Original Article
Formononetin targets the MAPK and PI3K/Akt pathways to induce apoptosis in human nasopharyngeal carcinoma cells in vitro and in vivo

Chenglin Qi, Mao Xie, Juan Liang, Heng Li, Zhenhua Li, Shujing Shi, Xuemin Yang, Zhi Wang, Jie Tang, Anzhou Tang

1Department of Otorhinolaryngology Head and Neck Surgery, The Frist Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China; 2Department of Traditional Chinese Medicine, The First Affiliated Hospital of Guangxi Medical University, Nanning, China. *Equal contributors.

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Abstract: Background/Aims: Formononetin is one of the main active components of Astragalus membranaceus and is considered a phytoestrogen. Previous studies have demonstrated that formononetin has antitumor activity in various cancer cell lines. However, the activity of formononetin against nasopharyngeal carcinoma (NPC) cells remains largely unclear. In the present study, we explored whether formononetin could exert effective inhibitory activity on NPC cells and investigated the underlying mechanism. Methods: CNE1 and CNE2 cells were treated with formononetin. Cell proliferation was examined using an MTT assay. Cellular apoptosis was examined using the Hoechst 33258 assay. The activation of the mitochondrial apoptotic pathway, the mitogen-activated protein kinases (MAPK) pathway and the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway was evaluated using quantitative qRT-PCR and western blotting. A tumor inhibition effect was analyzed using ectopic tumor implantation models. Results: We found that formononetin inhibited the proliferation of both NPC cell lines and induced apoptosis of CNE1 cells in vitro. Furthermore, formononetin treatment significantly suppressed Akt phosphorylation in CNE1 cells but upregulated the phosphorylation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38MAPK. In addition, we found that the pro-apoptotic factors BCL2-associated X protein (Bax) and caspase-3 were upregulated, and the anti-apoptotic B-cell CLL/lymphoma 2 (Bcl-2) was downregulated. Moreover, formononetin retarded the tumor growth rate in vivo. Conclusions: Our findings indicate that the potent anticancer effects of formononetin on NPC cells may be mediated by the mitochondrial apoptotic pathway under the regulation of the MAPK and PI3K/Akt pathways. This study lays the foundation for formononetin as a novel chemotherapeutic agent in NPC clinical treatment.

Keywords: Formononetin, nasopharyngeal carcinoma, apoptosis, MAPK, Akt

Introduction

Although it is rare in most parts of the world, nasopharyngeal carcinoma (NPC) is a distinctive type of head and neck cancer whose highest incidence occurs in South China and Southeast Asia [1]. The current treatment strategies for NPC include radiation, surgery or chemotherapy, with each treatment option having associated complications and side effects. Because of the high chemosensitivity of NPC, chemotherapy plays an important role in the treatment of this disease, especially among distant metastatic NPC patients [2]. However, drug resistance and the toxicity of current chemotherapeutic drugs, such as cisplatin, 5-fluouracil and bleomycin, may hamper their efficacy. Therefore, novel compounds with a high therapeutic potential need to be identified.

The root of Astragalus membranaceus is a traditional Chinese herbal medicine that has a long history and wide uses in clinical treatment. As the main active component of Astragalus membranaceus, formononetin [7-hydroxy-4'-methoxyisoflavone; the molecular formula is
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C_{16}H_{12}O_{4}, Figure 1] is a phytoestrogen and belongs to the isoflavonoid family. Formononetin promotes wound repair [3], inhibits bone loss [4], accelerates fracture healing [5], protects against acute lung injury [6] and has antihypertension effects [7]. Currently, research has focused on the antitumor and cytotoxic activities of formononetin to evaluate its potential as a novel anticancer agent. The antitumor pharmacologic properties of formononetin have been demonstrated in several human cancers, including breast cancer, cervical cancer, prostate cancer, colon cancer and hepatoma [8-12]. However, the effects of formononetin on NPC have not been well established.

