Inhibition effect of Guizhi-Fuling-decoction on the invasion of human cervical cancer

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ABSTRACT

Aim of the study: Guizhi-Fuling-decoction (GZFLD), a traditional Chinese medical formulation, exerts an anti-tumor effect, but the mechanisms of its action on invasive tumor inhibition have not been documented. The aims of this study were to identify the inhibitory effect of GZFLD on the invasive of cervical cancer and to elucidate the extensional mechanisms of its action.

Materials and method: The invasive ability of HeLa cells was tested with Transwell chamber. The expressions and activities of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) were measured by zymography/reverse zymography, RT-PCR and Western blot analysis. Establish tumor-bearing mice model to assess the ability of GZFLD to inhibit tumor growth and angiopoiesis in vivo.

Results: We have found that GZFLD suppressed the invasive ability of HeLa cells, inhibited MMPs expressions and activities, increased TIMPs expressions and activities, and furthermore restored the MMPs–TIMPs balance in HeLa cells in a concentration-dependent manner. Meanwhile in vivo, GZFLD had significantly inhibited tumor growth and angiopoiesis.

Conclusion: In general, our results showed that GZFLD had inhibited the invasion of cervical cancer both in vitro and vivo. The inhibitory effects may be associated with restoring the MMPs–TIMPs balance, and then suppressing the degradation of extracellular matrix.

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1. Introduction

Cervical cancer remains the second most common cancer worldwide among women, and the first most common cause of death of all gynecological cancers (Schoell et al., 1999). Invasion is the primary feature of cervical cancer, which frequently occurs in the early stage of cervical cancer, and the degradation of extracellular matrix (ECM) is one of the key steps in the process of cancer invasion (Simpson-Haidaris and Rybarczyk, 2001). Matrix metalloproteinases (MMPs) are a family of enzymes that are capable of degrading the constituents of ECM, and their activities are controlled by their endogenous inhibitors, which are known as the tissue inhibitors of metalloproteinases (TIMPs) (Klein et al., 2004).

It has been proposed that when MMPs increase, TIMPs decrease, the balance of MMPs and TIMPs, which is usually on a 1:1 molar basis under physiologic conditions, was broken in the tumor cells (Hojilla et al., 2003). A better understanding of the balance of MMPs and TIMPs in tumor invasion may result in the design of improved therapy.

However, no specific and effective therapy that decreases the expressions and activities of MMPs, increases the expressions and activities of TIMPs, and further restores the balance between them for cervical cancer invasion is currently available. In China, Korea, and Japan, extensive experience and abundant clinical data on the treatment of cervical cancer invasion have been documented with traditional Chinese medicine, which has been developed over a period of thousands of years (Yu et al., 1991; Deng et al., 2006).

Guizhi-Fuling-decoction (GZFLD) is a traditional Chinese medical (Kampo) formulation. The formula of GZFLD is originated from Guizhi Fuling Wan, first described in the Jingui Yaolue (220 AD) written by a famous doctor of Han Dynasty named Zhang Zhongjing. GZFLD is composed of five kinds of medicinal plants, Cinnamomum cassia BLUME (Cinnamomi Cortex), Paeonia lactiflora PALL (Peonies Radix), Paeonia suffruticosa ANDREWS (Moutan Cortex), Prunus persica BATSCH (Persicae Semen), and Poria cocos WOLF (Hoelen).

Previous studies have revealed that GZFLD could inhibit the development of cancer (Wang et al., 2004). Clinically, Guizhi Fuling Wan (Keishi-bukuryogan in Japanese) has been used to treat gynecological malignant tumor in China, which promoted softening and absorption of the proliferative lesion, elevated immunity, and enhanced chemotherapeutics efficacy. However, the anti-cancer
Fig. 1. Effect of Guizhi-Fuling-decoction (GZFLD) on cervical cancer HeLa cells invasion. Cells were incubated with the following chemoattractants for 24 h: 3.125 mg/l DDP as positive control (DDP), 0 mM GZFLD as blank control, 10 mg/l GZFLD (GFD1), 5 mg/l GZFLD (GFD2), and 2.5 mg/l GZFLD (GFD3). Cells were stained and the number of cells invading the lower side of the filter was measured and taken as the invasive activity. (A–E) Microphotographs of invasive HeLa cells, 40-fold. (F) The number of invasive cells. Data are expressed as mean cells invaded ± S.D. of at least three independent repeated experiments. Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments as P<0.05.
molecular mechanism of GZFLD was not fully elucidated. The inhibitory effects of GZFLD on the invasion of cervical cancer have not yet been studied.

