Original Article

miR-4739/ITGA10/PI3K signaling regulates differentiation and apoptosis of osteoblast

Yibo Song a, Zhaolei Meng b, Shanshan Zhang c, Nianguo Li d, Wei Hu a, Hong Li e, *

a Spinal Department of Orthopedics, Jinan Zhangqiu District Hospital of TCM, Jinan, Shandong, China
b Hand and Foot Department Ward 2, Jinan Zhangqiu District Hospital of TCM, Jinan, Shandong, China
c Thoracic Surgery Ward, Jinan Zhangqiu District Hospital of TCM, Jinan, Shandong, China
d Medical Department, Jinan Zhangqiu District Hospital of TCM, Jinan, Shandong, China
e Fourth Middle School of Zhangqiu District, Jinan, Shandong, China

1. Introduction

Osteoporosis (OP) is an osteopathy featured by a decrease in bone mass and density, which increasing the risk of cataclasis [1].

standardized diet and quality care are the main methods to treat OP, but they still cannot effectively control the occurrence and progress of OP [4]. In addition, some OP drugs also have side effects. For instance, using bisphosphonates to inhibit osteoclast activity may disrupt normal bone remodeling, and it may lead to osteonecrosis of the jaw or atypical femur fracture in a small number of patients [5,6]. Meanwhile, hormone replacement therapy is very effective in preventing bone loss, but may also increase the risk of venous thromboembolism and invasive breast cancer [7]. Therefore, it has very important clinical significance to explore the molecular mechanism underlying OP progression and develop effective markers and therapeutic targets.

Integrins, which composed of an alpha subunit and a beta subunit, were heterodimeric transmembrane receptors. They participated in the cell adhesion as well as signaling. Because members of the integrin family play an important role in many
basic biological processes, their abnormal expression was associated with many diseases [8–10]. Integrin alpha 10 (ITGA10) was proved to play a crucial part in adhesion, migration as well as the regulation of the inflammatory responses [11]. More importantly, the type I collagen is the main component of osteoblastic matrix [12]. Although relevant studies have shown that ITGA10 was involved in collagen formation through the connection with TNC gene [13], it is still unclear whether ITGA10 will play a role in the occurrence and development of OP so far.

MicroRNAs (miRNAs), which consisted of 19–24 nucleotides, are small non-coding RNAs [14]. Meanwhile, many crucial biological processes were strongly associated with miRNAs, such as cell differentiation, apoptosis, as well as proliferation [15]. Some research showed that certain miRNAs can be involved in bone metabolism, bone turnover as well as bone development, so as to regulate the occurrence and progress of OP [16]. A study have shown that miR-133 expression is increased during estrogen deficiency, regulating osteogenic differentiation of mesenchymal stem cells, and causing postmenopausal OP [17]. Evidence from other studies suggests that miR-34a could block OP via suppressing osteoclastogenesis and tgf[t2] [18]. miR-221 inhibited bone formation as well as osteoblast differentiation via directly targeting RUNX2 in the OP model [19]. Interestingly, in recent years miR-4739 has been widely reported in different differentiation via directly targeting RUNX2 in the OP model [19]. Yet, the specific effect of miR-4739 on OP has not been reported. Through bioinformatics analysis, we found that miR-4739 was up-regulated in osteoporosis and predicted as an upstream miRNA of ITGA10, implying that it may be involved in the development of this disease and arousing our interest in studying the function of miR-4739/ITGA10 in osteoporosis. Therefore, in this study, we probed the expression and roles of miR-4739/ITGA10 as well as their association in OP cell lines in vitro. The results found that miR-4739 regulates cell proliferation, differentiation and apoptosis by targeting ITGA10 and regulating PI3K/AKT signaling, indicating that miR-4739/ITGA10 might be candidate biomarkers for the diagnosis and treatment of OP.