Unlimited cell proliferation and dysregulation of apoptosis plays critical roles in cancer genesis and development, including NPC. As two classic signal transduction pathways that transduce extracellular signals from the membrane to the nucleus, the mitogen-activated protein kinases pathway (MAPK) and the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signal pathway are well-established regulators of cell apoptosis in different types of human cancers [13, 14]. The MAPK family is composed of seven main subgroups: the extracellular signal-regulated protein kinase 1/2 (ERK1/2); ERK3/4; ERK5; ERK7/8; Nemo-like kinase (NLK); c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK); and p38MAPK. ERK1/2 is frequently associated with cellular differentiation and survival and is strongly activated by growth factors [14, 15]. ERK3/4, ERK7/8 and NLK are atypical MAPK members, and ERK5 is expressed at high levels in specific organs, such as the thymus, spleen, and brain [15]. In contrast, JNK and p38MAPK respond strongly to chemotherapeutic agents [16, 17]. The JNKs contain three haplotypes: JNK1, JNK2 and JNK3. JNK1 and JNK2 are widely expressed among tissues, but JNK3 is only found in the brain, cardiac myocytes and testis [18]. Both JNK and p38MAPK can induce cellular apoptosis by regulating the pro-apoptotic protein Bax [19]. The PI3K/Akt pathway plays an important role in many physiological and pathological cell processes. Akt (also known as PKB) can suppress cellular apoptosis by reducing the level of pro-apoptotic protein Bax while also elevating the levels of the anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) [20].

Therefore, the aims of this study were to investigate the cellular responses of formononetin treatment in NPC cells and to explore the possible underlying mechanisms. We initially assessed the effects of formononetin on cell proliferation in NPC cells. We then examined whether formononetin alters cellular apoptosis regulators. Finally, we evaluated the anticancer effects of formononetin via the ectopic implantation of CNE1 cells. In this manner, we aimed to determine the therapeutic potential of formononetin for the intervention of NPC.

Materials and methods

Ethics statement

All animal experimental procedures were approved by the institutional animal research ethics committee of the Guangxi Medical University, document number 201505002. The certification number for the use the housing facility for laboratory animals is 0000345. The BALB/c nude mice were supplied by the Guangxi Medical University Laboratory Animal Center (Guangxi, China).

Reagents

Formononetin (purity >99%) was purchased from Shilan Technology (Tianjin, China). It was dissolved in dimethyl sulfoxide (DMSO) to create a 100 mM/L stock solution and stored at 4°C until further use. The final concentration of DMSO was 0.1% in cell experiments.

Cell culture

The human NPC cell lines, CNE1 and CNE2, were obtained from the Cancer Research Institute of the Central South University (Changsha, China). The cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 media (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 kU/L penicillin and 100 mg/L streptomycin.
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Figure 2. Proliferation inhibition of CNE1 and CNE2 NPC cells treated with formononetin. After being treated with indicated concentrations of formononetin separately for 24, 48 and 72 h, the viability of CNE1 (A) and CNE2 (B) cells were measured in an MTT assay. The data are presented as a ratio with the mean ± SD of three independent experiments with duplicate each time. *P<0.05 and **P<0.01 indicate significant difference compared with untreated cells.

at 37°C in 5% CO$_2$ and a humidified atmosphere.

Cell growth inhibition analysis using an MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cell viability. CNE1 cells were plated in 96-well plates at 5×10$^3$ cells per well and cultured for various times (24 h, 48 h, 72 h) in either a control solution (0.1% DMSO) or in one of series of concentrations of formononetin (5, 10, 20, 40, 80, 160 μM/L). Then, 20 μL of MTT solution (Sigma, St. Louis, MO, USA) was added to each well (5 mg/mL) and incubated for 4 h at 37°C. After removing the supernatants, the MTT-formazan product was dissolved in 150 μL of DMSO. The optical density was measured with an absorbance microplate reader (Thermo Scientific, Rockford, IL, USA) at wavelength of 490 nm.

Cell apoptosis analysis by Hoechst 33258

CNE1 cells were plated in 6-well plates and treated with control (0.1% DMSO) and various concentration of formononetin (5, 10, 20, 40 μM/L) for 48 h. Then, cellular morphology was observed using light microscopy. After washing three times with phosphate-buffered saline (PBS; pH 7.4), the cells were fixed in 4% formaldehyde for 10 min and stained with Hoechst 33258 (Sigma, St. Louis, MO, USA) for 15 min. The cells were observed under a fluorescence microscope at a wavelength of 350 nm. The cells with fragmented or condensed nuclei were defined as apoptotic.