Accordingly, in the following study, we have used human cervical cancer HeLa cells and HeLa cell xenografts in nude mice as our study model, to investigate the changes of MMPs and TIMPs when both of them were treated with GZFLD. Our study results had demonstrated that GZFLD could affect the expressions and activities of MMPs and TIMPs, restore MMPs–TIMPs balance, and thus, suppress the invasive ability and tumor growth of cervical cancer.

2. Materials and methods

2.1. Reagents

*Cinnamonum cassia* BLUME (*Cinnamomi Cortex*), *Paeonia lactiflora* PALL (Peonies Radix), *Paeonia suffruticosa* ANDREWS (Moutan Cortex), *Prunus persica* BATSCH (Persicae Semen), and *Poria cocos* WOLF (Hoelen) were kindly provided by Liaoning University of Traditional Chinese Medicine (Shenyang, China). Equal doses of the above each pharmaco were added with suitable water soaked for 2 h, and then decocted in slow fire followed by strong fire for twice. After using filtering method removed the residues, condensed the decoction to 1 mg/ml by water bath, and regulated the pH to 7.0. At last the GZFLD was stored away from light exposure at 4 °C. Working dilutions were made directly in the cell culture medium. RPMI 1640 was from Gibco Life Technologies (Burlington, Ontario, Canada); fetal calf serum was purchased from Hangzhou Season's Co. (Hangzhou, China); total RNA extracting reagent kit and AMV one-step RT-PCR system were purchased from Takara Biotechnology (Dalian, China); Rabbit polyclonal antibody against MMP-2, MMP-9, TIMP-1, TIMP-2, CD34 and β-actin were purchased from Boster Biotechnology (Wuhan, China); Transwell plate, cell culture flasks and 6-, 24-, 96-well cell culture plates were from Corning Chemical Co. (New York, USA); All other chemical reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

Human cervical carcinoma cell line HeLa was kindly provided by the research center of medical genomics of China Medical University. Human leukemia cell line U937 and mouse normal fibroblast cell line NIH 3T3 were purchased from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in a humidified incubator containing 95% air, 5% CO2 at 37 °C. Cells were incubated with the following as chemoattractants for 24 h: 3.125 mg/l DDP as positive control (DDP), 0 mg/l GZFLD as blank control (Control), 10 mg/l GZFLD (GFD1), 5 mg/l GZFLD (GFD2), and 2.5 mg/l GZFLD (GFD3). Medium was collected and loaded into a gelatin-containing zymogram gel. Levels of detection represent the zymogen activities of MMP-2 and MMP-9 in HeLa cells. MMPs bands are noted in (a). Densitometric analysis is shown in (b). Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments (P < 0.05). All the data listed above represents the results of three independent repeated experiments.

2.3. Transwell chambers analysis on the effects of GZFLD on HeLa cells invasion

The inhibitory effect of GZFLD on cell invasion was demonstrated in modified Transwell chambers. Polyvinyl pyrrolidone-free polycarbonate filters (pore sizes of 8 μm) were coated with matrigel basement membrane matrix diluted to 1 μg/μl with 100 μl PBS per well, incubated at 37 °C for 24 h, and then reconstituted with serum-free medium. Confluent cells were exposed to GZFLD (0 mg/l, 2.5 mg/l, 5 mg/l, and 10 mg/l) and diaminodichloroplatinum (DDP) (3.125 mg/l) for 24 h. The cells at the density of 5 × 10^6 cells/ml were cultured on serum-free medium, and then added to the upper chamber. The lower chamber contained 250 μl complete medium and 250 μl supernatant of rat NIH3T3 cells cultured on serum-free medium (as a chemo attractant). Specimens in the chambers were incubated for 12 h in 5% CO2–95% air at 37 °C. Following incubation, media from the upper chamber was removed and cells on the upper surface were carefully rinsed and then removed with a cotton swab. The chambers were fixed in 10% formalin and stained with H&E solution following a gentle rinse in PBS solution. Invading cells were counted from four random fields of view from three independent experiments using an Olympus AX80 microscope equipped with a DP-70 digital camera and a computer equipped with NIH Image 1.61, and values shown are the mean number of cells from an equal number of view fields.

