Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

The effect of Chaihu-shugan-san on cytotoxicity induction and PDGF gene expression in cervical cancer cell line HeLa in the presence of paclitaxel +cisplatin

Xue Jianfang[1], Zhu Ling[1], Jiao Yanan[1], Guan Yanliang[2*]

[1] Department of Gynaecology, Huangdao district Chinese Medicine Hospital, Qingdao, China
[2] Department of Oncology, Huangdao district Chinese Medicine Hospital, Qingdao, China

*Correspondence to: guanyanliangcd@163.com

Received July 29, 2021; **Accepted** September 30, 2021; **Published** November 22, 2021

Doi: http://dx.doi.org/10.14715/cmb/2021.67.3.21

Abstract: Chaihu-shugan-san, as a traditional Chinese herbal formula, is composed of seven different herbs. This medicine can treat cancer due to its antioxidant compounds. In this study, the effect of Chaihu-shugan-san was considered on cytotoxicity induction and PDGF gene expression in cervical cancer cell line HeLa at different concentrations and at different times, by the MTT method. Paclitaxel + cisplatin were used as a control in this study. The expression of the PDGF gene was quantitatively evaluated in treated cells by real-time PCR, and a generalized linear model was used to evaluate the effect of the medicine, and Duncan's multiple range tests were used to evaluate the data. The results of the MTT test showed that Chaihu-shugan-san had antitumor properties in different concentrations, but there was a significant difference between this medicine and paclitaxel +cisplatin. Also, examination of gene expression showed that this medicine reduced the expression of the PDGF gene in the HeLa cancer cell line ($P \leq 0.04$). Therefore, Chaihu-shugan-san could be suggested as an effective factor in preventing the growth of cervical cancer cells and controlling angiogenic factors that play an important role in the metastasis of cancerous tumors.

Key words: Cervical cancer; Chaihu-shugan-san; Cytotoxicity induction; PDGF gene.

Introduction

Tumor cells are part of the body cells that lose their ability to regulate proliferation and therefore increase indefinitely (1, 2). Therefore, these cells induce the formation of new veins from the existing capillary network through a process that is very similar to natural angiogenesis to supply the oxygen and nutrients they need. In fact, as the size of the tumor increases, the environment of the tumor cells becomes hypoxic and acidic, and they begin to increase the production of growth factors that result in the formation of localized blood vessels (3, 4). The PDGF gene is an important factor that plays a key role in angiogenesis and cancer progression, and therefore, study on the expression of this gene in cancer research can help treat cancer (5, 6).

Cervical cancer results from the increased and irregular growth of the epithelial cells of the cervix and the constant shedding of these cells. It is the fourth most common cancer and the fourth leading cause of cancer death in women (7). In 2020, out of 528,000 cases of this cancer, 226,000 cases resulted in death. Typically, 70% of cervical cancers occur in developed countries, and about 8% of all cancer deaths belong to it (8).

The use of conventional methods for the treatment of cancer, including chemotherapy, although has been able to reduce the progress of the disease to some extent, the many side effects of these methods is a major challenge in the treatment of cancer (9). Therefore, researchers are looking for natural compounds with anti-cancer properties in order to increase the life quality of cancer patients by reducing the side effects of cancer treatment methods (10). Chinese medicinal herbs with anti-tumor compounds can be a good substitute for therapeutic chemotherapeutic compounds to fight cancer cells (11, 12). In a study by Xiao *et al.*, the effect of Chaihu-shugan-san on metastasis-inducing genes such as Fibronectin, MMP9, and VEGF in the breast cancer cell line indicated a reduction in the expression of these genes and a reduction in tumor cell metastasis (13). Therefore, it seems that Chaihu-shugan-san can be considered as a suitable inhibitory agent to prevent the spread of tumor cells.

