Pharmacogn. Mag.
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ORIGINAL ARTICLE

Formononetin Induces Apoptosis of PC-3 Human Prostate Cancer Cells via Regulating Long Noncoding RNA H19 and the Mitochondrial Apoptosis Pathway

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Submitted: 26-Jul-2019 Revised: 04-Sep-2019 Accepted: 17-Feb-2020 Published: 15-Jun-2020

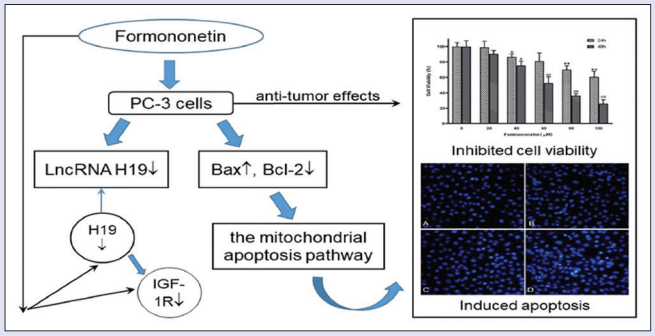
ABSTRACT

Background: Prostate cancer is a life-threating disease with high incidence and mortality in male. Formononetin, the main active component of some natural products, has been hypothesized as a promising anticancer agent in previous studies. **Objectives:** We investigated the toxic effects and potential molecular mechanism of formononetin in PC-3 prostate cancer cells to further understand the pharmacological effects of formononetin and provide more references for intensive research. **Materials and Methods:** PC-3 cells were incubated with different doses of formononetin for 24 h or 48 h. After that, cell viability was measured by Cell Counting Kit-8, and apoptosis was analyzed by Hoechst 33258 stains. The expression levels of tumor-related factors such as long noncoding RNA (LncRNA) H19, Bax, and Bcl-2 were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot methods. Subsequently, PC-3 cells were infected with a lentiviral vector to overexpress or knock down H19, and then, the expression of insulin-like growth factor-1 receptor (IGF-1R) mRNA was measured by RT-qPCR. **Results:** Formononetin significantly inhibited the viability of PC-3 cells and promoted apoptosis in a time-dose-dependent manner. We observed that the expressions of lncRNA H19 and Bcl-2 were significantly downregulated compared with the untreated group, while an opposite pattern was observed for Bax. According to the results of gene interaction experiments, IGF-1R may be a downstream target of H19 in PC-3 cells. **Conclusion:** Our results present evidence that formononetin induced apoptosis of PC-3 cells by regulating lncRNA H19 and the mitochondrial apoptosis pathway. Furthermore, we put forward the hypothesis that formononetin has an interference effect on the H19/IGF-1R pathway, which remains to be further confirmed.

Key words: Bax, Bcl-2, formononetin, H19, insulin-like growth factor-1 receptor, PC-3 cells

SUMMARY

- Formononetin inhibited the viability of PC-3 cells and induced cell apoptosis
- Formononetin activated the mitochondrial apoptotic pathway in PC-3 cells
- Formononetin down-regulated long noncoding RNA H19 in PC-3 cells
- Formononetin was likely to have an interference effect on the H19/insulin-like growth factor-1 receptor pathway in PC-3 cells, which remains to be further confirmed.



Abbreviations used: LncRNA: Long noncoding RNA; IGF-1R: Insulin-like growth factor-1 receptor; IGFs: Insulin-like growth factors.

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DOI: 10.4103/pm.pm_320_19

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INTRODUCTION

Prostate cancer is one of the most prevalent malignancies worldwide. According to the American Cancer Society, prostate cancer is the most frequently diagnosed cancer among 36 cancers in 185 Countries for men in 2018.[1] Traditional therapies for prostate cancer such as surgical operation, radiotherapy, and chemotherapy undertake the risk of causing unavoidable side effects and complications. According to previous studies, medicinal plants represent great resources for cancer-targeted drugs, which cause little toxic effects on normal cells.[2] Consequently, natural products research is a meaningful work.

