MicroRNAs in Serum Exosomes as Circulating Biomarkers for Postmenopausal Osteoporosis with Fragility Fractures

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Abstract

Background

Postmenopausal osteoporosis (PMOP) brings a heavy burden to society and seriously affects the quality of life of elderly people. Circulating biomarkers based on miRNAs, especially exosomal miRNAs, are widely studied. However, miRNAs in serum exosomes have not yet been reported in PMOP. The present study aimed to investigate the differences in miRNA expression profiles in PMOP with fragility fractures to identify the key circulating miRNAs in serum exosomes and to validate these molecules as potential biomarkers.

Methods

Postmenopausal women with osteoporotic fracture (severe osteoporosis, SOP group) and normal bone mass (Ctrl group) were selected from community. Serum exosomes were isolated by traditional differential ultracentrifugation from participants. Isolated exosomes were identified by electron microscopy, western blotting and nanoparticle-tracking analysis and then examined for exosomal miRNAs sequencing. Target gene pathways enrichment of differently expressed miRNAs were performed by the online bioinformatics tools TargetScan and DAVID. Moreover, the function of miRNAs associating with bone mineral density (BMD) on bone turnover were further confirmed by transfecting these miRNAs into human bone marrow mesenchymal stem cells (hBMSCs) to detect ALP activities.

Results

Compared to control group, 170 miRNAs were found to be significantly upregulated and 69 miRNAs were downregulated in SOP group. Three miRNAs (miR-324-3p, miR-766-3p and miR-1247-5p) were found to be associated with BMD of L1-L4, FN and TH, while miR-330-5p and miR-3124-5p were associated with BMD of FN and TH. Target gene signaling pathways analysis of these five miRNAs showed that Wnt signaling pathway was the most enrichment pathway relating to bone metabolism. Furthermore, miR-330-5p was found to promote the ALP activity of hBMSCs, while mir-3124-5p showed the opposite result.

Conclusion

Serum exosomal miRNAs were differentially expressed in postmenopausal osteoporosis patients with fragility fractures. Our study provides the first evidence that exosomal miRNA profiling revealed aberrant circulating miRNA in postmenopausal osteoporosis. MiR-324-3p, miR-766-3p, miR-1247-5p, miR-330-5p and miR-3124-5p, which were associated with BMD, may serve as candidate biomarkers for the diagnosis of PMOP with fragility fractures and provide future research directions.

Background

Osteoporosis (OP) is a systemic bone disorder characterized by an imbalance between bone formation and resorption, which leads to a reduction in bone mass^[1]. In women, postmenopausal osteoporosis (PMOP) is characterized by low bone mass and consequent fragility fractures, which have impaired quality of life and increased mortality in the population^[2, 3]. Although the measurement of BMD by dual energy X-ray absorptiometry (DXA) has been regarded as the “gold standard” and current approaches for predicting fractures are largely based on the measurement of BMD, BMD is associated with only 30–50% patients with major fragility fractures^[4]. In addition, the change in bone mass by DXA is gradual, and a period of 1 or 2 years is usually necessary to identify significant changes, which is inadequate to monitor bone loss^[5]. It is urgent to find a more accurate way to diagnose OP and predict fracture risk.

In recent years, miRNAs have attracted extensive attention for their roles in many biological processes, such as cell proliferation, differentiation, apoptosis and migration^[6–9]. It is known that miRNAs in serum may be associated with biological processes and play an important role in the progression of diseases. Regarding bone metabolism, some researches have shown that miRNAs were associated with bone metabolic disorders^[10] and several miRNAs were proved to regulate the osteogenic differentiation of BMSCs by mediating β -catenin-dependent migration^[11] or contribute to the regulation of Smad5^[12] and Runx2^[13]. Additionally, it was revealed that miRNAs might be biomarkers with diagnostic and prognostic potential in cancer and other diseases^[14]. However, the complexity and inherent heterogeneity of miRNAs in the circulation make it difficult to develop biomarkers and let alone evaluate the prognosis of diseases. Exosomes are membranous vesicles with a diameter of approximately 30–150 nm. Lipids, proteins and miRNAs are encapsulated by exosomes and play important roles in different biological processes^[15, 16]. Regarding the transfer of the cargos, exosomes could be considered as a novel vehicle of numerous disease biomarkers^[17, 18]. Moreover, it was reported that miRNAs in exosomes were stable enough under different storage conditions even at 4°C for a short time^[19], suggesting that miRNAs contained in exosomes may be potential biomarkers for the detection of fragility fractures and the assessment of disease progression.

