloid burden [103]. Moreover, another *Panax ginseng* component, the gintonin, has been reported to increase sAPPα release [104]. Further combination of several TCM herbs into a TCM mixture arguably enhances its potential. For example, an herbal mixture, composed of *Panax ginseng, Ginkgo biloba, and Crocus sativus* L., improves cognitive functions in double-transgenic APP (K670N/M671L)/PS1 (M146L) mice through reducing the amyloid burden [105]. The same study also concluded that the effects of this mixture are greater than the *Ginkgo biloba* extract alone. Therefore, a TCM approach could be more effective compared to a WM approach for treating a disease where the etiology is unknown, such as AD [13].

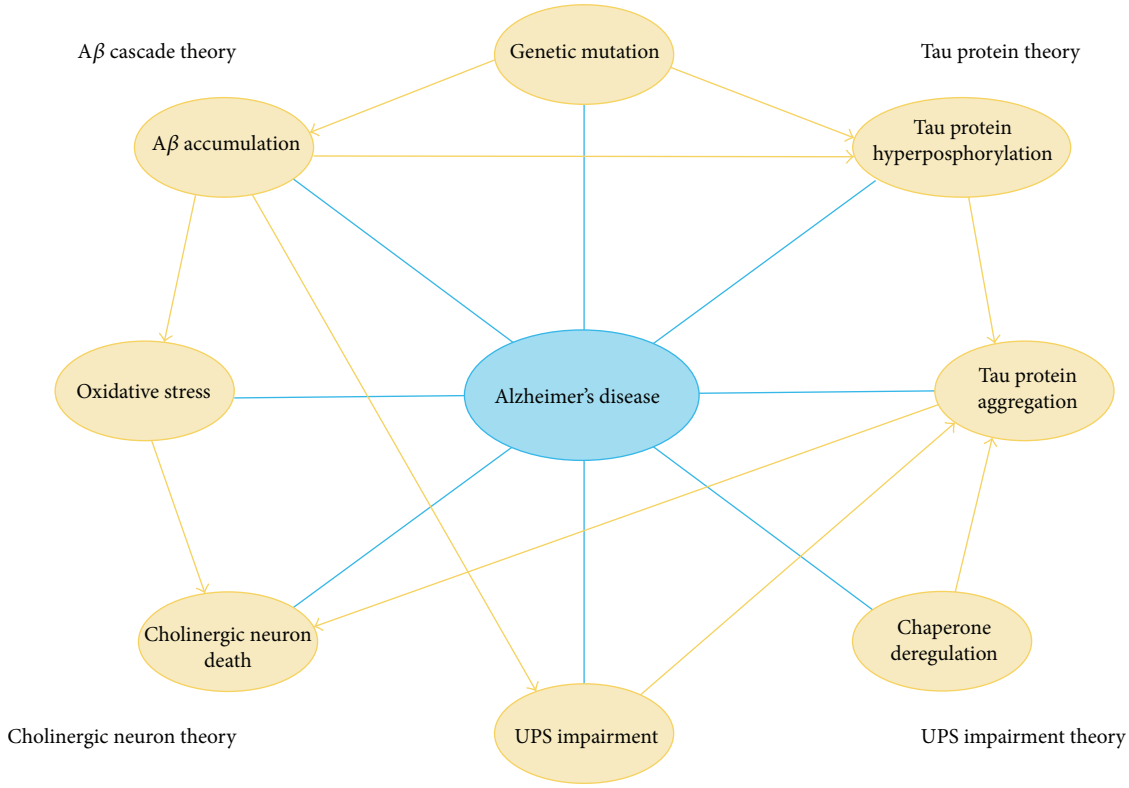

Figure 1: The multifaceted molecular pathology of AD. AD has been linked to many possible causes on genetic, molecular, and cellular levels. Each node in this figure represents a possible cause of AD. These causal events may work in concert and form an intricate cross-talking network, eventually resulting in neuronal death among patients.

There is abundant literature describing TCM methods for treating AD. We provide an extensive list of AD-related TCM studies in Supplementary Table S1.

6. Comparative Proteomics of AD-TCM Research

Because TCM treatments often consist of several active ingredients that work in synergy to promote the activities of each component, conventional experiment methods, such as Western blotting or measuring single enzymatic activities, are often inadequate to study the effects of TCM treatment. To address this problem, TCM research has recently applied various "omics" techniques, such as proteomics. Comparative proteomics is able to shed new light on the possible mechanisms of several known natural ingredients used in TCM for treating AD and other cognitive dysfunctions. Thus far, comparative proteomics studies regarding AD-related TCM treatments are very limited.

6.1. Huperzia serrata. Huperzine A is sesquiterpene alkaloid compound that can be isolated from *Huperzia serrata* [106]. *Huperzia serrata* is traditionally used in TCM as a treatment for fever, inflammation, blood disorders, myasthenia gravis, and schizophrenia [106, 107]. Huperzine A is also a promising drug candidate for the treatment of AD, with three meta-analysis studies reporting positive effects of the compound on patients with AD [108, 109].

In 1986, it was discovered that *Huperzia serrata*-derived Huperzine is a strong cholinesterase inhibitor [110, 111]. Cholinesterase is an enzyme that hydrolyzes the neurotransmitter acetylcholine into inactive metabolites [112]. Because it is suggested that AD is caused by acetylcholine deficiency, cholinesterase inhibitors, such as Huperzine A, are prescribed for AD treatment in China [107]. Emerging medicine, however, suggests that Huperzine A may also ameliorate AD via other mechanisms in addition to cholinesterase inhibition [107].

A proteomics study of Huperzine A on neuroblastoma N2a cells, which uses label-free liquid chromatography- (LC-) MS/MS, has discovered that Huperzine A has a neuroprotective effect on $A\beta_{1-42}$ oligomers-induced toxicity [113]. The study compares the proteomics profiles of untreated N2a cells, Aβ-induced N2a, and Aβ-induced N2a treated with Huperzine A. The results reveal that 198 proteins are differentially regulated among the groups. The study finds that the Trp53 protein is downregulated by a fivefold measure in the group treated with Huperzine A. Additionally, among the 198 differentially regulated proteins, 15 proteins are found to interact directly with Trp53 in a protein-protein interaction network analysis.

The same study also reveals that proteins involved in the UPS are differentially regulated between the groups of

patients with Aβ-induced N2a and Aβ-induced N2a treated with Huperzine A. The 26S proteasome core subunits, PSMB5 and PSMA5, and the 26S regulatory subunit, PSMD1, are found to be underexpressed upon Aβ induction and are rescued by the addition of Huperzine A. Other UPS components, such as E2 (UBE2K and CDC34) and E3 (UHRF1 and UBR4) ubiquitin ligases, are also regulated differentially. This argument is internally supported by the study's observation that Tau proteins are found to be lower in groups treated with Huperzine A. Tau proteins have been identified as the targets of the UPS. Thus, increased expression of proteins involved in the UPS ultimately leads to lower Tau protein accumulation. Another component of the UPS, UCHL3, which is a member of the ubiquitin C-terminal hydrolase (UCHL) family, is also shown to be upregulated in the Huperzine A-treated groups in the study. The implications of this protein are elucidated in Discussion of the present review.

An alternative possible mechanism of the Huperzine A-mediated amelioration of insults due to Aβ is the regulation of molecular chaperones and cochaperones, notably heat shock proteins 90 (HSP90) and HSP105, and the FK506 binding protein 8 (FKBP8, also known as FKBP38). HSP90, a pivotal chaperone for proper Tau protein folding and degradation, which has been shown to be downregulated in AD brains, has been found to be upregulated in the presence of Huperzine A [113, 114]. It has been suggested that HSP90 promotes the degradation of Tau proteins by recruiting the C-terminus of the HSP70-interacting protein (CHIP) E3 ligase and the promotion of Tau protein clearance [115]. The molecular cochaperone FKBP8 belongs to a group of proteins, so-called immunophilins, which are gaining attention for their role in AD pathogenesis [116]. The FKBP8 has been shown to promote apoptosis via interaction with presenilins [117]. In this study, FKBP8 is found to be inhibited by Huperzine A in Aβ-affected cells.

Furthermore, this study shows that Huperzine A may also support neuronal survival by upregulating PRDX3 [113], which is a member of the peroxiredoxin family of antioxidant enzymes that reduce hydrogen peroxide and alkyl hydroperoxides [118]. Upregulation of this protein may provide protection from oxidative insults induced by Aβ.

6.2. Ginkgo biloba. The earliest records of using *Ginkgo biloba* for the treatment of human diseases are documented in Li Tung-wan's *Shiwu Bencao* (Edible Herbal) and Wu-rui's Ri *Yong Ben Cao* (Herbal for Daily Usage) during the Yuan dynasty (1280–1368 A.D.), which chronicle the use of seeds for treatment of chronic bronchitis, asthma, enuresis, and tuberculosis [119]. Currently, extracts from *Ginkgo biloba* leaves are applied for the treatment of cognitive dysfunction, dementias, and AD [120]. *Gingko biloba* is an excellent example of TCM that has been successfully commercialized and accepted as a food supplement in Western countries. *Ginkgo biloba* extract has been standardized as EGb 761 and is sold under several commercial names, including Tebonin, Tanakan, Rokan, Ginkoba, and Ginkgold [121]. Standardized EGb 761, comprising 24% flavonol glycosides and 6% terpenoids, is composed of bilobalide and the ginkgolides A, B, C, M, and J [122].

Clinical studies of *Ginkgo biloba* extract show different results. An electrophysiological study argues that chronic administration of *Ginkgo biloba* extract improves cognitive performance and increases steady-state visually evoked potential amplitudes in the frontal and occipital lobes of middle-aged people for the sake of solving working memory tasks, as recorded by scalp electrodes [123]. Some clinical trials also prove the effectiveness of EGb 761 over placebos [124]. In contrast, however, another study with an older median age of patients shows negative results [125]. Meta-analyses studies of *Ginkgo biloba* extract claim that *Ginkgo biloba* extract improves cognitive function in patients with dementia and AD [126, 127]. Many theories have been developed on how *Ginkgo biloba* extract acts on cognitive function [121]. The most widely accepted theory is that *Ginkgo biloba* extract-derived terpenoids act as antioxidants and radical scavengers [128–132]. *Ginkgo biloba* extract also demonstrates neuroprotective ability against nitric oxide- (NO-) mediated toxicity by inhibiting protein kinase C (PKC) [133].

Vascular dementia (VD), the second leading cause of dementia, has some pathological overlap with AD and is suggested to have synergistic effects on AD [134–136]. In existing rodent models of VD, it has been observed that rodent brains demonstrate AD-characteristic pathogenesis following brain ischemia, such as the upregulation of APP [137], cleaving of APP into amyloid product [94, 138, 139], and Tau protein hyperphosphorylation [94, 140]. Due to this relationship, the data from VD studies can be carefully related to AD.

One proteomics study was carried out to determine the effects of *Ginkgo biloba* extract in rat brains after middle cerebral artery occlusion in a VD model [141]. Proteomics data from this study may shed light on protein regulation induced by a single dose of EGb 761. In the study, the author used 2DE and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS for quantitative proteomics analysis. The proteomics analysis reveals that 23 proteins are differentially regulated (with a measure of change greater than 2.5) by EGb 761. The study emphasizes the deregulation of PRDX2 and PP2A subunit B (i.e., a protein with an official name of PPP2R4) following EGb 761 treatment upon occlusion of the middle cerebral artery in patients. The upregulation of PPP2R4 and PRDX2 may provide some insight into the beneficial effects of EGb 761 in the treatment of AD. PRDX2 is a member of the family of peroxiredoxin proteins, which has antioxidative functionality (see *Huperzia serrata*) [118].

The activator PPP2R4 works on the PP2A protein. With regard to AD, it has been theorized that Aβ may assert toxicity by activating cellular kinases that cause Tau protein hyperphosphorylation, eventually leading to aggregation [49]. PP2A is among the types of Tau protein-targeting phosphatases that prevent the aggregation of Tau proteins by dephosphorylating aggregation-prone hyperphosphorylated Tau proteins [61, 62]. It has been demonstrated that Tau protein dephosphorylation can restore the ability of Tau proteins to bind to microtubules [49, 54]. This finding is

consistent with another study that reveals that EGb 761 is able to upregulate mRNA expression, as well as the activation of other phosphatases such as tyrosine/threonine phosphatase [142, 143].

Furthermore, this study also observes the downregulation of the HSP60 chaperone. The implication of HSP60 in AD is yet to be discovered. However, the modulation of HSPs is a common event in AD [144].

6.3. Acanthopanax senticosus. *Acanthopanax senticosus*, also known as *Eleutherococcus senticosus* or Siberian ginseng, has been used for treating various nervous and cardiovascular disorders [145]. This herb is reported to have positive effects on NDs such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and AD [146–149]. *Acanthopanax senticosus* extracts have been demonstrated to be neuroprotective both *in vitro* and *in vivo* [146, 149]. Additionally, *Acanthopanax senticosus* extract also prevents neuritic atrophy and potentially regenerates dystrophic neurites [147, 148]. Deeper investigation using proteomics methods may reveal the mechanisms of *Acanthopanax senticosus* extract in terms of AD treatment.

The work of Li et al. uses an iTRAQ quantitative proteomics method to decipher the effects of *Acanthopanax senticosus* extract on an *in vitro* PD model [150]. The authors prepare a crude extract derived from root and rhizomes of the herb using 80% ethanol, which is subsequently characterized by ultra-performance LC- (UPLC-) TOF/MS analysis. According to the results, the eleutheroside B and eleutheroside E content in *Acanthopanax senticosus* extract are $7.63 \pm 0.34\%$ (w/w) and $10.90 \pm 0.22\%$ (w/w), respectively.

In the study, the *Acanthopanax senticosus* extract is applied to SH-SY5Y neuroblastoma cells expressing A53T mutant α-synuclein. Although disturbed α-synuclein is more commonly observed in PD, a recent paradigm establishes a strong relationship between α-synuclein and Tau protein in AD as well [73, 151–153]. Accordingly, the results of this study may be carefully inferred to AD. The study finds that 84 proteins are differentially regulated between normal and A53T mutant α-synuclein-expressing cells. Among the 84 proteins, the expression of 16 proteins is reversed upon treatment with *Acanthopanax senticosus* extract.