Cell apoptosis analysis by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA of CNE1 cells was obtained using TRIZOL reagent (Life Technologies, Carlsbad, CA, USA) after treatment in a control solution (0.1% DMSO) or a solution with formononetin (10, 20, 40, or 80 μM/L) for 48 h. Then, following the manufacturer’s instructions of the Reverse Transcription Kit (Thermo Scientific, Rockford, IL, USA), total RNA was converted to cDNA. Quantitative RT-PCR was performed in 96-well plates with a SYBR Green PCR Kit (Roche, Germany) using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with the following primer sequences: Bax gene, forward 5'-GGTTGTGCACCTTTTCTA-3' and reverse 5'-CGGAGGAAGTCCAAATGTC-3'; caspase-3 gene, forward 5'-TGCACTCCACAGCACCCTGGTTA-3' and reverse 5'-CACTCGACAAAGCGACTGGATGAA-3'; and GAPDH gene, forward 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse 5'-TGGTGAAAGACGCCAGTGGA-3'. The mRNA levels were analyzed using the ΔΔCT method with GAPDH mRNA as the internal control of each sample.

Signal pathway analysis by western blot

CNE1 cells were cultured as described above. After incubation in a control solution (0.1%
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DMSO) and in formononetin (5, 10, 20, or 40 μM/L) for 48 h, cellular proteins were collected after lysing the cells with RIPA buffer containing protease and phosphatase inhibitors. The BCA Kit (Beyotime, China) was used to measure the total protein concentrations. Then, an equal amount of protein (30 μg) from each sample was separately subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (0.22 μm, Millipore, MA, USA). Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skim milk powder was used to block the membranes, which were then incubated separately overnight at 4°C with the primary antibodies caspase-3 (1:800), Bax (1:2,000), Bcl-2 (1:1,000), GAPDH (1:10,000) (Proteintech, Chicago, IL, USA); AKT (1:1,000), p-AKT (1:1000), p38 (1:1,000), p-p38 (1:1,000), JNK1/2 (1:1,000) and p-JNK1/2 (1:1,000) (Cell Signaling Technology, Danvers, MA, USA). After washing with TBST three times, the membranes were immunoblotted with an IRDye 680RD secondary antibody (1:10,000, room temperature for 1 h; Licor, NE, USA). Each protein band intensity was scanned and quantified using the Odyssey infrared imaging system (Licor, NE, USA) and normalized to the intensity of total AKT, total p38, total JNK1/2 and GAPDH.

**In vivo analysis of anticancer effects using tumor xenograft mouse models**

18 Five- to six-week-old (weight 18±2 g) male nude BALB/c mice were purchased from the Guangxi Medical University Laboratory Animal Center (Guangxi, China). The mice were fed under sterile conditions on a 12 h light: dark cycle and at a constant temperature of 22-24°C with 40-50% relative humidity in isolated pathogen-free ventilation chambers. A total of 2×10^6 CNE1 cells were resuspended in PBS and injected subcutaneously near the left armpit. When tumors reached a 5 mm diameter in length, tumor-bearing mice were randomly divided into three groups with six mice per group. Two groups were injected with either a high dose or a low dose (20 and 10 mg/kg, respectively) of formononetin intraperitoneally every 2 days for 10 days. The control group was treated with the same volume of 0.1% DMSO in PBS. All animals were sacrificed under anesthesia after treatment for 22 days. During the experiment, the long and the short diameter of tumors were measured every 2 days. To calculate the tumor inhibition rate (IR), the mass (in grams) of tumor was weighed on the twenty-second day. Characteristics to describe the tumors included the tumor growth curve, tumor mass and IR.
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Statistics

The data are expressed as the mean ± standard deviation (SD). The differences of the mean values were analyzed in a one-way analysis of variance (ANOVA) and Student’s t-test using the Statistical Package for Social Sciences (SPSS) 16.0 software (Chicago, IL, USA). A *p*-value of <0.05 was considered significant.

Results

Cytotoxicity of formononetin against CNE1 and CNE2 cells in vitro

Two human NPC cell lines, CNE1 and CNE2, were treated with 0-160 µM/L formononetin for 24, 48 and 72 h (CNE1, Figure 2A; CNE2, Figure 2B). Compared with the control vehicle group (0 µM/L), formononetin significantly reduced the viability of the two tested NPC cell lines in a dose-dependent manner (*P*<0.05), while the time-dependent effects of formononetin were not significant (*P*>0.05). The most pronounced effect was observed in CNE1 cells with the highest dose of 160 µM/L.

Effects of formononetin on apoptosis of human CNE1 cells

The Hoechst 33258 staining (Figure 3) assay was used to analyze the effect of formononetin on inducing apoptosis of CNE1 cells. After treatment with different concentrations of formononetin (0, 5, 10, 20 or 40 µM/L, shown as Figure 3A-E, respectively) for 48 h, the cells were observed through a fluorescence microscope to evaluate the presence of morphological changes indicative of apoptosis. As shown in Figure 3 (marked with arrows), the apoptotic cells were smaller and had characteristic condensed and fragmented nuclei. Taken together, these results indicate that formononetin could trigger apoptosis in CNE1 cells, which is consistent with the results of the MTT assay.