Table 1

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>Sense 5'-GGG ACA AGA ACC AGA TCA CAT AC-3'</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Sense 5'-CTT CTC AAA GGT GTA GGT GGT GC-3'</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Sense 5'-CTC TTC TTC TGG TGG TGG TGG TCA-3'</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Sense 5'-CTC TTC AGA CCC TGG TGG GAC-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense 5'-GGT GGG CCC AGG CAC CA-3'</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of Guizhi-Fuling-decoction (GZFLD) on MMP-2 and MMP-9 activities in cervical cancer HeLa cells. Cells were incubated with the following as chemoattractants for 24 h: 3.125 mg/l DDP as positive control (DDP), 0 mg/l GZFLD as blank control (Control), 10 mg/l GZFLD (GFD1), 5 mg/l GZFLD (GFD2), and 2.5 mg/l GZFLD (GFD3). Medium was collected and loaded into a gelatin-containing zymogram gel. Levels of detection represent the zymogen activities of MMP-2 and MMP-9 in HeLa cells. MMPs bands are noted in (a). Densitometric analysis is shown in (b). Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments (P < 0.05). All the data listed above represents the results of three independent repeated experiments.
Fig. 3. Effect of Guizhi-Fuling-decoction (GZFLD) on MMP-2 and MMP-9 expressions of mRNA and protein in cervical cancer HeLa cells. Cells were incubated with the following as chemoattractants for 24 h: 3.125 mg/l DDP as positive control (DP), 0 mg/l GZFLD as blank control (C), 10 mg/l GZFLD (G1), 5 mg/l GZFLD (G2), and 2.5 mg/l GZFLD (G3). Total RNA of HeLa cells was isolated and RT-PCR analysis was performed. Levels of detection represent the expressions of MMP-2 and MMP-9 in HeLa cells are noted in (a). Densitometric analysis is shown in (b). Total HeLa cells lysates were blotted with anti-MMP-2 and anti-MMP-9 antibody with Western blot assay. Immunoreactive bands are noted in (c). Densitometric analysis is shown in (d). Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate no significant differences among those treatments ($P<0.05$). All the data listed above represents the results of three independent repeated experiments.

2.4. RT-PCR analysis on the effects of GZFLD on MMP-2, MMP-9, TIMP-1, and TIMP-2 mRNA expression

Semiquantitive reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was used to measure mRNA expression. Briefly, after pretreatment with GZFLD and DDP, HeLa cells and xenografts tissue were collected and their total RNA was extracted using Trizol reagent according to its manufacturer’s instruction. cDNA synthesis and subsequent PCR reaction were performed using Superscript II One-Step system in a volume of 25 μl according to the system's manufacturer’s instruction. The cycling conditions set for RT-PCR were as follows: 50 °C for 30 min (reverse transcription), 94 °C for 2 min (pre-denaturation), followed by 25 cycles of PCR amplification process including denaturing at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 45 s, and by 1 cycle of final extension at 72 °C for 10 min. The sequences of the PCR primers for MMP-2, MMP-9, TIMP-1, TIMP-2, and β-actin were shown in Table 1. PCR products were separated with 1.5% agarose gel electrophoresis. Gels were stained with 0.5 μg/ml solution of ethidium bromide for 30 min followed by subsequent 30 min destained with water. Gel images were scanned using an image analysis system; the intensities of specific PCR bands were quantitated in relation to β-actin bands from the same sample.