The most significantly altered protein upon treatment with *Acanthopanax senticosus* extract is shown to be α-fetoprotein (AFP). AFP is upregulated in the SH-SY5Y overexpressing mutant α-synuclein, which is reversed upon treatment with the herbal extract. The authors conclude that the regulation of AFP is related to the apoptosis signal regulation. As it turns out, an additional insight can be deduced from this finding. AFP, together with α-synuclein and TDP-43, both of which are downregulated upon treatment with *Acanthopanax senticosus* extracts, are IDPs that are persistently found in NDs [68, 154]. Accumulation of IDPs is a pivotal indicator for the impaired clearance of misfolded proteins. The downregulation of IDPs upon treatment with the herbal extract suggests that *Acanthopanax senticosus* extracts have mechanisms for clearing misfolded proteins. Indeed, this is achieved by the drastic upregulation of PSMD7, a regulatory subunit of the 26S proteasome, in the *Acanthopanax senticosus* extract-treated cells. Overexpression of PSMD7 may recover the activity of the 26S proteasome, thus ensuring the proper clearance of misfolded proteins. In addition, the study shows that treatment with *Acanthopanax senticosus* extract causes an upregulation of USP5 (also known as UCHL5), which is another component of the UPS. The potential repercussions of the upregulation of UCHLs are elaborated on in Discussion of the present paper.

Another interesting finding from the study involves the upregulation of PPP2R5E, a regulatory subunit of PP2A. As described above (see *Ginkgo biloba*), PP2A prevents the aggregation of Tau proteins by dephosphorylating Tau proteins [61, 62].

Two members of HSP70 protein families, HSPA5 and HSPA9, are also found to be upregulated in *Acanthopanax senticosus* extract-treated cells. These HSPs are involved in protein processing in the endoplasmic reticulum and in protein folding [155]. However, the exact function of specific HSP molecules in AD is still controversial and is further assessed in our Discussion.

In addition, a comparative proteomics study for this herb has been carried out in an activated microglial cell line [156]. The authors of the study used bacterial LPS to stimulate inflammatory activation of the microglia. Activated microglia play an important role and may have interactive actions with the diseased neurons in AD pathogenesis [157, 158]. Therefore, assessing the effects of TCM extracts on microglia is critical to gain a better understanding of the effects of TCM on the AD brain. In the abovementioned study, proteome expression of cells treated with *Acanthopanax senticosus* extracts and a control group were compared using the 2DE method. The *Acanthopanax senticosus* extract significantly upregulated the expressions of PSMD13, FKBP4 (also known as FKBP52), and PRDX1. Although the specific functions of these proteins in microglia are not well characterized, this data suggests that there may be an interactive regulation of misfolded proteins between activated microglia and neurons during the progression of NDs [159–162]. Upregulation of the 26S proteasome subunit in microglia may help to degrade the misfolded proteins from the neighboring neurons, while the upregulation of PRDX proteins may help to battle oxidative insults [118]. FKBP4 has been demonstrated to inhibit Tau protein aggregation in neurons [163]. In sum, quantitative proteomics studies have shown that, in theory, *Acanthopanax senticosus* extracts have positive effects on AD patients by regulating protein clearance and Tau protein dephosphorylation in neurons and microglia.

6.4. Gastrodia elata. *Gastrodia elata*, commonly known as Tianma, is a member of the Orchidaceae family and is native to East Asian countries. Tianma has been used as traditional medicine for almost 2,000 years, as it was first described in an ancient Chinese medical text *Shennong Bencao Jing* (The Classic of Herbal Medicine) and also in *Bencao Gangmu* (Compendium of Materia Medica) [164, 165]. The tuber of Tianma has been used in TCM for centuries to treat dizziness, paralysis, and epilepsy [166]. Tianma is also used as an ingredient of *Tianma Gouteng Yin* and *Baizhu Tianma Tang* concoctions, which are prescribed for treating hypertension

[167]. In addition to these health benefits, the use of Tianma has been suggested for treating cognitive dysfunction and NDs [168–170]. Tuber extracts from Tianma contain active ingredients of phenolic phytochemicals, where gastrodin and 4-hydroxybenzyl alcohol are the primary components [166].

Several publications indicate the potential benefits of Tianma extracts for the treatment of AD or VD [171–175]. A study using a rat model in which the rodents are injected with $A\beta_{25-35}$ shows that long-term administration of crude Tianma water extract is partially effective for reversing $A\beta$-induced memory impairment [170]. Further, the study finds that Tianma extracts reduce the AP deposits and increase the expression and the activity of choline acetyltransferase. Similar results have been obtained by a different group of researchers using gastrodin instead of Tianma crude extracts [169]. Gastrodin also has been shown to be anti-inflammatory in the brains of mice and in cultured microglia cells. Gastrodin works by regulating the synthesis of proinflammatory cytokines [169, 176]. It is also reported that a water extract of Tianma has been known to modulate APP processing pathways, favoring the production of nonamyloidogenic products and enhanced cognitive functionality [171].

A comparative proteomics study using Tianma water extract has been carried out on neurons derived from human SH-SY5Y cells by using iTRAQ proteomics [177]. The study reveals that treatment with Tianma significantly alters the expression of 26S proteasome subunits, including PSMA1, PSMA2, PSMA3, PSMA4, PSMA5, PSMA6, PSMB7, PSMC3, PSMC5, PSMC6, PSMD1, PSMD2, PSMD3, PSMD8, PSMD11, PSMD12, PSMD13, and PSME3 [177]. As described, upregulation of 26S subunits may restore the protease abilities of the 26S proteasome [85]. Furthermore, the study demonstrates that TRIM28, a member of the E3 ligase protein family, is upregulated with treatment by Tianma. TRIM28 has been shown to target p53 for degradation; thus we assume that it has antiapoptotic effects [178]. Upregulation of these proteins may decrease the accumulation of misfolded proteins, thus preventing neurodegeneration. Similar to the active mechanism of *Acanthopanax senticosus* extracts, Tianma-treated cells have a threefold lower level of AFP in comparison to a control group of cells. This indicates that Tianma extracts may restore functionality in the UPS, which promotes the degradation of AFP. Two proteins from ubiquitin C-terminal hydrolase, UCHL3 and UCHL5, are also upregulated by treatment with Tianma [172]. The role of UCHLs in mediating TCM-treatment effects in AD is elaborated on in Discussion of the present review.

The same study also reveals that Tianma extracts may act on the regulation of molecular chaperones and cochaperones. Proteins from the HSPs families, such as HSP60, HSP70, HSP90, and HSP105, are found to be significantly upregulated by treatment with Tianma. Additionally, two FKBP immunophilins, FKBP3 and FKBP4, are also found to be upregulated by treatment with Tianma. We have discussed how HSP90 is involved in Tau protein degradation pathways (see *Huperzia serrata*), and it has been suggested that FKBP4 inhibits the aggregation of Tau proteins [163].

The effects of Tianma on the brain proteome have been elucidated *in vivo* in rats [171]. In accordance with the *in vitro* model, the investigation of brain proteomics reveals the involvement of HSP90 as a mediator protein in stimulation using Tianma. The brain proteomics of *in vivo* models, however, does not reveal the upregulation of 26S subunits, as found in *in vitro* models [171, 177]. This fact may be explained by the nature of the brain, in that the brain consists of a heterogeneous population of cells (i.e., mostly nonneuronal cells), which may mask the upregulation of neuron-enriched proteins [179, 180]. Additionally, tissue processing from *in vivo* studies involves treatments that are more elaborate, which may decrease the sensitivity of the proteomics assay. The study also reveals that PARK7 (also known as DJ-1), which is associated with autosomal recessive early-onset PD, is upregulated by Tianma [181]. It is suggested that the function of PARK7 is to protect cells from oxidative stress, and PARK7 may also act as a chaperone protein [182].

7. Discussion

There is a great deal of literature available to explain the mechanisms of TCM extracts in treating AD or improving general brain health. Meta-analyses and clinical trials of TCM-based treatment have shown the usefulness of TCM when used as prophylactic treatment for AD due to its improvement of cognitive function, improvement of daily life, and delay of cognitive decline in patients [124, 127, 183–186]. Thus far, available data often attributes the observed benefits of TCM limited to antioxidative action, free-radical scavenging, cholinesterase inhibition, or the antiapoptotic effects of TCM extracts (Supplementary Table S1). However, this is inadequate to fully explain the link between treatment and disease pathogenesis. The roles of antioxidants and cholinesterase inhibitors have been proven to be ineffective in treating or delaying AD [187–189], while apoptosis can be triggered by myriad direct or indirect causes, and therefore defining the apoptosis triggers (e.g., the inhibition of macroautophagy, DNA damage, accumulation of misfolded proteins, and other examples), rather than apoptosis itself, would be more beneficial for further mechanistic studies of TCM [190–192]. For these reasons, comparative proteomics studies of TCM are critical to discover further molecular mechanisms and to bridge the gap between TCM and WM.

The comparative proteomics data presented in this review reveals important mechanisms that are regulated upon treatment with TCM (Figure 2 and Table 1). The consistent involvement of the 26S proteasome and UPS are consistently observed in all experiments, with the exception of experiments related to *Ginkgo biloba*. Apart from this discrepancy, the data shows strong agreement on the upregulation of one or more 26S proteasome subunits in all cases. The ubiquitin ligases of the UPS are also found to be upregulated in the Huperzine A and Tianma studies, further suggesting that the UPS is a target of TCM phytochemicals. UPS pathways have been identified to degrade misfolded Tau proteins [115, 193–195]. Consequently, improving UPS activities by the overexpression of PA28γ (PSME3) recovers proteasome activities and bolsters cell survival of Huntington's disease-patient derived neurons [85]. Overexpression of PA28α (PSME1) also enhances the removal of misfolded and oxidized proteins

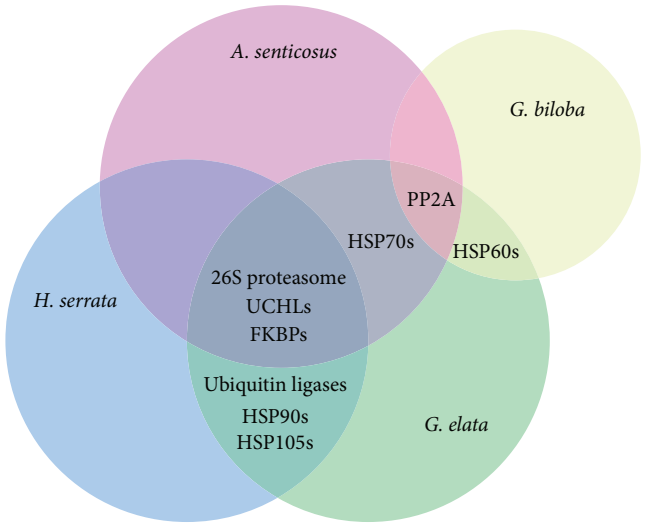

Figure 2: Diagram of the protein groups modulated by TCM treatment analyzed using proteomics methods. The UPS, consisting of the 26S proteasome, ubiquitin ligases, and ubiquitin hydrolases, is the main target of TCM treatments. Active TCM ingredients also target molecular chaperones and cochaperones such as HSPs and FKBPs. Additionally, TCM treatments modulate PP2A actions that regulate the dephosphorylation of Tau proteins.

and protects against oxidative stress in cardiomyocytes [196]. Thus, theoretically, the upregulation of UPS components by treatment with TCM is beneficial for AD patients.

Proteomics approaches are particularly beneficial for studying NDs, because NDs are characterized by the accumulation of protein aggregates, especially IDPs [70]. Through the use of proteomics, research is able to readily detect disordered proteins and to exploit them as internal indicators in support of the effects of TCM treatments. For example, in the Huperzine A study, Huperzine A upregulates the expression of UPS components, resulting in the more active proteasomal degradation of Tau proteins, thereby leading to lower observed levels of Tau proteins [113].

Additional findings from the proteomics data involve molecular chaperones and cochaperones. The most commonly occurring chaperones and cochaperones are HSP90, HSP70, HSP60s, HSP105s, and FKBPs. However, the role of HSPs in AD is still largely unknown. This ambiguity is caused by the ability of HSPs to bind promiscuously to a wide range of client proteins. Moreover, the actions of HSPs are often dictated by various regulator proteins, forming an HSP-client protein complex [144]. For instance, HSP70 and HSP90 have been known to form complexes with CHIP, an E3 ligase, to target Tau proteins for degradation by the UPS [115, 197–199]. One study claims that the forced induction of HSP70 and HSP90 decreases the aggregation of Tau proteins by promoting the binding of Tau proteins to microtubules [200]. Although HSP70 and HSP90 assist Tau protein degradation by recruiting CHIP, they also, to some extent, stabilize the binding of Tau proteins to microtubules, thus preventing the degradation of Tau proteins. In a seeming contradiction, it has been reported that the inhibition of HSP70 and HSP90 leads to the elimination of Tau protein aggregation [201–204]. Generally speaking, however, the expression of HSPs is inversely related to Tau protein aggregation, thus suggesting that the upregulation of HSPs by TCM is a favorable outcome [200].

Similar to HSPs, the role of FKBPs in AD is yet to be fully understood. A preliminary study argues that FKBP5 (FKBP51) promotes Tau protein accumulation, while FKBP4 (FKBP52) has a contrasting action by inhibiting the accumulation of Tau proteins [163]. It has also been suggested that FKBP4 protects against Aβ toxicity [205]. Accordingly, the upregulation of FKBP4 seems to be beneficial for treating AD, although there is no enough data to be conclusive about the benefits of other FKBPs. Smaller FKBPs, such as FKBP1A (FKBP12), have been found to coaggregate in NFTs [206]. It has been argued that the interaction between HSP and FKBP is important for Tau protein regulation [197]. Elucidating this link, however, is out of the scope of this review, and readers are directed to other reviews [197]. Because the function of FKBPs in AD is yet to be elucidated, the significance of FKBP modulation by TCM treatment is yet to be understood. However, it is important to recognize that the functions of individual members of FKBPs are very specific and cannot be generalized. For instance, FKBP4 and FKBP5 have almost identical protein domains, yet their actions on Tau protein regulation are antagonistic.

The proteomics data reviewed herein indicates that UCHL proteins are consistently modulated in most cases [113, 156, 177]. Although the potential involvement of this group of proteins in AD has been proposed, no strong conclusions have been reached [76, 91, 95, 207–218]. The UCHL protein group is important in maintaining availability of ubiquitin proteins for the UPS by recycling ubiquitin tags [211]. The best-known example of this group of enzymes is UCHL1 (also known as PARK5), which is known to serve a dual role in the UPS as a deubiquitinating enzyme in monomeric form or as an E3 ligase in dimeric form [215]. The loss-of-function of this particular protein has been demonstrated to impair the UPS and has been linked to the early-onset form of various progressive NDs, including PD [207, 219]. Furthermore, overexpression of UCHL1 reduces the production of Aβ by downregulating the protein level of β-secretase and APP [213, 214]. On the other hand, it has been reported that UCHL1, together with Parkin, promotes mitochondrial and synaptic failure by excessive activation of mitophagy in a truncated-Tau protein expressing cell line model [218]. UCHL3 is an important protein for functional working memory [212]. UCHL3 is also downregulated in an accelerated-senescence mouse model, suggesting that UCHL3 expression decreases with aging [216]. The implications of UCHL5 to proteasomal degradation are contradictory. It has been proposed that the expression of UCHL5 (UCH37) impedes proteasomal degradation by releasing polyubiquitin tags from the targeted proteins prior to introduction to the 26S proteasome [209]. One study reports that inhibition of UCHL5 with RNAi does not affect the proteolytic activity of the proteasome, but it does reduce the accumulation of polyubiquitinated proteins [217]. In contrast, other studies show that the inhibition of UCHL5 with the use of chemical inhibitors leads to

Table 1: AD-related protein families regulated by TCM as shown in various proteomics studies.