Formononetin is derived from natural products such as *Trifolium pratense* L., *Pueraria lobata* (Willd.) Ohwi, *Astragalus membranaceus* (Fisch.) Bunge var. Mongholicus (Bunge) Hsiao, and *Spatholobus suberectus* Dunn. Plenty of studies have demonstrated that formononetin has an anti-proliferation effect in various neoplasms.[3-10] Although formononetin has already been proved to have the potential of combating prostate cancer cells *in vitro* and *in vivo*, the molecular mechanisms have not been thoroughly elucidated. In the present study, we gave a further investigation into the toxicity of formononetin

Cite this article as: Wang R, Li K, Xie Z, Wang B, Dai Y, Chen J, *et al*. Formononetin induces apoptosis of PC-3 human prostate cancer cells via regulating long noncoding RNA H19 and the mitochondrial apoptosis pathway. Phcog Mag 2020;16:435-40.

and its potential pharmacological mechanism in PC-3 human prostate cancer cells.

First, we focused on the expression level changes of tumor-related factors such as long noncoding RNA (LncRNA) H19, Bax, and Bcl-2 caused by the drug. Bcl-2 family proteins play a crucial role in the cell mitochondrial apoptotic pathway, Bcl-2 has a protective effect on cell survival, while Bax antagonizes the effects of Bcl-2. Detecting the expression of Bcl-2 and Bax is a commonly used method for evaluating the apoptosis of cells.[11-13] LncRNA is a class of RNA molecules with a length of more than 200 nucleotides. They do not code for proteins but serve crucial functions in disease progression. LncRNA H19 is derived from the imprinted gene H19. Some studies have indicated that lncRNA H19 is likely to act as a carcinogenic factor in malignant neoplasms, when H19 was knocked down, the proliferation and migration of cancer cells could be inhibited.[14-17] Insulin-like growth factor-1 receptor (IGF-1R) is the binding site of IGFs and generally upregulated in various malignant tumors.[18-23] Former studies have revealed that IGF-1R is a molecular target of formononetin and targeting IGF-1R is a candidate strategy for inducing cancer cells apoptosis.[4,24,25] Furthermore, the relationship between H19 and IGF-1R in mammalian cells has been illustrated in several researches. It is likely that H19/IGF-1R signal pathway participates in the regulation of animal's normal growth and disease generation. For example, in the study of Lei *et al.*, LncRNA H19 could regulate the proliferation of bovine male germ line stem cells via interfering the expression of IGF-1R.[26] Another study has indicated that IGF-1R may be a downstream target of H19 in HSC-T6 cells, through this pathway cell proliferation was interfered.[27] To explore the relationship between the two drug targets, we used lentiviral vectors to overexpress or knock down H19 to explore the connection between H19 and IGF-1R in PC-3 cells. By conducting gene interaction experiments, we put forward the hypothesis that the H19/IGF-1R signal pathway could be activated by formononetin in PC-3 cells based on former studies.

MATERIALS AND METHODS

Drug preparation and cell culture

Formononetin (purity >98%) was purchased from Desite (Chengdu, China). It was dissolved with DMSO and diluted to final concentrations with RPMI-1640 before use. PC-3 human prostate cancer cells were obtained from Guangdong Medical University (Zhanjiang, China). Phosphate-buffered saline, RPMI-1640, and fetal bovine serum (FBS) were purchased from Gibco (USA). The complete growth medium was composed of 90% RPMI-1640 medium and 10% FBS.

Proliferation assay

Proliferation assays were conducted by Cell Counting Kit-8 (CCK-8, Code No. C0037, Beyotime, China). PC-3 cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and provided with a 37°C and 5% CO_2 environment for 24 h before the drugs were added. When the cell growth density reached 30%, different concentrations of formononetin solutions (0, 20, 40, 60, 80, and 100 μM) were added. After drug treatments, culture liquids were replaced with CCK-8 solutions for additional 2 h. Optical density values of cells were determined on a microplate reader at the wavelength of 450 nm and 650 nm (Thermo Scientific, USA).

