Systematic Analysis of Plasma Exosome MiRNA in Patients with Postmenopausal Osteoporosis

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Research

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Abstract

Background: More and more studies have shown that exosomes are involved in many aspects of bone metabolism. As the content of exosomes, miRNA plays an important role in the early diagnosis, treatment and prognosis evaluation of osteoporosis.

Objective: To determine the potential molecular markers of osteoporosis by analyzing the differences of plasma exosome miRNA expression between postmenopausal women with osteoporosis and healthy controls.

Methods: Serum samples of postmenopausal osteoporosis (PMOP) patients over 65 years old (n = 12) and healthy controls (n = 12) were collected. The exosomes were separated by molecular size exclusion chromatography (SEC). The differentially expressed miRNAs were analyzed by cluster analysis. The target genes of miRNAs were predicted and annotated by relevant software. The target genes were classified by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). The appropriate biomarkers were screened out.

Results: The miRNA spectrum of the patients was significantly different from that of the control group. Validation studies showed that 14 up-regulated miRNAs had a high probability of prediction, which could distinguish osteoporosis patients from healthy controls. Thirty-one genes were predicted to be targets for these miRNAs. GO enrichment analysis showed that miRNAs were mainly concentrated in protein binding, carbohydrate-binding, chromatin binding and protein kinase binding. KEGG enrichment analysis showed that miRNAs were mainly concentrated in the p53 signalling pathway, mineral absorption, glycerin metabolism, insulin secretion, glycosaminoglycan biosynthesis heparin sulfate /heparin pathway. This study identified a large number of differentially expressed miRNAs in plasma samples from postmenopausal women with osteoporosis. It may help to obtain new diagnosis and treatment strategies for PMOP.

Conclusion: Our study identified the characteristics of plasma miRNAs in patients with osteoporosis and identified 14 candidate miRNAs, which may be useful biomarkers of osteoporosis. We speculate that these differentially expressed miRNAs may play a key role in the process of bone marrow mesenchymal stem cells osteogenic metabolism and transformation.

Introduction

Osteoporosis (OP) is a common metabolic bone disease. It is characterized by decreased bone mass, destruction of bone microstructure, increased brittleness and decreased mechanical strength, which easily increases the risk of fracture^[1]. With the development of the ageing population, the incidence rate of osteoporosis is gradually increasing and has become an important public health problem facing our country. It is associated with age, especially with hip, vertebrae, distal forearm and humerus fractures. By 2050, the number of patients with hip fractures in Asia will more than double, accounting for 50% of the total number of hip fractures in the world^[2]. It is predicted that by 2050, the number of osteoporotic

fractures in China will reach 5.99 million, and the corresponding medical expenditure will be as high as 174.5 billion yuan^[3]. Therefore, in the daily clinical treatment of osteoporosis, we need more efficient, timely, accurate and perfect tools to understand the progress of osteoporosis and assess the risk of osteoporotic fracture.

Exosomes are extracellular vesicles (EVs) with a diameter of 30-100nm, which are secreted by the multivesicular body (MVB) of the endocytosis system fused with the cell membrane. The density of exosomes in sucrose solution ranged from 1.13 g/ml to 1.19 g/ml. The density of exosomes was related to cell origin and varied with protein content^[4]. Exosomes were first discovered by Johnstone et al. in the study of reticulocyte to mature erythrocytes. Exosome is rich in nucleic acids, including DNA, mRNA, miRNA, lncRNA and so on. It plays an important role in the transmission of genetic material between cells. It can regulate biological information and participate in a variety of physiological and pathological processes^[5]. Seeliger et al. Found that serum miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b were significantly up-regulated in patients with osteoporosis^[6]. Kocijan et al. Showed that eight miRNA combinations (miR-152-3p, miR-30e-5p, miR-140-5p, miR-324-3p, miR-19b-3p, miR-335-5p, miR-19a-3p, miR-550a-3p) can be used as good markers for the diagnosis of osteoporosis^[7]. AUC under the diagnostic curve of the model is greater than 0.9 regardless of age and gender. In 2013, three scientists won the Nobel Prize for discovering the regulation mechanism of exosome transport. In 2015, Professor Raghu kalluri of MD Anderson Cancer Center found that the exosome marker Recombinant Glypican 1 (GPC1) is a perfect diagnostic tool for pancreatic cancer^[8]. As a membrane structure, the miRNA in exosomes is more stable. Compared with the direct detection of miRNA in serum, the detection of blood exosomes miRNA has a smaller fluctuation range, and it is possible to obtain higher diagnostic performance. In other geriatric related diseases, including Alzheimer's disease^[9-10], Parkinson's disease^[11] and amyotrophic lateral sclerosis^[12], exosomes miRNA related research has been carried out. Researchers from the University of Pennsylvania and the University of Southern California have found that the exosomes of bone marrow mesenchymal stem cells (MSC) in osteoporotic mice can effectively treat osteoporosis^[13]. At the same time, it has been found that miRNA from the exosomes of osteoclasts can inhibit the activity of osteoblasts^[14]. In this study, we analyzed the differential expression of serum exosome miRNA in elderly postmenopausal women with osteoporosis and healthy people, and bioinformatics analysis of its target gene, rich KEGG pathway and GO function, to understand its role in the occurrence of osteoporosis.