Protein function	Protein name	References
26S proteasome	20S proteasome subunits alpha	[113, 177]
	20S proteasome subunits beta	[113, 177]
	26S proteasome regulatory subunits ATPase	[177]
	26S proteasome regulatory subunits non-ATPase	[113, 150, 156, 177]
	26S proteasome activator subunit	[113]
Ubiquitin ligases	Ubiquitin-conjugating enzyme E2K	[113]
	Ubiquitin-conjugating enzyme E2-CDC34	[113]
	E3 ubiquitin-protein ligase UHRF1	[113]
	E3 ubiquitin-protein ligase UBR4	[113]
	Tripartite motif containing 28	[177]
Ubiquitin C-terminal hydrolase	Ubiquitin carboxyl-terminal esterase L3	[113, 177]
	Ubiquitin carboxyl-terminal hydrolase L5	[150, 177]
Chaperone sand cochaperones	Heat shock protein 60 kDa	[141, 177]
	Heat shock protein 70 kDa	[150, 177]
	Heat shock protein 90 kDa	[113, 171, 177]
	Heat shock protein 105 kDa	[113, 177]
	FK506 binding protein 3, 25 kDa	[177]
	FK506 binding protein 4, 59 kDa	[156]
	FK506 binding protein 8, 38 kDa	[113]
Antioxidant	Peroxiredoxin 1	[156]
	Peroxiredoxin 2	[141]
	Peroxiredoxin 3	[113]
Phosphatases	Protein phosphatase 2A activator, regulatory subunit	[141]
	Protein phosphatase 2, regulatory subunit B	[150]

accumulation of ubiquitin-positive aggregates [208, 210]. This may suggest that UCHL5 needs to be maintained at certain levels to be able to recycle ubiquitin without impeding proteasomal degradation. Ultimately, the impact of TCM treatment in the expression of UCHLs must be assessed further in order to elucidate the benefits of TCM treatment for NDs, including AD and PD.

Another key point from the studies reviewed herein is that the proteomics data from *in vitro* experiments provides more detailed proteomics profiles than the proteomics data from *in vivo* experiments. *In vitro* proteomics data, in general, covers more proteins and overestimates the expression levels of control and treatment groups in comparison to *in vivo* experiments. Such a case occurred in the *in vivo* experiments using *Ginkgo biloba* and Tianma, which, having fewer numbers of covered proteins, may have caused our analysis to misjudge the regulation of interesting proteins. This fact is possibly due to the heterogeneity of the cells in the brain used in *in vivo* studies, especially in the brains of rodents, which have approximately four times the number of nonneuronal cells compared to neurons [179]. This may cause neuron-enriched proteins, such as some of the component proteins of the UPS, to be underestimated by quantitative proteomics [180]. Moreover, this discrepancy may arise due to the process of tissue cell preparation, in which the process of protein extraction from *in vivo* tissue is lengthier and potentially harsher (e.g., tissue homogenization and liquid nitrogen grinding), thereby potentially reducing the yield of proteins relative to *in vitro* experiments. Nevertheless, future proteomics technologies may overcome such problems. Ultimately, it is important to realize that it is impossible to conclude that one model is more meaningful than another, particularly when studying a complex and systematic disease such as AD.

8. Conclusion

In conclusion, TCM has a long history of treating dementias, including AD. Western medicine may stand to benefit from the centuries worth of TCM knowledge about AD, provided scientific explanation is available to validate and/or fully elucidate the findings of TCM. Proteomics is an essential tool in providing important insights to explain the effects of TCM treatment on AD. Based on the proteomics data reviewed herein, we conclude that TCM is a useful complementary and alternative medicine (CAM) for treating or delaying the onset of AD in patients, particularly when utilized as prophylactic treatment in the form of food supplements before the onset of the disease. Proteomics data reveals that the potential mechanisms of action of TCM for the prevention of AD pathogenesis involve overexpressing antioxidant proteins, reducing the accumulation of misfolded proteins by improving the UPS, modulating the expression of protein chaperones and cochaperones (notably HSPs and FKBPs), and overexpressing Tau protein phosphatase.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Antiosteoporotic Effects of Huangqi Sanxian Decoction in Cultured Rat Osteoblasts by Proteomic Characterization of the Target and Mechanism

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Huangqi Sanxian decoction (HQSXD) is routinely used for the treatment of osteoporosis in the Chinese traditional healthcare system. However, the targets and mechanism underlying the effect of HQSXD on osteoporosis have not been documented. In the present study, seropharmacology and proteomic approaches (two-dimensional gel electrophoresis combined with mass spectrometry) were used to investigate the effects and possible target proteins of HQSXD on osteoblast. We found that HQSXD-treated rat serum significantly enhanced osteoblast proliferation, differentiation, and mineralization. In HQSXD-S-treated osteoblasts, there were increases in the expression of N-formyl peptide receptor 2 and heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 and reduction in the expression of alpha-spectrin, prohibitin, and transcription elongation factor B (SIII), polypeptide 1. The identified proteins are associated with cell proliferation, differentiation, signal transcription, and cell growth. These findings might provide valuable insights into the mechanism of antiosteoporotic effect affected by HQSXD treatment in osteoblasts.

1. Introduction

Osteoporosis is the most frequent bone remodeling disease and its incidence increases with advancing age. This disease is characterized by a reduction in bone mass and microarchitectural deterioration of bone tissue, resulting in high risk of fractures [1]. Globally, osteoporosis and associated bone fractures have become a major health hazard afflicting millions of people [2]. Current treatment options for osteoporosis include bisphosphonates, estrogens, selective estrogen receptor modulators, calcitonin, denosumab, and teriparatide. However, many of these drugs generate side effects [3, 4] and their costs are too high to benefit a large population in the developing and developed countries, which may limit their applications.

Traditional Chinese medicine has been a part of healthcare in China for thousands of years and has recently been reevaluated for clinical approach [5]. The traditional Chinese medicine has fewer adverse reactions and is more suitable for long-term administration than synthetic drugs and antibiotics. Popular commercially available prescriptions include Jinkui Shenqi Wan (JKSQW), which exerts a therapeutic effect on the kidney-Yang deficiency and osteoporosis indicated in Chinese Pharmacopoeia (2010), and Huangqi Sanxian decoction (HQSXD), a traditional Chinese formula, which is composed of *Radix Astragali*, *Epimedii Folium*, *Cistanche Herba*, *Radix notoginseng*, *Radix Salviae Miltiorrhizae*, *Corydalis Rhizoma*, Radix Angelicae Sinensis, and *Radix Clematidis*. Our previous study revealed that Huangqi Sanxian decoction treatment significantly increased sex estrogen

level and bone mineral density (BMD) and repressed bone absorption function in postmenopausal women [6]. This suggested that HQSXD has beneficial effects in the treatment of osteoporosis. However, little is known about the mechanisms and targets underlying the effects of HQSXD on osteoporosis.

The Chinese traditional medicine theory believes that bone activities are controlled by the kidney. Strong "kidney" can nourish bones, but the weak "kidney" might hasten bone deterioration [7]. Kidney deficiency and blood stasis are the main pathological basis of osteoporosis. Huangqi Sanxian decoction is composed of eight Chinese medicinal herbs; of these, *Epimedii Folium* and *Cistanche Herba* strengthen kidneys, while *Radix Salviae Miltiorrhizae* and *Radix notoginseng* invigorate the circulation of blood. These herbs are an excellent combination for highlighting their superiority in the treatment of osteoporosis [8].

Jinkui Shenqi Wan, an ancient Chinese herbal formula, is indicated in the Chinese Pharmacopoeia (2010) for the treatment of Yang insufficiency of kidney, weakness and soreness of the loins and the knees, cold feeling in the limbs, and frequent urination. Human clinical studies have certified that Jinkui Shenqi Wan exerts a therapeutic effect on the kidney-Yang deficiency [9]. Hence, we used Jinkui Shenqi Wan as a positive control in this study.

In the present study, seropharmacology and functional proteomics technology were used to explore the multiple proteins associated with the antiosteoporotic effect. The results suggest a basis for the clinical use of HQSXD in the treatment of patients with osteoporosis.

2. Materials and Methods

2.1. Animals. Thirty Sprague-Dawley rats (fifteen male and fifteen female), weighing about 250–300 g, were purchased from the Animal Experimental Center of Guangdong Medical College (Dongguan, China). The animals were housed individually in a regulated environment (24 ± 0.5°C), with a 12-hour light/dark cycle (under light: 08:00–20:00 h). Food and water were given *ad libitum* throughout the experiment. After three days of acclimation, male and female SD rats were randomly divided into three groups: blank-control group, experimental group treated with Huangqi Sanxian decoction, and positive control group treated with Jinkui Shenqi Wan. The Committee of Experimental Animal Administration of the University approved the study, and the procedures of the experiment were in accordance with generally accepted international rules and regulations.

2.2. Preparation of HQSXD and JKSQW. HQSXD included eight plant extracts, including *Radix Astragali* (root, Chinese herbal name: Huang-Qi), *Epimedii Folium* (leaf, Chinese herbal name: Yin-Yang-Huo), *Cistanche Herba* (succulent stem, Chinese herbal name: Rou-Cong-Rong), *Radix notoginseng* (root and rhizome, Chinese herbal name: San-Qi), *Radix Salviae Miltiorrhizae* (root and rhizome, Chinese herbal name: Dan-Shen), *Corydalis Rhizoma* (rhizome, Chinese herbal name: Yan-Hu-Suo), Radix Angelicae Sinensis (root, Chinese herbal name: Dang-Gui), and *Radix Clematidis* (root and rhizome, Chinese herbal name: Wei-Ling-Xian) in a ratio of 15 : 10 : 10 : 5 : 10:10 : 8 : 10 [6]. The above eight medicinal extracts were obtained from Dongguan Sinopharm (Dongguan, China) and identified by Professor Zhou (Department of Pharmacy, Guangdong Medical College, Dongguan, China). The eight medicinal plant materials in the mixture (270 g) were powdered and coboiled with 1000 mL water for 2 hours. The extraction was repeated twice. The filtrates were concentrated to 200 mL under reduced pressure and kept at 4°C.

JKSQW consisted of *Radix Rehmanniae Preparata* (root, Chinese herbal name: Di-Huang), *Dioscoreae Rhizome* (root, Chinese herbal name: Shan-Yao), *Fructus Corni Officinalis* (fructus, Chinese herbal name: Shan-Zhu-Yu), *Sclerotium Poriae Cocos* (dried sclerotia, Chinese herbal name: Fu-Ling), *Cortex Moutan Radicis* (root bark, Chinese herbal name: Mu-Dan-Pi), *Rhizoma Alismatis Orientalis* (stem, Chinese herbal name: Ze-Xie), *Ramulus Cinnamomi Cassiae* (twig, Chinese herbal name: Gui-Zhi), *Radix Aconiti Lateralis Praeparata* (root, Chinese herbal name: Fu-Zi), *Radix Achyranthis Bidentatae* (root, Chinese herbal name: Niu-Xi), and *Plantaginis Semen* (seed, Chinese herbal name: Che-Qian-Zi) [9]. As per the instructions, 5 mg Jinkui Shenqi Wan (Beijing Tong Ren Tang Pharmaceutical Technology Development Inc., Dongguan, China) was dissolved in 100 mL distilled water before administration. The experimental dose for HQSXD and JKSQW in the present study was equivalent to the corresponding clinical prescription dose for a human subject weighing 60 kg.

2.3. Drug Administration and Sample Collections. Rats were randomly divided into three groups of 10 animals each (each group included five males and five females). Chinese medicine HQSXD solution (14 g/kg) and medicine JKSQW solution (0.520 g/kg) were administered orally every day for three days, twice a day. The blank-control group was orally administered distilled water, and they were monitored concurrently with the HQSXD-experimental groups. At the end of the experiment, sixty minutes after the last treatment, the animals were exposed to ether anesthesia; blood samples were collected by heart puncture under aseptic conditions and then centrifuged for 15 min, to obtain serum samples. Serum of HQSXD-treated rats and control serum were inactivated at 56°C in a water bath for 30 min and filtered through a 0.22 μm filter membrane, termed HQSXD-S, JKSQW-control-S, and BLANK-control-S, respectively, and then stored at −80°C.

2.4. Primary Osteoblasts Culture and Assay for Osteoblast Proliferation. Primary rat osteoblast cells were obtained from 1-day-old neonatal Sprague-Dawley rats as described previously [10]. Primary osteoblasts were cultured by seeding 96-well plates with a density of 1×10^4 per well and incubated for 24 h. After adhesion of cells, Dulbecco's Modified Eagle Medium (DMEM) was added and incubated for another 24 hours. Next, after discarding the medium, test (HQSXD-S) and control (JKSQW-control-S, BLANK-control-S) samples

were added at a concentration of 10% (v/v), and the cells were incubated at 37°C in a humid atmosphere containing 5% CO_2 for 72 h. Thereafter, 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 hours, after which the medium was discarded, and dimethyl sulfoxide (DMSO) (150 μL) was added. Absorbance was measured at 490 nm using a Synergy 2 multifunctional microplate reader (Bio-Tek) to assess osteoblast proliferation.

2.5. ALP Activity and Staining Assay. Osteoblasts were suspended in DMEM to obtain a cell density of 5×10^5/mL; 2 mL aliquots of the cell suspension were added to 6-well plates. After 24 h of incubation, the medium was changed and cells were incubated with test (HQSXD-S) and control (JKSQW-control-S, BLANK-control-S) at a concentration of 10% (v/v) for 3 days. Alkaline phosphatase (ALP) activity was measured using an ALP assay kit (Sigma) as described previously [11]. For ALP staining, after incubation with HQSXD-S, JKSQW-control-S, and BLANK-control-S (concentration of 10% (v/v)) for three days, the cells were fixed in 70% ethanol for 15 min, washed, and then incubated with ALP staining buffer, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Beyotime Institute of Biotechnology, China), at 37°C for 30 min, air dried, and photographed.

2.6. Mineralization Assay. After 21 days of differentiation, the mineralization of osteoblasts was analyzed as described previously [12]. Briefly, the cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol for one hour. The cells were then rinsed in distilled water, stained with 0.5% Alizarin Red S (ARS) at pH 4.2 with rotation for 30 min at 37°C, and subsequently washed with distilled water and dried in air. Stained cultures were photographed. To analyze ARS activity, the ARS in stained cells was destained with 10% cetylpyridinium chloride (CPC) monohydrate solution (Sigma) for 30 min with shaking. The absorbance was measured at 562 nm using a Synergy 2 multifunctional microplate reader.

