MiR-491-3p is down-regulated in postmenopausal osteoporosis and affects growth, differentiation and apoptosis of hFOB1.19 cells through targeting CTSS

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DOI: 10.5603/FHC.a2020.0001

Article type: ORIGINAL PAPERS

Submitted: 2019-09-23

Accepted: 2020-02-06

Published online: 2020-03-16

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MiR-491-3p is down-regulated in postmenopausal osteoporosis and affects
growth, differentiation and apoptosis of hFOB1.19 cells through targeting CTSS

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The running title: **miR-491-3p/CTSS pairs function in PMO**

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Abstract

Background. Postmenopausal osteoporosis (PMO) is a common disease related to aging, which has been paid increasing attention in recent years because of its serious complications. MiR-491-3p has been illustrated to play a crucial role in several diseases. However, the role of miR-491-3p in PMO has been poorly reported. Our research intends to explore the impacts of miR-491-3p on PMO.

Material and methods. The expression patterns of miR-491-3p and cathepsin S (CTSS) in patients with PMO were acquired from the GEO database. The human osteoblast cell hFOB1.19 was used to detect the function of miR-491-3p and CTSS in PMO. The viability and apoptosis of hFOB1.19 cells were measured by cell counting kit 8 and flow cytometry assays. The apoptosis and differentiation-related proteins were analyzed by Western blotting. The interrelation between miR-491-3p and CTSS was predicted by biological software and affirmed by luciferase assay.

Results. Our outcomes indicated that miR-491-3p was lower expressed in patients with PMO and up-regulation of miR-491-3p increased the viability and differentiation of hFOB1.19 cells, and inhibited the apoptosis of hFOB1.19 cells. CTSS, which highly expressed in patients with PMO, was confirmed as a direct target of miR-491-3p and inversely modulated by miR-491-3p. And the rescue assays discovered that overexpression of CTSS suppressed the promoting effects of miR-491-3p mimic on hFOB1.19 cells proliferation and differentiation, and repressed the inhibitory effects of miR-491-3p mimic on hFOB1.19 cells apoptosis.

Conclusions. Our outcomes illustrated that miR-491-3p could ameliorate hFOB1.19 cells biological characteristics through reducing CTSS, suggesting that miR-491-3p/CTSS might be potential biomarkers for the diagnosis and treatment of PMO.

Key words: target; proliferation; flow cytometry; biomarker
**Introduction**

In recent years, an increasing number of women over the age of 50 are being affected by the postmenopausal osteoporosis (PMO), which leads to an increased economic and social burden [1]. Bone health is mainly dependent on the dynamic balance between bone formation and bone resorption. Disorders of bone metabolism easily induce bone-related diseases, especially osteoporosis, which can result in a decrease in bone density and an increase in the risk of fracture [2, 3]. Bone homeostasis is a remodeling procedure regulated by three types of osteocytes, including osteoclast, osteoblast, and osteocyte [4]. PMO is considered to be a direct result of the decrease in endogenous estrogen in postmenopausal women, accompanied by a significant reduction in bone mass [5, 6]. Therefore, estrogen was widely used to treat PMO for a period of time. After hormonal therapy has been shown to increase the risk of heart disease and breast cancer, the use of estrogen dropped dramatically, even though the risk was low [7]. Several drugs have been found to treat PMO, including bisphosphonates, calcitonins, etc., but these drugs are more or less irritating to the gastrointestinal tract [7]. Therefore, it is imperative to understand the pathogenesis of PMO and develop effective therapeutic strategies.