Materials And Methods

Study population and blood collection

Participants included 12 patients with osteoporosis (dual-energy X-ray examination: T-value of ≤ -2.5 and below) and 12 healthy people (control group) from October 2019 to January 2020 in Lu'an Affiliated Hospital of Anhui Medical University. During diagnosis and treatment in our hospital, HBV / HIV samples were excluded. 8ml blood was collected by EDTA anticoagulant tube and stored temporarily at 4 ° C. The

collected blood was 500 g at 4 ° C and centrifuged for 20 min to remove the cells in the blood; The supernatant was collected, 3000 g at 4 ° C, centrifuged for 15 min, and the supernatant (plasma) was collected and stored at -80 ° C.

Exosome separation

Exosupur was used to separate exosomes. The collected plasma was filtered with a membrane, and the column sealing solution was eluted with PBS twice the volume of the column. The filtered plasma was continuously eluted with PBS, and the exosomes were collected according to the instructions. After that, the fractions were collected by a 100kD ultrafiltration tube, centrifuged for 5 min, and the exosomes were concentrated to about 200ul. Finally, the liquid is transferred to the EP pipe.

Verification of exosomes by QRT PCR

QRT PCR can be used to verify the target RNA markers (miRNA, mRNA, lncRNA) of exosomes, and to study the differences in their expression in different samples. When detecting differentially expressed miRNAs, it is necessary to select appropriate differential expression analysis software according to the actual situation. The DESeq is suitable for experiments with biological duplication, which can be used for differential expression analysis between sample groups to obtain differential expression miRNA sets between two biological conditions; The ldeg6 is suitable for experiments without biological duplication to obtain differentially expressed miRNAs between two samples. The edgeR is suitable for differential expression analysis between samples (groups) and can be used for differential analysis with or without biological duplication. In this analysis, we use edgeR software.

miRNA targeting gene prediction, GO classification and KEGG functional annotation

According to the known miRNA and newly predicted miRNA and gene sequence information of corresponding species, TargetFinder software was used for plant target gene prediction, Miranda and RNAhybrid were used for animal target gene prediction. Blast software was used to compare the predicted target gene sequences with NR, Swiss-Prot, GO, COG, KEGG, KOG and Pfam databases to obtain the annotation information of target genes. GO database is a structured standard biological annotation system, which establishes a standard information system for gene and protein research, which applies to all species. The topGO software was used to analyze the enrichment of differential genes among sample groups, and the hierarchical relationship of a significantly enriched term in the GO system was visually displayed in the form of a directed acyclic graph. The database structure is divided into several levels, the lower the level, the more specific the function. In organisms, different genes coordinate with each other to perform biological functions. The annotation analysis of differentially expressed miRNA target genes is helpful to further understand the function of genes. KEGG database is an important public database related to metabolic pathways. The path enrichment analysis of differentially expressed miRNA target genes is to analyze whether the differentially expressed miRNA target genes have been over presented in a certain pathway. Enrichment factor was used to analyze the enrichment degree of the pathway, and Fisher exact test method was used to calculate the enrichment significance.

Results

Differential expression of miRNA

In the detection of differentially expressed miRNAs, $|\log_2(\text{FC})| \geq 0.584962500721156$; $P\text{-value} \leq 0.05$ were used as screening criteria. The fold change (FC) was the ratio of expression between the two samples (groups). The P-value obtained by the original hypothesis can be expressed as the probability of no difference in expression. In addition, considering that the low expression miRNA has little biological significance, and to obtain more meaningful functional annotation analysis results, we only retain miRNAs with an average expression level higher than 1 as the final differential expression miRNA. After analysis: a total of 1004 miRNAs were obtained from all samples, including 984 known miRNAs and 20 newly predicted miRNAs. Among them, there were 14 kinds of miRNAs with different types in each group (Table 1).