2.7. Protein Extraction. Osteoblasts cells were incubated in 50 mL culture flasks and grown to subconfluence (approximately 60%–70%) and then treated with test (HQSXD-S) and control (JKSQW-control-S, BLANK-control-S) at a concentration of 10% (v/v) for 72 h, respectively. At the end of the incubation period, osteoblasts were collected by centrifugation at 1,000 rpm (4°C). The osteoblasts were washed twice with cold PBS, after discarding the medium. Total proteins were extracted in a chilled lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 40 mM dithiothreitol (DTT), 2% (v/v) IPG buffer, pH 3–10, 4 μg/mL protease inhibitor mixture, and 4 μg/mL phosphatase inhibitors. After addition of the chilled lysis buffer, the cell solution was kept oscillating for 1 h at 4°C to solubilize the proteins. The homogenate was subsequently centrifuged for 15 min at 14,000 rpm at 4°C. Total protein concentration was quantified using the Bradford assay (Amresco).

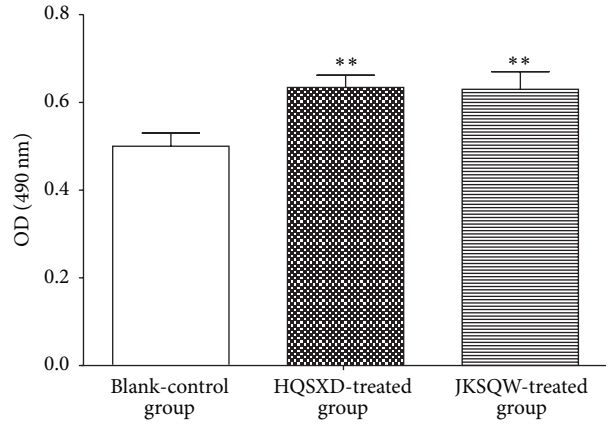

Figure 1: Effects of drugs on proliferation of osteoblasts. Although there was some improvement in the proliferation of osteoblasts in the HQSXD-treated group, it was not statistically significant when compared to the values observed in the group given JKSQW-control-S. Data are expressed as the mean ± SD.

2.8. Two-Dimensional Electrophoresis (2DE) and Image Analysis. Electrophoresis was carried out at least thrice for reproducibility. Two-dimensional electrophoresis (2DE) was carried out as described previously [13] with some modifications. For the first-dimension isoelectric focusing gel, 250 μg of protein was used for isoelectric focusing (IEF) using 17 cm nonlinear (NL) IPG strips (pH 3–10), which were immersed in rehydration buffer (8 M urea, 2% CHAPS, 25 mM DTT, 0.2% (w/v) Bio-Lyte, 0.1% bromophenol blue, and 1% IPG buffer). The strips were covered with mineral oil (GE Healthcare) to prevent samples from evaporation and rehydrated for 10–20 hours. Isoelectric focusing gel electrophoresis was performed by using an electrophoresis apparatus (GE Healthcare). The running conditions of the IEF process were as follows: 100 V, 1 h; 200 V, 1 h; 300 V, 1 h; 500 V, 1 h; 1,000 V, 1 h; 2,000 V, 1 h; and 8,000 V, up 60,000 V. After the IEF, the IPG strips were equilibrated with equilibration buffer-1 (6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl [pH 8.8], bromophenol blue dye, and 1% [w/v] DTT) for 15 min and then were repeated for an additional 15 minutes in 5 mL of equilibration buffer-2, except that DTT was replaced by 2.5% [w/v] iodoacetamide. The second dimension was done with a 12.5% acrylamide gel at 10 mA/gel for one hour and then at 38 mA/gel until the bromophenol blue dye reached the bottom of the gel. After electrophoresis, the gels were visualized by silver nitrate staining. Gels were scanned using the ImageScanner (GE Healthcare). The images were analyzed with ImageMaster 2D Platinum v7.0 software (GE Healthcare, San Francisco, CA) including spot detection, background subtraction, gel matching, and normalization. Spots were detected and matched automatically to a master gel and then edited manually. Matched spots from triplicate gel sets that showed overlap ratio with an absolute value ≥2 were recognized as differentially expressed. Differentially

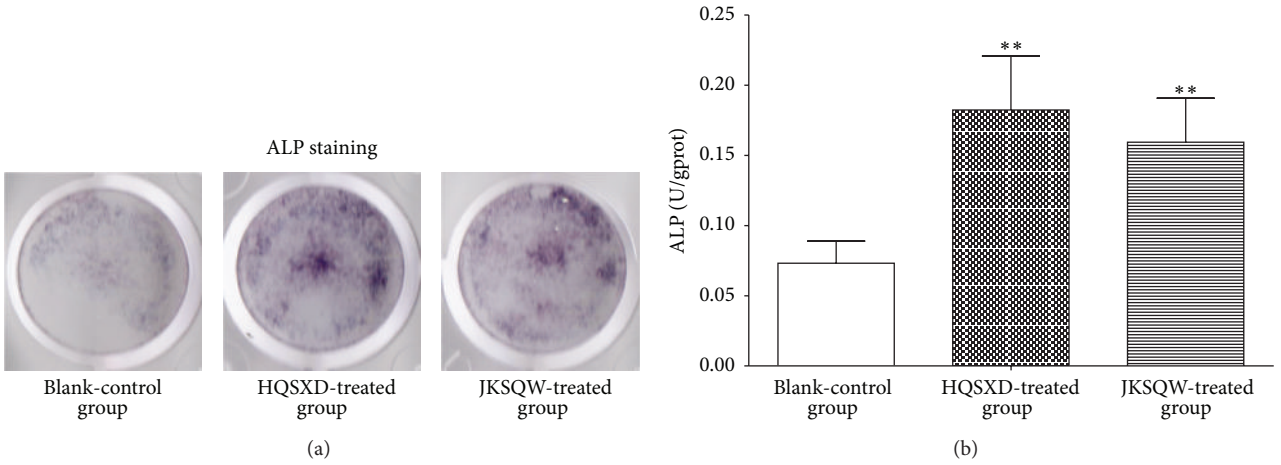

Figure 2: HQSXD-S enhances primary osteoblast differentiation. (a) Primary osteoblasts were treated with various drug-treated rat serums for 3 days. ALP-positive cells were stained with *ALP* solution. (b) Effect of HQSXD-S on alkaline phosphatase activity in primary culture osteoblasts. Data are means ± SD of six replicates. $^{**}p < 0.01$ versus control.

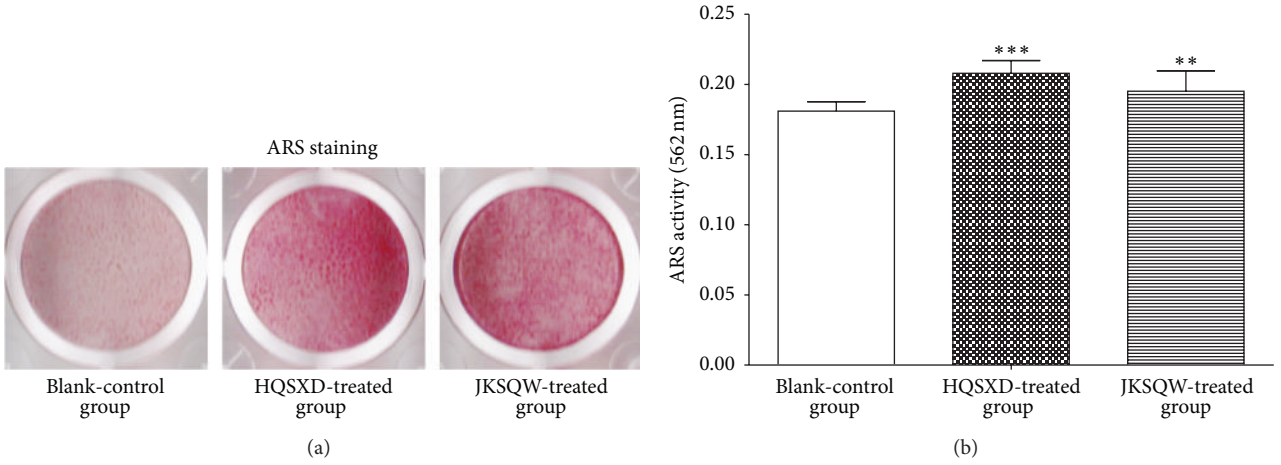

Figure 3: HQSXD-S promotes bone mineralization during osteoblastogenesis. (a) Calcium deposits stained with Alizarin Red solution seen in primary osteoblasts treated with various drug-treated rat serums for 21 days. (b) Stained calcium deposits were destained with 10% CPC buffer to measure the level of staining. Data are means ± SD of six replicates. $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

abundant spots were selected for mass spectrometry (MS) analysis.

2.9. Mass Spectrometry and Database Search. Protein spots were excised from the 2DE gels using a pipette tip. Gel pieces were destained in a solution of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (1 : 1), washed with deionized water, and dehydrated in 100% acetonitrile (ACN). Samples were rehydrated for digestion with trypsin (12.5 mg/mL) at 4°C for 30 min. Excess trypsin solution was replaced with 25 mM ammonium bicarbonate. The samples were incubated overnight at 37°C. Peptides were then extracted twice with 50% ACN/5% TFA followed by 100% ACN for 15 min each. After drying, the peptide extracts were desalted with ZipTip Pipette Tips (Millipore). Mass spectrometry was done using an ultraflex III MALDI-TOF/TOF-MS (Bruker) with a high voltage of 20 kV, and spectra were externally calibrated using the peptide standard Maker. Protein identification was determined by matching the peptide mass fingerprinting (PMF) and MALDI-TOF/TOF-MS results via MASCOT (version 2.2, Matrix Science) against NCBInr database with BioTools software. Database searches were performed using the following parameters: taxonomy, rice; enzyme, trypsin; and one missed cleavage allowed. Carbamidomethylation was selected as a fixed modification, and the oxidation was allowed as a variable. PMF tolerance set to 100 ppm, and MS/MS tolerance set to 0.7 Da. A protein was regarded as identified if the MASCOT protein score was above the 5% significance threshold for the database (score >64).

2.10. Western Blotting Analysis. To verify the results of 2DE of the identified proteins, we randomly chose four proteins for Western blot: FPR2, TCEB1, PHB, and alpha-spectrin. Cytosolic extracts were prepared from cells, and the protein in

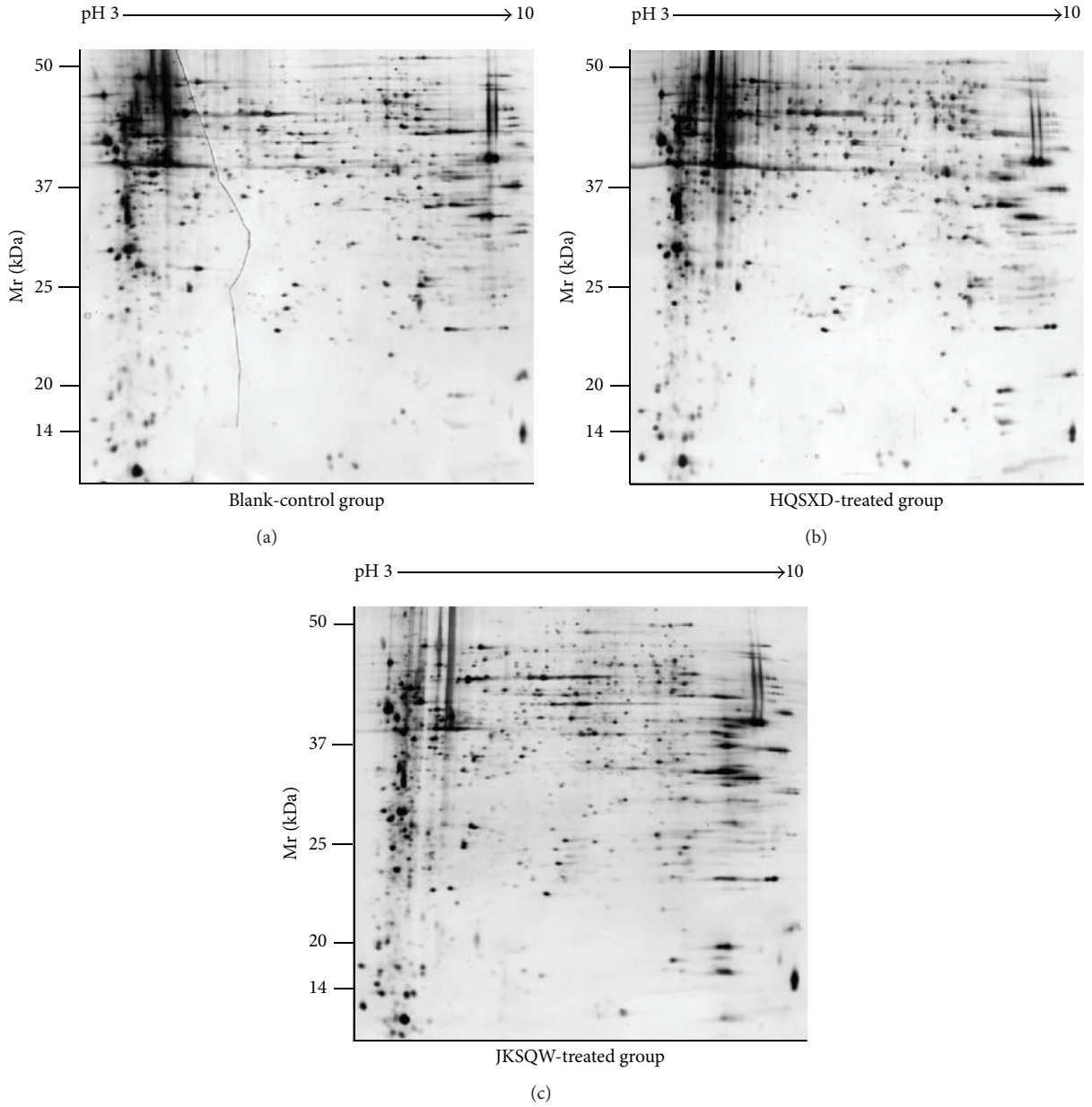

Figure 4: Representative silver nitrate stained gels showing two-dimensional electrophoresis protein profiles of (a) blank-control group, (b) HQSXD-treated group, and (c) JKSQW-treated group. Molecular weight (MW, kDa) and isoelectric point (P_I) are indicated along the y- and x-axes, respectively.

the supernatant was quantified using the BCA protein assay kit (Beyotime Institute of Biotechnology, China). A sample (50 μg) was electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred onto a PVDF membrane (Millipore). After blocking with 5% nonfat dry milk, the membranes were incubated with anti-FPR2 (M-73, sc-66901, Santa Cruz Biotechnology), anti-alpha-spectrin (C-11, sc-46696, Santa Cruz Biotechnology), anti-TCEB1 (ProteinTech Group, Inc., China), and anti-PHB (ProteinTech Group, Inc., China). The bound antibodies were detected using a horseradish peroxidase- (HRP-) conjugated secondary antibody and visualized by an enhanced chemiluminescence detection system, followed by quantification using the Image J2x.

