Effects and significance of formononetin on expression levels of HIF-1α and VEGF in mouse cervical cancer tissue

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Abstract. Effects and significance of formononetin on the expression levels of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in mouse cervical cancer tissue were investigated. The animal models of Balb/c nude mice with cervical cancer were established by the inoculation of HeLa cells, and randomly divided into positive control (n=10), cisplatin (n=15) and formononetin group (n=15). Mice were all sacrificed on the 31st day after administration, and their tumors were excised and weighed to calculate tumor inhibition rate. At the same time, their cancer tissues were obtained. RT-qPCR was used for detecting the mRNA expression levels of HIF-1α and VEGF, and western blotting for detecting the protein expression levels. During the medication intervention, mice in the formononetin group had no obvious adverse reactions, and were in good condition, whereas mice in the cisplatin group had poor appetite, drooping spirits and decreased activity. Mice in the cisplatin and the formononetin groups had significantly lower tumor mass and volume than those in the positive control group (P<0.05). The tumor inhibition rate of mice was 56.24% in the cisplatin group, and 50.17% in the formononetin group. Cervical cancer mice in the formononetin and the cisplatin groups had significantly lower mRNA and protein expression levels of HIF-1α and VEGF in tissues than those in the positive control group (P<0.05). Formononetin can inhibit the growth of cervical cancer and reduce the mRNA and protein expression levels of HIF-1α and VEGF in mouse cervical cancer. Formononetin has an inhibitory effect on cervical cancer tumors similar to that of cisplatin, but the former has smaller side effects, providing data for the clinical use in cervical cancer.

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Introduction

Cervical cancer is a malignant tumor and its incidence is second only to breast cancer. There is a gradual trend for younger age, seriously threatening life and health of females around the world and causing serious economic burden on families and society (1,2). There are 528,000 new patients with cervical cancer worldwide each year with 266,000 deaths, exceeding any other gynecologic tumor (3,4). According to reports in the literature, the mortality of cervical cancer is the eighth in malignant tumors in China. Compared to the 1970s, the mortality has decreased in the past 10 years, but the incidence in young females has increased. In addition, the mortality is still high in rural areas (5).

Hypoxia-inducible factor-1α (HIF-1α), a subunit of HIF-1 that regulates angiogenesis, and the growth, metastasis and apoptosis of tumors, promotes angiogenesis by regulating vascular endothelial growth factor (VEGF). It also improves oxygen carrying capacity, and maintains cell oxygen stability in hypoxic tissues and tolerance to hypoxia (6,7). The high expression of HIF-1α regulates cellular energy metabolism and promotes tumor angiogenesis (8). VEGF is by far the most potent pro-angiogenic factor, which is synthesized and secreted by various tumor cells (9). Stimulating the growth of endothelial cells to enhance vascular permeability, it promotes the expression of various cathepsins, which degrades extracellular matrix and promotes tumor angiogenesis (10,11).

At present, advanced cervical cancer is mainly treated by operation, radiotherapy and chemotherapy in clinical practice (12). Patients with stage II B or above are mainly comprehensively treated based on radiotherapy, with a five-year disease-free survival rate of approximately 67% and greater side effects (13,14). Even if radiotherapy technology and equipment are constantly updated and improved, the growth of primary tumors still cannot be effectively controlled (15). Therefore, it is of great significance to seek targeted drugs for treating cervical cancer. *Astragalus membranaceus* is a traditional Chinese medicine with invigorating *qi* and diuretic efficacy, detoxification and myogenic efficacy (16). As a phytoestrogen, one of main components of *astragalus membranaceus*, formononetin has the effect of regulating estrogen, metabolism, inflammation, and lowering blood pressure (17-19). Currently, studies have shown that it can inhibit bladder cancer and breast cancer (20,21).

In this study, the effects and significance of formononetin on the expression levels of HIF-1α and VEGF in mouse cervical cancer tissue were investigated, to provide references for clinical use.

Materials and methods

Subjects, grouping and modeling. A total of 45 healthy, female, Balb/c nude mice were selected, aged 6-8 weeks and weighing 15-20 g. They were purchased from Changzhou Carvins Laboratory Animal Co., Ltd. (Changzhou, China) with an animal certificate number of SCXK (Su) 2011-0003. They were reared in cages at room temperature between 23 and 25˚C with a humidity of 55-62%, 12/12 light cycles, free access to food and drink *ad libitum*. Modeling experiments were performed after 1 week of adaptive feeding in all mice.