Table 1
The 14 kinds of miRNAs with different types in each group.

miRNA	FDR	log2FC	P-Value	regulated
miR-190b-5p	1.000	0.876	0.031	Up
miR-196a-5p	0.381	1.677	0.001	Up
miR-196b-5p	0.381	1.677	0.001	Up
miR-205-5p	0.400	1.594	0.002	Up
miR-224-5p	0.381	1.010	0.001	Up
miR-335-3p	0.639	1.085	0.006	Up
miR-34a-5p	0.381	0.752	0.002	Up
miR-375-3p	0.623	1.791	0.005	Up
miR-320c	0.918	0.501	0.014	Up
miR-152-3p	0.966	0.407	0.015	Up
miR-664a-5p	0.970	0.524	0.016	Up
miR-320d	1.000	0.457	0.019	Up
miR-320e	1.000	0.466	0.021	Up
miR-98-5p	1.000	0.305	0.033	Up
miRNA: miRNA number; FDR: error detection rate; log2 (FC): logarithm of multiple of expression difference; p-value: the probability of expression difference; regulated: up-regulated miRNA (up) or down-regulated miRNA (down).				

Identification of miRNA target genes

We used Miranda and RNAhybrid for target gene prediction. The predicted target gene sequences were compared with NR, Swiss-Prot, GO, COG, KEGG, KOG and Pfam databases using blast software. A total of 31 target genes were predicted (Table 2).

Table 2
The 31 target genes.

Gene	FDR	FC	P-Value	regulated
TMEM43	0.399554	1.594083265	0.002446828	up
STAMBP	0.380968	1.010035972	0.001199966	up
DNAJC5B	0.380968	0.752404132	0.0015178	up
ATP1A3	0.399554	1.594083265	0.002446828	up
GEMIN5	0.380968	0.752404132	0.0015178	up
DEPDC5	0.380968	1.677325316	0.000966045	up
WDFY3	0.380968	0.752404132	0.0015178	up
PIDD1	0.380968	0.752404132	0.0015178	up
MILR1	0.399554	1.594083265	0.002446828	up
C16orf96	0.380968	0.752404132	0.0015178	up
NFXL1	0.623309	1.790590971	0.005321061	up
LGR6	0.380968	0.752404132	0.0015178	up
PHRF1	0.380968	0.752404132	0.0015178	up
PRR22	0.380968	0.752404132	0.0015178	up
SCN4A	0.380968	1.010035972	0.001199966	up
NLGN2	0.380968	0.752404132	0.0015178	up
PNPLA2	0.399554	1.594083265	0.002446828	up
TMEM151B	0.380968	0.752404132	0.0015178	up
NDST1	0.380968	0.752404132	0.0015178	up
RIMS2	0.380968	0.752404132	0.0015178	up
CABIN1	0.380968	0.752404132	0.0015178	up
COL6A6	0.380968	1.010035972	0.001199966	up
CCAR1	0.380968	0.752404132	0.0015178	up
SGSM2	0.380968	0.752404132	0.0015178	up
TRIM35	0.380968	0.752404132	0.0015178	up

Gene: Gene number; FDR: error detection rate; FC: ratio of expression amount between two samples (groups); p-value: the probability of expression difference; regulated: up-regulated miRNA (up) or down-regulated miRNA (down).

Gene	FDR	FC	P-Value	regulated
CEACAM20	0.380968	0.752404132	0.0015178	up
CCT3	0.399554	1.594083265	0.002446828	up
PPRC1	0.380968	0.752404132	0.0015178	up
FAM160A1	0.399554	1.594083265	0.002446828	up
VASN	0.380968	0.752404132	0.0015178	up
KIAA1109	0.380968	0.752404132	0.0015178	up
Gene: Gene number; FDR: error detection rate; FC: ratio of expression amount between two samples (groups); p-value: the probability of expression difference; regulated: up-regulated miRNA (up) or down-regulated miRNA (down).				

GO analysis and KEGG analysis of miRNA

We used the topGO software to analyze the enrichment of different genes among sample groups, and visually displayed the hierarchical relationship of a significantly enriched term in the GO system in the form of a directed acyclic graph. The database structure is divided into multiple levels, the lower the level, the more specific the function. In a directed acyclic graph, an arrow represents inclusion relation, that is, all genes of the term are annotated into its superior term. The enrichment levels of the miRNA target gene between samples were analyzed as follows (Fig. 1). The results of our analysis are as follows (Table 3).

