

Original Article

Six miRNAs identified serving as prognostic and predictive markers for osteoporosis by miRNA high-throughput method

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Abstract: Osteoporosis (OS) is the most common skeletal disease resulting in fracture. The aim of this study was to identify potential miRNAs that could function as specific biomarkers for identification and treatment of OS with miRNA-seq. MiRNA high-throughput data were downloaded from The Cancer Genome Atlas (TCGA) dataset. There were total 10 samples, including five normal specimen and five patient samples with spinal cord injury. Differentially expressed miRNAs were screened using SAMR package in R language with FDR<0.05 and Log FC>1. Target genes of OS were predicted by Targetscan software. And functional enrichment analysis was performed on the up- and down-regulated miRNAs by DAVID software basing on the hypergeometric analysis. Meanwhile, differentially expressed genes (DEGs) of mRNA were analyzed to construct the regulating network. Totally, 799 differentially expressed miRNAs were identified, among which six screened miRNAs, that is miR365, miR-10b, miR-129-3P, miRNA-671-5p, miR-141 and miR-25, had the potential to serve as biomarkers of OS. GO and KEGG analysis revealed that three pathways, including pathway in cancer, phosphatidylinositol signaling system and endocytosis, had the greatest effect on osteoporosis disease. Meanwhile, the miRNAs related with these pathways were almost up-regulated. Moreover, the most enriched GO and KEGG terms were both related to plasma membrane, indicating its association with the occurrence of OS disease. Six miRNAs identified in the present study could serve as prognostic and predictive markers for OS patients. Phosphatidylinositol signaling system, the most enriched GO term, possessed potential application in diagnosis and treatments of OS patients.

Keywords: Differentially expressed miRNA, osteoporosis, miRNAs-seq, functional analysis

Introduction

Primary osteoporosis is the most common and aggressive adult bone tumor over aged 65. It's estimated that half of all bone fracture are related to osteoporosis, causing it a major public health problem [1]. However, there were still many difficulties in prognostic and predictive strategies of OS since that the pathogenic factors were complicated and that the molecular mechanism of OS was still confusing. Accordingly, researches on the molecular mechanism of OS were in need and meaningful.

MicroRNA (miRNAs or miRs), are a growing class of small single-stranded noncoding RNAs

found in diverse organisms [2]. Recently, several studies indicate that expressions of miRNAs are associated with patients' survival and able to function as prognostic and predictive indicators. They can regulate translation of specific mRNA negatively. miRNAs were found to aberrantly expressed in a variety of tumor types and exert important regulations on tumor biology via acting as oncogenes or tumor suppressors [3, 4]. Although the biological functions of most miRNAs are not yet fully understood, their role in the regulation of cellular differentiation, proliferation, apoptosis and gene regulation, cancer development has drawn much attention in clinical management [5, 6]. In the bone tissue of osteoporosis patients, miRNAs also dis-

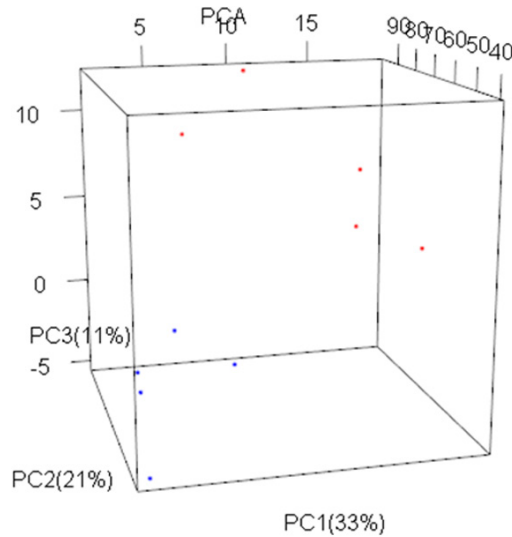


Figure 1. PCA of expression values of miRNA. The horizontal axis represents that the first component accounts for 33% while the vertical axis represents 21%. The red dots represent samples with osteoporosis while blue dots represent the normal ones.