In this study, we revealed the serum exosomal piRNA population between women suffering from PMOP with fragility fractures and same-age women with normal BMD to dissect the links between exosomal miRNAs and PMOP. The study of miRNA signatures will provide a deeper understanding of bone turnover mechanism to further identify potential diagnostic biomarkers of fragility fracture and assess fracture risk.

Methods

Patient serum samples

Postmenopausal women aged 65–75 years from two communities in Shanghai, China were enrolled and BMD of the lumbar, vertebra and hip were detected by DXA. This study was approved by the Medical Ethics Committee of Huadong Hospital (2019K055) and informed consent was obtained from all participants. Participants were divided into control group and severe osteoporosis (SOP) group according to BMD results and the history of fragility fractures. In control group, subjects had no fracture history and the T-score of BMD >-1.0 , while in SOP group, subjects suffered from fragility fractures in the vertebral spine and/or hip and the T-score of BMD ≤ -2.5 . Serum levels of calcium, phosphorus, 25-hydroxyvitamin D (25(OH)D), parathyroid hormone (PTH), P1NP and β -CTX were obtained to rule out secondary OP. Participants did not take insulin, sex hormones, glucocorticoids, anti-osteoporosis drugs, such as bisphosphonates, estrogen and progesterone replacement, selective estrogen receptor modulators (SERMs), parathyroid gland hormones or other drugs that will affect bone metabolism. Those who suffered from diabetes, severe cardiopulmonary disease, liver and kidney disease, endocrine and metabolic diseases, autoimmune diseases, malignant tumors and hyperlipemia were excluded. The participants information was listed in Table 1.

Table 1
Characteristics of the participants in this study.

Characteristics	Control, n = 18	SOP, n = 16	p-value
Age (year)	67.28 ± 5.2	67.0 ± 3.2	0.86
Height (cm)	157.53 ± 5.8	153.0 ± 6.8	0.04
Weight (kg)	59.62 ± 6.7	53.51 ± 7.4	0.02
BMI (kg/m ²)	24.02 ± 2.4	22.92 ± 3.6	0.31
Fracture of vertebra (%)	0	68.75	0.000
Fracture of hip (%)	0	43.75	0.000
Serum creatinine (umol/L)	61.8 ± 12.9	56.93 ± 8.9	0.27
AKP (U/L)	74.6 ± 17.8	80.23 ± 25.1	0.52
Serum calcium (umol/L)	2.41 ± 0.06	2.38 ± 0.11	0.53
Serum phosphorus (umol/L)	1.28 ± 0.23	1.19 ± 0.13	0.25
25(OH)D ₃ (ng/ml)	30.33 ± 15.11	28.79 ± 14.59	0.82
PTH (pg/ml)	31.8 ± 9.0	45.3 ± 20.2	0.14
β-CTX (pg/ml)	482.48 ± 209.58	476.79 ± 286.96	0.97
P1NP (ng/ml)	61.2 ± 12.4	61.1 ± 13.3	0.96
BMD of LS (g/cm ²)	0.884 ± 0.13	0.603 ± 0.06	0.000
BMD of FN (g/cm ²)	0.705 ± 0.11	0.511 ± 0.10	0.000
BMD of TH (g/cm ²)	0.752 ± 0.21	0.612 ± 0.16	0.04

Serum exosomes isolation

A 3 ml peripheral serum from each participants was collected and exosomes were isolated by traditional differential ultracentrifugation with four steps. At first, serum was diluted with sterile phosphate-buffered saline to 50ml, centrifugation at 3000×g for 30 min was performed, then supernatant was centrifuged at 12,000×g for 45min followed by ultracentrifugation for 2h at 120,000 ×g in 4 °C. The exosome pellet was re-suspended in lysis buffer or sterile PBS, depending on subsequent experiments.

