



Formononetin inhibits osteosarcoma cell proliferation and promotes apoptosis by regulating the miR-214-3p/phosphatase and tensin homolog pathway

Kang Li^{1,2}, Hongyuan Shen², Minghui Lu³, Jiarong Chen², Qingshui Yin², Pingyue Li²

¹Southern Medical University, Guangzhou 510515, China; ²Department of Orthopedics, General Hospital of Southern Theatre Command, Guangzhou, China; ³The University of the South: Sewanee, TN, USA

Contributions: (I) Conception and design: K Li, Q Yin; (II) Administrative support: Q Yin; (III) Provision of study materials or patients: M Lu, P Li; (IV) Collection and assembly of data: K Li, H Shen, M Lu; (V) Data analysis and interpretation: K Li, J Chen, M Lu; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Prof. Qingshui Yin. Department of Orthopedics, General Hospital of Southern Theatre Command, Liuhua Road, NO. 111 Street, Guangzhou 510010, China. Email: lk99199@163.com.

Background: Phytoestrogens have a similar molecular structure to estrogens which can produce either estrogenic or anti-estrogenic effects. It is generally believed that phytoestrogens combine with the estrogen receptor of osteosarcoma cells, affecting a variety of signal transduction pathways and cell metabolism, resulting in altered cell proliferation, differentiation, apoptosis, invasion and migration ability. Formononetin (FN) is the active ingredient of traditional Chinese medicine astragalus, angelica, and Pueraria lobate. Our study aims to detect the role of FN on MG-63 cell viability and apoptosis through regulating phosphatase and tensin homolog (PTEN) expression via MicroRNA-214-3p (miR-214-3p).

Methods: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Caspase 3 assay evaluated cell viability and apoptosis, respectively. Real-time quantitative polymerase chain reaction (qRT-PCR) and western blot evaluated the mRNA and protein expressions, respectively. The binding site of miR-214-3p/PTEN was detected via dual luciferase assay.

Results: FN suppressed cell viability and induced apoptosis, and decreased miR-214-3p level and promoted PTEN expression. PTEN was then regarded as a target of miR-214-3p, and FN improved PTEN level via inhibiting miR-214-3p. Further analysis showed that overexpressed miR-214-3p improved cell viability and suppressed apoptosis of MG-63 cells by inhibiting PTEN expression.

Conclusions: Finally, our results revealed that FN inhibited cell viability and induced apoptosis by regulating miR-214-3p. FN acted as a new treatment for MG-63 cells via increasing PTEN level by inhibiting the miR-214-3p level.

Keywords: PTEN; formononetin (FN); MicroRNA-214-3p; cell viability and apoptosis; osteosarcoma cell

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Introduction

Osteosarcoma is a malignant tumor originating from mesenchymal tissue (1). The metaphysis of long bones in adolescents has a high malignancy, and lung metastasis can occur early, with a poor prognosis (2). At present, comprehensive treatment with chemotherapy and surgical

resection as the core have been used to prolong the survival time of patients (3). However, because many mechanisms have not been resolved in the process of osteosarcoma development, osteosarcoma still has postoperative severe tumor recurrence and metastasis. Problems affecting the prognosis of patients (4). Most patients with osteosarcoma

have tumor suppressor gene suppression, including retinoblastoma (Rb) and phosphatase and tensin homolog (PTEN), high activation of the protein kinase B (AKT) pathway, and mutation of TP53 in some patients.

MicroRNAs (miRNAs) are non-lengths of 18 to 25 nt and encode small molecule RNAs (5). miRNAs combine with the target gene mRNA through base-pairing to degrade the target mRNA or inhibit its translation (6). During the development of osteosarcoma, the expression of various miRNA changes and the upregulated or downregulated miRNAs are widely involved in the occurrence, development of osteosarcoma (7). After searching the GEO database, we found that miR-214-3p was significantly upregulated in osteosarcoma (8). Moreover, miR-214-3p is one of the miRNAs with oncogene properties that are important in the process of various cancers (9). The miR-214-3p level is markedly increased in osteosarcoma (9,10).