2. Materials and methods

2.1. Bioinformatic analysis

The Gene Expression Omnibus (GEO) datasets of GSE35956 and GSE93883 were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). The GSE35956 datasets included 5 primary OP and 5 control specimens, and the gene difference analysis between the two groups was performed using the R program limma package. For the comparison of the expression levels of the two groups, the threshold value was set to |logFC|>1, and the p value was <0.05. Differentially expressed genes (DEGs) were obtained between the OP group and the control group, there were 2246 genes, including 2012 up-regulated differential genes and 234 down-regulated differential genes. Then, KEGG pathway enrichment analysis was performed on the above differential genes in the DAVID database, and 32 meaningful pathways were obtained with P < 0.05 as the screening condition. The enrichment results showed that the PI3K-Akt signaling pathway was enriched in multiple genes, namely IKB, CSF1R, ITGA10, COL1A1, ITGB1, CHAD, CCNE2, FN1A7, ITGB6, COL6A1, ANGPT2, EPO, ITGA10, NR4A1, FGFR, VWF, ITGA2, DDT4, GTH2, VEGFB, GH1, VWF, CDKN1A, GNGT2, PRLR, IFNB1, CHRM1, ITGA7, VEGFA, JAK1, NGF. Among them, the genes with low expression in OP are CCNE2, ITGA10, and other genes are highly expressed. Comprehensive literature analysis found that ITGA10 expression was down-regulated in the osteoporotic phenotype (glucocorticoid-induced osteoporosis) induced by prednisolone through zebrafish larvae, so ITGA10 was selected for analysis.

The GSE93883 datasets contained 6 health control samples and 12 osteoporotic patients with and without vertebral fractures. The R program limma package was used to analyze the difference of miRNA between the two groups. For the comparison of the expression levels of the two groups, the threshold value was set to |logFC|>1, and the p value was less than 0.05. DEGs were obtained between the osteoporosis group and the non-osteoporosis group, there were 435 miRNAs, of which 115 were up-regulated and 320 were down-regulated. Targetscan was used to predict miRNAs regulated upstream of ITGA10, and 597 miRNAs were obtained. The up-regulated miRNAs analyzed in the GSE93883 dataset were intersected with the predicted miRNAs to obtain 30 common miRNAs. According to the logFC ranking, the literature background was combined, and the miR-4739 with a large fold difference was selected for analysis.

2.2. Cell culture

The human osteoblastic cell line hFOB 1.19 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM/F12 containing 10% fetal bovine serum for 5 days. hFOB cells were subcultured every 2 days. We seeded cells at a density of 1×10^5 cells/well in 96-well plates. After the cells were adherent to the wall, the culture medium was refreshed and added 10 mmol/L β-glycerophosphate and 50 μg/ml ascorbic acid. Then transfected the cells with different transfections. Then added 20 μl of CCK-8 solution to every well 48 h later and cultured the cells in an incubator at 37 ℃ for 1.5 h. Finally, measured the absorbance value (OD value) of each well with a microplate reader at 450 nm.

2.3. CCK-8 assay

We seeded cells at a density of 1×10^5 cells/well in 96-well plates. After the cells were adherent to the wall, the culture medium was refreshed and added 10 mmol/L β-glycerophosphate and 50 μg/ml ascorbic acid. Then transfected the cells with different transfections. Then added 20 μl of CCK-8 solution to every well 48 h later and cultured the cells in an incubator at 37 ℃ for 1.5 h. Finally, measured the absorbance value (OD value) of each well with a microplate reader at 450 nm.
2.6. Luciferase reporter assay

To construct dual luciferase reporter plasmids, we cloned wild-type and mutant ITGA10 (with mutated miR-4739 binding sites) into a pmirGLO dual luciferase vector. HEK293T cells were co-transfected with wild-type pmirGLO-ITGA10 (or mutant) and miR-4739 inhibitor (or negative control) or mimic (or mimic control). And the luciferase activity was measured by Dual-Luciferase Reporter Assay Kit 48 h after transfection.

2.7. qRT-PCR

Isolated total RNA with TRIzol (Invitrogen). mRNA and miRNA were reverse-transcribed into cDNA with PrimeScript RT Reagent Kit as well as Mir-X™ miRNA First Strand Synthesis Kit, respectively. Then qRT-PCR was conducted to detect the expression of mRNA and miRNA by SYBR Premix Ex Taq II and SYBR PrimeScript™ miRNA RT-PCT Kit. GAPDH was used as the internal reference for mRNA detection, while U6 for miRNA. And the comparative Ct method \((2^{-\Delta\Delta Ct})\) was applied to analyze the relative expression of mRNA and miRNA. The primers were listed as follows:

- **miR-4739**: F: 5'-AGCGGAGAGCGGGAG-3', R: 5'-GCAACTGCTCGATCTC-3';
- **U6**: F: 5'-CTGCCTTCGGAGCTGCT-3', R: 5'-AGGTCAGACGTAGGCTGTC-3';
- **ITGA10**: F: 5'-CTTACCTCAAGTCACCTC-3', R: 5'-CACGACCTAAATGCTCGAC-3';
- **GAPDH**: F: 5'-TGTTCCGTCGATCTGA-3', R: 5'-CGGTTACCACCTTCTGTA-3'.