Chaihu-shugan-san is a traditional Chinese herbal formula with the composition of seven different herbs such as 1.5g of gancao (*Glycyrrhiza radix*), 4.5g of shaoyao (*Paeonia radix*), 4.5g of zhiqiao(*Citrus aurantium fructus*), 4.5g of xiangfu (*Cyperus rhizome*), 4.5g of chuanxiong (*Ligusticum chuanxiong rhizome*), 6g of Chaihu (*Bupleuri radix*), and 6g of chenpi (*Citrus reticulata pericarp*) (14). Chemical constituents of this Chinese medicine are ferulic acid, tangeretin, nobiletin, glycyrrhizic acid, saikosaponin A, Synephrine, neohesperidin, hesperidin, paeoniflorin, naringin, benzoic acid, narirutin, meranzin hydrate, oxypaeoniflorin, liquiritin, quercetin, benzoylpaeoniflorin, formononetin, isoliquiritigenin, liquiritigenin, albiflorin, and gallic acid (14-16).

In this study, the effect of Chaihu-shugan-san was evaluated on cytotoxicity induction and PDGF gene ex-

pression in cervical cancer cell line HeLa. Cisplatin and Paclitaxel are chemotherapy regimens for cervical cancer. Therefore, in this study, we use these two chemical medicines as a control, during the experiment.

Materials and Methods

Cell line and culture medium

This study was performed *in vitro* using a cervical cancer cell line (HeLa). HeLa cell line was obtained from the cell bank Australia. Cells of this cell line were cultured in RPMI-1640 culture medium, 10% FBS, at 37°C with 5% of CO_2 pressure and 95% humidity.

Determination of cytotoxicity and cell viability

In the present study, an MTT assay was used to evaluate the cytotoxicity. In order to measure the toxicity of Chaihu-shugan-san and paclitaxel +cisplatin, cervical cancer cells (Hela) were individually poured into 10^4 cells per well from a 96-well plate and cultured for 24 hours. Then, concentrations of 2.5, 5, 10, 25, 50, 75, 100, 250, 500, and 1000µg/ml were prepared from Chaihu-shugan-san and the cells in 96-well plates were treated with these concentrations for 24, 48, and 72 hours. Then 20µl of MTT solution with a concentration of 5mg/ml was added to each well and incubated for 3 hours. The supernatant was then removed and 200µl of DMSO was added to dissolve the formazone crystals. Absorption was recorded at 570 nm by ELISA reader and finally calculated according to the following ratio of cell viability.

$$\frac{\text{Mean light absorption of the experimental group} - \text{Planck average light absorption}}{\text{Mean light absorption of the control group} - \text{Planck average light absorption}} \times 100$$

IC50 Calculation

Origin software was used to calculate the concentration at which Chaihu-shugan-san and paclitaxel +cisplatin reduced cell viability by 50% (IC50). For this purpose, the values of concentrations and survival percentage were given to Origin software and after drawing a graph for each hour and according to the considered concentrations, the desired calculations were performed and IC50 was calculated.

Extraction and quality control of RNA

In the present study, in order to extract RNA, RNA purification column kit (Jena Bioscience) was used and according to the kit protocol, the steps of RNA extraction were performed by column method. For quantitative analysis, Nanodrop was used to measure the concentration and purity of extracted RNA, and the ratios obtained from the nanodrop indicated the purity of the RNA. In order to prevent genomic DNA contamination and false-positive response, RNase-free DNaseI (Fermentas, USA) was performed.

The cDNA synthesis

This step was performed by a cDNA synthesis kit (Sigma-Aldrich, USA) and PCR device. The cDNA synthesis was performed according to the protocol provided by the company.

Real-time PCR process and evaluation of gene expression

A real-time PCR test was used for quantitative analysis of PDFG gene expression. Materials required for this test are cDNA samples; SYBR Green Master Mix distilled water and sterile primer with a final volume of 20µl of solution. In this process, GAPDH was used as an internal control gene and the qRT-PCR reaction temperature cycle was performed at 94 °C for 2 minutes and during 40 cycles including 94 °C for 5 seconds and 60 °C for 10 seconds and 72 °C for 5 seconds, and finally, it was placed at 72 °C for 1 minute. After examining the melting curve and quality control of temperature and gene expression in qRT-PCR, due to the high efficiency of 95%, CT of the gene and internal control entered the Pfaffl formula and the expression of the desired gene was reported as relative (17). The characteristics of the primers used in this research are given in Table 1.