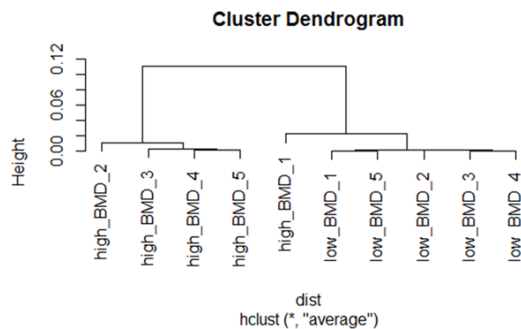


Figure 2. Cluster analysis of miRNA expression value. The vertical axis represents the height, revealing the differences among samples. The cluster tree suggests that samples with the similar expression value will be gathered.

played a significantly altered expression, besides, several research have proven that miRNAs were involved in osteoblast differentiation and bone formation [7, 8], but the role of miRNAs in osteoblasts still remains unclear, which need further research.

In this study, we aim to identify specific miRNA markers that are closely related with tumor progression and survival for OS patients by analyzing significantly altered miRNAs in a large dataset. And finally six RNAs and three signaling pathways were found to function as prognostic

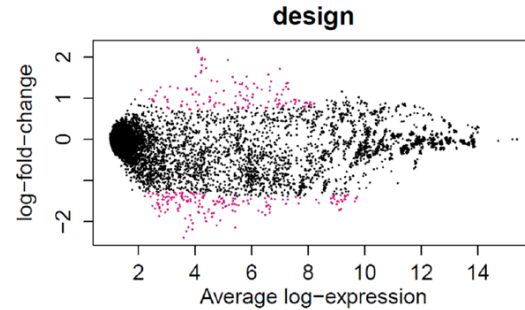


Figure 3. Differentially expressed analysis of miRNA. The black dots represent the no differentially expressed miRNAs while the red dots represent the differentially expressed miRNAs. The red dots above the figure stand for the miRNAs with the increased expression values while the below ones represent those with the decreased expression values.

and predictive markers for survival of OS patients, which may also be used as targets for treating bone loss and optimizing fracture healing in osteoporosis disease.

Materials and methods

Data source and data preprocessing

Microarray data of miRNA and mRNA were downloaded from Genome expression omnibus (GEO) dataset, with the accession number of GSE63446. Ten samples were included, including five normal bone tissues and five samples with osteoporosis. The data platform was Affymetrix Human Gene 1.0 ST Array. Data with low quality and batch difference was eliminated. Another miRNA dataset of GSE60230 was collected, including 14 osteoporosis samples of post-menopausal women. Then the two datasets were integrated to obtain the factors affecting bone density of women before and after menopausal.

Screening of differentially expressed miRNAs and mRNAs

SAMR package [9] in R language was used to screen the differentially expressed miRNAs between the normal bile duct tissue and samples with osteoporosis. $FC=2$ and $FDR<0.05$ were used as the cut-off criterion. Cluster analysis was performed on these differentially expressed miRNAs to ensure whether the difference between normal and osteoporosis specimens was significant. Cluster heatmap was drawn out afterwards. Principal Component