2.8. Western blotting

Extracted total protein from cultured cells with RIPA lysis buffer, and analyzed the protein content by BCA protein assay kit. Heated the protein at 95 °C for 5 min, and separated 20 μg protein on SDS-PAGE gels, next transferred them onto a PVDF membrane. The membranes were blocked with 5% skimmed milk powder for 1 h, then incubated together with the primary antibodies overnight at 4 °C. After washed the membranes with TBST buffer for 5 min three times, then incubated together with a corresponding secondary...
antibody for 1 h. After washing them with TBST three times, added ECL reagents to visualize the protein-antibody bound bands. Taking GAPDH as the internal reference, the relative protein expression was expressed by the ratio of the gray value of the target protein band to the gray value of the internal reference. The primary antibodies were as follows: anti-ITGA10 (AB6030, 1:1000, Merck), Alkaline phosphatase (ALP; ab229126, 1:1000, Abcam), Runx 2 (ab76956, 1:1000, Abcam), Osterix (ab209484, Abcam), Osteopontin (OPN; #88742, 1:1000, Cell Signaling Technology), p-PI3K (ab278545, 1:1000, Abcam), PI3K (#4249, 1:1000, Cell Signaling Technology), p-AKT (#4060, 1:2000, Cell Signaling Technology), AKT (#4691, 1:1000, Cell Signaling Technology), and GAPDH (ab8245, 1:5000, Abcam).

2.9. Statistical analyses

The experimental data was analyzed with SPSS22.0 statistical analysis software. Student’s t test was performed to analyze differences between 2 groups. Multiple comparisons were conducted by one-way ANOVA followed by Dunnett’s post-hoc test. And p < 0.05 was considered statistically significant.

3. Results

3.1. Differential gene analysis

Firstly, based on the GEO databases (GSE35956), we investigated the differential genes in OP. The dataset contained 5 primary OP and 5 cases of control. A total of 2246 differentially-expressed genes were obtained from OP group and normal group, including 2012 upregulated and 234 downregulated genes. Then we performed KEGG pathway analyses on the differential genes using the DAVID database. The result showed that multiple genes were enriched in the PI3K-Akt signaling pathway (Fig. 1 A), including IBSP, CSF1, FGFR1, IFNA21, FGF18, CSF2, PGDFB, PGIF, CSF1, EFNA3, ITGA10, COL2A1, ITGB1, CHAD, CCNE2, IFNA7, ITG6, COL6A1, ANGPT2, EPO, ITGA10, NRG4A1, FGF23, VWF3AE, EPHA2, DDT1, GH2, VEGFB, GH1, VWF, CDKN1A, GNGT2, PRLR, IFNBI, CHRM1, ITGA7, VEGFA, JAK1, NGF. Among them, CCNE2 and ITGA10
miR-4739 regulated osteoblast proliferation and apoptosis by targeting ITGA10

(A) Cell proliferation was measured with CCK8 assay. **P < 0.01 versus NC group, && P < 0.01 versus inhibitor group and ##P < 0.01 versus si-ITGA10 group. (B) The apoptosis of osteoblast was measured by flow cytometry.
were down-regulated in OP, while the remaining genes were all up-regulated. Huo et al. demonstrated that prednisolone contributes to glucocorticoid-induced OP by down-regulating ITGA10 in zebrafish larvae [19]. Furthermore, we evaluated the ITGA10 expression using GSE35956 data, and the results indicated that the expression of ITGA10 in OP tissues was significantly down-regulated compared with the control (Fig. 1B), suggesting the involvement of ITGA10 in OP. Therefore, we selected ITGA10 for subsequent experimental analysis.

3.2. Prediction of miRNAs targeting ITGA10

We screened out the intersection of the miRNAs predicted by TargetScan and the upregulated miRNAs in GEO dataset (GSE93883) for the prediction of upstream miRNAs targeting ITGA10. The result indicated that 30 common miRNAs were obtained from the intersection data (Fig. 1C). Among them, miR-4739 has a more significant difference, as we observed that the expression of ITGA10 in OP tissues was significantly down-regulated compared with the control (Fig. 1B), suggesting the involvement of ITGA10 in OP. Therefore, we selected ITGA10 for subsequent experimental analysis.