MicroRNAs (miRNAs), as 22–25 nucleotides non-coding small RNAs, play an indispensable role in regulating genes expression in organisms [8]. Emerging evidences discovered that miRNAs are implicated in regulating numerous biological processes, such as cell growth, apoptosis, autophagy, differentiation, inflammation, invasion and migration [9–12]. In addition, the function of miRNAs in the development of PMO has attracted much attention. Recent work performed by Li et al. demonstrated that miR-133a participated in the procedure of PMO by facilitating osteoclast differentiation [13]. Moreover, it has been reported by Liu et al. that miR-210 was implicated in the development of PMO through increasing vascular endothelial growth factor (VEGF) expression and osteoblast differentiation [14]. Moreover, circulating miR-133a-3p might be regarded as an underlying non-invasive biomarker and therapy target in PMO [15]. However, in addition to several reports on the function of miR-491-3p in cancers, for example, miR-491-3p reduced multidrug resistance of hepatocellular carcinoma, inhibited the growth and invasion of osteosarcoma cells, regulated the chemo-sensitivity of human tongue cancer, and
participated in the pathogenesis of clear-cell renal cell carcinoma [16–19], little research focused on its role in PMO.

Our data found that miR-491-3p was lower expressed in patients with PMO, while the expression of CTSS in PMO patients showed an opposite trend. Meanwhile, CTSS was confirmed as a target of miR-491-3p. More importantly, we revealed that miR-491-3p could promote the growth and differentiation of hFOB1.19 cells, and inhibit the apoptosis of hFOB1.19 cells through reducing CTSS expression. Our research affords novel pairs of molecules and basic support for the diagnosis and therapy of PMO.

Materials and methods

Data sources. The expression patterns of miR-491-3p and CTSS in PMO were acquired from the GEO database following accession numbers GSE74209 and GSE56116. The GSE74209 dataset including 6 patients with PMO fractures (osteoporotic) and 6 patients with osteoarthritis (healthy) was used to analyze the expression of miR-491-3p. The GSE56116 dataset containing 10 patients with PMO fractures (osteoporotic) and 3 patients with osteoarthritis (healthy) was utilized to analyze the expression of CTSS.

Cell culture and transfection. Human osteoblast cell line hFOB1.19 was purchased from the Shanghai cell bank of the Chinese Academy of medical sciences (Shanghai, China) and cultivated in the α-MEM medium including 10% fetal bovine serum, 100 U/mL penicillin and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Overexpression of miR-491-3p and CTSS were achieved by transfection of miR-491-3p mimic and pcDNA3.1-CTSS (GenePharma, Shanghai, China) into cells, respectively. The transfection concentrations were 50 nmol/L for the miR-491-3p mimic, miR-491-3p mimic NC, pcDNA3.1-CTSS and pcDNA3.1. All of them were transfected into hFOB1.19 cells by Lipofectamine 3000 reagent (Invitrogen).
Cell viability test. Cell counting kit-8 (CCK-8) test was carried out to detect the viability of hFOB1.19 cells. Cells were inoculated in 96-well plates with the density of $1 \times 10^3$ cells/well. After cells adhesion, the culture medium was changed, miR-491-3p mimic or miR-491-3p mimic NC was added, and the cultivation was continued for 48 h. Then, the cells were added with 20 μL of CCK-8 agent and cultured for another 1.5 h. Finally, microplate reader SpectraMax®iD3 (Molecular Devices, San Jose, CA, USA) was utilized to measure the optical density (OD) values.

Cell apoptosis test. The apoptosis of treated cells was detected by flow cytometry. Collected cells into a centrifuge tube and centrifuged them. Then, suspended cells with pre-cooling phosphate buffer saline (PBS) and centrifuged again. Subsequently, sucked out the supernatant and re-suspended cells with $1 \times$ binding buffer. Adjusted cell density to $1–5 \times 10^6$ cells/mL. Mixed 5 μL of Annexin V/FITC and 100 μL of cell suspension into 5 mL flow tube, and then the mixture was cultured in the darkroom for 5 min. Before detection, 10 μL of PI and 400 μL of PBS were added into the flow tube. The results were analyzed by Flowjo software 7.6.1.

MiR-491-3p target gene prediction website. The biological prediction websites including MiRanda (http://www.microrna.org), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/), and TargetScan (http://www.targetscan.org/), miRDB (http://www.mirdb.org/) were used to predict the target genes of miR-491-3p.