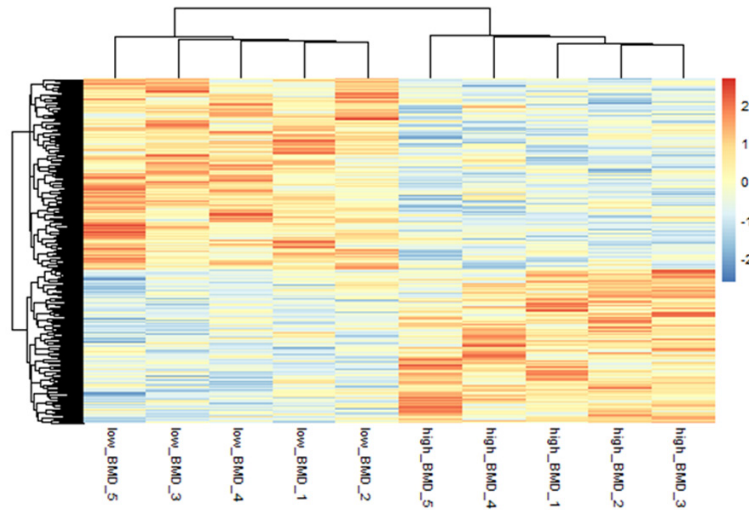


Figure 4. Cluster analysis of differentially expressed miRNA. The horizontal axis below represents sample names. The vertical axis on the right stands for the name of miRNA, while the left ones represent the cluster of miRNA. The red color stands for the up-regulation of miRNA while the blue color represents the down-regulation of miRNA. Two clusters of samples are included; one is the normal bile duct tissue while the other is the osteoporosis tissue. MiRNA can also be divided into two kinds, one is the down-regulated miRNAs in osteoporosis and the other is the up-regulated miRNAs.

character was analyzed by Network Analyzer, plug-in of Cytoscape [17]. Besides, clusterone, another plug-in of Cytoscape, was used to module functions in the network. *P*-value less than 0.05-0.5 was used as the threshold and modules ranging the top three was selected out to carry the functional analysis. Correlation coefficient of miRNA-mRNA interactions was calculated out and the network structure was observed by constructing NP network. Function of each cluster tree was obtained by analyzing the cluster tree with different network structure.

Results

Data source

Analysis (PCA) [10] was used to justify whether normal bile duct tissues can clearly differentiate from those tissues with osteoporosis. Meanwhile, differentially expressed mRNAs were screened out in the same way.

Screening of target genes and functional enrichment analysis

Target gene data of differentially expressed miRNA were extracted out from Mirtarbase [11] dataset. Then, gene ontology (GO) [12] functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) [13] pathway analysis were performed by DAVID (Database for Annotation, Visualization, and Integrated Discovery). The enriched *p* value and the corrected *p* value after multiple test (Benjamini rectification) were obtained by DAVID [14, 15] analysis. Potential targets of miRNA were predicted by Targetscan software, and then GO and KEGG functional analysis were performed on these genes in order to obtain the potential therapy targets [16].

Construction and analysis of miRNA-mRNA interaction network

Network of target genes and protein was constructed by Cytoscape and then the topology

A total of 20023 miRNA expression values of 10 samples were downloaded from the dataset. The difference between normal samples and osteoporosis specimens were figured out by PCA (**Figure 1**) and cluster analysis (**Figure 2**).

Differentially expressed analysis of miRNA

A total of 799 differentially expressed miRNAs were found between normal and osteoporosis specimens, including 441 up-regulated and 358 down-regulated miRNAs accounting for 55.2% and 44.8%, respectively (**Figure 3**). Hereinto, six miRNAs (miR365, miR-10b, and miR-129-3P, up-regulated; miRNA-671-5p, miR-141 and miR-25, down-regulated) were detected to serve as the potential prognostic or predictive markers of osteoporosis. As for the samples of women patients, there were five differentially expressed miRNAs, including 2 up-regulated and 3 down-regulated genes.

Functional analysis of target genes of miRNA

3372 target genes of down-regulated miRNAs were predicted while 2110 target genes of up-regulated miRNAs were found out (**Figure 4**). GO and KEGG network annotation were performed by DAVID on these target genes