3.3. ITGA10 was regulated by miR-4739

Evidence from the TargetScan website suggested that miR-4739 could potentially target ITGA10 (Fig. 2B). In order to validate the targeting relationship between miR-4739 and ITGA10, we constructed two kinds of luciferase reporters for ITGA10. The wild type reporter (WT ITGA10) contained a wild 3’UTR of ITGA10, while the mutant-type (MUT ITGA10) reporter contained a mutant 3’UTR designed by the miR-4739 binding site mutation sequence. Our results indicated that luciferase activity of WT ITGA10 3’UTR was significantly increased by miR-4739 inhibitor (p < 0.05), while that of MUT ITGA10 3’UTR had no significant difference (p > 0.05, Fig. 2C); luciferase activity of WT ITGA10 3’UTR was significantly decreased by miR-4739 mimic (p < 0.05), while that of MUT ITGA10 3’UTR had no significant difference (p > 0.05, Fig. 2D), indicating that ITGA10 could be targeted by miR-4739.

We then transfected osteoblasts with miR-4739 inhibitor, si-ITGA10, and the corresponding si-NC to detect whether miR-4739 regulates the expression of ITGA10. The qRT-PCR and Western blotting results indicated that the mRNA as well as protein expression levels of ITGA10 was decreased significantly in si-ITGA10 group, while upregulated significantly in cells transfected with miR-4739 inhibitor. Meanwhile, we cotransfected miR-4739 inhibitor and si-ITGA10 into osteoblasts and detected the ITGA10 expression. The results indicated that compared with the si-ITGA10 group, the mRNA and protein levels of ITGA10 were increased significantly, but they were significantly lower than miR-4739 inhibitor groups (Fig. 2E~G). In conclusion, these conclusions indicated that miR-4739 inhibits ITGA10 expression in a targeted manner.

3.4. The effect of miR-4739/ITGA10 on osteoblast proliferation, apoptosis and differentiation

The CCK-8 assay was performed to evaluate the impacts of miR-4739/ITGA10 on the osteoblast proliferation. Our results indicated
that compared with the NC, the cell proliferation was significantly increased in miR-4739 inhibitor groups at 48 h and 72 h, while in cells transfected with si-ITGA10, the cell proliferation was decreased significantly. Besides, si-ITGA10 significantly reversed the promoting effects of miR-4739 inhibitor (Fig. 3A).

The impacts of miR-4739/ITGA10 on the osteoblast apoptosis was measured with flow cytometry analysis. The apoptosis rate of miR-4739-inhibitor groups was significantly decreased, while increased significantly in si-ITGA10 group. However, the apoptosis rate in the miR-4739 inhibitor+si-ITGA10 group was higher than that in miR-4739-inhibitor group, but it was lower than that in si-ITGA10 group (Fig. 3B).

Finally, we detected the impacts of miR-4739/ITGA10 on cell differentiation. The results showed that the expressions of osteogenic proteins such as ALP, OPN, Osterix and Runx 2 were all significantly increased in miR-4739 inhibitor groups relative to NC, whereas the si-ITGA10 group showed a significant decrease. Likewise, the expression of these proteins in co-transfection groups was between miR-4739 inhibitor group and si-ITGA10 group (Fig. 4A and B). Generally speaking, these research results suggested that the inhibition of miR-4739 could accelerate the proliferation, differentiation and suppress cell apoptosis of osteoblasts by targeting ITGA10.

3.5. The effect of miR-4739/ITGA10 on osteoblast function mediated by PI3K/AKT signaling pathway

The qRT-PCR and Western blot results indicated that in the group with the transfection of miR-4739 inhibitor, the PI3K/AKT signaling pathway was significantly activated by increasing the phosphorylation levels of PI3K and AKT proteins, while the PI3K/AKT signaling pathway was inhibited in si-ITGA10 groups (Fig. 5A and B). Meanwhile, the activation degree of the PI3K/AKT signaling pathway in the group that co-transfected with miR-4739 inhibitors and si-ITGA10 was between miR-4739 inhibitor group and si-ITGA10 group.