Dual luciferase reporter assay. The fragments of CTSS-mutant-type (mut) and CTSS-wild-type (wt) were synthesized and inserted into a pmirGLO vector (GenePharma). The cells were inoculated to 24-well plates. When cells grew to 80% confluence, luciferase vector containing wt-CTSS or mut-CTSS and miR-491-3p mimic or mimic NC were co-transfected into HEK293 cells by Lipofectamine 3000 reagent (Invitrogen). After 48 h, the protein was extracted and the luciferases activity was detected by Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) following the manufacturer’s specification.
RNA extraction and Quantitative Real-time PCR (qRT-PCR). The whole RNA was obtained from the treated cells by TRIzol (Invitrogen) and reverse transcribed into cDNA by PrimeScript RT Reagent Kit and Mir-X™ miRNA First Strand Synthesis Kit (Invitrogen). Then the expression of mRNA and miRNA were analyzed by real-time PCR with the help of SYBR Premix Ex Taq II and SYBR PrimeScript™ miRNA RT-PCT Kit (TaKaRa, Tokyo, Japan), accordingly. Subsequently, the real-time PCR was performed on a 7900HT real-time PCR system with the following procedures: 40 circles consist of 95°C for 5 min, 95°C for 30 s, then 60°C for 45 s, 72°C for 30 min. GAPDH was considered as the internal standard for mRNA detection, and U6 was regarded as the internal standard for miRNA calculation. $2^{-\Delta\Delta C_t}$ method was used to analyze the relative expression of mRNA and miRNA. The sequences of the primers were synthesised as below: MiR-491-3p, forward: 5’-AGTGGGGAACCCTTCC-3’, reverse: 5’-GAACATGTCTGCG-TATCTC-3’; U6, forward: 5’-AAAGCAAATCATCGGACC-3’, reverse: 5’-GTACAACACATTTCTCAGGA-3’; CTSS, forward: 5’-TGGATCACCACTGGCATCTCTG-3’, reverse: 5’-GCTCCAGGTTGTGAAGCATCAC-3’; GAPDH, forward: 5’-TGTGGGGATCAATGGGATTTGG-3’, reverse: 5’-CCCTCCAGGGGATCTGTTTG-3’.

Western blotting. Protein samples were extracted from the treated cells by RIPA lysis buffer with protease inhibitor. 20 μg of protein within 1 × loading buffer was put into each well, isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto PVDF membranes under wet condition. Then 5% non-fat milk dissolved in TBST (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.05% Tween-20) was utilized to block the membranes. The membranes were then incubated with primary antibodies including CTSS (1:2000), Bcl-2 (1:1000), Bax(1:2000), Cleaved caspase-3 (1:3000), Cleaved caspase-9 (1:3000), ALP (1:3000), Runx2 (1:1000), Osterix (1:5000), OPN (1:1000) and GAPDH (1:10000) (all from Abcam, Cambridge, UK) at 4°C overnight. Next, the membranes were incubated with the IgG-HRP antibody at about 25°C for 1 h. Finally, the signals were developed by ECL (HRP Substrate Luminol Reagent: HRP Substrate Peroxide Solution = 1:1) and scanned by QUANTITY ONE software 4.6.6. GAPDH was regarded as the internal standard.
**Statistical analysis.** All experimental data were repeated at least three times and analyzed by SPSS22.0 and Graphpad Prism 5.0 software. Student’s t-test was used to analyze the difference between two samples, while the comparison among multiple groups was calculated by ANOVA followed by Dunnett (compare all groups vs. control group) and Bonferroni (compare selected pairs of groups) *post hoc* test. P value less than 0.05 was considered to indicate a statistically significant difference.

**Results**

*MiR-491-3p was lower expressed in patients with PMO*

Firstly, GEO dataset was used to detect the expression of miR-491-3p in PMO. The data in Figure 1A manifested that miR-491-3p was lower expressed in patients with PMO fracture (osteoporotic, n = 6) than that of the patients with osteoarthritis (healthy, n = 6, P = 0.0087). Subsequently, to investigate the effects of miR-491-3p on hFOB1.19 cells, we attempted to up-regulate miR-491-3p with mimic. The data from Figure 1B indicated that miR-491-3p expression was significantly upregulated in hFOB1.19 cells after treated with miR-491-3p mimic compared with that of the control and miR-491-3p mimic NC groups (P < 0.01).