Apoptosis analysis

PC-3 cells were seeded into a 6-well plate at a density of 1×10^5 cells/well and treated with different doses of formononetin (0, 25, 50, and 100 μM) for 48 h. Adherent cells were fixed with a fixative solution (Code No. C0003, Beyotime, China) for 3 h and stained with Hoechst 33258 solution (Code No. C0003, Beyotime, China) for 5 min in the dark condition. Cell apoptotic morphology images were captured by a fluorescence microscope (Olympus IX71, Japan). Cells showing enhanced stain changes were defined as the apoptotic cell.

RNA isolation and reverse transcription-quantitative polymerase chain reaction

PC-3 cells were treated with various doses of formononetin (0, 25, 50, and 100 μM) for 48 h; after that, total RNA in each group was isolated by the RNAiso Plus lysis solution (Code No. 9108, Takara, Japan) and then subjected to reverse transcription procedure according to the kit protocol (PrimeScript RT reagent Kit with gDNA Eraser, Code No. RR047A, Takara, Japan). Next, relative expression level analyses of tumor-related factors (lncRNA H19, Bax and Bcl-2) were conducted by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) kit SYBR Premix Ex TaqII (Code No. RR820A, Takara, Japan) in an Applied Biosystems 7500 Instrument (Thermo Scientific, USA). β-Actin was set as the internal reference. Primers' sequences were listed in Table 1.

Western blot analysis

After treated with various concentrations of formononetin (0, 25, 50, and 100 μM) for 48 h, PC-3 cells were collected and decomposed with cytolysis buffer (Solarbio, China). After 30 min, total proteins in each group were obtained. Bax and Bcl-2 proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, USA) in

Table 1: Primers used for quantitative reverse transcription polymerase chain reaction

Primer name	Sequence 5′-3′
β-actin	Forward: CCTGGCACCCAGCACAAT
	Reverse: GGGCCGGACTCGTCATAC
IGF-1R	Forward: GCTTGCCGCCACTACTACTATGC
	Reverse: CGCAGAAGTCACGGTCCACAC
H19	Forward: ACTCAGGAATCGGCTCTGGAAGG
	Reverse: GATGTGGTGGCTGGTGGTCAAC
Bcl-2	Forward: GAGCATCAGGCCGCCACAAG
	Reverse: CTTCCAGACATTCGGAGACCACAC
Bax	Forward: GATGCGTCCACCAAGAAGCTGAG
	Reverse: CACGGCGGCAATCATCCTCTG

IGF-1R: Insulin-like growth factor-1 receptor

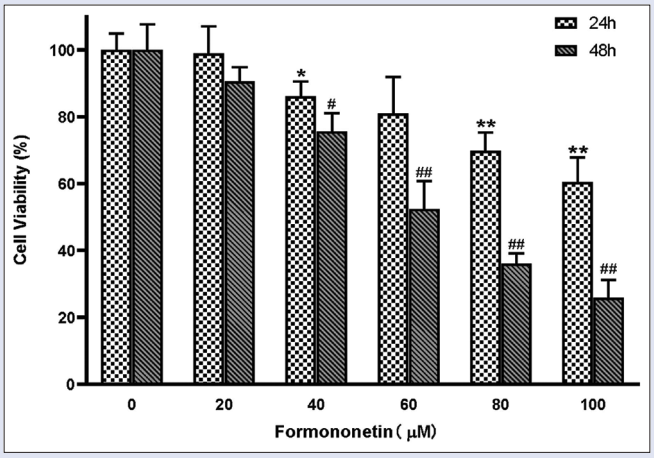

Figure 1: Formononetin inhibited the viability of PC-3 cells in a time-dose-dependent way. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the untreated group in 24 h. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the untreated group in 48 h, $n = 5$