Transmission electron microscopy (TEM)

The suspension was mixed with an equal volume of 4% paraformaldehyde, and 25ul of the solution was taken up to the loaded copper mesh, dried at room temperature for 20 minutes, and the liquid on the filter screen was blotted from one side with a filter paper, and 30ul of phosphotungstic acid solution was

added, stained for 5 min at room temperature, and then was blotted with a filter paper and dried at room temperature. The exosomes were photographed under a transmission electron microscope.

Western blot analysis

Exosomes were lysed in RIPA buffer with 1% phenylmethylsulfonyl fluoride (PMSF) and placed on ice for 10 minutes. Protein was quantified by using BCA protein quantitative kit (Sangon Biotech, Shanghai) according to the instruction. The concentration was adjusted by appropriate amount of radioimmunoprecipitation assay (RIPA) buffer, and sodium dodecyl sulfate (SDS) loading buffer of 1/4 volume was added. Protein samples were loaded, separated on 10% SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membranes, followed by blocking for 1 hour in 5% non-fat skimmed milk in tris buffered saline with tween (TBST) solution. After blocking, membranes were incubated with primary antibodies against TSG101 (1:1000 dilution, ab125011, Abcam) and CD63 (1:1000 dilution, ab216130, Abcam) respectively overnight at 4°C. Membranes were then washed using TBST for three times and incubated in secondary antibody for 1 hour in room temperature. At last, membranes were washed and developed by Tanon3500 gel imaging and photographing system (Tanon Science & Technology Co, Ltd.)

Nanoparticle tracking analysis (NTA)

Isolated pellets were analyzed by the Nanosight NS300 System (Malvern Instruments, UK) to determine the size and quantity of particles. Laser-irradiated nanoparticles are captured for 60 seconds and particle were analyzed by NTA software.

miRNA library construction and sequencing

Serum exosomes were isolated, prepared and sent to BGI-Wuhan (Wuhan, China) for miRNA library construction and next-generation sequencing. For each sample, Clean reads were obtained via removing the low quality reads and aligned with the human genome. Clean reads were further mapped to sRNA in the GenBank and Rfam to analyze their distribution and annotate small RNA sequences. After sequencing by an Illumina sequencer, image analysis, and base identification, the raw reads after quality control were harvested. Clean reads were aligned against known miRNA precursors and mature miRNAs in the miRBase to identify conserved miRNAs.

MiRNA Target Prediction and Relevant Signaling Pathway

Targets of miRNA were predicted by using Targetscan (<http://www.targetscan.org>) and Gene Ontology (GO) enrichment analysis of target genes was performed using DAVID online tool(<https://david.ncifcrf.gov/summary.jsp>). The relevant signaling pathways were analyzed using the KEGG database in DAVID.

Cell cultures, transfection and osteogenic differentiation

Bone Mesenchymal Stem Cells (BMSCs) were purchased from Cyagen Bioscience Inc and cultured in human bone marrow mesenchymal stem cell basal medium with 10% fetal bovine serum, penicillin-streptomycin and glutamine at 37°C with 5% CO₂. Cells were transfected with 20nM microRNA mimics on day 0 and cultured in human mesenchymal stem cell osteogenic differentiation basal medium with 10% fetal bovine serum, 1% glutamine, 1% ascorbate, 0.2% β-Glycerophosphate and 0.01% dexamethasone from day 1 to day 7 to induce osteogenic differentiation. Mediums were changed every 2 days.

Alkaline phosphatase (ALP) activity assay

ALP activity was examined by using Alkaline Phosphatase Assay Kit (ab83369, Abcam) in bone mesenchymal stem cells on day 7 after transfecting with related miRNA mimics or vehicles. 5mg pNPP was dissolved in solution with 0.1 M glycine, pH 10.4, 1 mM MgCl₂ and 1 mM ZnCl₂. Cell culture medium was discarded and 100ul pNPP solution was added 15 minutes. The absorbance was examined at 405 nm.