*Overexpression of miR-491-3p could inhibit the apoptosis of hFOB1.19 cells and promote the proliferation and differentiation of hFOB1.19 cells*

To further explore the impacts of miR-491-3p on hFOB1.19 cells proliferation, apoptosis and differentiation, CCK-8, flow cytometry and Western blotting assays were carried out. The data from CCK-8 showed that up-regulation of miR-491-3p could increase the OD values of hFOB1.19 cells compared with that of the control, and the difference was significant at 48 h and 72 h (Figs. 2A, P < 0.01). The results from flow cytometry demonstrated that the apoptosis rate of hFOB1.19 cells was reduced nearly 60% after upregulation of miR-491-3p compared with that of the control (Figs. 2B–C, P < 0.01). Subsequently, the apoptotic-related proteins were analyzed by Western blotting. The results indicated that the expression of anti-
apoptotic protein Bcl-2 was reduced, and the expression of pro-apoptotic proteins including Bax, Cleaved Caspase-3 and Cleaved Caspase-9 were increased in hFOB1.19 cells after transfected with miR-491-3p mimic (Figs. 2D–E, P < 0.01). To study the effects of miR-491-3p on hFOB1.19 cells differentiation, we analyzed the changes of differentiation-related proteins with Western blotting. The results indicated that after miR-491-3p mimic treatment, the expression of ALP, Runx2, Osterix and OPN significantly increased to 2.05, 2.13, 2.9, and 2.3 times of the control group, respectively (Figs. 2F–G, P < 0.01). All consequences indicated that up-regulation of miR-491-3p could increase the proliferation and differentiation of hFOB1.19 cells, and reduce the apoptosis of hFOB1.19 cells.

**CTSS was highly expressed in patients with PMO and negatively regulated by miR-491-3p**

Based on the results from target gene prediction website and bioinformatics analysis, CTSS was selected as a target of miR-491-3p for further investigation. As presented in Figure 3A, the wt 3’UTR of CTSS contains the complementary binding sequence of miR-491-3p. Subsequently, dual luciferase reporter assay was performed to confirm the association between miR-491-3p and CTSS. The data in Figure 3B showed that the luciferase activity in wt 3’UTR of CTSS was clearly decreased after transfected with miR-491-3p mimic compared with that of the miR-491-3p mimic NC group (P < 0.01). However, miR-491-3p had little effect on luciferase activity of the mut 3’UTR of CTSS. The results further confirmed that miR-491-3p directly binds with CTSS in osteoblast. The data from GEO database indicated that CTSS was significantly up-regulated in patients with PMO fracture (osteoporotic, n = 10) compared with that of the patients in osteoarthritis (healthy, n = 3, P = 0.02, Fig. 3C). And the expression of CTSS was clearly reduced in hFOB1.19 cells after transfected with miR-491-3p mimic (Figs. 3D–E, P < 0.01). The results implied that CTSS was a direct target of miR-491-3p and negatively modulated by miR-491-3p.
CTSS suppressed the positive effects of miR-491-3p on hFOB1.19 cells

