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Original Research

Effect of isofebrifugine on the proliferation and invasion of human gastric cancer cells via MMP

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Abstract: Gastric cancer (GC) is one of the most common fatal cancers among gastrointestinal malignancies. At present, the treatment of gastric cancer involves a combination of surgery and chemotherapy. Isofebrifugine (IFE) is an alkaloid with many biological properties. In this study, results from MTT, scratch and invasion assays showed that IFE significantly inhibited the proliferation, migration and invasion of SGC7901 gastric cancer cells. Through RT-PCR and Western blot experiments, it was revealed that IFE significantly inhibited the mRNA and protein expressions of MMP-2, MMP-9 and SDF-1 which are closely related to cancer invasion and metastasis. Thus, IFE possesses anti-gastric cancer properties.

Key words: Isofebrifugine; Proliferation; Invasion; Gastric cancer; MMP.

Introduction

Gastric cancer (GC) is a fatal epithelial malignant tumor of the digestive tract, and the second leading cause of cancer-related death in the world (1, 2). Due to continuous progress in medical research, great achievements have been made in the treatment of gastric cancer through early diagnosis/operation, radiotherapy and chemotherapy. However, although the prognosis of GC patients has been improved, the 5-year survival is still very low (28-31%) due to the high invasion and metastasis capacities of gastric cancer which limit the effect of treatment (3, 4). The prognosis of metastatic gastric cancer is poor, and the median survival time is about 12 months (5). Therefore, there is need for a safe, efficient and low-toxicity therapeutic drugs, to reduce the mortality of clinical gastric cancer patients. In recent years, natural drugs have become the research focus for cancer treatment because of their high safety, as well as multi-level, multi-target and multi-link treatments (6, 7).

Changshan (*Dichroa febrifuga* Lour.) is the dry root of Saxiaceae Changshan which contains mainly alkaloids, coumarins, steroids, polyphenols and other chemical constituents. Isofebrifugine (IFE) which is one of its main alkaloids, has a variety of biological effects, including anti-malaria, anti-tumor, anti-inflammation, and wound-healing properties (8-11). Studies have shown that IFE is used to antagonize bladder, prostate, breast, skin and lung tumors through inhibition of tumor angiogenesis, proliferation and migration (11-14). However, there are few studies on its anti-gastric cancer properties. In this study, the inhibitory effect of IFE on the proliferation and invasion of human gastric cancer SGC-7901 cells was studied, and its related molecular mechanism was elucidated.

Materials and Methods

Cell lines, drugs, equipment and major reagents

Gastric cancer cell SGC-7901 was purchased from Shanghai cell bank of Chinese Academy of Sciences. Isofebrifugine was bought from Chengdu Ruifensi Biotechnology Co. Ltd; RPMI-1640 complete culture solution and FBS were products of Gibco Biological Co., Ltd, while dimethyl sulfoxide (DMSO) and penicillin streptomycin mixture were purchased from Beijing Soleberg Technology Co. Ltd. Trypsin was bought from Hyclon Company; MTT was product of Biyantian Institute of Biotechnology, and Matrigel was bought from BD (United States). The other equipment and reagents were transwell cell (Corning, USA); UV spectrophotometer (Beckman, USA); Trizol, DEPC, reverse transcription kit, and SYBR PrimeScript RT-PCR Kit (TaKaRa, Japan); DNA Marker (Tiangen Biochemical Technology Co., Ltd), and Real-time PCR ABI 7500 (ABI Company, USA). The PCR primers were synthesized by Shanghai Shenggong Biological Engineering Co. Ltd). Rabbit anti-human MMP-2 monoclonal antibody, rabbit anti-human MMP-9 monoclonal antibody and rabbit anti-human GAPDH monoclonal antibody were purchased from Santa Cruz Company, USA. Rabbit anti-human SDF-1 polyclonal antibody was bought from Wuhan BOSTER Biological Co. Ltd.