2.11. Statistical Analysis. Data are presented as mean ± SD of triplicate samples. Comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test, and the difference was considered statistically significant if $p < 0.05$.

3. Results

3.1. Effects of HQSXD-Treated Rat Serum on Proliferation of Osteoblasts. The proliferation of primary osteoblasts showed an upward trend compared to that of blank control. However, there was no significant difference detected in the proliferation between HQSXD-treated and JKSQW-treated groups.

Table 1: Effects of drugs on proliferation of osteoblasts.

Groups	N	Oral administration dose ($g \cdot kg^{-1}$)	Serum additive volume (%)	OD value
Blank-control	5	—	10	0.50 ± 0.03
HQSXD-treated	5	14	10	$0.634 \pm 0.028^{**}$
JKSQW-treated	5	0.520	10	$0.63 \pm 0.04^{**}$

Note: $^{**}p < 0.01$ compared with BLANK-control-S.

Similar changes in proliferation between JKSQW-treated group and HQSXD-treated group indicated that HQSXD had an influence on osteoblasts (Table 1, Figure 1).

3.2. HQSXD-Treated Rat Serum Enhances Primary Osteoblast Differentiation. ALP is an important biochemical marker of differentiated osteoblasts, and the effects of drug on ALP activities in osteoblasts were first determined. Results of ALP staining showed that HQSXD-S and JKSQW-control-S stimulated osteoblast differentiation (Figure 2(a)). The cells cultured with HQSXD-S showed a significantly higher ALP activity than that cultured with BLANK-control-S (Figure 2(b)). ARS staining in osteoblasts was assessed after 21 days of incubation to examine whether HQSXD enhanced bone mineralization during osteoblastogenesis. In osteoblasts treated with BLANK-control-S, the calcium deposition in the mineralized matrix was minimal. The proportional areas of Alizarin Red-positive staining in the HQSXD-treated group and JKSQW-treated group were higher than that in the Blank-control group (Figure 3(a)). As shown in Figure 3(b), the level and intensity of ARS staining indicated the extent of mineralization, which increased upon treatment with HQSXD-S.

3.3. Protein Expression Profile in HQSXD-Treated and HQSXD-Untreated Osteoblasts. Two-dimensional electrophoresis and gel sliver nitrate staining were conducted to further investigate the differential protein expression between HQSXD-S-treated and HQSXD-S-untreated osteoblasts. After optimization of the 2DE gels, with representative 2DE gel images shown in Figure 4, approximately 938 ± 26, 875 ± 34, and 904 ± 22 protein spots were detected in blank protein sample, HQSXD protein sample, and JKSQW protein sample, respectively. During analysis with ImageMaster 2D Platinum, spots with an overlap ratio absolute value ≥ 2 were recognized as differentially expressed. Thirty-eight protein spots were found to be significantly regulated among three groups, of which 15 spots were downregulated and 23 spots upregulated. Ten of these 38 spots exhibited a more than twofold increase or decrease in abundance as observed in all replicate gels. These 10 regulated proteins were indicated by the circle in Figure 5, and the selected regions that showed significant differences in protein expression profile of osteoblasts among three groups were shown in Figure 6. All of them were excised from the gels for further identification by MALDI-TOF/TOF-MS analysis.

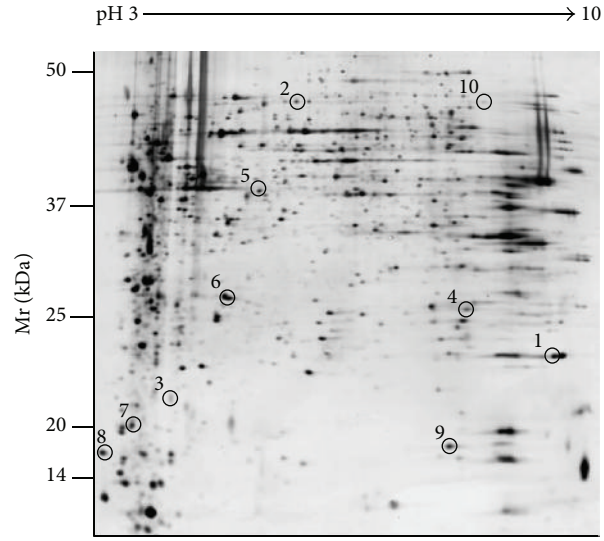

Figure 5: Representative 2DE gel image. Ten spots that were statistically significant ($p < 0.05$) are shown in the map and indicated by numbers. All were cut from the gels for further identification by MALDI-TOF/TOF-MS analysis, outlined in Table 2.

3.4. Identification of the Differentially Expressed Proteins. Proteins were identified by MALDI-TOF/TOF-MS. Ten peptide mass fingerprints (PMFs) and 50 peptide fragment fingerprints (PFF) were successfully obtained. A selected PMF of protein spot 6 is displayed in Figure 7(a), and the TOF/TOF analysis is shown in Figures 7(b)–7(f). All PMFs were evaluated with the Mascot software in NCBInr database to identify the protein spots. The result had high confidence if the protein was ranked as the best hit with a significant score and high sequence coverage. Finally, we identified eight proteins in these spots. Properties of the identification of eight selected protein spots are summarized in Table 2.

3.5. Effect of HQSXD on the Expression of Proteins That Regulate Antiosteoporotic Activity. To further investigate the influence of HQSXD on the expression of antiosteoporotic proteins, we examined the expression of FPR2, alpha-spectrin, PHB, and TCEB1 by Western blotting. The expression of FPR2 was increased by treatment with HQSXD-S in comparison to the blank-control group. However, HQSXD remarkably decreased alpha-spectrin, PHB, and TCEB1 protein levels compared with blank-control group (Figure 8). Results from Western blot manifested the same trend as from proteomic analysis.

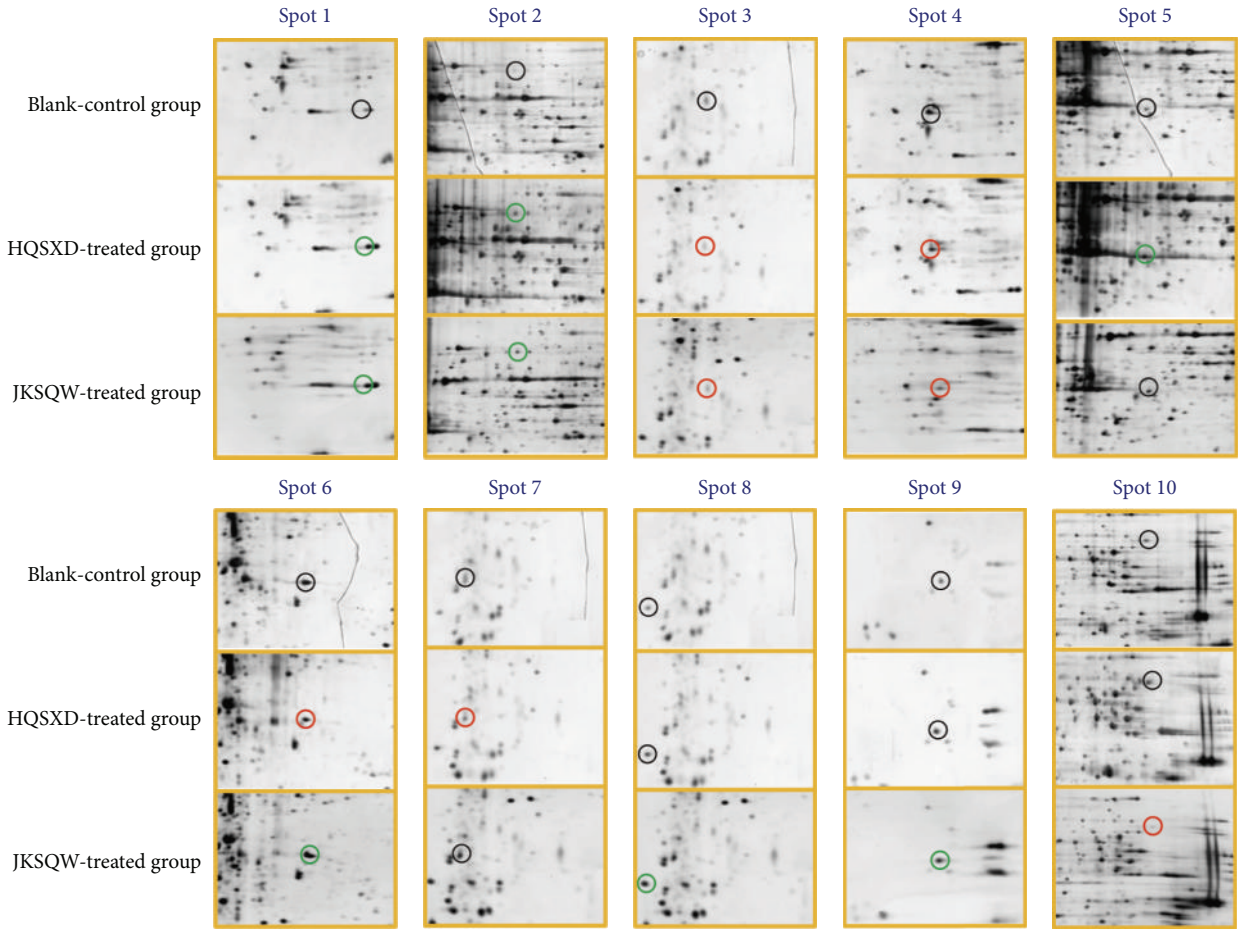

Figure 6: The ten protein spots of osteoblasts that were treated with and without HQSXD-S. Selected regions showed significant differences in the protein expression profile of osteoblasts among the three groups. Upregulated spots are indicated by green circles and downregulated ones by red circles.

Table 2: Summary of differentially expressed proteins in osteoblasts treated with HQSXD.

Spot number[a]	Protein score[b]	Matching peptides (number)	Theoretical P_I[c]	Theoretical Mr (Da)[c]	Target protein	Species
1	70	6	9.27	39299	N-Formyl peptide receptor 2	*Rattus norvegicus*
3	67	7	5.65	54851	Alpha-spectrin	*Rattus norvegicus*
5	76	6	10.08	43712	Heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1	*Rattus norvegicus*
6	173	12	5.57	29859	Prohibitin	*Rattus norvegicus*
7	67	4	4.59	12752	Transcription elongation factor B (SIII), polypeptide 1	*Rattus norvegicus*
8	70	11	4.98	127444	Chromosome segregation protein	*Rattus norvegicus*
9	101	4	6.91	17386	Nucleoside diphosphate kinase	*Rattus norvegicus*
10	84	12	8.86	48199	Mast cell carboxypeptidase A	*Rattus norvegicus*

[a]Protein spot number according to Figure 3.
[b]Protein scores were based on combined mass and mass/mass spectra from MALDI-TOF/TOF identification MS.
[c]Theoretical molecular mass (Mr) and isoelectric point (P_I) from the NCBInr database.

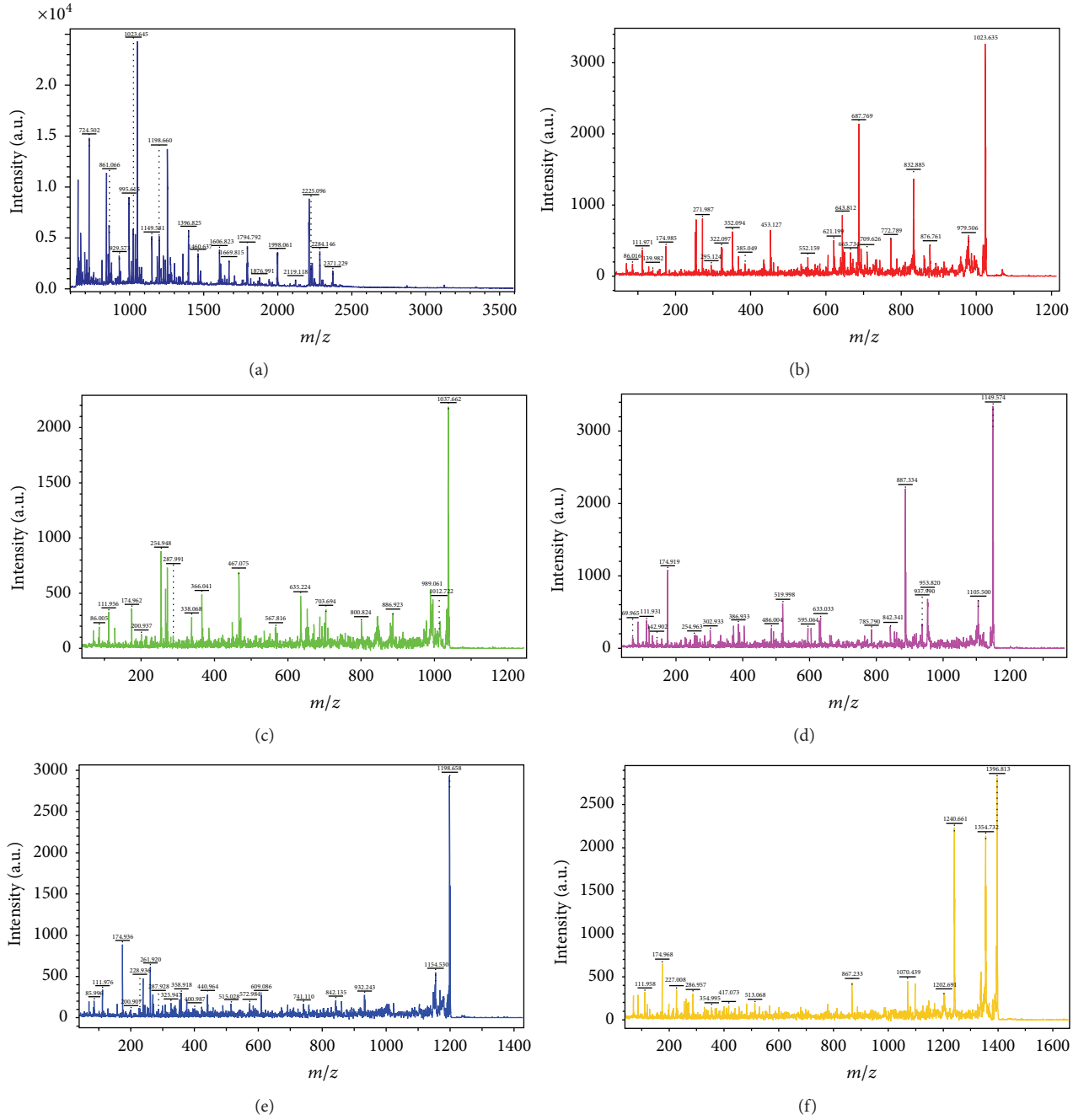

Figure 7: The results of the MALDI-TOF/TPF-MS analysis of protein spot 6. (a) Peptide mass fingerprinting and ((b)–(f)) peptide fragment fingerprinting of spot 6.