Formononetin (FN) is the active ingredient of traditional Chinese medicine astragalus, angelica, and Pueraria lobate (11,12). It belongs to isoflavone phytoestrogen and has pharmacological activities, including anti-oxidation, enhanced immunity, and anti-inflammatory (13,14). Studies have found that FN can show satisfactory anticancer effects in ovarian cancer, colorectal cancer, and gastric cancer by suppressing cell viability and inducing apoptosis (15,16). However, the effect and mechanism of FN on osteosarcoma cell proliferation and apoptosis are not apparent.

Therefore, this study hypothesized that the function of FN in MG-63 cells might be related to miR-214-3p/PTEN. The *in vitro* experiments were performed in MG-63 cells. Our experiment shows crucial opinions into the role of FN on the progress and development of osteosarcoma cells.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-2296>).

Methods

Cell culture and Transfection

All media contained penicillin (100 IU/mL), 10% (v/v), FBS and streptomycin (100 mg/mL). Inoculate MG-63 cell (5×10^5 cells/well) in a 24-well plate, and then incubate overnight. GenePharma (Shanghai, China) synthesized the miR-214-3p mimic or PTEN plasmid. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) transfected miR-214-3p mimics (50 nM), PTEN plasmid (50 nM), and the corresponding negative controls in differentiated podocytes

following with the manufacturer's instruction.

Cell viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution measured the cell viability (Dojindo, Kumamoto, Japan). The MG-63 cells (2,000 cells/well) were seeded in 96-well plates. Then, the MG-63 cell was incubated in 10% MTT. And incubate in an incubator (37 °C, 5% CO₂) for 2 h. The OD value was evaluated at 450 nm.

Apoptosis

A caspase-3 assay kit evaluated the apoptosis (Beyotime, Shanghai, China). Caspase 3 belongs to the CED-3 subfamily of the Caspase family and is a protease that plays an important role in the process of apoptosis (17). Caspase 3 Assay Kit is based on Caspase 3 which can catalyze the substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) to produce yellow p-nitroaniline (pNA), which has strong absorption around 405 nm. The activity of Caspase 3 can be detected by measuring absorbance (yellow product is proportional to the activity of Caspase 3 enzyme). The MG-63 cells were treated with paraffin in 96-well plates for 90 minutes and passed through 80 µL of reaction buffer (caspase-3 substrate, 2 mmol/L, 10 µL). Ten µL of cell lysate protein was incubated in each sample in an incubator (37 °C, 5% CO₂) for 2 h. Used R&D Systems' colorimetric assay kit (Thermo Scientific, USA) to measure samples at 405 nm.

Luciferase reporter assay

The miR-214-3p putative binding site with wild type (WT) and mutant type (MUT) of PTEN 3'-UTR were combined and cloned into the pmirGLO dual-luciferase reporter vector (YouBio, Changsha, China). The reporter vectors (NC-mimic, miR-214-3p mimic, MUT, and WT 3'-UTR of PTEN) were co-transfected into HEK293T cells. After 48 h, the activity of luciferase was evaluated by a dual luciferase assay (Promega, Madison, USA), standardized as Renilla luciferase.

Real-time quantitative polymerase chain reaction (qRT-PCR)

Trizol reagent (YouBio) extracted the total RNA of cell samples. Then, the SuperScript RT kit (YouBio) reverse

transcribed 2 µg of total RNA. ABI Prism7900 Sequence Detection System with SYBR Green PCR Master Mix (Applied Biosystems, USA) was used to perform qRT-PCR. Corresponding primers were synthesized by GenScript company (Nanjing, China) and showed as follow: PTEN-forward: AGAC CATA ACCC ACCA CAGC; PTEN-reverse: TACA CCAG TCCG TCCT TTCC. GAPDH-forward: CGGC AAGT TCAA CGGC ACAG; GAPDH-reverse: CGCC AGTA GACT CCAC GACA T. miR-214-3p-forward: ACAG CAGG CACA GACA GGCA G; miR-214-3p-reverse: GTGC AGGG TCCG AGGT ATTTC. 5S-forward: GATC TCGG AAGC TAAG CAGG; 5S-reverse: TGGT GCAG GGTC CGAG GTAT. Among them, GAPDH and 5S rRNA were applied as internal references of above-mentioned genes and miR-214-3p respectively. Calculating targeted gene expression following the $2^{-\Delta\Delta CT}$ method (18). GAPDH and U6 snRNA are used as reference genes, respectively.