Cell culture

The gastric cancer SGC-7901 cells were cultured in

RPM11640 broth containing 10% fetal bovine serum in an atmosphere of 5% CO_2, 95% saturated humidity at 37°C, with replacement of culture medium every 1-2 days. At 80% confluence, the gastric cancer SGC-7901 cells were subjected to trypsin digestion treatment.

MTT assay on the effect of IFE on the proliferation of SGC-7901 cells

Cells in logarithmic growth phase were inoculated in 96-well plates at a density of 5×10^4 cells/mL, (100μL per well) and incubated overnight in an incubator at 37°C and 5% CO_2 according to a previously reported method (15). Different concentrations of IFE (2.5, 5, 10, 20, 40μM) were added to the treatment group, while blank control contained culture medium in place of IFE. The cells were cultured for 24, 36 and 48 h. At each predetermined time, the drug-containing medium was carefully removed, and 100μL 0.5% MTT solution was added to each well, followed by incubation for 4h. Thereafter, the MTT solution was carefully removed, and 150μL DMSO was added to each well to dissolve the formazan crystals formed. The absorbance of the resultant solution was read at 570nm in a microplate reader after 5min oscillation at a low speed. The percentage inhibition of cell proliferation was calculated as follows:

$$Proliferation\ inhibition\ (\%) = \frac{(OD_{control} - OD_{drug\ treatment}) \times 100\%}{OD_{control}}$$

Effect of IFE on migration of GC7901 gastric cancer cells

SGC-7901 cells of gastric cancer at logarithmic growth stage were taken and digested by trypsin into suspension at a density of 5×10^8 L^{-1} single cell, and incubated 24 h in an incubator at 37°C and 5% CO_2, 95% saturated humidity. Scratch test can be carried out when the cell density reaches 80%, using the sterilized 200μL pipette tips to mark vertically, control the scratch speed and force, ensure the same width of scratch (660-680 mum). After scratch, the cell fragments were washed and marked with PBS, washed for 3 times, added into the culture medium, and cultured at 37°C in the incubator with volume fraction of 5% CO_2 and 95% saturated humidity for 0 h, 24 h and 48 h respectively after scratche, and then photographed and observed under an inverted microscope.

Effect of IFE on the invasion of GC-7901 Cells

Gastric cancer SGC-7901 cells were digested and counted. Then, 3.0×10^5 cells were placed in a sterilized 1.5-ml EP tube, and 200 microns of blank culture medium or IFE medium (5 or 20 μM) were added to the suspended cells, in strict compliance with the instructions in Transwell chamber kit (note: the invasion test Transwell chamber is coated with Matrigel gel). After regular training for 24 h, the transwell chamber was taken out and fixed by 95% (v/v) alcohol for 15 min. Washed by PBS liquid for three times after air drying and stained by 0.1%(v/v) crystal violet solution 30 min, then placed in a glass slide sterilization. Each sample randomly selected five view to picture and count under 200x microscope. The number of cells in the lower chamber of Matrigel is the number of penetrating Matrigel cells.

Assay of effect of IFE on mRNA expressions of MMP-2, MMP-9 and SDF-1 using RT-PCR

The cells were cultured in 6-well plates. When the cells had grown to 80% confluence, they were separately incubated with two different concentrations of IFE (5 and 20μM) for 24 hours. Cells without IFE served as control. After digestion with 0.25% trypsin, the cell suspensions were collected in labeled centrifuge tubes. Total RNA was extracted with TRIzol reagent, and an appropriate amount of RNA (as a reverse transcription template) with 1μL olig dt (100μM) was added into PCR tube, deoxidized at 70°C for 5 min. The PCR tube was then removed and placed on ice to cool down. Then, 4μL 5x buffer, 1 μL RNase inhibitor, and 2μL dNTP mixture (10 $mmol\cdot L^{-1}$) were added successively. The reaction conditions were: 42°C for 60 min, and 70°C for 10 min. The PCR tube was taken out and cooled on ice, and kept at -20°C in a refrigerator. The cDNA from the reverse transcription was used as the template for the assay of MMP-2, MMP-9 and SDF-1 mRNA expressions using RT-PCR. The designed primers of MMP-2, MMP-9, SDF-1 and internal reference gene GAPDH were synthesized by Shanghai Sangon Co. Ltd.