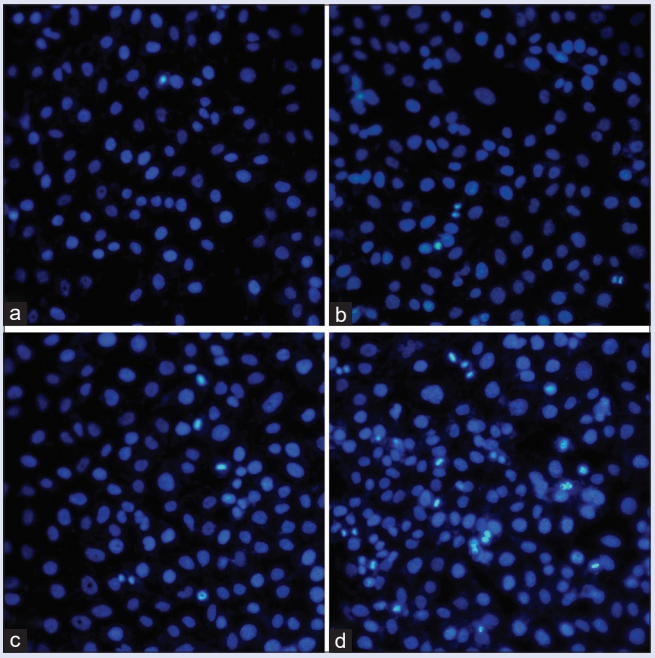

Figure 2: Formononetin induced PC-3 cells apoptosis in a dose-dependent manner. (a) 0 μM formononetin (b) 25 μM formononetin (c) 50 μM formononetin (d) 100 μM formononetin

electrophoresis apparatus (LiuYi, China). Subsequently, protein bands were blocked with 5% non-fat milk (Solarbio, China) and incubated with antibodies (Bax and Bcl-2 rabbit monoclonal antibodies: Cell Signaling Technology, USA, 1:1000 dilution; goat anti-rabbit IgG antibody: Beyotime, China, 1:1000 dilution). β-Actin protein antibodies (Bioss, China, 1:5000 dilution) were used as the internal control. Protein

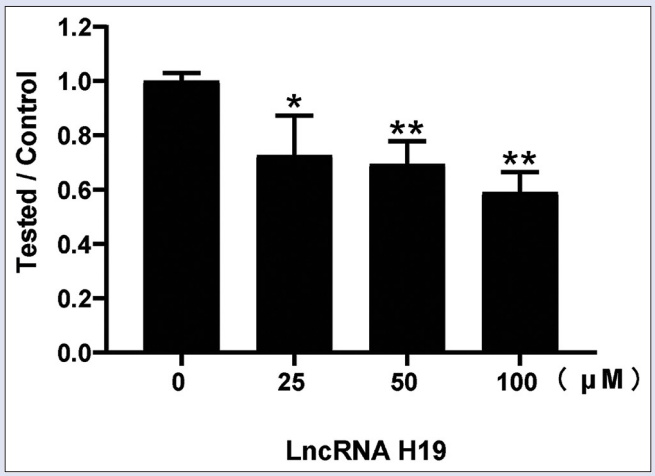

Figure 3: Formononetin decreased the expression of lncRNA H19 in PC-3 cells. *$P < 0.05$, **$P < 0.01$ versus the untreated group, $n = 3$