4. Discussion

In this study, we found that the proliferative activities of osteoblasts between HQSXD-S-treated group and JKSQW-control-S-treated group were similar. This investigation demonstrates that HQSXD can significantly facilitate bone formation through increasing the number of osteoblasts, which is beneficial to the treatment of osteoporosis. By measuring ALP activity, HQSXD was first screened for its ability to induce osteogenesis. HQSXD is capable of significantly promoting osteoblast differentiation, as well as increasing osteoblast mineralization.

Results of the identification of the selected protein spots are summarized in Table 2. The molecular weight (Mr) and isoelectric point (P_I) of each protein spot shown in Table 2 are theoretical values. The eight protein spots were identified as (1) N-formyl peptide receptor 2 (FPR2); (2) alpha-spectrin; (3) heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 (HS3ST3A1); (4) prohibitin (PHB); (5) transcription elongation factor B (SIII), polypeptide 1 (TCEB1); (6) chromosome

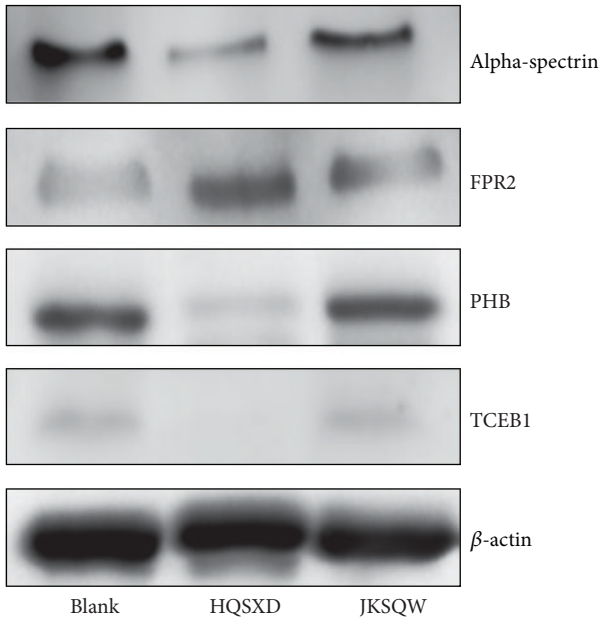

Figure 8: HQSXD-S treatment decreased alpha-spectrin, PHB, and TCEB1 expression and increased FPR2 expression. Cell lysates (50 μg) were processed for Western blot analysis. Normalization performed to β-actin. The bands shown here were from a representative experiment repeated three times.

segregation protein; (7) nucleoside diphosphate kinase; and (8) mast cell carboxypeptidase A.We detected some proteins related to transcription, cell proliferation, differentiation, and apoptosis, such as FPR2, prohibitin, alpha-spectrin, heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1, and transcription elongation factor B (SIII), polypeptide 1, which varied greatly after HQSXD treatment.

Our results suggest that HQSXD can upregulate the expression of FPR2. N-Formyl peptide receptor (FPR) is a G protein-coupled receptor, which modulates stromal cell differentiation [14] and binds to N-formyl peptides, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP). Our study has shown that fMLP enhances the differentiation of MSCs into osteoblasts via an FPR-mediated signaling pathway and results in bone formation [15]. The FPR2 receptor belongs to the formyl peptide receptor family that is involved in signaling stem cell adhesion, migration, and homing for injured and inflamed tissues awaiting repair; this could potentially be exploited to direct the stem cells to target specific tissue site [16]. Therefore, the current results imply that treatment with HQSXD might promote the formation of osteoblasts by upregulating the expression level of FPR2.

We found that HQSXD could inhibit the expression of alpha-spectrin. Alpha-spectrin includes two genes encoding for alpha-I subunit and alpha-II subunit, each of them presenting its specific cellular expression pattern. Alpha-II spectrin deficiency is associated with cell proliferation defects, due to cell cycle arrest in the G1 phase (first gap phase) [17]. Spectrin and protein kinase C theta were observed in aggregates during the early stage of apoptosis [18]. Hence, we presume that alpha-spectrin is involved in the antiosteoporotic effect of HQSXD.

In this study, PHB was downregulated in HQSXD-treated osteoblasts. Prohibitin, a highly conservative protein, regulates the cohesion of sister chromatids, cellular signaling, mitochondrial biogenesis [19], cell proliferation, differentiation, apoptosis, and gene transcription [20, 21]. PHB blocks the transition of cells from G1 phase to S phase of the cell cycle, thereby arresting cell proliferation [22, 23]. PHB has been reported to affect the apoptotic pathways by repressing the transcriptional activity of E2F1 [24, 25]. As mentioned above, it is possible that HQSXD induces proliferation, differentiation, and apoptosis partly through downregulating expression of PHB in osteoblasts. It is likely that PHB might be the novel candidate in the new antiosteoporotic drug screening.

The expression of TCEB1 was downregulated in HQSXD-treatment osteoblasts. TCEB1, a 13 kDa protein also named elongin C, was originally identified as a member of the mammalian transcription factor SIII that increases the rate of transcription by suppressing RNA polymerase II pausing [26]. As part of a family of separate complexes containing elongin B and various substrate specificity factors, it acts as an E3 ubiquitin ligase [27]. Elongin B (ELB 1) and elongin C (ELC1) form a stable complex, and that depletion of either gene product by RNA-mediated interference (RNAi) causes pronounced defects in the second meiotic division and arrest of germ cell proliferation in G1 [28]. Therefore, it is possible that that TCEB1 is involved in the antiosteoporotic effect of HQSXD.

Although this study has thrown some light on the mechanism of HQSXD action, we failed to characterize the well-identified protein (e.g., HS3ST3A1) closely involved in osteoporosis. HS3ST3A1, a member of the heparan sulfate biosynthetic enzyme family, possesses heparan sulfate glucosaminyl 3-O-sulfotransferase activity. Depletion of Hs3st-A in enterocytes results in increased intestinal stem cell proliferation and tissue homeostasis loss [29].

5. Conclusions

The results confirm that HQSXD has a beneficial effect on osteoblasts and alters the expression level of some proteins in osteoblasts. The protein expressed by osteoblasts treated with HQSXD may be involved in cell proliferation and differentiation and other physiological processes and in the regulation of cell activation. Further study is needed to investigate the effects of major active constituents in HQSXD on protein expression on osteoblast so as to demonstrate the interaction and synergistic mechanism.

Conflict of Interests

All the authors state that they have no conflict of interests to declare.

Authors' Contribution

Chong-Chong Guo and Li-Hua Zheng contributed equally to this work.

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Research Article

Moxibustion Reduces Ovarian Granulosa Cell Apoptosis Associated with Perimenopause in a Natural Aging Rat Model

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In recent years, concerns about the adverse effects of hormone replacement therapy have increased interest in alternative therapies for the management of the symptoms of perimenopause. Here, we investigated the effects of moxibustion, a traditional Chinese practice that is involved in heated *Artemisia vulgaris* (mugwort) stimulation, on hormonal imbalance and ovarian granulosa cell (GC) apoptosis in a rat model of perimenopause. Our results showed that mild warm moxibustion (MWM) modulated the circulating levels of estradiol and follicle-stimulating hormone and their receptors and inhibited apoptosis in the ovaries of perimenopausal rats, similar to the effect of estrogen. Further investigation revealed that the effects of MWM on ovary tissues and cultured GCs were mediated by the modulation of the activity of Forkhead box protein O1 and involved the JAK2/STAT3 pathway. Our results provide information on the factors and pathways modulated by MWM and shed light on the mechanism underlying the beneficial effect of moxibustion on the symptoms of perimenopause.

1. Introduction

Perimenopause refers to the period surrounding the transition into menopause, which is defined as the end of menstruation and fertility [1]. Menopause is characterized by the loss of estrogen production by the ovaries, which can have a wide range of effects on women's health and quality of life. The menopausal decrease in estrogen levels contributes to decreased bone mass and density, the accumulation and redistribution of adipose tissue, and an increased risk of metabolic disorders and cardiovascular disease [2–4]. Hormone replacement therapy, which was used widely in the 1960s and early 1970s, was found to be associated with an increased risk of endometrial and breast cancer, triggering an increased interest in alternative therapies for the treatment of perimenopause-related symptoms [5, 6].

Apoptosis is a critical mechanism for cellular homeostasis by which cells that are damaged, senescent, or no longer useful are eliminated via programmed cell death. During follicular growth and development, more than 99% of follicles undergo a degenerative process known as atresia that has been associated with apoptosis of granulosa cells (GCs) [7–9]. Although atretic follicles show the biochemical and morphological features of apoptosis, the exact molecular mechanisms and signal transduction pathways involved in the apoptosis of GCs remain unclear. The Forkhead box O (FOXO) family of transcription factors regulates the expression of genes associated with apoptosis, cell cycle progression, cellular homeostasis, and mitochondrial metabolism among others [10]. FOXO1 has been suggested to be involved in follicular development, and its levels and localization change during follicular atresia [11]. In GCs, follicle-stimulating hormone (FSH) functions as a survival factor by preventing apoptosis, whereas FOXO1 induces apoptosis and follicular atresia, and several signaling cascades are involved in the regulation of FOXO1 activity in response to FSH stimulation [12, 13]. In addition, FOXO1 has been suggested to be involved in the regulation of FSH and luteinizing hormone (LH)

levels, whose interactions with ovarian regulatory factors are essential for GC proliferation, differentiation, and apoptosis [13, 14].

Moxibustion is a traditional Chinese therapy that consists of direct or indirect acupuncture-point stimulation using burned dried *Artemisia vulgaris* (mugwort). Moxibustion has been used for a wide range of conditions including arthritis, gastrointestinal disorders, gynecological complaints, and pain. However, clinical trial-based evidence supporting the effectiveness of moxibustion is limited [15].

In the present study, we examined the effects of moxibustion on ovarian GC apoptosis and explored the underlying mechanisms. Our results indicate that moxibustion inhibits the apoptosis of GCs via a mechanism involving FOXO1 and the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway and reveal a potential novel mechanism modulating the effects of hormonal imbalances associated with perimenopause.

2. Materials and Methods

2.1. Animals. Immature female (3-4 weeks), young female (3-4 months), and perimenopausal female (11 months, weighting 180 ± 20 g) Sprague-Dawley rats were obtained from the Experimental Animal Center at the Shanghai Jiangwan Hospital, China. All rats were kept under controlled temperature (30 ± 2°C) and light (14 h light, 10 h dark) conditions with rat chow and water ad libitum. All experiments were approved by the Institutional Animal Research Ethics Committee of the Shanghai Jiangwan Hospital.

2.2. Groups and Treatment. Perimenopausal female (11 months) Sprague-Dawley rats were randomly divided into the following three groups ($n = 10$ per group): control, mild warm moxibustion (MWM), and conjugated equine estrogen (CEE) groups. In addition, 10 adult female rats (3-4 months) comprised the young group. Prior to the initiation of experiments, vaginal smears of the female rats were obtained at 8 : 30 am every morning to confirm the perimenopausal status of the older rats according to the estrous cycle. In the MWM group, moxa sticks were ignited 1-2 cm above Guanyuan (CV4) and Shenshu (BL23) for 20 min once daily for 6 days with 1 rest day, and the temperature of the local area was kept at 43 ± 2°C. The BL23 acupoints on both sides were used interchangeably. The CEE group received 0.1 mg/kg/d CEE (Wyeth Pharmaceuticals Inc., Lotio 071207) by gavage administration once a day. The rats in the control and young groups did not receive any treatment. Five rats from each group were randomly blooded from the aorta abdominalis and then sacrificed after 4 and 8 weeks, and their ovaries and uterus were removed.

2.3. Radioimmunoassay (RIA) for Estradiol, FSH, and LH. The collected blood samples were coagulated for 2 h and centrifuged at 4°C, 4000 rpm, for 10 min, to separate the serum. One part of the separated serum was used for the measurement of estradiol (E_2), FSH, and LH and stored at −80°C until radioimmunoassay according to the kit manuals. E_2, LH, and FSH kits were purchased from Beijing Kemei biotechnology Co., Ltd. (Lotio 081025). Other samples were stored at −20°C for culturing GCs *in vitro*.

2.4. TUNEL Assay. TUNEL staining was performed using a fluorescence detection kit (Roche, Indianapolis, IN, USA). Paraffin embedded sections (5 μm) were stained with terminal deoxynucleotidyl transferase and fluorescein-dUTP after proteinase K treatment and incubated for 60 min at 37°C in a humidified chamber in the dark. Sections were rinsed three times with PBS and visualized with a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a digital camera. The percentage of apoptotic cells was determined in micrographs of TUNEL positive and DAPI-stained nuclei using Image J software in 10 random fields at 400x magnification. At least 100 cells were counted in each field.

2.5. Immunohistochemistry. For immunohistochemistry, paraffin embedded sections (5 μm) of the ovaries of rats in the different groups were deparaffinized, treated with 1% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity, washed in Tris-buffered saline, blocked with 10% normal rabbit serum, and incubated overnight at 4°C with rabbit-anti-human polyclonal antibodies against Bcl-2, Bax (Santa Cruz Biotechnology, Santa, CA, USA), and active caspase-3 (Abcam, Cambridge, MA, USA). After washing in TBS, slides were incubated with biotinylated goat anti-rabbit IgG in TBS with 0.05% BSA. Sections were then washed in TBS and incubated with avidin and biotin (Vectastain ABC-Elite kit, Vector Laboratories, Burlingame, CA, USA), and bound antibody was visualized after the addition of a solution of 3,3′-diaminobenzidine tetrachloride (DAB, Sigma, St. Louis, MO, USA) in Tris-HCl with 0.03% H_2O_2. DAPI staining for the cell nucleus was used to calculate the total number of cells. The stage of each section was scored based on THE staining density and the percentage of positively stained cells. The mean staining intensity for each slice was calculated. The intensity of immunostaining was statistically analyzed using semiquantitative scores of "1" for weak or no staining; "2" for mild staining; "3" for moderate staining; and "4" for strong staining when, respectively, <1, 1–10, 10–50, and >50 percent of the cells in the follicular or corpus luteum cross section stained positively.

2.6. Granulosa Cell Isolation and Culture. Rats (female, 3-4 weeks) were injected intraperitoneally with 60 units of pregnant mare serum gonadotropin (PMSG, Animal Drugs Factory, Huangzhou, Zhejiang, China). After 48 h, the rats were sacrificed, the ovaries were removed, and GCs were isolated by needle puncture under an inverted microscope. After obtaining single cell suspensions, they were washed twice with PBS and cultured with complete medium, including DMEM/F12 (1 : 1) medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C and 5% CO_2.