Formononetin increased the mRNA expression of Bax and caspase-3

Bax is a key gene in the intrinsic pathway that mediates apoptosis, and caspase-3 is a key executor of cellular apoptosis. To further investigate formononetin-induced apoptosis in human CNE1 cells, mRNA levels of Bax (Figure 4A) and caspase-3 (Figure 4B) were evaluated using qRT-PCR after treatment with formononetin (0, 10, 20, 40, or 80 µM/L). The mRNA expression levels of Bax and caspase-3 were significantly increased compared with the control group (0 µM/L; *P*<0.05). These results further confirmed that formononetin could stimulate apoptosis in CNE1 cells through activation of the mitochondrial apoptosis pathway in a dose-dependent manner.

Formononetin increased the protein expression of p-JNK1/2, p-p38, Bax and caspase-3 and decreased the protein expression of p-Akt and Bcl-2

Cellular apoptosis is usually regulated by two main pathways, PI3K/Akt and MAPK. Hence,
Formononetin targets the MAPK and PI3K/Akt pathways and regulation of downstream apoptosis-related proteins. When CNE1 cells were pretreated with specific concentrations of formononetin (0, 5, 10, 20, or 40 µM/L) for 48 h, protein expression was evaluated by western blot. The phosphorylation levels of Akt (A), JNK1/2 (B) and p38 (C) were normalized to total Akt, total JNK1/2 and total p38, respectively, while the downstream proteins caspase-3 (D), Bax (E) and Bcl-2 (F) used GAPDH as a control. The results are the expression of three independent experiments (n=3), *P<0.05 and **P<0.01 vs. control.

Figure 5. Formononetin inhibits in vivo growth of ectopically implanted CNE1 tumors

To further investigate the antitumor effect of formononetin in vivo, a nude BALB/c mice xenograft model was adopted. A CNE1 cell suspension was subcutaneously injected into mice. After tumor induction, tumor-bearing mice were randomly divided into three groups (n=6) and treated with diluent vehicle (1% DMSO in PBS) or formononetin (10 and 20 mg/kg). The tumor volumes (Figure 6B) were measured every 2 days. There were no deaths during the experimental period. All animals were sacrificed, and the tumor tissue samples were excised (Figure 6A) and weighed two days after the final treatment. Tumor mass (Figure 6C) was reduced in a dose-dependent manner (0.9001±0.1801 g
in control, 0.6289±0.1934 g and 0.1635±0.0844 g in the low- and high-dose group, respectively), which was consistent with the tumor volume (1533±270 mm$^3$, 966±353 mm$^3$ and 289±132 mm$^3$ in the control, low- and high-dose groups, respectively). The tumor IR was calculated according to average tumor weight of each group (Figure 6D). These data suggested that formononetin might have anticancer activity in vivo, similar to established chemotherapeutic drugs.

Discussion

The main etiological factors of NPC include genetic susceptibility, environmental factors and latent infection of the Epstein-Barr virus (EBV). Because the cancer growth occurs in a painless area (mostly arising in the fossae lateral pharyngeal and lateral walls) without the typically symptoms of the early stages of the disease, most NPC patients present at a late stage at first diagnosis [1]. Therefore, early diagnosis and efficient treatment is of paramount importance to NPC patients. As previously mentioned, it is imperative to identify new anticancer agents. A previous study demonstrated that traditional Chinese Medicine, as an adjunctive therapy, offers benefits to NPC patients, such as minimizing side-effects, maximizing tumor control and improving quality of life [21]. However, because of the complexity of traditional Chinese medicine, these results have not been widely recognized by the international community. Previous studies have demonstrated that molecular targeting is a novel and promising therapy for NPC [22, 23]. Hence, as a single agent of Chinese herbal medicine, we chose to study the potential anticarcinogenic activity of formononetin (Figure 1; purity >99%) in a series of in vitro and in vivo experiments. The results confirmed our hypothesis that formononetin has significant antitumor activity on NPC, which has not been previously reported. Moreover, investigating the underlying mechanism may offer help in the discovery of novel anticancer drugs.
Sustained proliferation and resistance to cell death are two of the six biological capabilities of human cancer cells that cause uncontrolled tumor growth [24]. Accordingly, a potential anticancer agent must possess suppress growth and induce apoptosis. In our study, we exposed two human NPC cells to concentrations of formononetin and found that formononetin inhibited the growth of CNE1 and CNE2 cells, depending on the dosage but not the exposure time (Figure 2). However, treatment with drugs for 24 h was too short of an exposure period for the cells to adapt to the microenvironment, whereas 72 h of drug treatment induced a response due to a change in cell metabolism. Hence, a 48 h treatment was chosen in all subsequent in vitro experiments. In addition, CNE1 cells were more sensitive to formononetin. Chemotherapy is necessary to treat an advanced-stage tumor, particularly highly differentiated tumors. This study used CNE1 cells that originated from a highly differentiated tumor tissue for treatment with formononetin for 48 h.