2.5. Western blot analysis on the effects of GZFLD on MMP-2, MMP-9, TIMP-1, and TIMP-2 protein expression

Western blotting analysis was used to measure the expression of proteins. Briefly, equal protein concentrations of conditional culture medium and xenografts tissue protein extract (50 μg) were loaded onto SDS-polyacrylamide gels and run at 40 mA. Then electrophoretically, separated polypeptides were transferred onto PVDF membranes by electroblotting, and the membranes were incubated in blocking buffer (5% skim milk in TBS) overnight. After washing, the membranes were incubated with specific primary antibodies against MMP-2, MMP-9, TIMP-1, TIMP-2, and β-actin (1:200 dilutions) for 1 h at room temperature. Blots were rinsed three times in TBS and 0.05% Tween-20 for 10 min each before adding secondary antibodies diluted in blocking buffer for 3 h at room temperature. Finally, after washing three times, bands were visualized using enhanced chemiluminescence. Comparison between different treatment groups was made by determining the
specific protein/β-actin ratio of the immunoreactive area with densitometry.

2.6. Gelatin zymography analysis on the gelatinolytic activities of MMP-2 and MMP-9

The gelatinolytic activities of secreted MMP-2 and MMP-9 were analyzed with zymography on gelatin-containing polyacrylamide gels. For this purpose, equal protein concentrations of conditional culture medium were loaded onto a 10% SDS-polyacrylamide gel that contained 1 mg/ml gelatin and subjected to electrophoresis. After electrophoresis, the gels were washed in 50 mM Tris buffer that contained 2.5% Triton X-100. The gels were incubated for an additional 24 h in 50 mM Tris buffer (pH 7.4) that contained 5 mM CaCl₂, 0.02% NaN₃, and 2 μM ZnCl₂. After that, the gels were stained with Coomassie Blue and subsequently destained in an equilibrated buffer (3% glycerol, 10% acetic acid and 40% methanol). Proteins having gelatinolytic activity were visualized as clear zones in an otherwise blue gel. Gels were scanned, and density analysis of the bands was performed using NIH-Image.

2.7. Reverse zymography analysis on the enzyme activities of TIMP-1 and TIMP-2

Reverse zymography was used to detect TIMP-1 and TIMP-2 activities. For this purpose, equal protein concentrations of conditional culture medium were then subjected to SDS–10% polyacrylamide gel electrophoresis on gels containing 1 mg/ml of gelatin. After electrophoresis was completed, gels were washed in 2.5% Triton X-100 and incubated for 1 h at 37 °C with conditioned media from phorbol 12-myristate 13-acetate (PMA)-activated U937 cells, containing a mixture of activated MMPs. Then gels were incubated with 50 mM Tris–HCl, 50 mM NaCl, 10 mM CaCl₂, and 0.05% Brij for 18 h at 37 °C, stained in Coomassie Blue, and destained in acetic acid/methanol. Under these conditions, TIMPs inhibit gelatin digestion by activated MMPs and produce dark blue bands against a
bright background. For quantification, densitometric scanning was performed by using a Bio-Rad GS 690 Image Analysis software system.

2.8. MTT analysis on the effects of GZFLD on HeLa cells proliferation

The effect of GZFLD on cell proliferation was measured using an MTT-based assay. Briefly, the cells (10^4/well) were incubated in triplicate in a 96-well plate in the presence of various concentrations of GZFLD (1.25 mg/l, 2.5 mg/l, 5 mg/l, 10 mg/l, 20 mg/l, 40 mg/l) for 24 h. Thereafter, 20 μl of MTT solution (5 mg/ml) was added to each well and then incubated for 4 h at 37 °C. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved in 200 μl of DMSO and then the optical density (OD) value was measured at 490 nm by a multiscanner autoreader. The following formula was used: Inhibition rate (%) = (1 − OD of the experimental samples)/OD of the control × 100%.

2.9. HeLa cell xenografts in nude mice

Female BALB/c- nu mice, ages 4–6 weeks, were purchased from Institute of Laboratory Animal Sciences of Chinese Academy of Medical Sciences (Peking, China). The treatment protocol followed the guidelines of animal experimentation adopted by the Centre Animal Sciences of China Medical University. Mice were given injections subcutaneously into left infer-armpit with 1 × 10^7 viable HeLa cells, as determined by trypan blue staining, resuspended in 200 μl PBS. After 10 days, when tumors of 0.6–0.7 cm³ in volume were detectable, mice were randomized in five groups (n = 10): (1) GZFLD 20 mg/kg, (2) GZFLD 10 mg/kg, (3) GZFLD 5 mg/kg, (4) DDP 10 mg/kg as positive control and (5) 0.9% NS as blank control and were administered intraperitoneally once daily for the following 14 days consecutively. We sacrificed the mice on the 15th day after beginning intratumoral injections and removed the tumors for immunohistochemical. Tumor volume measurements were taken every 3 days using calipers and were calculated using the formula: π/6 × larger diameter × (smaller diameter^2).