GCs were plated in 24-well cultures dishes with complete medium overnight. The medium was removed and replaced

with the culture media supplemented with 10% of the separated sera from the different natural aging model groups (control, CEE, and MWM) and the young group at 8 weeks, separately. Validating the effects of MWM on cultured GCs was mediated by the modulation of the activity of FOXO1, before treatment with the MWM serum, and GCs were transfected with constructs containing the full length open reading frame of mouse FOXO1 (OriGene) to overexpress FOXO1. The cells were cultured at 37°C and 5% CO_2 for 48 h, and cells were collected for apoptosis rate analysis and western blot analysis.

2.7. Apoptosis Analysis by Flow Cytometry. Cells in suspension were washed in ice-cold PBS and incubated at 4°C for 5 min. After removal of the supernatant, cells were filtered with ice-cold binding buffer to a concentration of 5×10^5–5×10^6/mL. A 100 μL cell suspension was treated with 5 μL of Annexin V-FITC (100 U/mL, Sigma) and 2.5 μL of propidium iodide (50 μg/mL, Sigma), mixed, and incubated on ice in the dark for 10 min. Then, 400 μL of ice-cold 1x binding buffer was added to the suspension, mixed for 30 min, and analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA, USA).

2.8. Western Blot Analysis. Total lysates were prepared in radioimmune precipitation assay buffer and protein content was determined using the BCA method (Pierce, Rockford, IL, USA). Protein samples containing 15 μg of protein were separated in 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked for 2 h in 5% milk powder in PBS and then incubated in primary antibodies against ER, FSHR, LHR, Bcl-2, Bax, p-JAK2, JAK2, p-STAT3, STAT3, β-actin (Santa Cruz Biotechnology), active caspase-3, FOXO1, and p-FOXO1 (Cell Signaling Technology, Beverly, MA) overnight at 4°C followed by HRP-conjugated secondary antibodies for 2 h at room temperature. After washing, proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce). β-actin was used as the loading control.

2.9. Statistical Analysis. Results are expressed as the mean ± SD. Differences were analyzed using one-way analysis of variance (ANOVA) or t-test. The level of significance was set at $p < 0.05$. All experiments were repeated at least three times.

3. Results

3.1. Moxibustion Regulates the Levels of Circulating E_2, FSH, and LH in Aged Perimenopausal Rats. Serum levels of E_2, FSH, and LH were measured in control perimenopausal rats treated with or without MWM and CEE and compared with those in the young (3-4 months old) group. Serum E_2 levels were significantly lower in control than in young rats, and treatment with MWM significantly increased E_2 levels at 4 and 8 weeks in perimenopausal rats similar to the effect of CEE (Figure 1(a)). FSH levels were significantly decreased by MWM compared to those in the control group at 4 and 8 weeks, and CEE had a similar effect, although the decrease was only significant at 8 weeks (Figure 1(b)). MWM and CEE did not significantly affect the serum levels of LH in the aged group at 4 and 8 weeks (Figure 1(c)). Western blot analysis of the expression of the receptors for estrogen, FSH, and LH (ER, FSHR, and LHR) in the uterus of rats in the different groups showed that MWM and CEE upregulated the expression of ER and FSHR compared to the control (Figure 1(d)). At 4 weeks, FSHR expression was slightly increased in the MWM and CEE groups, whereas, at 8 weeks, MWM markedly increased ER expression compared to the control and CEE groups. LHR expression levels did not differ among the four groups of rats (Figure 1(d)). The changes in the expression levels of ER, FSH, and LHR were consistent with the observed hormonal changes and indicate that moxibustion modulates female hormones and their receptors in aged perimenopausal rats.

3.2. Moxibustion Reduces Apoptosis in the Ovaries of Aged Perimenopausal Rats. To examine the effect of moxibustion on cell apoptosis associated with perimenopause, ovarian tissues were obtained from middle-aged rats treated as indicated and cell apoptosis was evaluated using TUNEL staining. Figure 2(a) shows representative images of TUNEL staining in ovarian tissues of control, MWM-treated, CEE-treated, and young rats at 4 and 8 weeks after treatment, with green fluorescence indicating TUNEL positive nuclei. Analysis of staining intensity showed that apoptosis rates were higher in the natural aged perimenopausal rats than the young rats at both time points. MWM and CEE treatment slightly decreased the rate of apoptosis at 4 weeks and significantly decreased the number of TUNEL positive nuclei at 8 weeks in the ovaries of perimenopausal rats ($p < 0.05$) (Figure 2(b)).

Immunohistochemical analysis of the expression of the antiapoptotic protein Bcl-2 and the proapoptotic proteins Bax and caspase-3 showed that MWM and CEE upregulated the expression of Bcl-2 and downregulated that of Bax and caspase-3 in ovarian tissues of perimenopausal rats compared to the levels in untreated controls, with obvious differences observed at 8 weeks (Figure 3). Table 1 summarizes the relative immunostaining intensity of each gene.

3.3. Involvement of FOXO1 and the JAK2/STAT3 Pathway in GC Apoptosis Associated with Perimenopause. To explore the mechanisms underlying the MWM-induced inhibition of apoptosis in the ovary, the expression and activation of FOXO1, JAK2, and STAT3 were examined in the ovaries of rats in the different treatment groups. STATs are transcription factors that are activated in response to cytokines and growth factors, and activation of STAT3 is associated with cell proliferation and the inhibition of apoptosis [16]. Phospho-JAK2 phosphorylates STAT3, which translocates to the nucleus to regulate the expression of several genes. The JAK2/STAT3 pathway interacts with FOXO1 via the phosphoinositol 3 kinase (PI3K)/Akt pathway, and dephosphorylation and nuclear translocation of FOXO1 induce apoptosis. Western blot analysis showed that FOXO1 levels were higher in the ovary tissues of natural aged perimenopausal rats than in

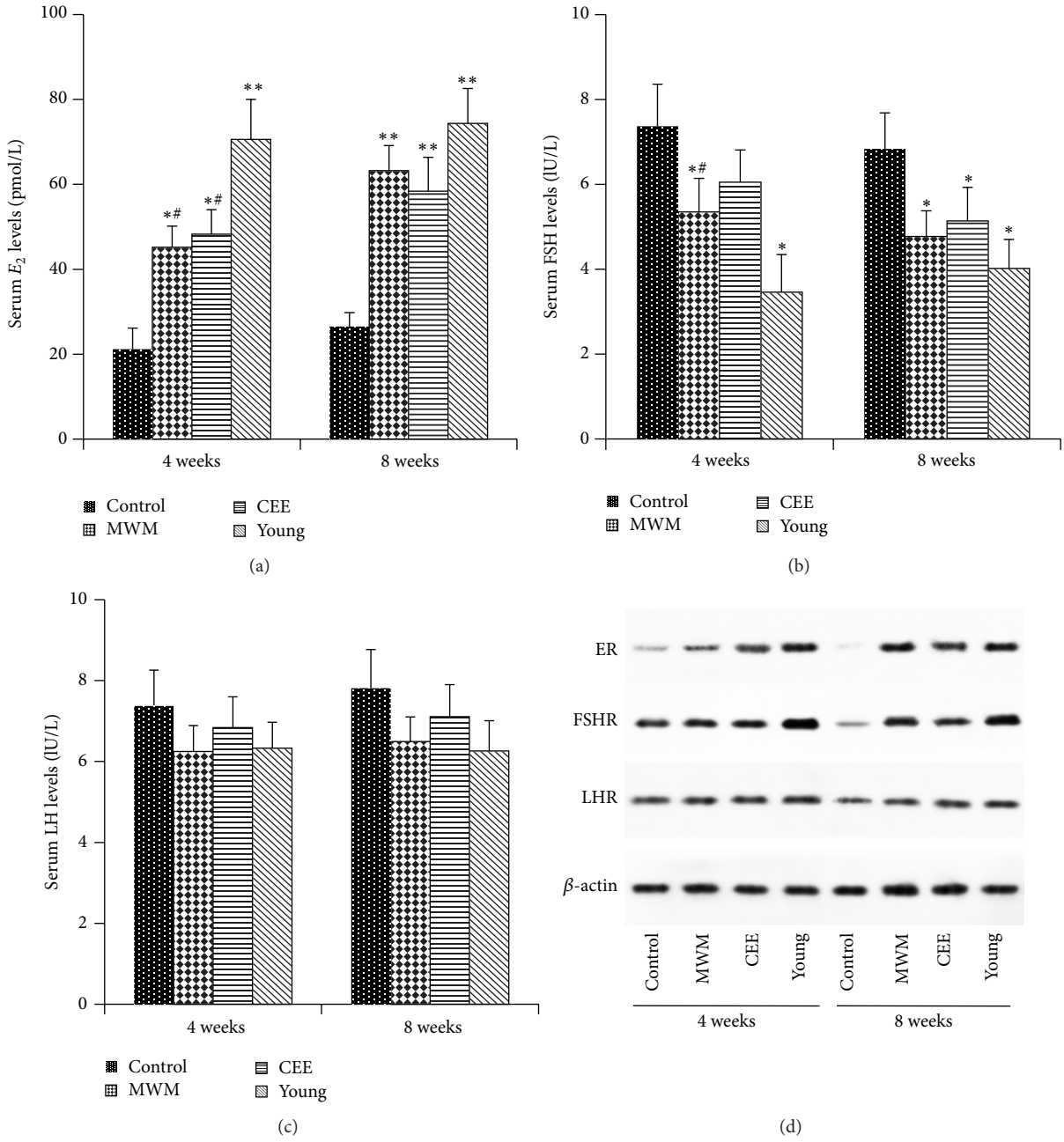


FIGURE 1: Serum levels of E_2, FSH, and LH and expression of the corresponding receptors. Serum levels of E_2 (a), FSH (b), and LH (c) were measured in control, MWM, CEE, and young groups at 4 and 8 weeks by radioimmunoassay. Data are presented as the mean ± standard error of the mean (n = 5 rats/group, 3 repetitions). ∗: versus control group, #: versus young group; $^{*,\#}p < 0.05$, $^{**}p < 0.01$.

those of young rats (Figure 4(a)). MWM and CEE markedly increased the levels of p-FOXO1 and downregulated FOXO1 expression at 4 and 8 weeks and increased the levels of phospho-JAK2 and phospho-STAT3 at 4 and 8 weeks, confirming the antiapoptotic effect of MWM in the ovaries of perimenopausal rats. In GCs cultured in medium supplemented with serum from rats in the different groups, FOXO1 overexpression reversed the effect of MWM on the inhibition of apoptosis (Figure 4(b)). Western blot analysis showed that the upregulation of Bcl-2 and the downregulation of Bax and caspase-3 induced by MWM were reversed by FOXO1 overexpression (Figure 4(c)). Similarly, FOXO1 overexpression reversed the effect of MWM on the activation of JAK2 and STAT3 phosphorylation in GCs from perimenopausal rats (Figure 4(d)). Taken together, these results indicate that the effect of MWM on the inhibition of apoptosis in GCs is mediated by the modulation of pro- and antiapoptotic proteins by the FOXO1/JAK2/STAT3 axis.

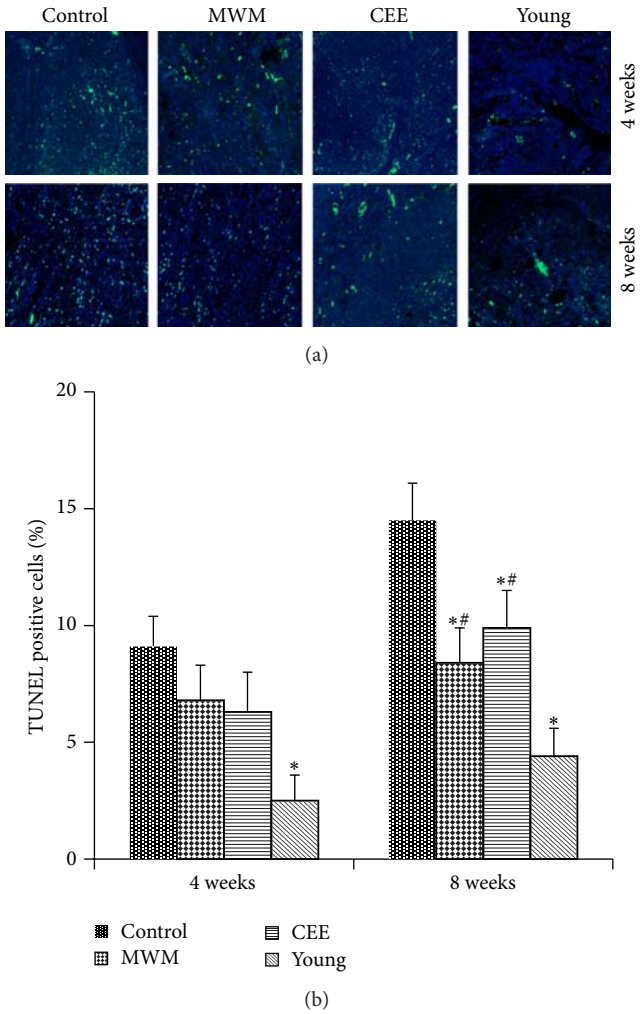


Figure 2: Effects of moxibustion on apoptosis in an *in vivo* model of perimenopause. (a) Representative images of TUNEL staining in rat ovarian tissues obtained from control, MWM, CEE, and young groups 4 and 8 weeks after the intervention (200x). TUNEL positive nuclei were visualized with fluorescein (green). Nuclei were stained with DAPI and are shown in blue. (b) Quantification of TUNEL positive cells. Data are expressed as the mean ± SD; $^{*}p < 0.05$ versus control group; $^{\#}p < 0.05$ versus young group.

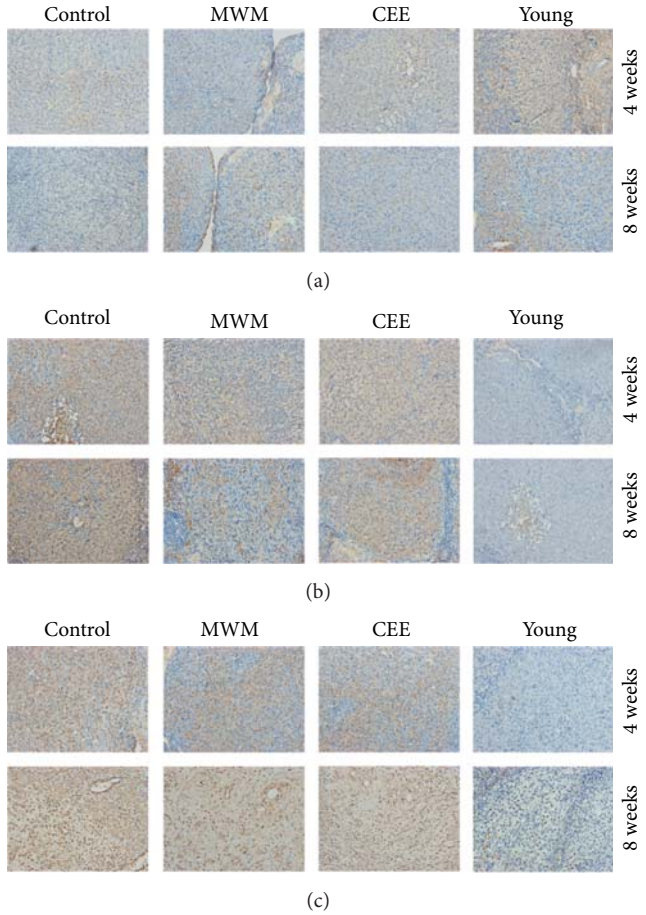


Figure 3: Immunohistochemical staining of apoptosis-related proteins in ovarian tissues. (a)–(c) Representative images of immunostaining of ovary tissues of rats in the control, MWM, CEE, and young groups against Bcl-2 (a), Bax (b), and caspase-3 (c). The antiapoptotic protein Bcl-2 shows higher staining intensity, whereas Bax and caspase-3 show lower staining intensity in MWM and CEE than in the controls at 8 weeks.