Western blot

Cells were lysed with pre-chilled radio-Immunoprecipitation Assay (RIPA) buffer (addition of cocktail and phosphatase inhibitor), 30 µg/sample was taken for sodium salt-Polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis, and transferred into a polyvinylidene fluoride (PVDF) membrane (0.45 µm), 1 × Tris- Buffered Saline Tween20 (TBST) The diluted 2% Bovine Serum Albumin (BSA) was blocked, and the primary antibody (PTEN, Caspase 3, Caspase 9, Bcl-2) was cultured (4 °C, 12 h). The goat Anti-Mouse IgG-HRP (1:3,000) (Solarbio) for β-actin is cultured (37 °C, 1 h). After 4 times of TBST washing, the images were added dropwise.

Statistical analysis

The GraphPad 8.0 was used for statistical analysis. All data were expressed as an independent experiment three times and shown as mean ± standard deviation (SD). Statistical analysis following with the ANOVA and the P<0.05 of data were considered significant.

Results

FN can down-regulate miR-214-3p expression and promote PTEN levels

To evaluate the inhibitory role of FN on the cell viability

of MG-63 cell treatment of FN (0, 15, 30, and 45 µM) for 48 h and then evaluated by MTT assay. As compared to the control group, as the FN concentration gradually increased, the cell viability was markedly inhibited (*Figure 1A*). After MG-63 cell treatment of FN, a definite increase in the number of apoptotic cells with 45 µM FN was found (P<0.05) (*Figure 1B*). The data showed that FN-induced apoptosis with dose-dependent. Then, qRT-PCR analysis on miR-214-3p and PTEN expressions after treatment of FN (0, 15, 30, and 45 µM) were also detected, and results exhibited the miR-214-3p level was markedly reduced and PTEN level was markedly improved by FN treatment with dose-dependent (*Figure 1C*). Differently, the PTEN level was improved after the treatment of FN (*Figure 1D*).

miR-214-3p can bind PTEN and inhibit PTEN expression

PTEN was whether predicted to the miR-214-3p by the TargetScan database, and the synthetic 3'-UTR fragment of PTEN was cloned into pmirGLO to construct WT or MUT dual-luciferase reporter plasmids (*Figure 2A*). Moreover, *Figure 2B* exhibited that the activity of luciferase was reduced after treatment of miR-214-3p mimic and WT-PTEN 3'-UTR vector as compared to that treatment of miR-214-3p mimic and MUT-PTEN 3'-UTR. MG-63 cells were treated with miR-214-3p mimic to verify the relationship, and the transfection efficiency was determined. The miR-214-3p level was markedly increased after the treatment of miR-214-3p mimic (*Figure 2C*). However, the PTEN level was significantly decreased by miR-214-3p mimic (*Figure 2D*). These data showed that miR-214-3p could bind PTEN and inhibit the PTEN level.

miR-214-3p improves MG-63 cell viability and inhibits apoptosis by down-regulating PTEN expression

To further determine whether PTEN was involved in the roles of miR-214-3p on the cell viability and apoptosis, MG-63 cell was treated with miR-214-3p mimic vector alone, or miR-214-3p mimic + PTEN vector for 24 h. The proliferation was inhibited, and the apoptosis was improved by the treatment of miR-214-3p mimic + PTEN vector as compared to that treatment of miR-214-3p mimic vector alone (*Figure 3A,B*). Moreover, the data of western blot exhibited that the Caspase 3, Caspase 9, PTEN protein expression was upregulated in the miR-214-3p mimic +