In order to ensure the absence of genomic DNA contamination in the RNA sample, negative RT minus control was used and to ensure the absence of exogenous contamination, non-template control NTC was used in real-time PCR. The processed product was then loaded on an agarose gel. No banding was observed on gel electrophoresis at this stage for genomic DNA contamination or exogenous contamination.

Statistical analysis of data

In this study, statistical analysis of data was performed by SPSS software version 17 and Duncan's multiple range tests at 5% level and the generalized GLM statistical model was used considering a dependent variable and independent variables. The significance level was considered 0.05.

Results

Cytotoxicity study of Chaihu-shugan-san on HeLa cells in compared to paclitaxel +cisplatin

MTT results of Chaihu-shugan-san treatments with different concentrations on HeLa cells in 24 hours of drug treatment showed that cell survival decreased from 1000 to 250µg/ml and again reduction was observed in survival cells from 250 to 2.5µg/ml (Figure 1, Table2). For the first 24 hours, the lowest survival rate was observed in 2.5µg/ml Chaihu-shugan-san concentration, in addition to the control sample. According to the statis-

Table1. Information of PDGF and GAPDH primers.

Gene		Sequence	Length	Cycle number
PDGF	Forward	3′-GCCAGGTTGTCTCCTGGTTA-5′	86bps	40
	Reverse	3′-TGCTTGGGACACATTGACAT-5′		
GAPDH	Forward	3′-TGCACCACCAACTGCTTAGC-5′	87bps	40
	Reverse	3′-GGCATGGACTGTGGTCATGAG-5′		

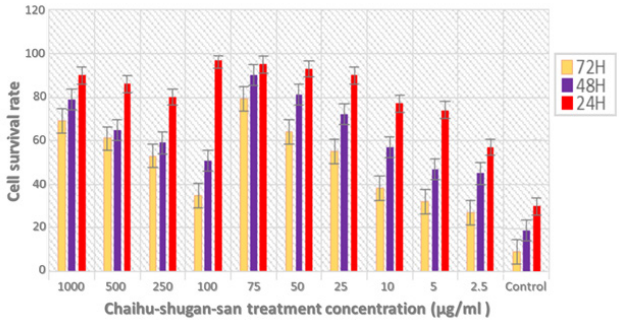

Figure 1. Graph of Chaihu-shugan-san and paclitaxel + cisplatin (as control) toxicity on HeLa cervical cancer cell line during 24, 48 and 72 hours; survival differences were significant in all groups with the control group (P <0.05).

Table2. Chaihu-shugan-san and paclitaxel + cisplatin (as control) toxicity on HeLa cervical cancer cell line during 24, 48 and 72 hours.

Concentration (µg/ml)	24 hour (%)	48 hour (%)	72 hour (%)
Control	30	19	10
1000	90	79	69
500	86	65	61
250	80	59	53
100	97	51	35
75	95	90	79
50	93	81	64
25	90	72	55
10	77	57	38
5	74	47	32
2.5	57	45	27

tical analysis, the survival chances due to all concentrations had significant differences with the control group (P≤0.001). The MTT results of Chaihu-shugan-san treatments on Hela cells in the 48-hour treatment showed that cell survival was reduced from 1000 to 100µg/ml and then it was reduced again from 75 to 2.5µg/ml. Based on a statistical analysis of 48-hour data, the survival chances due to all concentrations had significant differences with the control group (P≤0.001). The MTT results of Chaihu-shugan-san treatments during 72 hours also showed that the cell survival decreased from 1000 to 100µg/ml and then decreased again from 75 to 2.5µg/ml. Statistical analysis of the treatments applied in 72 hours showed that the reduction of cell survival in all concentrations was significant in comparison to the control group (P≤0.001).

According to the statistical analysis of Duncan's multiple range tests, there was a significant relationship between all concentrations and the control group during 24, 48 and 72 hours.

IC_{50} rate of Chaihu-shugan-san

Using graphs obtained from different concentrations of Chaihu-shugan-san in 48 and 72 hours using origin 2018 software, the IC50 of Chaihu-shugan-san concentrations at 200, 100 and 10µg/ml was calculated.