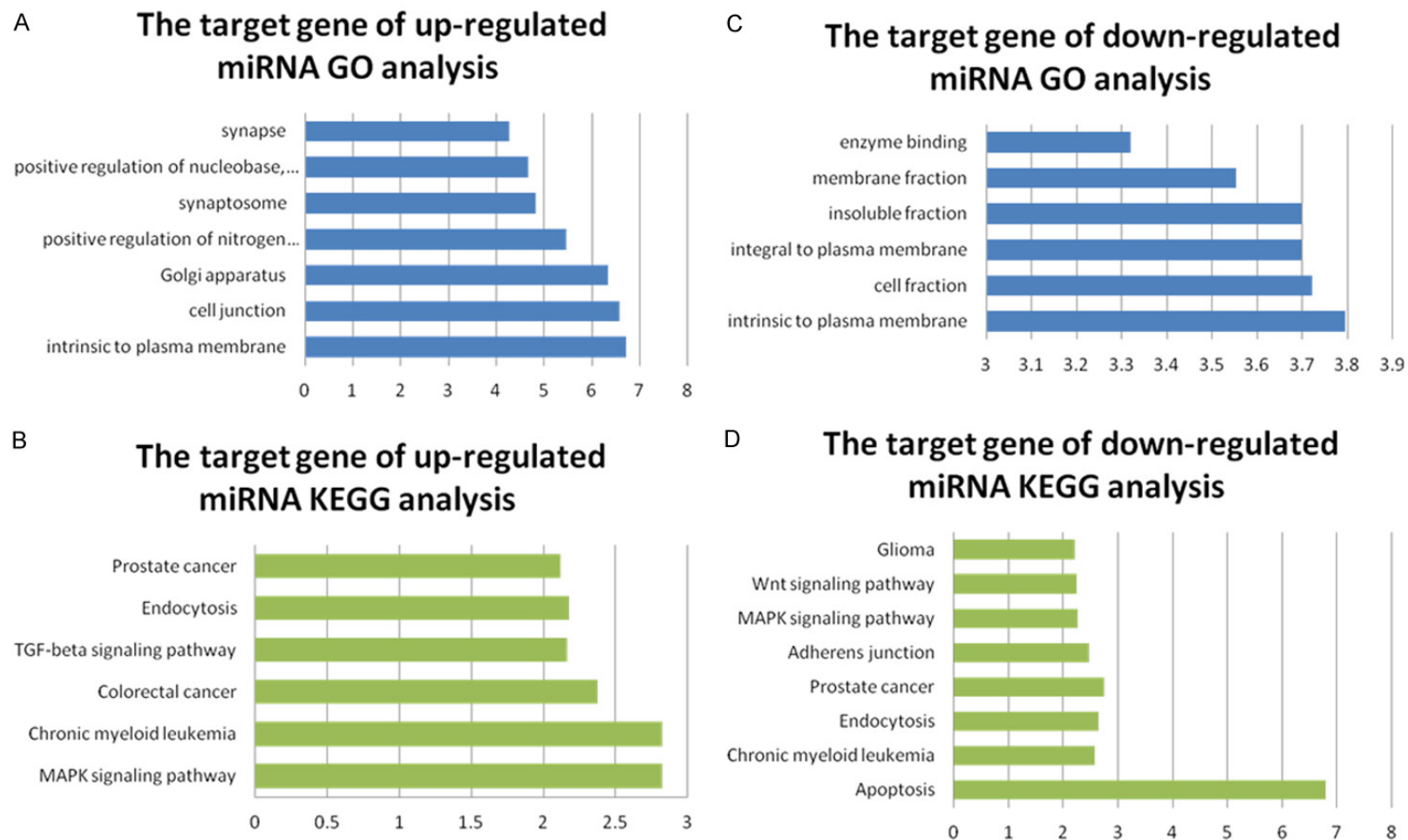


Figure 5. Go and KEGG analysis of target genes of differentially expressed miRNA. A, C are the GO analysis of target genes of up-regulated miRNA and B, D are the KEGG analysis of target genes. The horizontal axis represents the significant degree while the vertical axis stands for the function annotation. The larger the significant degree is, the closer of the relation between the target gene and the annotation.