Statistical analysis

Numerical data was presented as the mean ± standard deviation. Statistical analysis was performed using SPSS and correlations were analyzed using Spearman data. P<0.05 was considered statistically significant.

Results

Characterization of participants

18 participants were in control group who had a normal bone mass and 16 participants were in severe osteoporosis (SOP) group who suffered from vertebral fracture (68.2%) and/or hip fracture (43.9%). BMI, age, biochemical markers and bone metabolism markers, 25(OH)D, PTH, and BMD between the two groups were shown in Table 1. No statistical differences in the age were observed between the two groups. The mean values of weight and BMI were lower in participants with osteoporotic fracture (P < 0.05). In addition, the SOP group have significantly lower BMD at all measured sites (lumbar spine, femoral neck and total hip) as compared to control group.

Signaling Pathway Analysis Of Target Genes

We further analyzed and investigated the potential function of differently expressed top 10 miRNAs with online bioinformatics data analysis tools TargetScan and DIANA. Target genes of upregulated miRNAs were mainly involved in rheumatoid arthritis, maturity onset diabetes of the young, glycosphingolipid biosynthesis-glybo series, N-glycan biosynthesis, glycosphingolipid biosynthesis-lacto and neolacto series (Fig. 3A). Target genes of downregulated top 10 miRNAs were mainly involved in proteoglycans in

cancer, adrenergic signaling in cardiomyocytes, arrhythmogenic right ventricular cardiomyopathy (ARVC), and mucin type O-glycan biosynthesis (Fig. 3B).

Correlation Analysis Of Mirnas With Bone Mineral Density

To greater confirm the core exosomal miRNAs involved in the progression of PMOP, correlations between known miRNAs and BMD of lumbar L1-L4, FN, and TH were analyzed and the results were shown in Table 2. Three miRNAs were related to the BMD of L1-L4, FN and TH (miR-324-3p and miR-766-3p were positively correlated, while miR-1247-5p was negatively correlated). In addition, two miRNAs were associated with BMD of FN and TH (miR-330-5p was positively correlated, while miR-3124-3p was negatively correlated). Signaling pathway enrichment for target genes of these five miRNAs were investigated and the results showed that Wnt signaling pathway was the most enrichment pathway relating to bone metabolism and osteogenic differentiation as shown in Table 3. Hence, we further analyzed the potential role of these five miRNA candidates in Wnt signaling pathway and online bioinformatics tools as TargetScan and DAVID was applied to help to predict target genes that may be involved in Wnt signaling pathway (Fig. 4).

Table 2
Differently expressed miRNAs in serum seosomes related to BMD

	L1-L4		FN		TH	
miRNA-name	R	P	R	P	R	P
hsa-miR-324-3p	0.511	0.001	0.403	0.009	0.353	0.020
hsa-miR-766-3p	0.408	0.008	0.451	0.004	0.372	0.015
hsa-miR-1247-5p	-0.365	0.017	-0.341	0.024	-0.348	0.022
hsa-miR-330-5p	0.268	0.006	0.355	0.020	0.338	0.025
hsa-miR-3124-3p	-0.205	0.122	-0.339	0.025	-0.298	0.044
LS, lumbar spine; FN, femoral neck; TH, total hip						

Table 3

Signaling pathway enrichment for target genes of miRNAs related to BMD

Term	P-Value	Fold Enrichment
Regulation of actin cytoskeleton	1.37E-04	2.790967
Wnt signaling pathway	0.004861	2.682394
Estrogen signaling pathway	0.040353	2.492729
Ras signaling pathway	9.60E-04	2.456883
Regulating pluripotency of stem cells	0.014823	2.423734
Rap1 signaling pathway	0.003138	2.350288
Hippo signaling pathway	0.023872	2.247171
VEGF signaling pathway	0.027434	2.174344
Long-term depression	0.040105	2.148561
Calcium signaling pathway	0.029788	2.067991
PI3K-Akt signaling pathway	0.003639	1.967089
Cytokine-cytokine receptor interaction	6.07E-04	1.867280
Vascular smooth muscle contraction	0.036487	1.853134
MAPK signaling pathway	0.003362	1.721263
Endocytosis	0.039149	1.611420