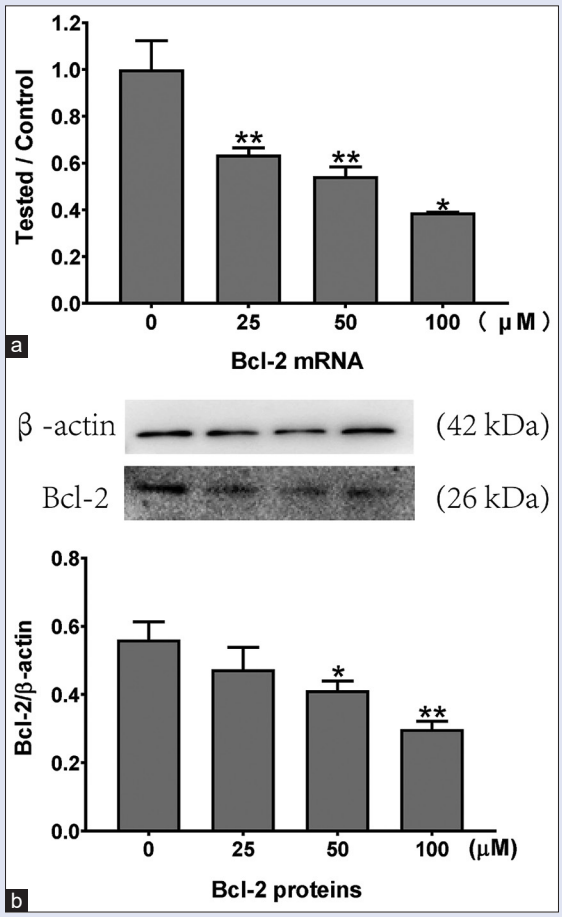

Figure 4: Formononetin had an inhibitory effect on Bcl-2. (a) Bcl-2 mRNA (b) Bcl-2 proteins. *$P < 0.05$, **$P < 0.01$ versus the untreated group, $n = 3$

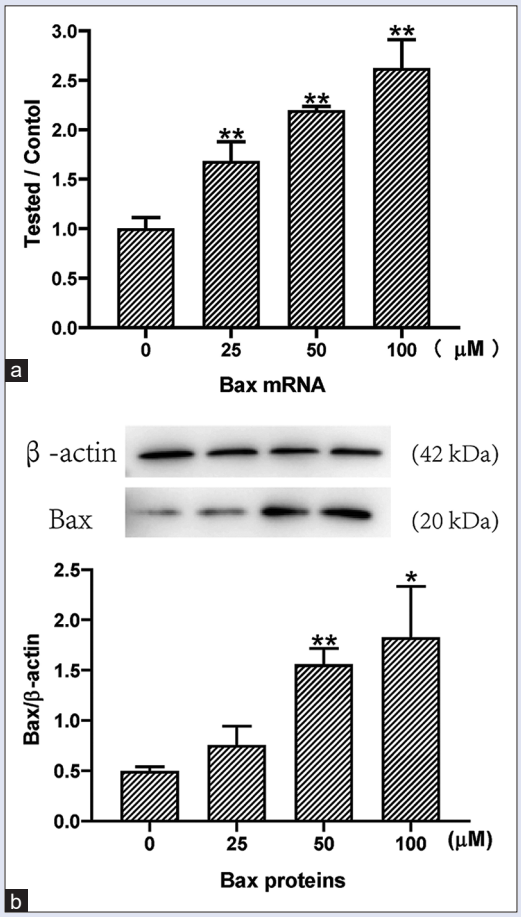

Figure 5: Formononetin increased the expression of Bax. (a) Bax mRNA (b) Bax proteins. *$P < 0.05$, **$P < 0.01$ versus the untreated group, $n = 3$

expressions were detected by a LAS500 Imaging system (GE Healthcare, USA).

Lentivirus vector constructions and gene expression detection after transfection

Viral vectors were constructed (Genechem, China) and transfected into PC-3 cells referring to the manufacturer's protocol. In the trial tests, we observed cells that presented the strongest fluorescence signals when multiplicity of infection was set to 50 and the infection duration was set to 96 h. Considering the above-mentioned experimental conditions, PC-3 cells in each group were co-incubated with H19 overpression vectors, H19 silencing vectors, or empty vectors for 96 h, respectively. Then, the transfection efficiency was confirmed by GFP fluorescence screening. The expression levels of lncRNA H19 and IGF-1R mRNA in each group were measured by RT-qPCR.