4. Discussion

OP is frequently connected with the increased risk of fracture as well as reduced bone quality [22]. Mounting evidence suggested that OP may be caused by the disequilibrium between osteoblasts and osteoclasts in bone formation as well as resorption [23]. Given the bone formation in the process of bone remodeling depends on osteogenic differentiation in bone tissue, so controlling osteoblast formation is subsequently an important target for the prevention of OP [24].

In our study, via bioinformatics analysis we found that the down-regulated gene ITGA10 was the significant differential gene in OP patient, and miR-4739 was the potential upstream miRNA for ITGA10. There was plenty of evidence proving that ITGA10 plays a crucial part in many diseases. Okada et al. found that ITGA10 could facilitate tumor cell survival via activation of TRIO-RAC-RICTOR-mTOR signaling, and they provided an underlying treatment strategy for patients with high-risk myxofibrosarcoma [25]. Research also indicated that compared with the normal human epidermal melanocytes, the transcription of ITGA10 was induced in melanoma cell lines [26]. Herein, we revealed for the first time that ITGA10 expression is down-regulated in OP and play a promoting role in cell proliferation and differentiation and an inhibitory role in cell apoptosis. More importantly, the expression of ITGA10 was increased in hFOB 1.19 cells after treatment with...
miR-4739 inhibitors, and the targeted binding between ITGA10 and miR-4739 was verified. Further cell functional experiments found that the inhibition of miR-4739 could promote osteoblast proliferation, differentiation, and inhibit cell apoptosis by affecting ITGA10 expression. Therefore, it is proved that miR-4739 could regulate the osteoblast biological process by targeting ITGA10. Accumulating evidence supports that the abnormal regulation of miRNAs is related to the occurrence and progression of OP. [27-29]. For instance, Li et al. studied whether miRNAs have changes in postmenopausal OP patients, and verified that compared with normal subjects, miR-21 was down-regulated and miR-133 A was up-regulated in patients with OP and osteopenia [30]. There were also studies found that miR-93–5p as well as miR–100–5p were significantly up-regulated in osteoblasts and osteoclasts of osteoporotic patients, which may injure the mineralization and maturation of osteoblasts [31]. Of note, Wang et al. found that up-regulated miR-4739 targets bone morphogenetic protein 7 to mediate pleural fibrosis [32]. A study also showed that miR-4739 could regulate osteogenic and adipocytic differentiation of immortalized human bone marrow stromal cells [33]. Combined with previous research results, our study indicated that perhaps miR-4739/ITGA10 axis play key regulatory roles in the progression of osteoporosis.

Many researches demonstrated that the PI3K/AKT cell signaling pathway is involved in regulation of osteoporosis [34,35]. It has been reported that the osteoblast function was affected by PI3K [36] and AKT [37] signaling pathways via the bone marrow mesenchymal stem cells proliferation and differentiation. The study of Dong et al. showed that the inhibitor of β-catenin transcription activity ICG001 could reduce proliferation, differentiation and mineralization of PI3K/AKT-induced osteoblasts. Furthermore, they verified that the PI3K/AKT pathway was closely related to fracture healing and could promote fracture repair [38]. Liu et al. also found that the ferric ammonium citrate could promote osteoclast differentiation through the Trem-2-mediated PI3K/AKT signaling pathway [39]. Based on the important role of this pathway in bone-related diseases, we further studied the effect of miR-4739 on the PI3K/AKT pathway in osteoblasts. Our research suggested that the activation of PI3K/AKT was significantly increased via the transfection with miR-4739 inhibitor, while the knockdown of ITGA10 abolished the promoting effect induced by miR-4739 inhibitor. These results intimated that miR-4739 suppressed the activation of PI3K/AKT partly through inhibiting the expression of ITGA10. Together with the osteoblast functional experiments results, we verified that the inhibition of miR-4739 could promote the proliferation, differentiation and suppress cell apoptosis of osteoblasts by regulating ITGA10 via PI3K/AKT signaling pathway. We believe that these results may provide novel targets for clinical prevention and diagnosis in OP. However, the effect of ITGA10 on OP needs to be further studied in animal experiments.

5. Conclusion

In conclusion, ITGA10 was significantly downregulated in OP. The inhibitor of miR-4739 could promote cell proliferation, differentiation, and inhibit cell apoptosis by up-regulating ITGA10 and activating the PI3K/AKT signaling pathway. Thus, miR-4739/ITGA10 might be a potential diagnostic marker and therapeutic target for OP.

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Declarations of competing interest

The authors declare no conflicts of interest.

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