This study was approved by the Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University (Wuxi, China). Patients who participated in this research had complete clinical data. Signed informed consents were obtained from the patients or the guardians.

Cervical cancer HeLa cells (item no. C015; Nanjing Laifusai Biotechnology Co., Ltd., Nanjing, China) were digested with trypsin (item no. EB04590; Shanghai Shifeng Biotechnology Co., Ltd., Shanghai, China) and prepared into single cell bacterial suspension with a serum-free medium, at a concentration of $1x10^8$/ml. Then, 0.3 ml of HeLa cell suspension was inoculated into the subcutaneous position of the left side of the armpit of the nude mouse near the back, and the tumor growth was recorded. Tumor volume = (longest diameter of the tumor) x (shortest diameter)^2 x 0.5 (22). On the 6th day of inoculation, 40 nude mice were tumorigenic. The tumor diameter exceeding 2.0 mm indicated successful modeling. Among the remaining 5 nude mice, 1 mouse was not tumorigenic, and the remaining 4 mice had tumor diameters <2.0 mm.

The 40 mice successfully modeled were randomly divided in formononetin group (n=15), cisplatin group (n=15) and positive control group (n=10). Mice in positive control group were administered with 0.1 ml of 0.9% saline (item no. PB180353; Wuhan Bafeier Biotechnology Service Co., Ltd., Wuhan, China) once a day. Mice in cisplatin group were intraperitoneally administered (3 mg/kg cisplatin dissolved in 0.9% saline) with 0.1 ml of cisplatin (item no. RB187; Shanghai Guangrui Biotechnology Co., Ltd., Shanghai, China) solution, once every 7 days after modeling. On the other days, 0.1 ml of 0.9% saline was intraperitoneally injected every day. Mice in formononetin group were intragastrically administered (10 mg/kg formononetin dissolved in 0.9% saline) with 0.1 ml of formononetin (item no. JKM0063; Shanghai Jingke Chemical Technology Co., Ltd., Shanghai, China) solution once a day. The mice were observed during the medication intervention. All the mice were sacrificed on the 31st day after the administration, and their tumors were excised and weighed to calculate the tumor inhibition rate. Tumor inhibition rate = (average tumor weight of mice in positive control group - that in medication intervention groups)/that in positive control group x 100%. At the same time, their cancer tissues were obtained. The mRNA and protein expression levels of HIF-1α and VEGF in tissues were detected.

RT-qPCR detection of mRNA expression levels of HIF-1α and VEGF. First 100 mg of cervical cancer tissue was obtained from mice, ground and pulverized, then 1 ml of TRIzol lysate (Shanghai Pufei Biotechnology Co., Ltd., Shanghai, China) was added to isolate total RNA from the tissues. After extraction, 1.5% agarose gel electrophoresis was used for analyzing RNA integrity, a micronucleic acid determinator (Beijing Meilin Hengtong Instrument Co., Ltd., Beijing, China) for detecting the purity and concentration of the extracted RNA. A260/A280 value was considered to meet experimental requirements between 1.8 and 2.0. Then, 2 μg of the total RNA was taken, and a reverse transcription kit (ReverAid TM First Strand cDNA synthesis kit, #k1622; Promega Corporation, Madison, WI, USA) was used to synthesize cDNA. Reaction system: 5XPrimerScript Buffer 2 μl, PrimerScript RT enzyme mix 0.5 μl, Random 6 mers (100 μM) 0.5 μl, Oligo dT Primer (50 μM) 0.5 μl, total RNA 2 μg, dd H_2O was added to 10 μl. Reaction conditions: 25˚C for 5 min, 42˚C for 60 min, 70˚C for 5 min. Products of 2 μl was subjected to PCR cycle with SYBR-Green PCCR kit (Beyotime, Shanghai, China), after pre-denaturation at 94˚C for 3 min, denaturation at 94˚C for 45 sec, renaturation at 58˚C for 30 sec, extension at 72˚C for 45 sec, for a total of 35 cycles, and then extension at 72˚C for 10 min after the cycles. β-actin was used as a reaction internal reference. All the samples were determined 3 times. $2^{-\Delta Cq}$ was used to calculate the mRNA expression levels of HIF-1α and VEGF in normal tissues of mice in the control group and in cancer tissues of mice in the formononetin, the cisplatin and the positive control groups (23). Primer sequences are shown in Table I.