Data analysis

All the results were repeated at least three times and presented as mean ± standard deviation. One-way ANOVA and Student's *t*-test were used for data analyses in a SPSS 13.0 system (SPSS, Chicago, IL, USA). Differences with a probability value of $P < 0.05$ between groups were considered significant.

RESULTS AND DISCUSSION

Using the active ingredients of natural products as supplements for chemotherapeutic drugs may be a recommendable option to enhance the curative effect of cancer chemotherapy, owing to the combined results of reducing drug resistance and side effects.[28-31] Here, we chose PC-3 human cells as the experimental model to further investigate the toxic effects and potential molecular mechanism of formononetin in prostate cancer.

CCK-8 assay validated that the cell viability of 40–100 μM formononetin groups was significantly lower compared with the untreated group at both 24 h and 48 h [Figure 1]. As shown in Figure 2, the apoptotic cells' (enhanced fluorescence stain) proportions accumulated gradually with the increase of drug doses. The results above suggested that formononetin could inhibit the viability of PC-3 cells and induce cells apoptosis in a time-dose-dependent manner, which added evidence to the conjecture that formononetin could be used as a candidate agent for suppressing the growth of prostate cancer cells.

Subsequently, to further investigate the internal effects of formononetin on PC-3 cells, we conducted RT-qPCR and Western blot experiments to detect the expression level changes of tumor-related factors such as lncRNA H19, Bax, and Bcl-2 after drug treatments. It was observed that the expressions of lncRNA H19 and Bcl-2 were significantly downregulated compared with the control group, while an opposite pattern was observed for Bax ($P < 0.05$) [Figures 3-5]. These data revealed that formononetin played antitumor effects by regulating lncRNA H19 and Bcl-2 family molecules. Bcl-2 and Bax proteins play a crucial role in the cell mitochondrial apoptotic pathway; consequently, formononetin may induce apoptosis of PC-3 cells through the mitochondrial apoptotic pathway.

As has been noted, H19 could be identified as a candidate target for cancer treatments.[32,33] In this experiment, we observed that formononetin could inhibit PC-3 cells by downregulating H19, so targeting H19 is likely to be an effective means to suppress PC-3 prostate cancer cells. Considering former studies, we provide complementary new insights into the interaction between H19 and IGF-1R in PC-3 cells. Considering former studies, we provide complementary new insights into the interaction between H19 and IGF-1R in PC-3 cells, and there might be a connection between H19 and IGF-1R in human cells.[34,35] Here, we investigated