Table 3
GO analysis of miRNAs.

GO term	Annotated	Significant	Expected	KS
cation-transporting ATPase activity	30	1	0.06	0.00025
NAD-dependent histone deacetylase activity (H3-K9 specific)	9	0	0.02	0.00024
protein kinase binding	308	0	0.65	0.000017
kinase activity	612	0	612	0.000045
kinase binding	339	0	0.72	0.00027
protein complex binding	452	0	0.96	0.00000048
enzyme activator activity	169	0	0.36	0.000081
cytoskeletal protein binding	480	0	1.02	0.00027
protein binding	5230	12	11.08	0.00000096
carbohydrate derivative binding	1485	3	3.15	0.00029
GO term: GO Annotated: the number of genes annotated to the function; significant gene: the number of differentially expressed miRNA target genes annotated to the function; expected gene: the expected number of differentially expressed miRNA target genes annotated to the function; KS: significant statistics of enriched term, the smaller the KS value, the more significant the enrichment.				

KEGG analysis was used to identify the key pathways of these miRNAs. These miRNAs are mainly enriched in glycolipid metabolism, p53 signalling pathway, insulin secretion, glycosaminoglycan biosynthesis heparin sulfate/heparin sulfate, mineral absorption, cell adhesion molecules, protein digestion and absorption, RNA transport, endocytosis, and protein processing pathways in the endoplasmic reticulum (Table 4). The annotation results of KEGG, the target gene of differentially expressed miRNA, were classified according to the pathway types in KEGG, and the classification map was obtained (Fig. 2). The analysis of over presentation of differentially expressed miRNA target genes in a certain pathway is the pathway enrichment analysis of differentially expressed miRNA target genes. Enrichment factor was used to analyze the enrichment degree of pathways, and Fisher exact test was used to calculate the significance of enrichment. The calculation formula of enrichment factor is as follows:

Table 4
KEGG analysis of miRNAs.

KEGG term	P-Value	Q-Value
Glycerolipid metabolism	0.00496955382331776	0.0496955382331776
p53 signalling pathway	0.0343229680682807	0.343229680682807
Insulin secretion	0.0343229680682807	0.343229680682807
Glycosaminoglycan biosynthesis - heparan sulfate/heparin	0.0415384579568064	0.415384579568064
Mineral absorption	0.0487053891427951	0.487053891427951
Cell adhesion molecules (CAMs)	0.076893317988859	0.76893317988859
Protein digestion and absorption	0.081517514583033	0.81517514583033
RNA transport	0.141936434199582	1
Endocytosis	0.146266254865688	1
Protein processing in the endoplasmic reticulum	0.150576402008529	1
KEGG term: KEGG Function description; p-value: the probability of expressing difference; Q-value: p-value corrected by multiple hypothesis tests. Therefore, the larger the ordinate, the more reliable the enrichment significance of differentially expressed miRNA target genes in this pathway.		

Enrichment Factor =

$$\frac{\text{number of differentially expressed miRNA target genes in the path/number of all genes in the path}}{\text{number of differentially expressed miRNA target genes in all KEGG/number of all genes in KEGG}}$$

The results are as follows. (Fig.3)

Discussion

In our study, we screened 14 miRNAs with significant differences by detecting the expression of total miRNAs in plasma samples of postmenopausal women with osteoporosis. Our study shows that the differential expression of miRNA is closely related to the molecular pathogenesis of PMOP. We also annotated the functions and related pathways of miRNA and its target genes. Our study suggests that miRNA may be considered as a potential biomarker of PMOP. We found that the differentially expressed miRNAs have been proved to be related to PMOP. A recent study showed that LncRNA LOXL1-AS1 controls osteogenic and adipogenic differentiation of BMSC in PMOP by regulating the mir-196a-5p/HMGA2 axis^[15]. At the same time, another study showed that overexpression of miR-196a accelerated osteogenic differentiation in osteoporotic mice through GNAS dependent Hedgehog signalling pathway^[16]. Another study also found that miR-335-5p overexpression promoted bone formation and

regeneration in mice^[17]. Therefore, this miRNA may be a potential target for the treatment of PMOP. Consistent with our study, changes in miRNA expression play a key role in PMOP.