To explore the interrelation between miR-491-3p and CTSS in PMO, the biological properties of hFOB1.19 cells were analyzed after treated with miR-491-3p mimic and pcDNA3.1-CTSS. The result from Figure 4A indicated that the OD values of hFOB1.19 cells were raised after treated with miR-491-3p mimic in contrast with that of the control, while the raising tendency was reversed in miR-491-3p mimic and pcDNA3.1-CTSS group (P < 0.01). The data indicated that CTSS could suppress the ascending proliferation ability of hFOB1.19 cells induced by miR-491-3p mimic. And the data from flow cytometry discovered that the apoptosis of hFOB1.19 cells was reduced nearly 50% after treated with miR-491-3p mimic compared with that of the control, and increased approximately two times after treated with miR-491-3p mimic and pcDNA3.1-CTSS compared with that of the miR-491-3p mimic group (Fig. 4B–C, P < 0.01). Moreover, the expression of anti-apoptotic protein Bcl-2 was reduced about 60%, and the pro-apoptotic proteins Bax, Cleaved caspase-3 and Cleaved caspase-9 were significantly increased after transfected with miR-491-3p mimic and pcDNA3.1-CTSS compared with that of the miR-491-3p mimic group (P < 0.01). Whilst, the raising tendency of ALP, Runx2, Osterix and OPN expression induced by miR-491-3p mimic was reversed after transfected with miR-491-3p mimic and pcDNA3.1-CTSS (P < 0.01). All the data implied that CTSS could suppress the promoting effects of miR-491-3p on hFOB1.19 cells proliferation and differentiation, and repress the inhibitory effects of miR-491-3p on hFOB1.19 cells apoptosis.

Discussion

PMO is an insidious disease without any obvious symptoms in its early stage. Once the signs of PMO appear, the fracture has already occurred [20]. Therefore, it is essential to search effective and preventive measures and molecular markers for early diagnosis. With the development of more and more validated miRNA signatures and mature medium-throughput methods in the clinical setting, specific miRNA markers are raised potentially to conduce to human health [21]. Recently, several researchers have discovered that some miRNAs including miR-491-3p were modulated by
enterovirus 71 which leads major outbreaks of hand, foot, and mouth diseases [22]. MiR-491-3p has been reported to be involved in the pathogenesis of major depression or suicide [23]. In addition, previous study has found that miR-491-3p played a vital role in the modulation of multidrug resistance in hepatocellular carcinoma through regulating ABCB1 and Sp3 expression [19]. Moreover, miR-491-3p was discovered to suppress the growth and invasiveness of osteosarcoma [18] and glioblastoma [24] cells. However, study of miR-491-3p in osteoporosis has not been well reported. In the current study, the data indicated that miR-491-3p was lower expressed in PMO patients. Importantly, some researches have reported that proliferation and differentiation of osteoblast are important for the development of osteoporosis [25]. ALP, Runx2, Osterix and OPN have been demonstrated to play positive roles in promoting osteogenesis [26]. In our study, the upregulation of miR-491-3p increased the expression of ALP, Runx2, Osterix and OPN, indicating that miR-491-3p can promote osteoblast differentiation. Cell apoptosis is an important biological process of diseases, including PMO [27]. In our study, up-regulation of miR-491-3p attenuated the occurrence of apoptosis. These results insinuated that miR-491-3p inhibited the development of PMO through promoting the growth and differentiation of osteoblast, and suppressing the apoptosis of osteoblast.

It is well known that miRNAs perform their functions through binding to mRNAs and blocking protein expression. Based on the biological prediction, bioinformatics analysis and dual luciferase reporter assay, CTSS was selected and confirmed as a direct target of miR-491-3p. CTSS, as a lysosomal protease, can promote the degradation of damaged or unnecessary proteins in the lysosomal pathway [28], and has been reported to be involved in several diseases including atherosclerosis, tumor metastasis and osteoporosis [29]. In addition, growing evidences have indicated that CTSS regulated adipocyte and osteoblast differentiation, bone turnover, and bone microarchitecture [30]. Furthermore, CTSS was also used as a potential drug target for osteoporosis and rheumatoid arthritis [31]. In our study, CTSS was verified as a direct target of miR-491-3p, and negatively modulated by miR-491-3p. Moreover, data from GEO dataset showed that CTSS was highly expressed in patients with PMO. Rescue assays indicated that overexpression of CTSS could limit the positive effects of miR-491-3p on osteoblast. More in vivo experiments are needed to confirm these results, since in vitro experiments cannot fully simulate the situation in vivo.
In conclusion, our findings discovered that miR-491-3p showed a tendency of low expression in patients with PMO, while the expression trend of CTSS in PMO was just the opposite. What’s more, overexpression of miR-491-3p could promote osteoblast proliferation and differentiation, and inhibit osteoblast apoptosis. In addition, CTSS was verified as a target gene of miR-491-3p and negatively regulated by miR-491-3p in osteoblast. Further experiments implied that overexpression of CTSS could suppress the positive promoting effects of miR-491-3p on osteoblast. To sum up, miR-491-3p could promote the positive biological behaviors of osteoblast through reducing CTSS, providing a possible pair of molecules for the treatment of PMO.
References