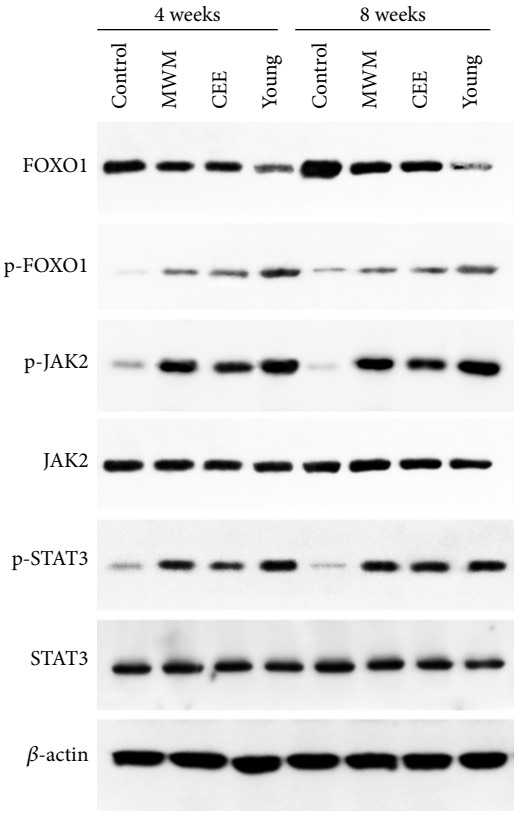
Table 1: Relative intensity of positive immunostaining of each gene in the rat ovary.

	Bcl-2	Bax	Caspase-3
4 weeks			
Control	1~2	3~4	3~4
MWM	1~2	2~3	3
CEE	1~2	2~4	3
Young	2~3	1	1
8 weeks			
Control	1	4~3	4
MWM	2	3	3
CEE	1~2	3~4	3~4
Young	2	1~2	1~2

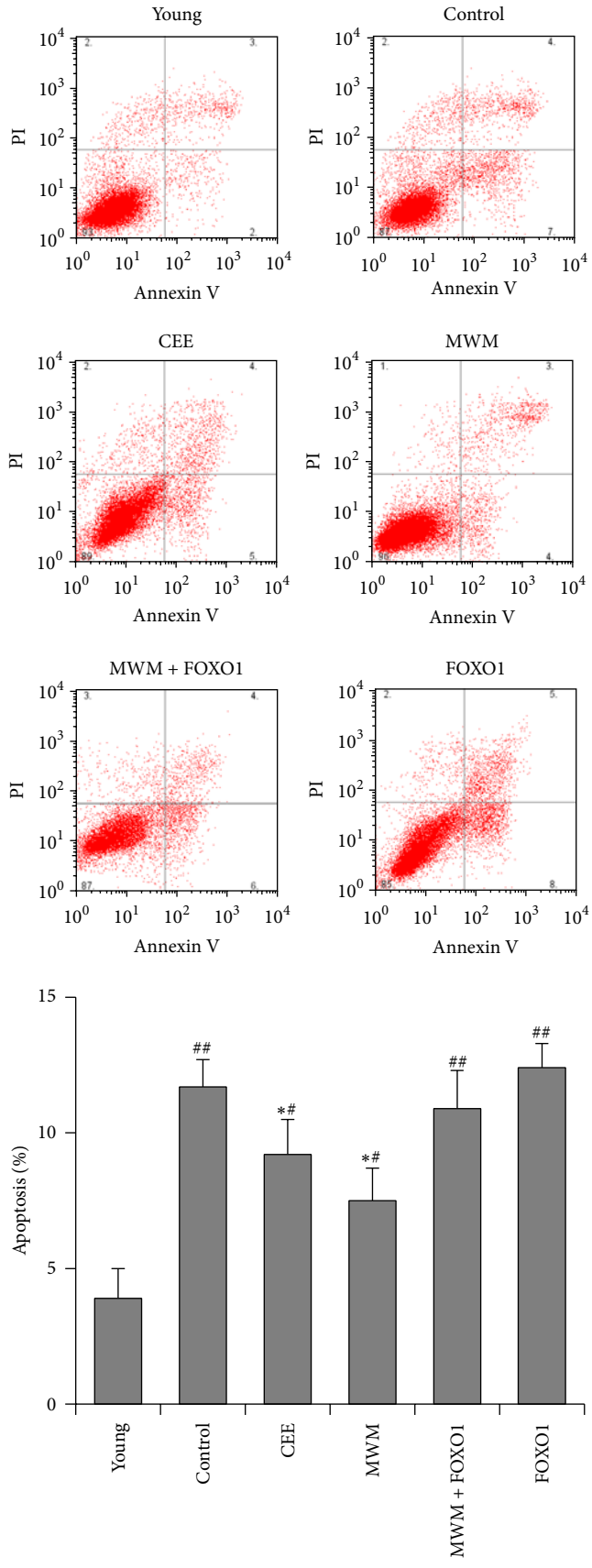
1: weak or no staining; 2: mild staining; 3: moderate staining; 4: strong staining.

4. Discussion

Follicular growth and atresia in the ovary are regulated by complex interactions between hormones, steroids, growth factors, cytokines, and proteins [17]. GCs play an essential role in follicular maintenance and atresia, as they provide molecules essential for follicular growth, and their self-killing by apoptosis is a central process leading to follicular atresia. The fate of GCs is regulated by interactions between gonadotropins (FSH and LH), and the activity of FSH in the ovaries has been shown to be partially mediated by FOXO1, suggesting that the role of GCs in the ovary involves a network of interacting molecules that includes FSH and FOXO1 [13]. In the present study, we examined the mechanism underlying the effect of MWM on the hormonal imbalance of perimenopause and elucidated a potential underlying mechanism involving FOXO1 and the JAK2/STAT3 pathway.



(a)



(b)

Figure 4: Continued.

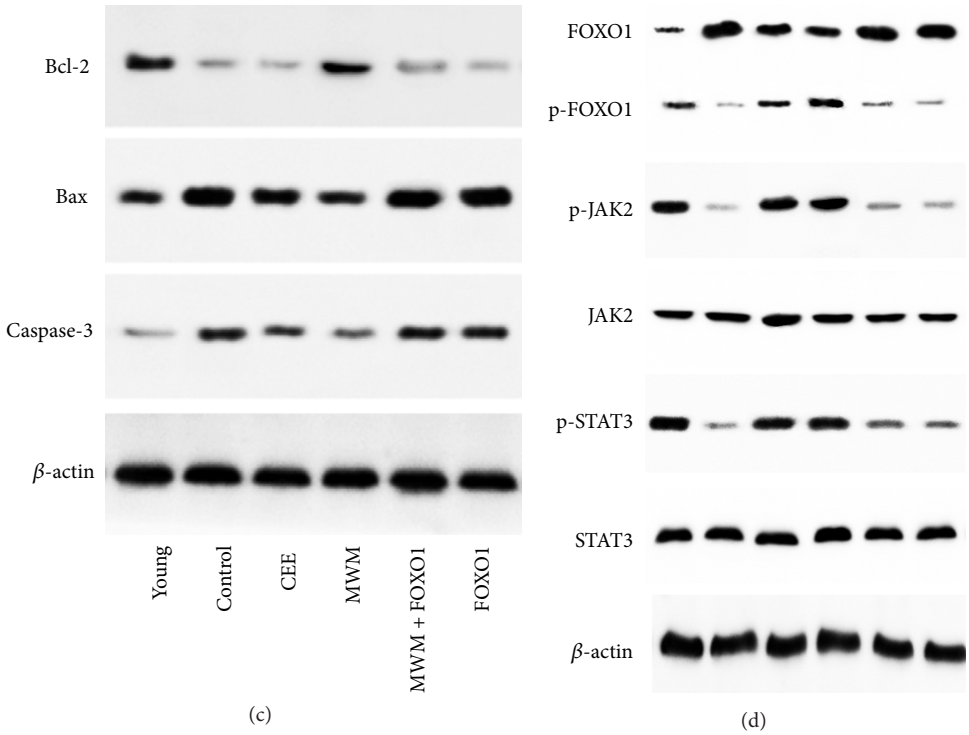


Figure 4: Involvement of FOXO1 in GC apoptosis associated with perimenopause. (a) Western blot detection of FOXO1, p-FOXO1, p-JAK2, JAK2, p-STAT3, and STAT3 protein levels in ovary tissues of natural aged perimenopausal rats. MWM downregulated FOXO1 and upregulated p-FOXO and activated the JAK2/STAT3 pathway. (b)–(d) GCs were cultured in DMEM/F12 medium containing 10% serum from the different perimenopausal model groups and young rats. (b) Effect of MWM and FOXO1 overexpression on GC apoptosis induced by natural aging rat serum assessed by flow cytometry. The rate of GC apoptosis was expressed as the mean ± SD. ∗: versus control group, #: versus young group; $^{*,\#}p < 0.05$; $^{\#\#}p < 0.01$. (c) Western blots analysis of the antiapoptotic protein Bcl-2 and the proapoptotic proteins Bax and caspase-3. (d) Western blot analysis of FOXO1, p-FOXO1, and JAK/STAT pathway protein levels. β-actin was used as the positive (loading) control. Representative images from three independent experiments are shown.

Many of the physical and psychological symptoms of perimenopause are attributed to the depletion of estrogen and the associated hormonal imbalance. Traditional Chinese medicine has been used for a long time for the treatment of gynecological disorders, and an increasing number of studies have focused on elucidating the pharmacological effects of herbal medicines. Here, we determined the effect of MWM on the serum levels of E_2, FSH, and LH in a natural aging rat model and showed that MWM increased the circulating levels of E_2 and decreased those of FSH without affecting the levels of LH, similar to the effects of CEE. Similar effects on E_2/FSH/LH levels were obtained in previous studies using a preparation of herbal medicines designated as RRF, which restored hormonal balance, alleviated reproductive organ and skeleton degeneration, and restored bone mineral density in a rat model [18, 19]. On the other hand, Danggui Buxue Tang (DBT), a traditional Chinese preparation used widely to alleviate perimenopausal symptoms, exhibits weak estrogenic properties and its activity was shown to be largely independent of the estrogen receptor in *in vivo* experiments [20]. Our present results showed that MWM upregulated the expression of the receptors for estrogen and FSH similar to the effect of CEE, suggesting that its function in the uterus is ER dependent. However, research on the mechanisms underlying the various effects of MWM is limited; therefore, further investigation of the effect of MWM on the different factors regulating follicular growth and atresia is warranted.

MWM inhibited apoptosis concomitant with the upregulation of antiapoptotic proteins and the downregulation of proapoptotic proteins in our natural aged rat model, similar to the effect of CEE. Moxibustion was previously shown to inhibit epithelial cell apoptosis by modulating Bcl-2/Bax expression in a rat model of ulcerative colitis [21]. In a rat model of Crohn's disease, moxibustion inhibited colonic epithelial cell apoptosis by downregulating tumor necrosis factor- (TNF-) alpha and TNF receptor-1 [22]. In the present study, further investigation revealed the involvement of FOXO1 and the JAK2/STAT3 pathway in the antiapoptotic effect of MWM in the ovary and the direct inhibition of GC apoptosis. FOXO1 is highly expressed in GCs, where its activity is modulated by steroid hormones [23]. FOXO1 activity is partially regulated by its Akt-mediated phosphorylation, which protects cells from apoptosis by inhibiting its transactivation of proapoptotic genes [24]. In the present study, MWM increased the levels of phosphorylated FOXO1 in ovary tissues in our aged rat model, suggesting that the effect of MWM is mediated by the regulation of FOXO1 activity. This was supported by the reversal of MWM-induced

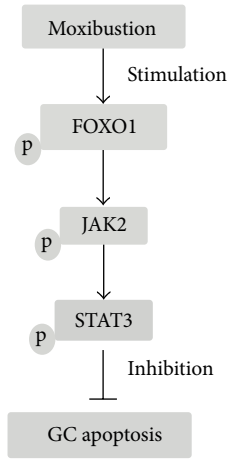


Figure 5: Role of FOXO1/JAK2/STAT3 pathway in the regulation of perimenopausal rats. MWM inhibition of apoptosis in GCs in a natural aging perimenopausal rat model, the JAK2/STAT3 pathway involved in the effect of MWM via FOXO1.

inhibition of apoptosis by FOXO1 overexpression in cultured GCs. The changes in the activation status of FOXO1 induced by MWM were accompanied by the upregulation of phospho-JAK2 and phospho-STAT3, and this effect was also reversed by FOXO1 overexpression indicating the involvement of the JAK2/STAT3 pathway in the effect of MWM via FOXO1.

FSH, which is required for the production of estrogen and the development of antral follicles, was shown to inhibit apoptosis in GCs via a mechanism involving FOXO1 [13]. The responses to FSH are achieved through the activation of several signaling cascades in GCs, including protein kinase A (PKA), protein kinase B (PKB/AKT), p38 mitogen-activated protein kinase (p38-MAPK), and extracellular signal-regulated kinases 1 and 2 (ERK1/2), which are involved in the regulation of FOXO1 activity. Our results showed that MWM modulated the levels of FSH and inhibited apoptosis via a mechanism involving FOXO1. MWM decreased the levels of circulating FSH and upregulated the expression of FSHR in the uterus, while decreasing the activity of FOXO1. However, the involvement of the PI3K/Akt pathway in modulating the activity of FOXO1 was not assessed. Therefore, the effect of MWM on FOXO1-dependent apoptosis mediated by the effect of FSH on the PI3K/AKT kinase cascade should be investigated further.

In conclusion, the present study examined the effect of moxibustion on GC apoptosis and its relation to hormonal imbalance in a natural aging perimenopausal rat model. Our results suggested that MWM inhibits GC apoptosis via a mechanism involving FOXO1 and the JAK2/STAT3 pathway. The schematic of this pathway was shown in Figure 5. However, further investigation is necessary to clarify the factors and pathways regulated by MWM, which would shed light on the mechanisms underlying the beneficial effects of moxibustion on the symptoms of perimenopause.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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