In our study, the function of these differentially expressed miRNAs was also predicted by GO enrichment analysis. Consistent with previous studies, miRNAs are involved in some key processes of PMOP. Studies have shown that by inhibiting adenosine monophosphate-activated protein kinase, the mineralization of osteoblasts induced by bone morphogenetic protein-2 (BMP-2) can be inhibited through the non-Smad mechanism^[18]. Adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine kinase, is involved in bone remodelling. AMPK is activated in the early stage of osteoblast differentiation. Studies have shown that AMPK activation promotes osteoblast differentiation and mineralization. Another study found that subchondral bone marrow mesenchymal stem cells showed high phosphorylation of extracellular signal-related kinase/mitogen-activated protein kinase signal transduction, which was closely related to the proliferation of MSC^[19]. In addition, osteoblasts can induce osteoclast formation, which is strongly promoted by IL-1 β ^[20]. Therefore, the current research may provide more evidence on the effect of miRNA on PMOP. The results of the KEGG analysis also support the previous study. Previous studies have shown that heparinized β -tricalcium phosphate (β -TCP) supports the adhesion and proliferation of rat MSC and is expected to differentiate into osteoblast lineage compared with β -TCP and control^[21]. In another study, natural bone ceramics/type I collagen could promote the expression of early osteogenic genes, while nano-hydroxyapatite (nHA)/COL could up-regulate the expression of late osteogenic genes^[22]. Liu Through cell-level research, LGR6 is highly expressed in osteoblast progenitor cells and enhances the Wnt signalling pathway by enhancing the stability of β -Catenin to promote osteogenesis^[23]. Therefore, these pathways obtained in our study may help us to understand the molecular pathogenesis of PMOP. Overall, this study identified a large number of differentially expressed miRNAs in blood samples from postmenopausal women with osteoporosis. The key functions and pathways of differentially expressed miRNAs were obtained by enrichment analysis of GO and KEGG. The exact function of this miRNA needs further study. This study may help to obtain new clinical applications for PMOP.

Conclusion

(1) 14 up-regulated miRNAs have a high predictive probability to distinguish osteoporosis patients from healthy controls; (2) GO enrichment analysis showed that miRNA was mainly enriched in protein binding, carbohydrate-binding, chromatin binding and protein kinase binding; (3) KEGG enrichment analysis showed that miRNAs were mainly enriched in p53 signalling pathway, mineral absorption, lipid metabolism, insulin secretion, glycosaminoglycan biosynthesis heparan sulfate/heparin pathway. This study identified a large number of differentially expressed miRNAs in plasma blood samples of postmenopausal women with osteoporosis, which may help to obtain new diagnosis and treatment strategies for PMOP.

Declarations

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CZ, JX, and SC analyzed the data and wrote the first draft. SS and QW designed the study, proofread, and revised the submission. RL directed the statistical analyses of the data. CY retrieved documents and extracted data. All authors contributed to the article and approved the submitted version.

Ethics approval and consent to participate

The study protocol was approved by the ethics committee of Anhui Medical University, and all participants had signed informed consent. All experiments were in line with the principles of The Code of Ethics of the World Medical Association (Declaration of Helsinki) for research experiments involving human subjects.

Consent for publication

I understand that the text and any pictures or videos published in the article will be freely available on the internet and may be seen by the general public. The pictures, videos and text may also appear on other websites or in print, may be translated into other languages or used for commercial purposes. I have been offered the opportunity to read the manuscript.

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Figures

Figure 1

The target gene TopGo of differentially expressed miRNA. For each GO node to be enriched, the most significant 10 nodes are represented in the box in the diagram, and the corresponding relations of each layer are also included in the graph. Each box (or ellipse) gives the content description and enrichment significance value of the GO node. Different colours represent different enrichment significance. The darker the colour, the higher the significance.

Figure 2

The KEGG pathway classification map of target genes. The ordinate is the name of the KEGG metabolic pathway, and the abscissa is the number of genes annotated to the pathway and its proportion to the total number of genes annotated.

Figure 3

The pathway enrichment analysis of target genes. Each figure in the figure represents a KEGG path. See the legend on the right for the pathname. The Abscissa is enrichment factor, which indicates the proportion of the number of differentially expressed miRNA target genes annotated to a certain pathway in the total number of genes annotated to the pathway. The larger the enrichment factor, the more significant the enrichment level of differentially expressed miRNA target genes in this pathway. The ordinate is $-\log_{10}(Q\text{-value})$, where Q-value is the P-value after multiple hypothesis test correction. Therefore, the larger the ordinate, the more reliable the enrichment significance of differentially expressed miRNA target genes in this pathway.