miRNA candidates relating to BMD could regulate ALP activity in hBMSCs

Signaling pathway enrichment results showed that 5 miRNA candidates relating to BMD were also involved in regulating pluripotency of stem cells. To further confirm the function of these five miRNA candidates on bone turnover imbalance, ALP activity were detected in bone mesenchymal stem cells by transfecting with miRNA mimics or vehicles. ALP activity results showed that miR-330-5p suppressed ALP activity and inhibited the osteogenic differentiation of BMSCs, while miR-3124-3p showed the opposite result. In aggregate, these observations suggest that these differentially expressed miRNAs may be involved in the progression of PMOP and have potential to be novel diagnostic biomarkers of PMOP.

Discussion

Osteoporosis and fracture have been strongly associated with women in post-menopausal age. Although clinical and basic research is constantly progressing, patients are still facing delayed diagnosis and fragility fractures, which indicate that exploration of circulating biomarkers is needed to provide a convenient and noninvasive diagnosis. MiRNAs are regarded as promising biomarkers to evaluate

disease progression and miRNAs in the serum of OP patients has been investigated, and the patterns of circulating miRNAs are likely to be diagnostic predictors of OP^[20]. However, few data about miRNAs, let alone circulating exosomal miRNAs, are available in PMOP with fragility fractures. Hence, we presented, for the first time, the serum exosomal miRNAs expression profiles in an elderly population between PMOP patients with fragility fractures and those with normal BMD and uncovered exosomal miRNAs with promising diagnostic values.

PMOP with fragility fractures is a complex biological process that involves complicated signaling pathways. In this study, we focused on miRNAs associated with BMD and related molecular mechanisms to gain insight into the link between PMOP with fragility fractures and miRNAs. We found five exosomal miRNAs (miR-324-3p, miR-766-3p, miR-1247-5p, miR-330-5p and miR-3124-5p) were related to BMD. Moreover, predicted target genes of these five miRNAs were highly associated with Wnt signaling pathway. Wnt signaling pathway is well known for its role in regulating self-renewal and differentiation in stem cells and bone metabolism^[21–24]. Among them, miR-324-3p, miR-766-3p and miR-1247-5p were found to be associated with BMD of the lumbar spine, femoral neck and hip sites, while miR-330-5p and miR-3124-5p were found to be associated with BMD of the hip. Previous studies^[25] proved that miR-324-3p was expressed at low levels in low-traumatic fractures, indicating that in elderly individuals, low expression of miR-324-3p may result in fractures by reducing bone density. MiR-766-3p could reduce the protein expression of Wnt3a^[26] and NF- κ B^[27], which play important roles in OP. In breast tumors, miR-1247-5p promotes tumor growth via the Dishevelled1 (DVL1)/Wnt/ β -catenin signaling pathway^[28], which promotes the differentiation of skeletal cells and accelerates bone regeneration^[29]. MiR-330-5p was found to silence SPRY2 expression and further influence the progression of tumors via Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling^[30], which is a regulator of osteoclastogenesis and plays an important role in bone loss^[31]. Although there were few studies on miR-3124-5p, it was found to induce the osteogenic differentiation of BMSCs. These results stated that the exosomal miRNAs candidates associated with BMD might play complex regulatory roles in the progression of PMOP by networking with cell signaling pathways. However, more studies are needed to clarify the molecular mechanisms of miRNAs in circulating exosomes in PMOP with fragility fractures.