The sequences of the primers used were:

GAPDH: Forward: 5'-TGATGACATCAAGAAGGTGAAG-3', Reverse: 5'-TCCTTGGAGGCCATGTGGGCCAT-3'; MMP-2: Forward: 5'- AGATCTTCTTCTTCAAGGACCGGT-3', Reverse: 5'- GGCTGGTCAGTGGCTTGGGGTA -3'; MMP-9: Forward: 5'- CTGGTCATAAGGGCTAAAT-3', Reverse: 5'- TCTTGGCGTCACTTCTCA -3'; SDF-1: Forward: 5'- ATTCTCAACACTCCAAACTGTGC -3', Reverse: 5'- ACTTTAGCTTCGGGTCAATGC-3'.

The reaction conditions were: pre-denaturation at 94°C for 2 min, denaturation at 94°C for 60 sec, annealing at 59°C for 60 sec, extension at 72°C for 60 sec, 30 cycles, and extension at 72°C for 10 min. Agarose gel electrophoresis was carried out using 5μL PCR reaction products. After the electrophoretic images were analyzed and processed with gel imager, the relative expressions of MMP-2, MMP-9 and SDF-1 mRNA were expressed as the percentage of the optical density value of GAPDH mRNA via:

$$Relative\ content\ of\ gene\ (\%)t = \frac{(Optical\ density\ of\ gene\ mRNA) \times 100\%}{(Optical\ density\ of\ GAPDH\ mRNA)}$$

Effect of IFE on protein expressions of MMP-2 and MMP-9

Total protein was extracted and quantified. Western blot was used to determine the expression levels of MMP-2, MMP-9 and SDF-1 proteins as described previously (16). The blots were visualized using enhanced chemiluminescence method, while Image Lab 5.2.1 software was used to analyze the grayscale value of Western blot strips.

Statistical analysis

Statistical analysis was performed using GraphpadPrism7.0 statistical software. Measurement data are expressed as mean ± SD, and one-way ANOVA and t-test were used for statistics. Values of $p < 0.05$ were considered statistically significant.

Results

Effect of IFE on the proliferation of SGC-7901 cells

As shown in Figure 1, MTT results showed that IFE significantly inhibited the proliferation of SGC-7901 cells at different time points in time and dose-dependent manner.

Effect of IFE on the migration ability of SGC-7901 cells

The results of the scratch test in Figure 2 show that IFE inhibited the migration of gastric cancer SGC-7901 cells in a dose-dependent manner ($p < 0.05$).

Effect of IFE on invasion of SGC-7901 cells

Treatment with IFE led to significant inhibition of the invasion ability of SGC-7901 gastric cancer cells in a dose-dependent fashion ($p < 0.05$, Figure 3).

Effect of IFE on mRNA expressions of MMP-2, MMP-9 and SDF-1 in SGC-7901 cells

The effect of IFE on the mRNA expressions of MMP-2, MMP-9 and SDF-1 was determined with RT-PCR. As shown in Figure 4, compared with the blank control group, IFE significantly inhibited the mRNA expressions of MMP-2, MMP-9 and SDF-1 in SGC-7901 gastric cancer cells ($p<0.05$) in a dose-dependent manner.

Effect of IFE on protein expressions of MMP-2, MMP-9 and SDF-1 in SGC-7901 cells

As shown in Figure 5, compared with the blank control group, IFE significantly and dose-dependently inhibited the protein expressions of MMP-2, MMP-9 and SDF-1 in SGC-7901 gastric cancer ($p<0.05$).