2.10. Immunohistochemical and morphometric analyses

Tumors were fixed in 10% formalin solution, paraffin-embedded, and cut into 5-mm-thick sections. Immunohistochemistry was carried out using CD34 (0.2 Ag/ml). Tissue sections were incubated with primary antibodies for 3 h at room temperature, followed by incubating with biotin-conjugated secondary antibodies for 30 min. Streptavidin–horseradish peroxidase conjugate was added and the peroxidase activity was made visible with iaminobenzidine. Counterstaining was done with hematoxylin. Morphometric analysis was done in five 20× fields in each tumor to determine the area occupied by CD34-positive endothelial cells using Image-Pro Plus software version 5.1 (Media Cybernetics, Silver Spring, MD). The vasculature density was calculated as the ratio of the area occupied by endothelial cells divided by the total area.

2.11. Data analysis

The results were expressed with mean ± S.D. All the data were processed with SPSS13.0 software. One-factor variance analysis was
used in comparison of several groups. The comparison of time course of tumor growth curves by two-way ANOVA with group and time. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of GZFLD on the invasiveness of HeLa cells

The effect of GZFLD on the ability of cells to invade through reconstituted basement membranes was analyzed using matrigel-coated Transwell chambers, as described in Section 2. Incubation with GZFLD inhibited HeLa cells invasion in a dose-dependent manner (Fig. 1). The numbers of invasive cells treated with 10 mg/l and 5 mg/l GZFLD were $9.00 \pm 1.58$ and $9.60 \pm 2.07$, respectively, and showed significant decrease ($P < 0.05$), if compared with of the vacant control group ($40.00 \pm 3.81$), however, they were similar with of the DDP group ($9.40 \pm 1.95, P > 0.05$).

3.2. Effect of GZFLD on MMPs mRNA, protein and enzyme activities expressions in HeLa cells

We have examined the important role of GZFLD in the regulation of MMPs activities, and found out that GZFLD at 5 mg/l and 10 mg/l did significantly suppress MMP-2 and MMP-9 activities with zymography assay ($P < 0.05$) (Fig. 2). At the same time, MMP-2 and MMP-9 mRNA and protein expressions were significantly inhibited by GZFLD at the concentrations of 5 mg/l and 10 mg/l compared with the blank control group, but there were no statistical significances about the differences among 5 mg/l and 10 mg/l GZFLD group and DDP group ($P > 0.05$) (Fig. 3).

3.3. Effect of GZFLD on TIMPs mRNA, protein and enzyme activities expressions in HeLa cells

TIMPs are preferably bound to the active center and inhibit MMPs protease activity. The balance between both molecules finally determines the net proteolytic activity. To examine whether GZFLD had decreased MMPs and increased the expressions and activities of their natural inhibitors or not, we have measured both TIMP-1 and TIMP-2 mRNA, proteins and enzyme activities. As shown in Fig. 4, in contrast to the down-regulated MMPs levels, TIMPs were slightly increased by GZFLD from 5 mg/l in HeLa cells compared with the blank control group, but there were no statistical significances about the differences among 5 mg/l, 10 mg/l GZFLD group and DDP group ($P > 0.05$).

3.4. Effect of GZFLD on restoring the MMPs–TIMPs expression imbalances in HeLa cells

There were some investigations indicated that the MMP:TIMP ratio in cancer cells was approximately 1 corresponded to those cancers of good prognosis. Here, we have examined the expression ratio of MMPs:TIMPs in cervical cancer HeLa cells treated with GZFLD (0 mg/l, 2.5 mg/l, 5 mg/l, and 10 mg/l) and 3.125 mg/l DDP. As shown in Fig. 5, the mRNA, proteins, and enzyme activities expression levels of MMP-2 and MMP-9 significantly decreased in 5 mg/l and 10 mg/l GZFLD group when compared with the untreated group. However, the mRNA, proteins, and enzyme activities expression levels of TIMP-1 and TIMP-2 were increased under the 5 mg/l and 10 mg/l GZFLD condition. Therefore, the ratio of MMP:TIMP reduced followed by the increased concentration of GZFLD. These results strongly suggest that GZFLD causes a recuperation of the molar ratio imbalance between MMPs and TIMPs by decreasing the expressions of MMPs but augmenting the expressions of TIMPs. These phenomena indicate that GZFLD in cervical carcinoma results in a shift of the balance between MMPs and TIMPs.