This study found that miR-330-5p could suppress ALP activity and inhibited the osteogenic differentiation of BMSCs. Recent researches^[32] found that miR-330-5p was upregulated in senescent MSCs compared with young MSCs. MSCs are known to have self-renewal and multi-differentiation abilities, and a reduction in osteogenic differentiation of MSCs leads to loss of bone mass and contributes to increased risk of fracture. An considerable amount of research has confirmed that miRNAs play roles in MSC proliferation, migration and differentiation^[33]. Knockdown of miR-330-5p facilitates osteogenesis through the biglycan-induced bone morphogenetic protein (BMP)/Smad pathway and further to influence the progression of OP^[34]. These findings implied that miR-330-5p in circulating exosomes could not only be a biomarker but also a functional molecule in the progression of PMOP. Our next experiment will focus on the effects and mechanism of miR-330-5p on BMSCs to extend the study.

Conclusions

In conclusion, this study used second-generation sequencing to provide the first information that the differential miRNA expression profiling in serum exosomes between PMOP people with fragility fractures and normal BMD. miR-324-3p, miR-766-3p, miR-1247-5p, miR-330-5p and miR-3124-5p were found to be associated with BMD and may serve as circulating biomarkers as well as therapeutic targets and treatment options for PMOP with fracture.

Abbreviations

osteoporosis (OP)

postmenopausal osteoporosis (PMOP)

microRNAs (miRNAs)

bone mineral density (BMD)

human bone marrow mesenchymal stem cells (hBMSCs)

dual energy X-ray absorptiometry (DXA)

procollagen type 1 N-terminal propeptide (P1NP)

beta-C-terminal cross-linking telopeptide of type 1 collagen (β -CTX)

25-hydroxyvitamin D (25(OH)D)

parathyroid hormone (PTH)

selective estrogen receptor modulators (SERMs)

Transmission electron microscopy (TEM)

Tumor Susceptibility Gene 101(TSG101)

Nanoparticle tracking analysis (NTA)

Alkaline phosphatase (ALP)

p-Nitrophenyl phosphate (pNPP)

Gene Ontology (GO)

lumbar spine (LS)

femoral neck (FN)

total hip (TH)

Dishevelled1(DVL1)

Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK)

Declarations

Ethics approval and consent to participate

Ethics approval and consent to participate.

This study was approved by the Medical Ethics Committee of Huadong Hospital (2019K055) and written informed consent was obtained from all participants prior to the study.

Consent for publication

Not applicable

Availability of data and materials

The data analyses in this current study are available from the corresponding author on reasonable request.

Competing interests

All authors have no conflicts of interest.

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Authors' contributions

QC designed the study. HS and QC drafted the manuscript. QC, JC and CX contributed to its refinement. HS, XJ and CX performed the statistical analysis. QC, HS and XJ interpreted the analytical data

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Not applicable

References

1. HADJIDAKIS D J, ANDROULAKIS I I. Bone Remodeling[J]. *Annals of the New York Academy of Sciences*, 2006,1092(1):385–396.
2. Baron R, Kneissel M: WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* 2013, 19(2):179–192.
3. Cooper C, Atkinson EJ, Jacobsen SJ, O'Fallon WM, Melton LJ, 3rd: Population-based study of survival after osteoporotic fractures. *Am J Epidemiol* 1993, 137(9):1001–1005.
4. C. E M C R. The impact of fragility fracture and approaches to osteoporosis risk assessment worldwide [J]. *bone*, 2017,104(2017 Nov):29–38.
5. Rossi F E, Diniz T A, Neves L M, et al. The beneficial effects of aerobic and concurrent training on metabolic profile and body composition after detraining: a 1-year follow-up in postmenopausal women[J]. *European journal of clinical nutrition*, 2017,71(5):638–645.
6. Johnson C D, Esquela-Kerscher A, Stefani G, et al. The let-7 microRNA represses cell proliferation pathways in human cells[J]. *Cancer research*, 2007,67(16):7713–7722.
7. Chen J, Mandel E M, Thomson J M, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation[J]. *Nature Genetics*, 2006,38(2):228–233.
8. Abouheif M M, Nakasa T, Shibuya H, et al. Silencing microRNA-34a inhibits chondrocyte apoptosis in a rat osteoarthritis model in vitro[J]. *Rheumatology*, 2010,49(11):2054–2060.
9. Zhang F, Jing S, Ren T, et al. MicroRNA-10b promotes the migration of mouse bone marrow-derived mesenchymal stem cells and downregulates the expression of E-cadherin[J]. *Mol Med Rep*, 2013,8(4):1084–1088.
10. Li C, Cheng P, Liang M, et al. MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation[J]. *Journal of Clinical Investigation*, 2015,125(4):1509–1522.
11. Long H, Sun B, Cheng L, et al. miR-139-5p Represses BMSC Osteogenesis via Targeting Wnt/ β -Catenin Signaling Pathway[J]. *DNA and Cell Biology*, 2017,36(8):715–724.
12. Wei F, Yang S, Guo Q, et al. MicroRNA-21 regulates Osteogenic Differentiation of Periodontal Ligament Stem Cells by targeting Smad5[J]. *Scientific Reports*, 2017,7(1).
13. Li X, Guo L, Liu Y, et al. MicroRNA-21 promotes osteogenesis of bone marrow mesenchymal stem cells via the Smad7-Smad1/5/8-Runx2 pathway[J]. *Biochemical and Biophysical Research Communications*, 2017,493(2):928–933.
14. Huang W. MicroRNAs: Biomarkers, Diagnostics, and Therapeutics[J]. *Methods Mol Biol*, 2017,1617:57–67.