Western blotting for the detection of protein expression levels of HIF-1α and VEGF. Cervical cancer tissue (100 mg) was obtained from mice, ground and pulverized. An appropriate amount of RIPA lysate (item no. EX6010-100 ml; Jinkelong Biotechnology Co., Ltd., Beijing, China) containing 100 mM PMSF was added, and shaken on ice for 30 min to fully lyse the cells to extract total protein. Then 8% SDS-PAGE (item no. LM0014A; Shanghai Lianmai Bioengineering Co., Ltd., Shanghai, China) was used for electrophoresis separation. PVDF (item no. 28416245; Suzhou Ruinuode Biotechnology Co., Ltd., Suzhou, China) was left at room temperature for 2 h after transmembrane. Rabbit anti mice HIF-1α monoclonal antibody (cat. no. 36169; dil, 1:700) and VEGF primary antibody (cat. no. 2479; dil, 1:1,000) both from Cell Signaling Technology, Inc., (Danvers, MA, USA) were respectively added, shaken for 30 min and incubated at 4˚C overnight. After the membrane was washed with TBST (item no. P0233; Beyotime Institute of Biotechnology, Shanghai, China) 3 times, HRP-labeled secondary antibody dilution (cat. no. SA00001-2; dil, 1:2,000; Wuhan Sanying Biotechnology, Wuhan, China) was added, incubated at room temperature for 120 min and then exposed in the dark. β-actin (cat. no. 8457; dil, 1:1,000; Cell Signaling Technology, Inc.) was used as an internal reference. A chemiluminescence imaging system (GelView 6000 Pro; Guangzhou Yunxing Scientific Instrument Co., Ltd.) was used for gray-light scanning, and photoshop software for analyzing the relative expression of the protein, repeated 3 times.

Statistical analysis. SPSS17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for analysis. Measurement data

Table I. Primers for HIF-1α mRNA and VEGF mRNA and internal reference sequences.

Genes	Upstream primers	Downstream primers
HIF-1α	5'-TCACGAGGGGTTCCCGCCTCGCA-3'	5'-TGCGAGGCGGGAAACCCCTCGTGA-3'
VEGF	5'-GGATCCATGAACTTTCTGCT-3'	5'-GAATCCACCGCCTCGGCTTGTC-3'
β-actin	5'-CCAGCCTTCCTTCTTGGGTAT-3'	5'-TTGGCATAGAGGTCTTTACGG-3'

HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

Table II. Inhibition rates of formononetin and cisplatin on cervical cancer.

Groups	n	Tumor mass (g)	Tumor volume (cm^3)	Tumor inhibition rate (%)
Positive control	10	8.73±2.15	10.91±4.58	0.00
Formononetin	15	4.35±0.86[a]	6.22±1.61[a]	50.17
Cisplatin	15	3.82±0.73[a]	5.76±1.26[a]	56.24
F		51.040	13.700	
P-value		<0.001	<0.001	

[a]P<0.05, compared to positive control group.

Table III. Effects of formononetin and cisplatin on mRNA expression levels of HIF-1α and VEGF in cervical cancer tissue.

Groups	n	HIF-1α	VEGF
Positive control	10	0.79±0.27	0.53±0.18
Formononetin	15	0.46±0.17[a]	0.32±0.15[a]
Cisplatin	15	0.38±0.12[a]	0.26±0.10[a]
F		15.750	11.370
P-value		<0.001	<0.001

[a]P<0.05, compared to positive control group. HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

were expressed as mean ± standard deviation, and tested by the t-test. One-way ANOVA was used for comparison among groups, paired t-test for comparison in the group. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibition rates of formononetin and cisplatin on cervical cancer tumors. During the medication intervention, mice in the formononetin group had no obvious adverse reactions, and were in good condition, whereas mice in the cisplatin group had poor appetite, drooping spirits and decreased activity. There were statistically significant differences in the tumor mass and volume of mice among the cisplatin, the formononetin and the positive control groups (P<0.001). Mice in the cisplatin and the formononetin groups had significantly lower tumor mass and tumor volume than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05). The tumor inhibition rate of mice was 56.24% in the cisplatin group, and 50.17% in the formononetin group (Table II).