RNA quality control

Examination of negative RT-PCR control and NTC results showed that there was no contamination from genomic and exogenous DNA in the extracted RNA.

Evaluation of PDGF gene expression

According to the amplification curve of PCR cycles, for the GAPDH gene, it can be seen that in the first 23 cycles, there is no noticeable change in the amount of reproduced products, but then, from cycle 24 onwards, the reaction enters an exponential phase (Fig 2). About the PDGF gene, the exponential phase for amplification happened from cycle 30.

The melting curves of the samples for the studied genes show that the melting curves all match and the temperature peaks of all the graphs are at the same temperature (Figure 3).

The results of PDGF target gene expression by Real-Time PCR in HeLa cervical cancer cells exposed to concentrations of 10, 100, and 200µg /l Chaihu-shugan-san showed that at a concentration of 200µg/ml, the relative expression of the gene was 0.26, at a concentration of 100µg/ml, the relative expression of the gene was 0.36, and at a concentration of 10µg/ml, the relative expression of the gene was 0.90. In the statistical analysis performed in the MTT section, there was a significant relationship between these concentrations of 200, 100, and 10µg/ml (P≤0.001). Also, In terms of gene expression, this relationship was statistically significant (P≤0.004). Therefore, the reduction of gene expression in these concentrations can be justified.

Since PDGF is an angiogenic gene that is involved in cancer metastasis, changes in gene expression were not directly related to cell survival. Therefore, changes in gene expression are independent of changes in cell survival and can be fully justified.

Statistical analysis of the data showed that the results of PDGF gene expression under the influence of Chaihu-shugan-san were significant in comparison to the negative control sample (P≤0.004)(Figure4), and exposure to different concentrations of Chaihu-shugan-san could reduce angiogenic factor expression (PDGF). In addition, the increase in concentration caused a decrease in the rate of gene expression.

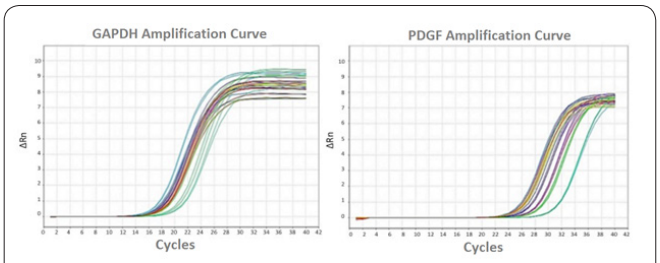

Figure 2. Amplification curves of GAPDH and PDGF genes in the studied group.

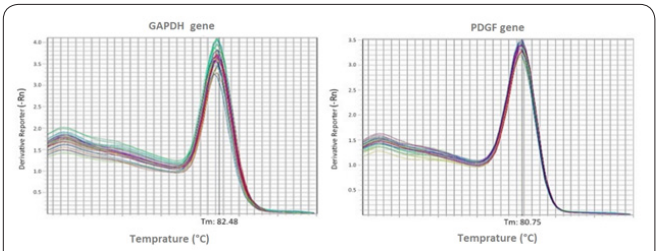

Figure 3. Melting curves of GAPDH and PDGF genes in the studied group.

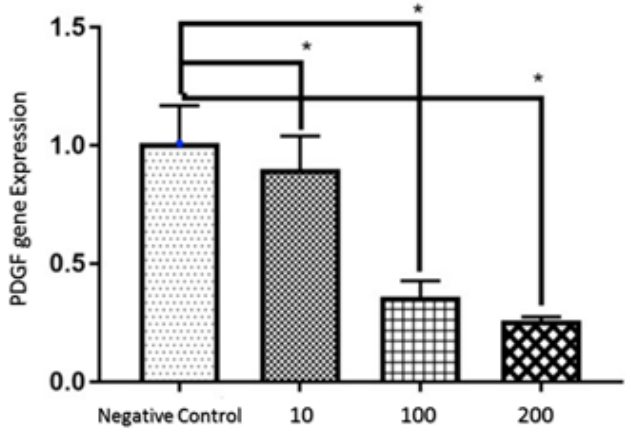

Figure 4. Changes in PDGF gene expression after 24 hours of treatment with different concentrations of Chaihu-shugan-san (µg/ml).