3.5. Effect of GZFLD on suppressing proliferation of HeLa cells

The effects of Guizhi-Fuling-decoction (GZFLD) on suppressing proliferation of HeLa cells line determined by MTT assay are shown in Fig. 6. HeLa cells were cultured in complete medium in the absence (control) or presence of various concentrations of GZFLD (1.25 mg/l, 2.5 mg/l, 5 mg/l, 10 mg/l, 20 mg/l, 40 mg/l) for 24 h. Untreated cells (control) were considered as the baseline (100%) for the analysis. GZFLD inhibited cervical cancer HeLa cell growth, and the inhibition was concentration dependent. This was evident at a concentration as low as 2.5 mg/l (9.78%). Maximum inhibition (63.69%) was observed at 40 mg/l GZFLD. So from the results of MTT assay we could deem
that the concentrations of GZFLD in the above experiments had the effects only a little.

3.6. Effect of GZFLD on reducing HeLa xenograft tumor growth and invasion

Given the ability of GZFLD to suppress cervical cancer cell growth and invasion in vitro, we examined whether GZFLD could inhibit cervical carcinoma growth and invasion in vivo. Continuous daily administered intraperitoneally with GZFLD, starting 10 days after HeLa cells injection, was analyzed on nude mice bearing HeLa cervical carcinoma xenografts. Intraperitoneal administration of 20 mg/(kg d) and 10 mg/(kg d) GZFLD significantly \( (P < 0.05) \) reduced the tumor growth compared with controls receiving 0.9% NS alone (Fig. 7a and b). Mice treated with GZFLD showed a 71.41% and 55.05% reduction in tumor volume and weight, respectively. As shown in Fig. 8, 20 mg/(kg d) and 10 mg/(kg d) GZFLD caused a significant reduction of MMP-2 and MMP-9 expressions at both mRNA \( (P < 0.05) \) and protein \( (P < 0.05) \) levels as evaluated by RT-PCR and Western blot analysis on multiple samples \( (n = 10) \) of xenografts. We also observed a marked elevation of TIMP-1 and TIMP-2 mRNA and protein expressions in animals that received 20 mg/(kg d) and 10 mg/(kg d) GZFLD (Fig. 9).

To determine whether the reduction in tumor growth was associated with a reduction of angiogenesis, tissue sections of cervical tumors on day 25 after tumor cell injection were analyzed by immunohistochemistry (Fig. 10a). As summarized in Fig. 10b, tumor induced vascularization, which was quantified as microvesSEL density expression, in untreated xenografts were significantly \( (P < 0.05) \) higher than the corresponding values in 20 mg/(kg d) and 10 mg/(kg d) GZFLD-treated xenograft. These results suggest that intraperitoneal administration of GZFLD induces cervical tumor growth and invasion inhibition that is associated with changed MMPs and TIMPs expressions and decreased the angiogenesis in vivo.