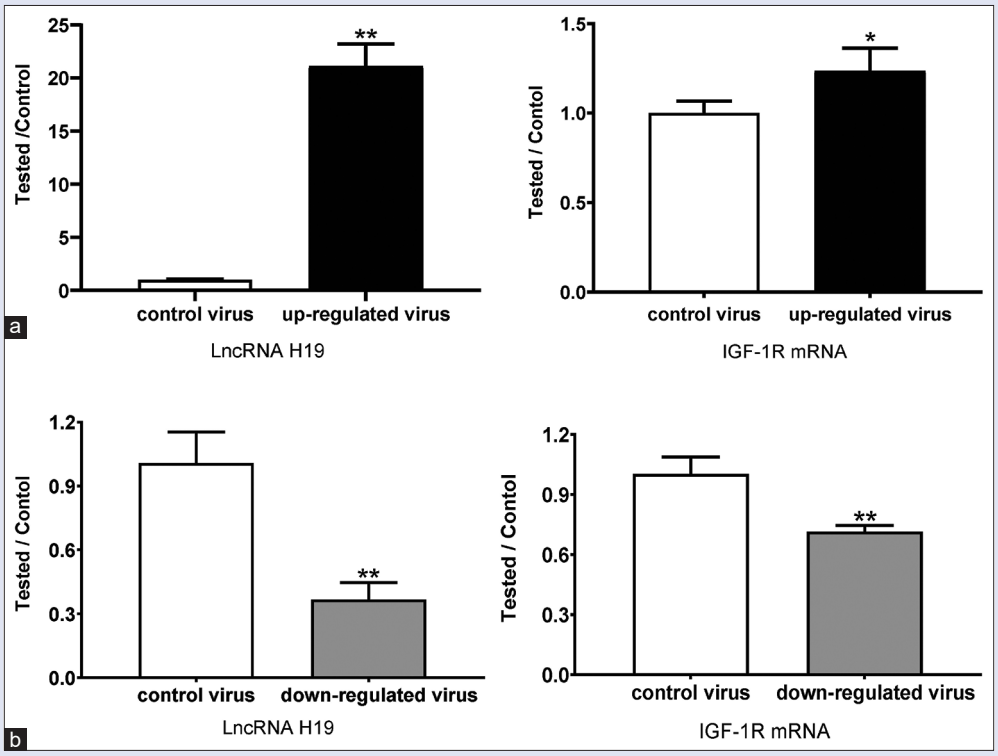

Figure 6: H19 may have a regulatory effect on insulin-like growth factor-1 receptor in PC-3 cells. (a) H19 upregulated significantly increased the expression of insulin-like growth factor-1 receptor mRNA. (b) H19 downregulated significantly decreased the expression of insulin-like growth factor-1 receptor mRNA. *$P < 0.05$, **$P < 0.01$ versus the untreated group, $n = 3$

the relationship between H19 and IGF-1R in PC-3 cells. Interestingly, compared with the empty vector groups, H19 upregulated in PC-3 cells significantly increased the expression of IGF-1R mRNA, while H19 downregulated significantly decreased the expression of IGF1R mRNA [Figure 6a and b], which means IGF-1R may be a downstream target of H19 in PC-3 cells. Consequently, we presented the hypothesis that there is a H19/IGF-1R regulatory pathway in PC-3 cells. Previous studies have reported that formononetin had a remarkable inhibitory effect on IGF-1R and could enhance the Bax/Bcl-2 ratios in PC-3 cells.[4,36] Our experiments gave a supplement to their findings and further investigated the connection between molecular targets of formononetin to establish the possible gene regulatory networks and lay a foundation of farther biological experiments. In consideration of the above findings, we speculated that formononetin inhibited the viability of PC-3 cells by targeting H19/IGF-1R regulatory pathway. However, there is a need for further studies to offer deeper insights into this pathway. In addition, according to numerous researches, in mammals, the effect of H19 on IGF-1R may be mediated by miR-675, which is derived by lncRNA H19 and could bind to the 3'-UTR of IGF-1R mRNA, consequently inhibits the translation of IGF-1R.[34,35,37-39] Besides, Ghazal *et al*. have reported that in the endometrial stromal cells, H19 altered IGF-1R expressions by acting as the molecular sponge of microRNA let-7.[40] Since H19 and IGF-1R are both potential anticancer targets, untangling the regulation mechanism of the two genes is worth exploring in future studies.

CONCLUSION

Taken together, the results of our study indicated that formononetin induces apoptosis of PC-3 human prostate cancer cells by regulating lncRNA H19 and the mitochondrial apoptosis pathway. Based on former studies, we put forward the hypothesis that formononetin has an interference effect on the H19/IGF-1R pathway, which remains to be further confirmed. In brief, our experiments furtherly investigated the pharmacological mechanism of formononetin and indicated the potential value of formononetin as an antiprostate cancer agent. However, more pharmacology studies are needed for the development of novel phytomedicine for cancer treatment.

Acknowledgements

Authors are thankful to National Natural Science Foundation of China.

Financial support and sponsorship

This work was supported by the National Natural Science Foundation of China (NO.81560418)..

Conflicts of interest

There are no conflicts of interest.

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