Discussion

In the current study, it was found that Chaihu-shugan-san, with its anti-tumor properties, in different concentrations inhibited the growth and proliferation of cancer cells at different times. In addition, the present study showed that Chaihu-shugan-san at concentrations of 10, 100, and 200µg/ml reduced the expression of the PDGF gene. Numerous studies have been conducted on the effect of traditional Chinese medicine, including Chaihu-shugan-san, on the induction of cytotoxicity on cancer cells (13, 18-24). According to the results of previous studies and the present study, Chaihu-shugan-san has the ability to control cancer cells due to its antioxidant properties.

The results of this study showed that after 72 hours of treatment of cells with different concentrations of Chaihu-shugan-san, with a decrease in concentration from 1000 to 2.5µg/ml, a significant reduction ($P < 0.05$) was observed in HeLa cells survival. According to the results, it seems that higher concentrations of Chaihu-shugan-san as a treatment, due to the presence of various compounds, may play the role of a nutrient for cells and thus increase cell survival. While decreasing the concentration of Chaihu-shugan-san in cellular treatments may have increased its cytotoxic role, so that a concentration of 5.5 µg/ml showed the greatest reduction in cell survival in cervical cancer cells. Statistical analysis of MTT test results related to treatments of 10, 100, and 200 µg/ml showed that the observed changes in the survival rate of these concentrations were significant ($P \leq 0.001$). In gene expression, the difference in the amount of gene expression is significant ($P \leq 0.004$). In MTT results, the reduction of cell survival at 10 µg/ml was greater than the concentration of 100 µg/ml, and at the concentration of 100 µg/ml was more than the concentration of 200 µg/ml, so the reduction of gene expression at these concentrations is justifiable. As stated in the results section, at a concentration of 200µg/ml of the drug compared to a concentration of 100 µg/ml and a concentration of 100 µg/ml compared to a concentration of 10µg/ml, there was a further decrease in gene expression. This means that the highest reduction in expression was obtained at a concentration of 200 µg/ml and these differences were statistically significant ($P < 0.05$). Considering that these concentrations were selected from IC_{50} results to evaluate the expression of the gene and the resulting changes are statistically significant at the 5% level, so the decrease in PDGF gene expression at these concentrations can be justified. Therefore, based on the results, Chaihu-shugan-san can be suggested as a traditional medicine with anti-tumor properties and with the ability to suppress angiogenic factors in tumor cells.

In the present study, it was found that Chaihu-shugan-san had acceptable toxicity on the cervical cancer cell line. Although there was a significant difference between Chaihu-shugan-san and paclitaxel+cisplatin, Chaihu-shugan-san can help these chemotherapeutic drugs to reduce their severe side effects, due to their natural nature and compatibility with the human body. Chaihu-shugan-san also decreased PDGF gene expression in the cervical cancer cell line. Therefore, Chaihu-shugan-san could be suggested as an effective factor in preventing the growth of cervical cancer cells and controlling angiogenic factors that play an important role in the metastasis of cancerous tumors.

References

1. Lu C, Guan J, Lu S et al. DNA sensing in mismatch repair-deficient tumor cells is essential for anti-tumor immunity. Cancer Cell 2021; 39(1): 96-108. e106.

2. Kazemi E, Kahrizi D. Lack of association between gastric cancer and hopq alleles in Helicobacter pylori. Genetika 2016; 48(3): 893-902.

3. Ahn JC, Teng PC, Chen PJ et al. Detection of Circulating Tumor Cells and Their Implications as a Biomarker for Diagnosis, Prognostication, and Therapeutic Monitoring in Hepatocellular Carcinoma. Hepatology 2021; 73(1): 422-436.

4. Kazemi E, Kahrizi D, Moradi M et al. Association between Helicobacter pylori hopQI genotypes and human gastric cancer risk. Cell Mol Biol 2016; 62(1): 6-9.

5. Guérit E, Arts F, Dachy G, Boulouadnine B, Demoulin J-B. PDGF receptor mutations in human diseases. Cell Mol Life Sci 2021: 1-15.