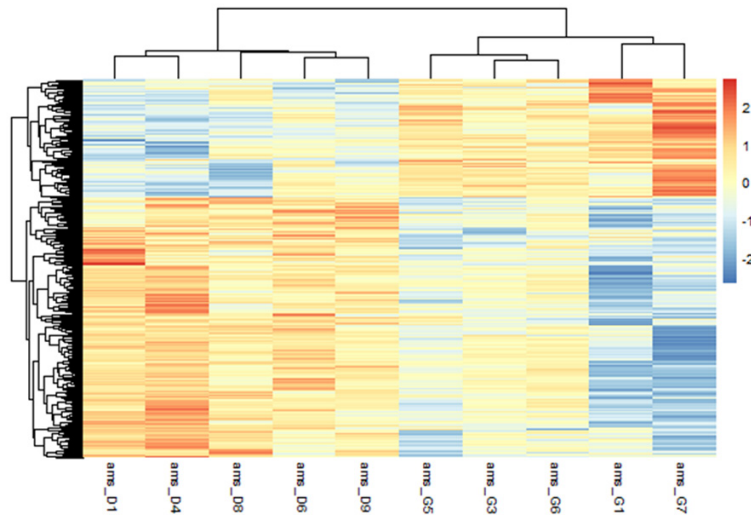


Figure 6. Differentially expressed mRNA in OS samples. The horizontal axis below is the sample names. The vertical axis on the right is the mRNA names while the left ones are the cluster situation of miRNAs. The red color represents the up-regulated miRNAs while the blue represents the down-regulated mRNA. There are two kinds of clusters, one is the normal tissue and the other is the osteoporosis ones. Expression of mRNAs can also be divided into two clusters, one is the down-regulated mRNA in osteoporosis and the other is the up-regulated mRNA.

($P=0.05$). There were 6 GO terms and 8 KEGG pathways enriched in target genes of down-regulated miRNAs (**Figure 5**). Hereinto, the intrinsic to plasma membrane was the most obvious in GO term and apoptosis was the most enriched KEGG pathway. Meanwhile, there were 7 GO terms and 6 KEGG pathways enriched in up-regulated miRNAs. Intrinsic to plasma membrane and MAPK were the most significant terms in GO annotation and KEGG annotation, respectively. The most enriched terms in up- and down-regulated miRNAs were both related with plasma membrane, which indicated its association with the occurrence of the disease.

Differentially expressed mRNAs analysis

There were 526 differentially expressed mRNAs between the normal samples and patients with osteoporosis by R package, including 164 up-regulated mRNA and 362 down-regulated mRNA, accounting for 29.2% and 70.8%, respectively (**Figure 6**).

Network analysis of differentially expressed miRNA and target genes

Topology calculation of the network was conducted by the properties of node degree distribution,

the shortest path distribution, the closeness centrality, and the topology degree (**Figure 7**). Codes degree distribution of protein-protein interaction (PPI) network of these miRNAs was in the form of power rate and had the structure of small-world, revealed by the character of average shortest path and the larger average accumulation character. The top three miRNAs with the largest degree of nodes were selected out for KEGG functional analysis (**Table 1**). There were 267 target genes in has-miR-142-3p, the top miRNA with the node in down-regulated miRNAs; there were 304 target genes in has-miR-448, 192 target genes in has-miR-521 and 95 target genes of up-regulated miRNAs in has-miR-761. Then, KEGG

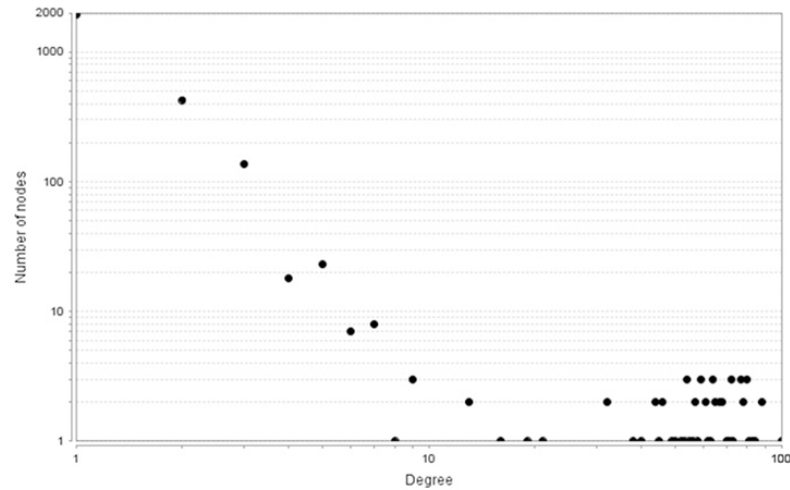
annotation was performed on these target genes. Three pathways, including pathway in cancer, phosphatidylinositol signaling system and endocytosis, were closely related with osteoporosis disease.