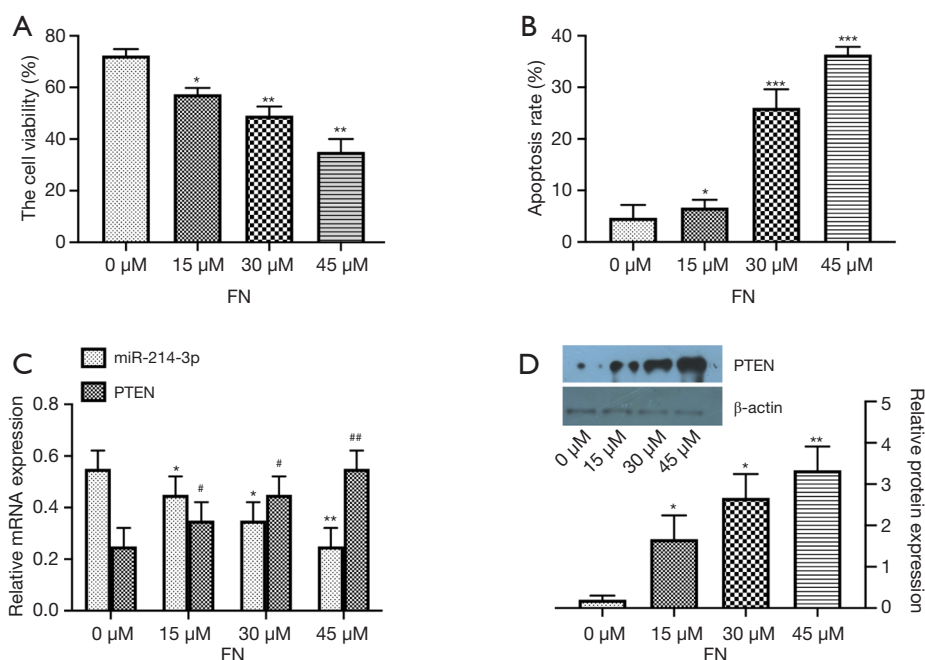


Figure 1 Effect of formononetin (FN) on the miR-214-3p and PTEN expression. (A) The cell viability was evaluated by MTT assay. (B) Caspase 3 kit was used to evaluate the apoptosis rate of cells. (C) The miR-214-3p and PTEN mRNA expressions were evaluated by real-time quantitative polymerase chain reaction (qRT-PCR). (D) Western blotting evaluated the PTEN level. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. FN (0 μ M); # $P < 0.05$, ## $P < 0.01$ vs. FN (0 μ M) PTEN.