6. Kazemi E, Kahrizi D, Moradi M, Sohrabi M, Yari K. Gastric cancer and helicobacter pylori: impact of hopQII gene. Cell Mol Biol 2016; 62(2): 107-110.

7. Park YR, Kim YJ, Ju W, Nam K, Kim S, Kim KG. Comparison of machine and deep learning for the classification of cervical cancer based on cervicography images. Sci Rep 2021; 11(1): 1-11.

8. Kjaer SK, Dehlendorff C, Belmonte F, Baandrup L. Real-world Effectiveness of Human Papillomavirus Vaccination Against Cervical Cancer. J Natl Cancer Inst 2021.

9. Redd WH, Burish TG, Andrykowski MA. Aversive conditioning and cancer chemotherapy. *Cancer, Nutrition, and Eating Behavior*: Routledge; 2021: 117-132.

10.Alilu L, Heydarzadeh L, Habibzadeh H, Rasouli J. The effect of peer education on management of chemotherapy side effects in patients with cancer. Iran J Nurs Midwifery Res 2021; 26(1): 81.

11.Shirzad M, Abbassian A. A new glance at the role of traditional medicines in treatment of cancers. J Cancer Res Clin Oncol 2021: 1-2.

12.Tourang M, Fang L, Zhong Y, Suthar R. Association between Human Endogenous Retrovirus K gene expression and breast cancer. Cell Mol Biomed Rep 2021; 1(1): 7-13.

13.Xiao K, Li K, Long S, Kong C, Zhu S. Potential molecular mechanisms of Chaihu-Shugan-San in treatment of breast cancer based on network pharmacology. Evid Based Complement Alternat Med 2020; 2020.

14.Feng D-d, Tang T, Lin X-p et al. Nine traditional Chinese herbal formulas for the treatment of depression: an ethnopharmacology, phytochemistry, and pharmacology review. Neuropsychiatr Dis Treat 2016; 12: 2387.
15.Su Z-H, Zou G-A, Preiss A, Zhang H-W, Zou Z-M. Online identification of the antioxidant constituents of traditional Chinese medicine formula Chaihu-Shu-Gan-San by LC–LTQ-Orbitrap mass spectrometry and microplate spectrophotometer. J Pharm Biomed Anal 2010; 53(3): 454-461.
16.Bilal I, Xie S, Elburki M, Aziziaram Z, Ahmed S, Jalal Balaky S. Cytotoxic effect of diferuloylmethane, a derivative of turmeric on different human glioblastoma cell lines. Cell Mol Biomed Rep 2021; 1(1): 14-22.
17.Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research 2001; 29(9): e45-e45.
18.Efferth T, Li PC, Konkimalla VSB, Kaina B. From traditional Chinese medicine to rational cancer therapy. Trends Mol Med 2007; 13(8): 353-361.
19.Xiang Y, Guo Z, Zhu P, Chen J, Huang Y. Traditional Chinese medicine as a cancer treatment: Modern perspectives of ancient but advanced science. Cancer Med 2019; 8(5): 1958-1975.
20.Nie J, Zhao C, Deng L et al. Efficacy of traditional Chinese medicine in treating cancer. Biomed Rep 2016; 4(1): 3-14.
21.Kazemi E, Zargooshi J, Kaboudi M, Heidari P, Kahrizi D, Mahaki B, Mohammadian Y, Khazaei H, Ahmed K. A genome-wide association study to identify candidate genes for erectile dysfunction. Brief Bioinforma 2021;22(4):bbaa338. https://doi.org/10.1093/bib/bbaa338
22.Li X, Yang G, Li X et al. Traditional Chinese medicine in cancer care: a review of controlled clinical studies published in Chinese. PloS one 2013; 8(4): e60338.
23.Ye L, Jia Y, Ji K et al. Traditional Chinese medicine in the prevention and treatment of cancer and cancer metastasis. Oncol Lett 2015; 10(3): 1240-1250.
24.Yang Z, Zhang Q, Yu L, Zhu J, Cao Y, Gao X. The signaling pathways and targets of traditional Chinese medicine and natural medicine in triple-negative breast cancer. J Ethnopharmacol 2021; 264: 113249.