Discussion

Gastric cancer is the second deadliest malignant tumor in the world, and a great threat to human health. Due to the non-restrictive characteristics of tumor cell proliferation, migration and invasion, gastric cancer patients often have local metastasis and poor prognosis, resulting in death (2). Clinically, gastric cancer is characterized by frequent recurrence and poor prognosis, and the 5-year survival of advanced gastric cancer is about 30% (17). Therefore, it is of great clinical significance to find newer, safer, efficient and low-toxicity therapeutic drugs for reducing the mortality of gastric cancer patients and for improving the prognosis of the disease.

In recent years, natural products have attracted more and more attention in the treatment of cancer due to their mild effects, multi-target treatment and minimal adverse reactions (18, 19). Research on TCM monomers and their anti-tumor effects has become an important focus of attention in the medical field. One of the alkaloids with many physiological activities is IFE.

Studies have shown that MMPs are widely involved in important physiological processes such as tumor invasion and metastasis, tissue development and repair, and inflammatory responses. The MMPs not only have effective roles in clearing extracellular matrix, but also are involved in promoting tumor angiogenesis by par-

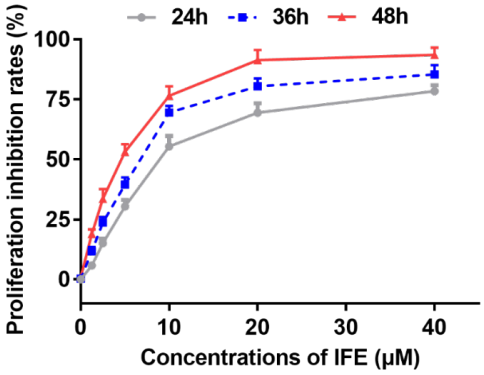

Figure 1. Effect of IFE on SGC-7901 cell proliferation**.** SGC-7901 cells were treated with various concentrations of MAC for 24, 36 and 48 h. Data are shown as mean ± SD of three independent experiments.

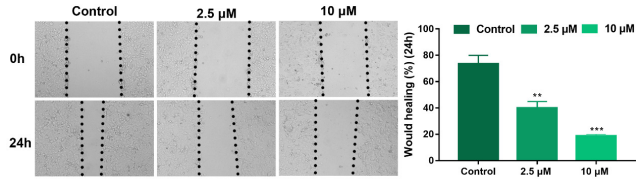

Figure 2. Effect of IFE on migration ability of SGC-7901 cells. Data are presented as mean ± SD. $^{**}p<0.01$, $^{***}p<0.001$ vs control.

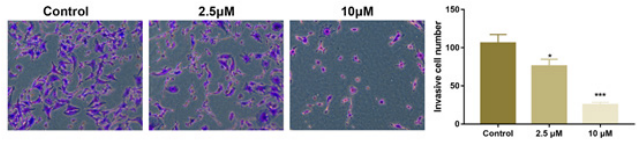

Figure 3. Effect of IFE on invasion of SGC-7901 cells, as determined with Transwell assay. Data are presented as mean ± SD. $^{*}p<0.05$, $^{***}p<0.001$ vs control.

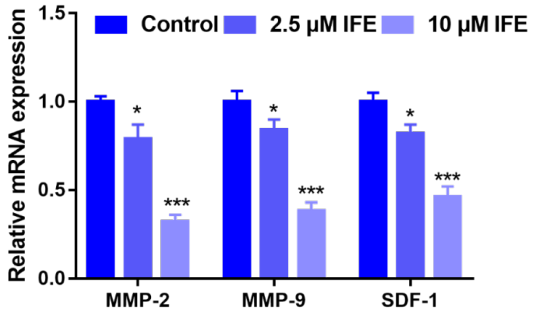

Figure 4. Effect of IFE on the expressions of MMP-2, MMP-9, SDF-1 mRNA in SGC-7901 cells (n =3). SGC-7901 cells were treated with various concentrations of IFE for 48 h. $^{*}p < 0.05$, ***, $p< 0.001$, vs control.