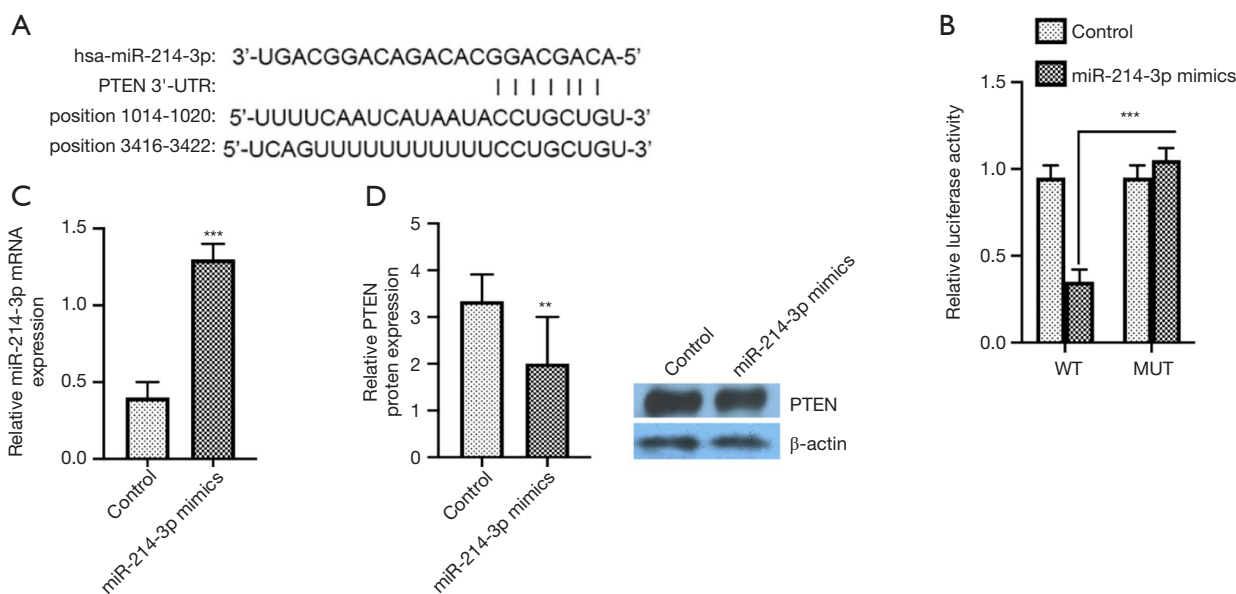


Figure 2 miR-214-3p binds phosphatase and tensin homolog (PTEN) and inhibit PTEN expression. (A) TargetScan predicts miR-214-3p targets the PTEN. (B) the miR-214-3p binding site on the 3'-UTR of PTEN mRNA was evaluated, and the activity of luciferase was examined in HEK-293T. (C) Real-time quantitative polymerase chain reaction (qRT-PCR) evaluated the miR-214-3p mRNA expression. (D) Western blot evaluated the PTEN level. ** $P < 0.01$, *** $P < 0.001$ vs. control.

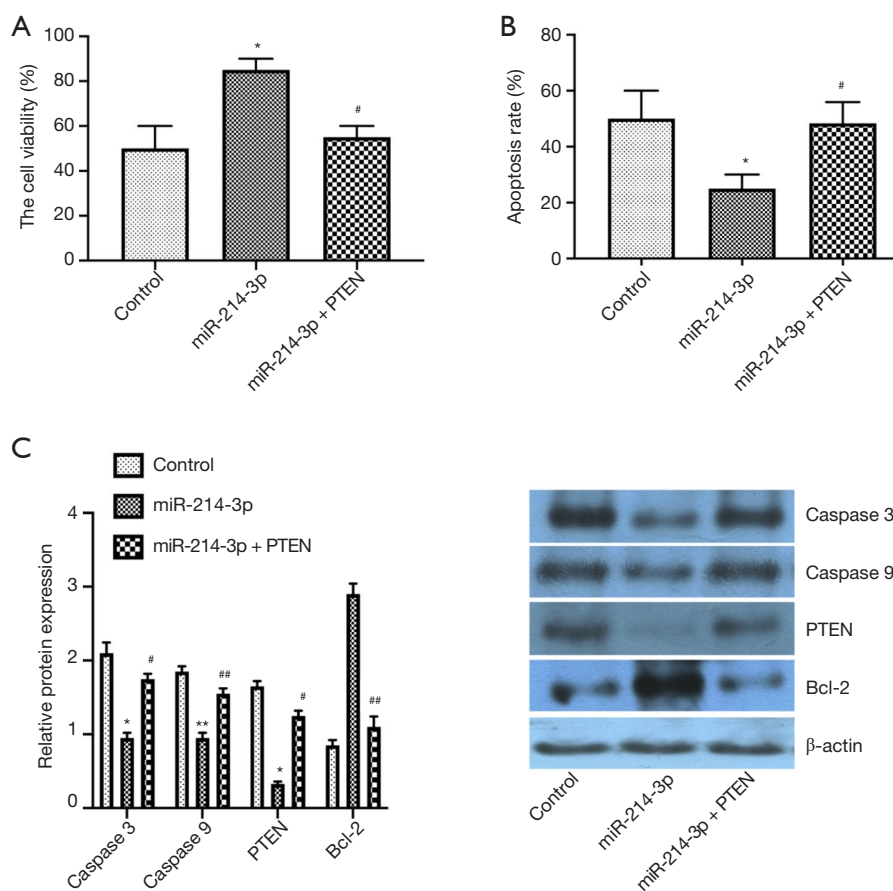


Figure 3 miR-214-3p promotes MG-63 cell viability and inhibits apoptosis through down-regulating phosphatase and tensin homolog (PTEN) expression. (A) The cell viability was evaluated by MTT assay. (B) Caspase 3 kit was used to evaluate the apoptosis rate of cells. (C) Caspase 3, Caspase 9, PTEN, Bcl-2 levels were evaluated by western blotting. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. miR-214-3p mimics.