4. Discussion

According to preliminary test, GZFLD at the doses of 2.5 ml/l, 5 ml/l and 10 mg/l were used in this study. The data presented in this work demonstrated that GZFLD could markedly decreased the invasive ability of human cervical cancer HeLa cell line, and the effect of treatment with GZFLD on decreasing invasive ability at doses of 5 ml/l and 10 mg/l were better than that of 2.5 ml/l \( (P < 0.05) \). Furthermore, analysis of matrix metalloproteinases and their tissue inhibitors in cervical cancer HeLa cell line showed that this inhibition was associated with significant down-regulated expressions of matrix metalloproteinases including MMP-2 and MMP-9 and up-regulated expressions of tissue inhibitor of metalloproteinase TIMP-1 and TIMP-2, both in mRNA and protein levels. The current study also showed the activities of these enzymes were regulated in the same way. Meanwhile, in this investigation, we also found that the ratio of MMP:TIMP reduced followed by the concentration of GZFLD raised. These results suggested that GZFLD inhibited cervical cancer HeLa cell line invasion by decreasing the expressions and activities of MMP-2 and MMP-9, increasing the expressions...
Fig. 8. Effect of GZFLD on MMP-2 and MMP-9 expressions of mRNA and protein of HeLa human cervical carcinoma xenografts. Mice were given injection of $1 \times 10^7$ HeLa cells subcutaneously into left infra-armpit. After 10 days, mice received intraperitoneal administration of 0.9% NS as blank control (C), 20 mg/kg GZFLD (G1), 10 mg/kg GZFLD (G2), 5 mg/kg GZFLD (G3) and 10 mg/kg DDP as positive control (DP) for 14 days. Each group consisted of 10 mice. The mice were sacrificed on the 15th day after beginning intratumoral injections and tumors were removed from control or GZFLD-treated mice. The effects of the treatment with GZFLD on MMPs mRNA and protein expression were measured in multiple samples ($n=10$) of xenografts by RT-PCR and Western blot (a and c), respectively. Densitometric analysis was evaluated by Scion image and results were expressed as relative units (b and d). Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments ($P<0.05$).

and activities of TIMP-1 and TIMP-2, restoring the MMPs–TIMPs balance, and thus protecting extracellular matrix from degrading.

Guizhi-Fuling-decoction (Keishi-bukuryogan in Japanese) is a traditional Chinese medical (Kampo) formulation, composed of five kinds of medicinal plants, *Cinnamomum cassia* BLUME (*Cinnamomi Cortex*), *Paeonia lactiflora* PALL (*Peonies Radix*), *Paeonia suffruticosa* ANDREWS (*Moutan Cortex*), *Prunus persica* BATSCH (*Persicae Semen*), and *Poria cocos* WOLF (*Hoelen*). In the theory of traditional Chinese medical, *Cinnamomum cassia* BLUME plays the most important role in the formulation, and the other four components in coordination with *Cinnamomum cassia* BLUME jointly exert the anti-tumor effect. But the effects of the formulation are not only the simple addition of the effect of the five components respectively. There were small or even no anti-cancer effects when using each composition of the formulation singly, but it was contrary that the five compositions compatibly applying in an appreciable proportion may exert stronger effects on anti-cancer not only in vitro as delineation as above, but also in vivo GZFLD would inhibit the tumor growth rate of tumor-bearing mice model, and the growth inhibiting rate could reach 71.41%.

Cancer cell–matrix interaction is a critical step that promotes cell migration, extracellular matrix degradation, and even cell invasion (Stetler-Stevenson et al., 1993). The proteolytic degradation of extracellular matrix is a critical event during tumor invasion. Although the breakdown of the basement membrane is achieved by several MMPs, MMP-2 and MMP-9 appear to be the most important for basement membrane type IV collagen degradation (Seiki, 1999). Similar to various malignant tumors, human cervical cancer HeLa cells express MMP-2 and MMP-9 at a high level (Garzetti et al., 1996; Davidson et al., 1999). Overexpression of MMP-2 or MMP-9 exacerbates the ECM degrading of invasive cervical cancer; whereas their inhibition has been reported to attenuate the ECM degraded process (Nouvo et al., 1995; Garzetti et al., 1996; Gilles et al., 1996; Davidson et al., 1999; Asha-Nair et al., 2003).
Fig. 9. Effect of GZFLD on TIMP-1 and TIMP-2 expressions of mRNA and protein of HeLa human cervical carcinoma xenografts. Mice were given injection of $1 \times 10^7$ HeLa cells subcutaneously into left infer-armpit. After 10 days, mice received intraperitoneal administration of 0.9% NS as blank control (C), 20 mg/kg GZFLD (G1), 10 mg/kg GZFLD (G2), 5 mg/kg GZFLD (G3) and 10 mg/kg DDP as positive control (DP) for 14 days. Each group consisted of 10 mice. The mice were sacrificed on the 15th day after beginning intratumoral injections and tumors were removed from control or GZFLD-treated mice. The effects of the treatment with GZFLD on TIMPs mRNA and protein expression were measured in multiple samples ($n = 10$) of xenografts by RT-PCR and Western blot (a and c), respectively. Densitometric analysis was evaluated by Scion image and results were expressed as relative units (b and d). Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments ($P < 0.05$).