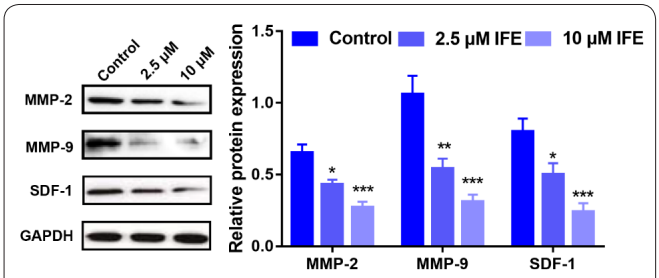

Figure 5. Effect of IFE on the expressions of MMP-2, MMP-9, SDF-1 protein in SGC-7901 cells. SGC-7901 cells were treated with various concentrations of IFE for 48 h. *, $p < 0.05$, $^{***}p < 0.001$ vs control.

ticipating in the formation of vascular growth factors (20-23). Two important members of the MMPs family, MMP-2 and MMP-9 effectively decompose the major components of basement membrane, and their over-expressions enhance the invasion and metastatic ability of cancer cells. A large number of studies have shown that MMP-2 and MMP-9 are highly expressed in gastric cancer tissues, and they can be used as important indicators for the occurrence, development and prognosis of gastric cancer (21).

Chemokines are secreted peptides capable of chemotactic cell migration, and their relative molecular weight is 8 -12 kD. They are secreted by a variety of cells in the body. The interaction between chemokines and their receptors plays an important role in inflammation, tissue damage, infection and cardiovascular diseases, and also participate in the occurrence and development of malignant tumors (24). One important member of the CXC subfamily of chemokines is SDF-1. It is located on the long arm of human chromosome 10 and has a high interspecies conservation. It is mainly secreted by bone marrow cells, stromal cells, and tumor cells (25) . In recent years, a large number of studies have confirmed that SDF-1 and its receptor chemokine receptor 4 (CXCR4) and CXCR7 play an important role in biological processes such as the growth and metastasis of various malignant tumors (gastric cancer, lung cancer and cervical cancer). Studies have shown that SDF-1 is the most important chemokine for tumor progression at present (26-28).

The results of this study showed that the inhibition of SGC-7901 gastric cancer cells treated with IFE was significantly increased with the extension of culture time and the increase in IFE concentration, and the proliferation ability of SGC-7901 gastric cancer cells was significantly decreased. These results suggest that IFE effectively inhibits the proliferation of SGC-7901 gastric cancer cells, and significantly reduces their migration and invasion. Moreover, IFE significantly inhibited the expression levels of mRNA and protein for MMP-2, MMP-9 and SDF-1 in SGC-790 gastric cancer cells in a dose-dependent manner. It is known that MMP-9 participates in tumor angiogenesis and in raising bone marrow-derived cells to tumor cells interstitial process, and also promotes new blood vessels formation and tumor growth. The over-expressions of MMP-2 and MMP-9 lead to the enhancement of invasion and metastasis of cancer cells. The protein SDF-1 is a component of SDF-1 /CXCR4 axis which plays an important role in promoting the growth, metastasis and invasion of tumor cells. Therefore, based on the results of the present study, it can be speculated that the mechanism involved in the anti-gastric cancer effect of IFE may be related to down-regulation of the expressions of MMP-2, MMP-9 and SDF-1 genes.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the authors named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Jianbo Wu, Chuankang Tang; Jin Xu, Jianbo Wu, Chuankang Tang collected and analysed the data; Jin Xu wrote the text and all authors have read and approved the text prior to publication. Jianbo Wu and Chuankang Tang contributed equally to this work as co- Corresponding author.

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