PTEN group as compared to the miR-214-3p mimic group, while the expression of Bcl-2 was down-regulated (Figure 3C). The above data showed that overexpressed miR-214-3p improved cell viability and inhibited apoptosis of MG-63 cells, at least partially, by inhibiting PTEN expression.

FN inhibits osteosarcoma cell viability and induces apoptosis by down-regulating miR-214-3p

Whether the miR-214-3p-mediated cell viability and apoptosis were modulated by the FN, MG-63 cells were treated with FN (30 μ M), miR-214-3p mimic, or FN (30 μ M) + miR-214-3p mimic. qRT-PCR exhibited that FN treatment markedly reduced the miR-214-3p expression as compared to the miR-214-3p mimic group (Figure 4A). MTT assay exhibited that the suppression role of FN in cell

proliferation was attenuated by miR-214-3p, revealing that the cell viability in FN (30 μ M) + miR-214-3p mimic group was markedly improved as compared to that in FN (30 μ M) group (Figure 4B). Moreover, the Caspase 3 assay results suggested that miR-214-3p suppressed FN-induced cell apoptosis (Figure 4C). Moreover, the results of the western blot showed that the Caspase 3, Caspase 9, PTEN protein expressions were upregulated in the miR-214-3p mimic group as compared to the miR-214-3p mimic group, while the expression of Bcl-2 was down-regulated (Figure 4D). The above results showed that the anti-tumor role of FN in MG-63 might be partially through regulating miR-214-3p.