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Figure 1. Determination of miR-491-3p expression. A. Data from GEO dataset revealed that miR-491-3p was lower expressed in PMO osteoporotic (n = 6) compared with that of the healthy people (n = 6), P = 0.0087. B. The relative expression of miR-491-3p in hFOB1.19 cells was measured after transfected with miR-491-3p mimic or miR-491-3p mimic NC, **P < 0.01 vs. control.

Figure 2. MiR-491-3p promoted proliferation and differentiation of hFOB1.19 cells, and inhibited their apoptosis. A. The proliferation of hFOB1.19 cells were measured by CCK-8 assay. B–C. The apoptosis of hFOB1.19 cells was detected by flow cytometry. D–E. The apoptosis related proteins in hFOB1.19 cells were analyzed by Western blotting. F–G. The differentiation related proteins in hFOB1.19 cells were measured by Western blotting. **P < 0.01 vs. control.

Figure 3. CTSS was highly expressed in PMO and negatively regulated by miR-491-3p. A. The sequences of wild type 3’UTR of CTSS, mutant type 3’UTR of CTSS, and miR-491-3p were presented. B. The luciferase activity of wt CTSS and mut CTSS were assessed after transfected with miR-491-3p mimic or NC. C. The data from GEO indicated that CTSS was clearly up-regulated in patients with PMO (n = 10) compared with that of the healthy people (n = 3), P = 0.02. D–E. The expression of CTSS was detected after transfected with miR-491-3p mimic or NC. **P < 0.01 vs. control.

Figure 4. CTSS suppressed the promoting effects of miR-491-3p on the proliferation and differentiation of hFOB1.19 cells, and the inhibitory effects of miR-491-3p on hFOB1.19 cells apoptosis. A. The hFOB1.19 cells proliferation was detected by CCK-8 after treated with miR-491-3p mimic/miR-491-3p mimic + pcDNA3.1-CTSS. B–C. Flow cytometry was utilized to detect the apoptosis of hFOB1.19 cells. D–E. The apoptosis related proteins were measured. F–G. The expression of ALP, Runx2, Osterix and OPN were detected by Western blotting. **P < 0.01, #P < 0.01, ** represented miR-491-3p mimic vs control, #represented miR-491-3p mimic and pcDNA3.1-CTSS vs miR-491-3p mimic.
Figure 1.

A

\[ P = 0.0087 \]

B

Relative expression level

Healthy (n=6) Osteoporotic (n=6)

Data from GSE74209

Figure 2.

A

OD Value

control

miR-491-3p mimic

B

PI

Anним死N 比TC

C

Relative expression level

G(V3) V3.3

D

Bcl-2

Bax

Cleaved caspase-3

Cleaved caspase-9

GAPDH

E

Fold change

Bcl-2

Bax

Cleaved caspase-3

Cleaved caspase-9

GAPDH

F

ALP

Runx2

Osterix

OPN

GAPDH

G

Fold change

ALP

Runx2

Osterix

OPN
Figure 3.

A
3' UTR of CTSS mRNA (wt) 5'... GUAAAGUAAGAGAAUGCAUAA... 
miR-491-3p 3'... CAUCUUCUUAAAGGAUGAUUC
3' UTR of CTSS mRNA (mut) 5'... GUAAAGUAAGAAAACUUGCCAA...

B

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C

Relative expression of CTSS

P = 0.02

D

CTSS
GAPDH

E

Fold change

control

miR-491-3p mimic

Data from GSE6116