15. Huang Y, Liu W, He B, et al. Exosomes derived from bone marrow mesenchymal stem cells promote osteosarcoma development by activating oncogenic autophagy[J]. *Journal of Bone Oncology*, 2020,21:100280.
16. Niu C, Wang X, Zhao M, et al. Macrophage Foam Cell–Derived Extracellular Vesicles Promote Vascular Smooth Muscle Cell Migration and Adhesion[J]. *Journal of the American Heart Association*, 2016,5(10).
17. Fang DY, King HW, Li JY, Gleadle JM: Exosomes and the kidney: blaming the messenger. *Nephrology (Carlton)* 2013, 18(1):1–10.
18. Fernandez-Messina L, Gutierrez-Vazquez C, Rivas-Garcia E, Sanchez-Madrid F, de la Fuente H: Immunomodulatory role of microRNAs transferred by extracellular vesicles. *Biol Cell* 2015, 107(3):61–77.
19. Ge Q, Zhou Y, Lu J, et al. miRNA in Plasma Exosome is Stable under Different Storage Conditions[J]. *Molecules*, 2014,19(2):1568–1575.
20. Seeliger C, Karpinski K, Haug A T, et al. Five Freely Circulating miRNAs and Bone Tissue miRNAs Are Associated With Osteoporotic Fractures[J]. *Journal of Bone and Mineral Research*, 2014,29(8):1718–1728
21. Rajakulendran N, Rowland K J, Selvadurai H J, et al. Wnt and Notch signaling govern self-renewal and differentiation in a subset of human glioblastoma stem cells[J]. *Genes & Development*, 2019,33(9–10):498–510.
22. Yang T, Bassuk A G, Fritzsche B. Prickle1 stunts limb growth through alteration of cell polarity and gene expression[J]. *Developmental Dynamics*, 2013,242(11):1293–1306.
23. Choi S, Kim H, Cha P, et al. CXXC5 mediates growth plate senescence and is a target for enhancement of longitudinal bone growth[J]. *Life Science Alliance*, 2019,2(2):e201800254.
24. Yamashita T, Hagino H, Hayashi I, et al. Effect of a cathepsin K inhibitor on arthritis and bone mineral density in ovariectomized rats with collagen-induced arthritis[J]. *Bone Reports*, 2018,9:1–10.
25. Kocijan R, Muschitz C, Geiger E, et al. Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures[J]. *The Journal of Clinical Endocrinology & Metabolism*, 2016,101(11):4125–4134.
26. You Y, Que K, Zhou Y, et al. MicroRNA-766-3p Inhibits Tumour Progression by Targeting Wnt3a in Hepatocellular Carcinoma[J]. *Mol Cells*, 2018,41(9):830–841.
27. Hayakawa K, Kawasaki M, Hirai T, et al. MicroRNA-766-3p Contributes to Anti-Inflammatory Responses through the Indirect Inhibition of NF- κ B Signaling[J]. *International Journal of Molecular Sciences*, 2019,20(4):809.
28. Zeng B, Li Y, Feng Y, et al. Downregulated miR-1247-5p associates with poor prognosis and facilitates tumor cell growth via DVL1/Wnt/ β -catenin signaling in breast cancer[J]. *Biochemical and Biophysical Research Communications*, 2018,505(1):302–308.
29. Minear S, Leucht P, Jiang J, et al. Wnt Proteins Promote Bone Regeneration[J]. *Science Translational Medicine*, 2010,2(29):29r-30r.