Discussion

FN has the effects of regulating estrogen, inflammation,

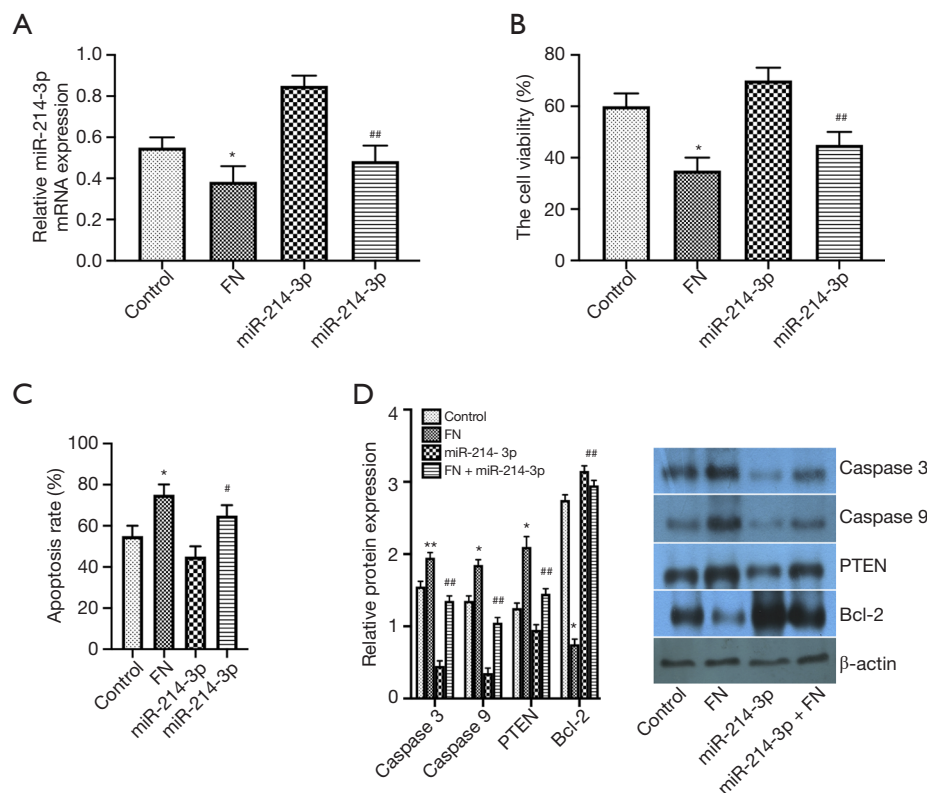


Figure 4 Formononetin (FN) inhibits osteosarcoma cell viability and induces apoptosis by decreasing miR-214-3p. (A) The miR-214-3p mRNA level was evaluated by real-time quantitative polymerase chain reaction (qRT-PCR). (B) MTT assay evaluated cell viability. (C) The apoptosis rate of cells was evaluated by the Caspase 3 kit. (D) Caspase 3, Caspase 9, phosphatase and tensin homolog (PTEN), Bcl-2 levels were evaluated by western blot. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ vs. miR-214-3p mimics.

and lowering blood pressure. Also, FN plays an anti-tumor effect in various ways (19). Wang and other studies pointed out that FN suppressed the cell viability of colon tumor cells via inhibiting the cell cycle at the G0/G1 phase by suppressing the Cyclin D1 protein level; also, FN inhibits the cell viability of colon tumor cells (20). The role of FN is also related to the up-regulation of miR-149 expression and suppression of PI3K/AKT and STAT3 signaling activation (20). Hu and others found that FN suppressed the cell viability of osteosarcoma cells *in vivo* and *in vitro*, and promoted the Bax expression, an apoptosis protein, by decreasing the miR-375 expression and the inhibitory protein Bcl-2 expression that induces apoptosis (21). In the present study, FN suppressed the cell viability and induced apoptosis, and decreased miR-214-3p level and promoted PTEN level.

miR-214-3p is one of the miRNAs with oncogene properties that are important in the process of various cancers (9). The miR-214-3p level is markedly increased in

osteosarcoma (9,10). Liver cancer has been shown to have oncogene capabilities. For example, in liver cancer, miR-214-3p binds directly with Wnt3a to improve the proliferation (22,23). Recent reports have found miR-214-3p regulates Wnt3a in osteosarcoma (9). miR-214-3p participates in the growth and metastasis of cancers through targeted regulation of the critical tumor suppressor gene PTEN (24). PTEN is a crucial tumor inhibitor gene and is closely related to various tumors (25). PTEN is the most critical upstream molecule in the protein kinase B (AKT) pathway, and it plays a role in inhibiting AKT phosphorylation through dephosphorylation/proximal interphalangeal joints 3 (PIP3) (26). Disturbance of the phosphatidylinositol 3 kinase (PI3K)/AKT signaling is widespread in many cancers, which inhibit apoptosis, promote cell viability, affect synthesis and sugar metabolism (27). PTEN was predicted to be a binding site of miR-214-3p by TargetScan database, and miR-214-3p levels were significantly increased in MG-63 cells transfecting with miR-214-3p mimic. However, the PTEN

level was significantly decreased by a miR-214-3p mimic. Moreover, the activity of luciferase was treatment with miR-214-3p mimic and WT-PTEN 3'-UTR vector as compared to that treatment of miR-214-3p mimic and MUT-PTEN 3'-UTR.

Classical apoptosis pathways include endogenous mitochondrial cytochrome release signaling and exogenous death receptor signaling. Existing research shows that these two pathways are not independent, but interrelated and intertwined, and these two pathways will eventually activate and wither end-of-life effect enzyme caspase 3 (28-30). The Bcl-2 and Bax expressions were changed markedly through western blot and qRT-PCR. The mitochondrial-mediated apoptosis pathway is regarded as a critical center of apoptosis and is only regulated by the proteins of the Bcl-2 family (31). Bcl-2 is one of the most critical inhibitors of apoptosis, and Bax promoted apoptosis, a critical factor that determines whether cells survive or die (32). In the current study, overexpressed miR-214-3p improved cell viability and suppressed apoptosis of MG-63 by inhibiting PTEN expression. Moreover, FN inhibited proliferation and induced apoptosis of MG-63 by regulating miR-214-3p.

Conclusions

Our results showed that FN might be acting as a novel treatment for MG-63 through the up-regulation of PTEN expression via miR-214-3p.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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