Evidence indicates that apoptotic cell death pathway plays a role in maintaining normal cellular homeostasis. Furthermore, insufficient apoptosis is a hallmark of cancer [24-26]. Previous research has suggested that tumor chemotherapy is closely related to the mitochondrial apoptosis pathway [27]. In accordance with these findings, our result obtained using Hoechst 33258 staining assay indicated that formononetin can promote apoptosis in CNE1 cells. Caspase is associated with the intrinsic (mitochondrial) apoptosis pathway [25]; furthermore, among the caspase family members, caspase-3 is an executor of the apoptotic program [28]. To further investigate the pro-apoptotic properties of formononetin on CNE1 cells at the molecular level, we investigated the mRNA expression levels of Bax and caspase-3 through qRT-PCR, and the protein expression of Bax, Bcl-2 and caspase-3 were evaluated using western blots. The results revealed that both the mRNA and protein expression of the pro-apoptotic Bax and caspase-3 genes increased, whereas the protein expression of the anti-apoptotic gene Bcl-2 decreased. In summary, formononetin can dose-dependently induce apoptosis in CNE1 cells through the mitochondrial pathway, which suggests that formononetin may be used as a candidate chemotherapy drug for the treatment of NPC.

Cooperation with oncogenic signals, such as PI3K/Akt signaling and the MAPK pathway, plays a key role in driving tumorigenesis and resistance to apoptosis for many cancers [27]. The PI3K/Akt pathway is a target of anticancer drugs [29]. Moreover, Akt can inactivate pro-apoptotic factors in cell survival [13]. MAPks are closely associated with the generation and development of several solid tumors [14]. Thus, we chose to evaluate the levels of phosphorylated p38MAPK and the ubiquitously expressed JNK1/2 to determine the involvement of the MAPK pathway. Likewise, Akt expression was evaluated to determine the involvement of the PI3K/Akt signaling pathway. Generally speaking, Akt activation is beneficial to the tumor [13], whereas p38MAPK suppresses cancer [15]. Interestingly, the JNK proteins appear to have a contradictory function, in that transient JNK activation can promote cell survival, whereas sustained activation leads to cell apoptosis [30]. Although prolonged JNK activation has been observed in NPC, the effect remains unclear [31]. Therefore, we focused on the phosphorylation of Akt, p38MAPK and JNK1/2. The results of the western blot assay indicated that treatment of CNE1 cells with formononetin dose-dependently increased the phosphorylation of p38MAPK and JNK1/2 while decreasing Akt phosphorylation. These results demonstrated that formononetin could activate MAPK signaling and suppress the PI3K/Akt pathway in CNE1 cells. In addition, the overactivation of JNK1/2 may promote apoptosis; however, additional cellular molecular experiments are needed to confirm this possibility.

To further substantiate the anticancer pharmacological effects of formononetin, we performed in vivo experimentation. Remarkably, formononetin could restrain tumor growth in CNE1 cell xenograft mice in a dose-dependent manner, in accordance with our hypothesis.

Taken together, our results demonstrate the significant anticancer effects of formononetin on human NPC in vitro and in vivo. Moreover, we identified the molecular mechanism, which included the activation of MAPK signaling and the suppression of the PI3K/Akt pathway to ultimately induce apoptosis through the mitochondrial apoptotic pathway. These results pro-
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vide preliminary evidence for the development of formononetin as a potential chemotherapeutic drug and for continuing the search for new anticancer agents for the clinical treatment of NPC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Anzhou Tang, Department of Otolaryngology Head and Neck Surgery, The First Affiliated Hospital of Guangxi Medical University, No. 6 Shuangyong Road, Nanning 530021, Guangxi, China. Tel: +86 771 5356511; Fax: +86 771 5356511; E-mail: anzhoutang@163.com

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