The study, the expressions and activities of MMP-2 and MMP-9 were both significantly down-regulated by GZFLD in a dose-dependence manner. This finding indicates that the inhibited effect of GZFLD on cervical cancer invasion is likely attributable to its anti-matrix metalloproteinases properties.

The controls of MMPs include genes transcribing, proteins translating, the activation of these degradative enzymes, and the production of their natural inhibitors TIMP-1 and TIMP-2 that could be bound with the active site of MMPs in the ratio of 1:1 what followed next inhibited the activation of MMPs and kept the dynamic equilibrium with MMPs. Indirect evidence and direct experimental data exist to suggest that the impairment of the balance of matrix metalloproteinases and their tissue inhibitors may result that the expressions and activities of MMPs were increased so that the cancer is progressing and a more severe outcome of cancer is emerging (Brew et al., 2000). The invasion of malignant tumor cells was inhibited by exogenously administering or over-expressions TIMP-1 and TIMP-2 in vivo and in vitro (Noël et al., 1998; Kruger et al., 1998; Sato et al., 1999). Furthermore, reduced expressions of TIMPs are observed concomitant with augmenting the production of MMPs in cervical cancer tissues, suggesting a host response to tumor invasion and metastasis in an effort to regulate the activity of MMPs for preventing ECM breakdown (Westermarck and Kähäri, 1999). Our present results also showed that productions of TIMP-1 and TIMP-2 were increased significantly and the invasive ability of cervical cancer HeLa cells was inhibited markedly in GZFLD-treated cells, suggesting a potential involvement of the anti-cancer effect of TIMPs in GZFLD treatment. To combine together with the effects of GZFLD on the decreased expression activities of MMPs, we found that GZFLD could recover the balances of the molar ratio between MMPs and TIMPs which may contribute to inhibit the progression of invasiveness of cervical carcinoma cells in vitro. Moreover, the...
action of GZFLD on the microvessel formation of HeLa human cervical carcinoma xenografts. Mice were given injection of 1 × 10^6 HeLa cells subcutaneously into left infer-armpit. After 10 days, mice received intraperitoneal administration of 0.9% NS as blank control, 20 mg/kg GZFLD (GFD1), 10 mg/kg GZFLD (GFD2), 5 mg/kg GZFLD (GFD3), and 10 mg/kg DDP as positive control (DDP) for 14 days. Each group consisted of 10 mice. The mice were sacrificed on the 15th day after beginning intratumoral injections and tumors were removed from control or GZFLD-treated mice. Immunohistochemical analysis of CD34 expression in HeLa tumor xenografts (a). Original magnification, 20 ×. (b). Quantitative assessment of immunohistochemical analysis for microvessel density. Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments (P < 0.05).

**Fig. 10.** Effect of GZFLD on the microvessel formation of HeLa human cervical carcinoma xenografts. Mice were given injection of 1 × 10^6 HeLa cells subcutaneously into left infer-armpit. After 10 days, mice received intraperitoneal administration of 0.9% NS as blank control, 20 mg/kg GZFLD (GFD1), 10 mg/kg GZFLD (GFD2), 5 mg/kg GZFLD (GFD3), and 10 mg/kg DDP as positive control (DDP) for 14 days. Each group consisted of 10 mice. The mice were sacrificed on the 15th day after beginning intratumoral injections and tumors were removed from control or GZFLD-treated mice. Immunohistochemical analysis of CD34 expression in HeLa tumor xenografts (a). Original magnification, 20 ×. (b). Quantitative assessment of immunohistochemical analysis for microvessel density. Treatments denoted by the same letter indicate no significant differences among those treatments. Treatments denoted by different letters indicate significant differences among those treatments (P < 0.05).

In summary, GZFLD has inhibition effects on invasive ability of human cervical cancer HeLa cell line. Part of the mechanism is that GZFLD can enhance the expressions and activities of TIMPs and down-regulate the expressions and activities of MMPs. These results also suggested that the expressions aimed at down-regulating MMPs, particularly up-regulating TIMPs, would eventually become an effective therapeutic strategy for the treatment of invasive cervical cancer.

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**References**