Effects of formononetin and cisplatin on mRNA expression levels of HIF-1α and VEGF in cervical cancer tissue. There were statistically significant differences in the mRNA expression levels of HIF-1α and VEGF in mouse tissue among the cisplatin, the formononetin and the positive control groups (P<0.001). Mice with cervical cancer in the formononetin and the cisplatin groups had significantly lower mRNA expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05; Fig. 1 and Table III).

Effects of formononetin and cisplatin on protein expression levels of HIF-1α and VEGF in tissues of cervical cancer. There were statistically significant differences in the protein expression levels of HIF-1α and VEGF in tissues of mice

Table IV. Effects of formononetin and cisplatin on protein expression levels of HIF-1α and VEGF in cervical cancer tissue.

Groups	n	HIF-1α	VEGF
Positive control	10	0.84±0.19	0.73±0.16
Formononetin	15	0.51±0.14[a]	0.36±0.12[a]
Cisplatin	15	0.41±0.13[a]	0.28±0.10[a]
F		25.630	42.330
P-value		<0.001	<0.001

[a]P<0.05, compared to positive control group. HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

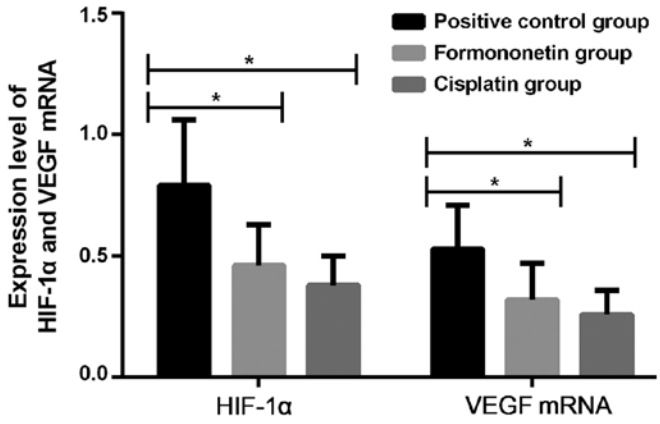

Figure 1. Effects of formononetin and cisplatin on mRNA expression levels of HIF-1α and VEGF in mouse tissue with cervical cancer. The results of RT-qPCR showed that there were statistically significant differences in the mRNA expression levels of HIF-1α and VEGF in mouse tissue among the cisplatin, the formononetin and the positive control groups (P<0.001). Mice with cervical cancer in the formononetin and the cisplatin groups had significantly lower mRNA expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05). *P<0.05. HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

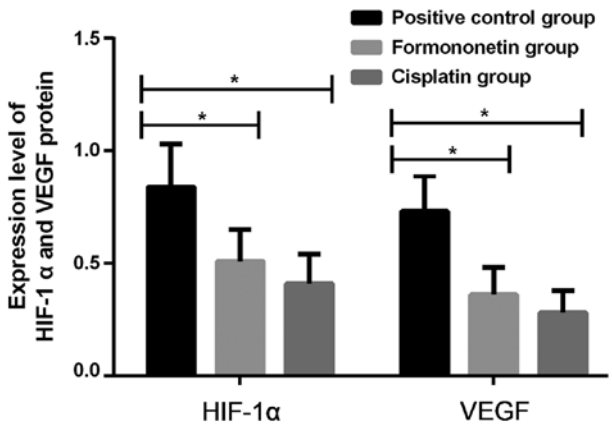

Figure 2. Effects of formononetin and cisplatin on protein expression levels of HIF-1α and VEGF in mouse tissue with cervical cancer. The results of western blotting showed that there were statistically significant differences in the protein expression levels of HIF-1α and VEGF in mouse tissue among the cisplatin, the formononetin and the positive control groups (P<0.001). Cervical cancer mice in the formononetin and the cisplatin groups had significantly lower protein expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05). *P<0.05. HIF-1α, hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor.

among the cisplatin, the formononetin and the positive control groups (P<0.001). Mice with cervical cancer in the formononetin and the cisplatin group had significantly lower protein expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05; Fig. 2 and Table IV).