30. Xiao S, Yang M, Yang H, et al. miR-330-5p targets SPRY2 to promote hepatocellular carcinoma progression via MAPK/ERK signaling[J]. *Oncogenesis*, 2018,7(11).
31. Jin H, Shao Z, Wang Q, et al. Sclareol prevents ovariectomy-induced bone loss in vivo and inhibits osteoclastogenesis in vitro via suppressing NF- κ B and MAPK/ERK signaling pathways[J]. *Food & function*, 2019,10(10):6556–6567.
32. Yoo J K, Kim C, Jung H Y, et al. Discovery and characterization of miRNA during cellular senescence in bone marrow-derived human mesenchymal stem cells[J]. *Experimental Gerontology*, 2014,58:139–145.
33. Tomé M, López-Romero P, Albo C, et al. miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells[J]. *Cell Death and Differentiation*, 2011,18(6):985–995.
34. Jin S, Bai Y, Zhao B, et al. Silencing of miR-330-5p stimulates osteogenesis in bone marrow mesenchymal stem cells and inhibits bone loss in osteoporosis by activating Bgn-mediated BMP/Smad pathway[J]. *European review for medical and pharmacological sciences*, 2020,24(8):4095.

Figures

Figure 1

Characterization and identification of serum exosome by ultracentrifugation. (A) Exosomes were analysed by electron microscopy (Scale bar=100 nm). (B) Size distribution of exosomes were analysed by the Nanoparticle tracking analysis. (C) Western blotting was applied to detect the exosomal markers TSG101 and CD63 in serum sample after exosome isolation and exosomes isolated from serum sample.

Figure 2

Differentially expressed miRNAs in serum exosomes between SOP and control library. (A)Volcano plot was applied to show differentially expressed exosomal miRNAs in severe osteoporosis group and control group. (B) Deletion of unknown miRNA and diverse miRNAs were analyzed with miRbase database. (C) Classification of potential target genes for differently expressed known miRNAs by GO analysis. X axis shows the number of target genes, and the Y axis shows the GO terms of biological process, cellular component and molecular function.

Figure 3

Heat map of signaling pathway enrichment for target genes of top 10 differentially expressed miRNAs. Data of top 10 upregulated (A) and downregulated (B) miRNAs were analyzed by online bioinformatics tool DIANA. Each row and column represent a miRNA and pathway respectively. The red color shades represent high relative levels and yellow shades represent lower relative levels.

Figure 4

The potential roles of miRNAs related to BMD in Wnt signaling pathway. Five differentially expressed miRNAs (including miR-324-3p, miR-776-3p, miR-1247-5p, miR-330-5p and mir-3124-3p) associated with BMD were predicted to play roles in Wnt signaling pathway through regulating their potential target genes.

Figure 5

The function of miRNAs related to BMD in regulating ALP activity in hBMSCs. Five differentially expressed miRNAs mimics (including miR-324-3p, miR-776-3p, miR-1247-5p, miR-330-5p and mir-3124-3p) associated with BMD were transfected into hBMSCs. ALP activity was examined on day 7 after transfection assay. Error bars represent SD of three independent experiments; ** $p < 0.01$ *** $p < 0.001$.

Supplementary Files

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- [Supplementarytable1.docx](#)