Discussion

Cervical cancer, a common malignant tumor in female, is caused by complex changes in multi-gene and multi-factor interactions (24). Its pathogenesis in tumor angiogenesis is particularly important in the hypoxic condition (25-27). HIF-1α promotes the proliferation of cancer cells, and VEGF induces the division of vascular endothelial cells to promote tumor growth (28). In addition to killing cancer cells, radiotherapy also causes damage to patients' immune function (29). Chemotherapeutics affect patients' quality of life, with greater toxic and side effects (30). Therefore, medical workers are increasingly concerned about new bio-targeted therapies. According to reports in the literature, formononetin can inhibit the proliferation of osteosarcoma cell line U2OS (31), colorectal cancer cell line HCT-116, DU-145, HeLa and gastric cancer cell line SGC-7901 (32), as well as promote apoptosis.

This study showed that during the medication intervention, mice in the formononetin group had no obvious adverse reactions, and were in good condition, but mice in the cisplatin group had poor appetite, drooping spirits and decreased activity. Mice in the cisplatin and the formononetin groups had significantly lower tumor mass and tumor volume than those in the positive control group, with statistically significant differences (P<0.05), but there was no significant difference in those between the formononetin and the cisplatin groups (P>0.05). The tumor inhibition rate of mice was 56.24% in the cisplatin group, and 50.17% in the formononetin group. Therefore, in this experiment, formononetin had an anti-tumor effect on treating mice with cervical cancer similar to cisplatin, but the former has no significant adverse reactions with more mildly effects. This may be due to the fact that as

a phytoestrogen with mild nature, small toxic and side effects, and diverse biological activities, formononetin has multiple effects such as anti-tumor, immune regulation, anti-oxidation, lowering blood lipid and cholesterol (33). According to research by Kim *et al* (34), the high expression of HIF-1α and VEGF in cervical cancer tissues is correlated with clinical stage, pathological grade and lymph node metastasis. In order to verify the correlation between the inhibition of tumor growth by formononetin and the expression levels of HIF-1α and VEGF, RT-qPCR and western blotting were performed. The results showed that mice with cervical cancer in the formononetin and the cisplatin group had significantly lower mRNA expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences ($P<0.05$), but there was no significant difference in those between the formononetin and the cisplatin groups ($P>0.05$). Mice with cervical cancer in the formononetin and the cisplatin groups had significantly lower protein expression levels of HIF-1α and VEGF in tissues than those in the positive control group, with statistically significant differences ($P<0.05$), but there was no significant difference in those between the formononetin and the cisplatin groups ($P>0.05$). In the study by Bachtiary *et al* (35), the results of immunohistochemistry show that patients with HIF-1α expression in cervical cancer tissues account for 72.1%. The HIF-1α expression occurred in the early stage of tumor formation, and it is speculated that HIF-1α may play an important role in the occurrence and development of tumors. The study by Birner *et al* (36) found that patients with high expression of HIF-1α have significantly lower overall survival time than patients with moderate or no expression of HIF-1α, and the high expression of HIF-1α is an important prognostic indicator of early cervical cancer. The study by Chen *et al* (37) shows that the expression of HIF-1α and VEGF are closely related to tumor angiogenesis, and HIF-1α may play an important role in the invasion and tumor angiogenesis of gastric cancer. Highly expressed HIF-1α is closely correlated with tumor recurrence and distant metastasis. The finding of Jin *et al* (38) are consistent with ours. Their results show that formononetin promotes apoptosis of cervical cancer HeLa cells, and has an anti-tumor effect. Our experiments have confirmed that formononetin can inhibit cervical cancer. It is speculated that it may inhibit cancer by inhibiting the expression levels of HIF-1α and VEGF. However, the specific mechanism is still unclear, which requires more in-depth research.

In summary, formononetin can inhibit the growth of cervical cancer tumors and reduce the mRNA and protein expression levels of HIF-1α and VEGF in mouse tissue with cervical cancer. Formononetin has an inhibitory effect on cervical cancer tumors similar to cisplatin, but the former has smaller side effects, and can provide useful data for clinical application.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ wrote the manuscript. YZ and JZ contributed to PCR and western blotting. CC and JZ were responsible for the model construction. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Wuxi People's Hospital Affiliated to Nanjing Medical University (Wuxi, China). Patients who participated in this research had complete clinical data. Signed informed consents were obtained from the patients or the guardians.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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