Discussion

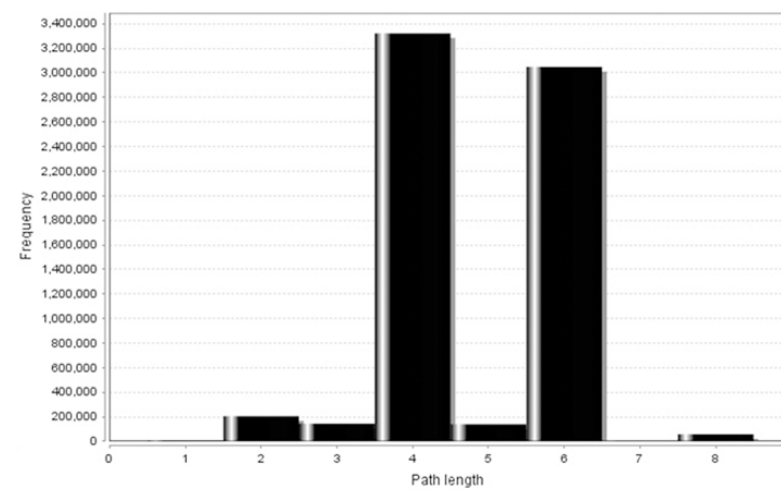
Osteoporosis is a chronic bone disease without any apparent symptom, extremely prevalent in the elderly and post-menopausal women. Patients with osteoporosis were more prone to bone fracture and what's worse, it can aggravate the complication of patients with cardiovascular or respiratory disease, causing great economic burden to the society. Unfortunately, nowadays, there are still many difficulties in the diagnosis and treatments of OS and many researchers have explored the pathogenesis of the disease.

As is known, the diagnosis and treatment of tumor is the key to decreasing the death rate and improving the post therapeutic. Research showed that miRNAs were involved in the process transformation and resistance to drugs of tumor. What's more, miRNAs were identified differentially expressed in the osteoporosis disease [5, 18-20]. Applying high-throughput

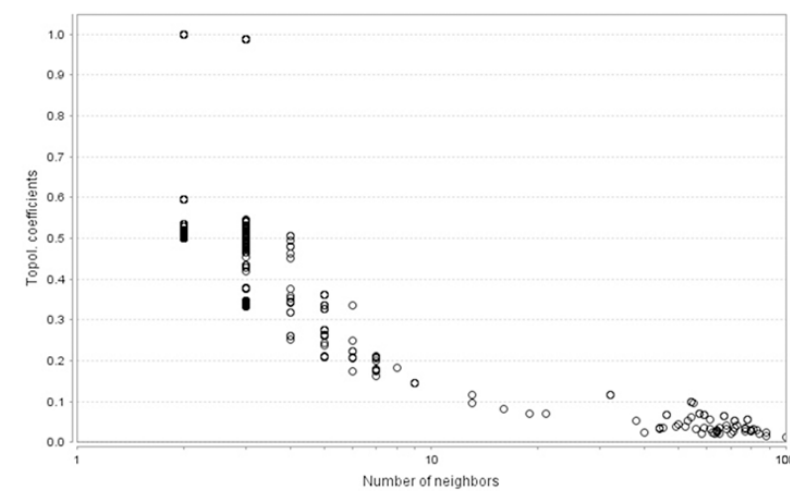
A The node degree distribution



B The shortest path length distribution



C The closeness centrality



D The topological degree

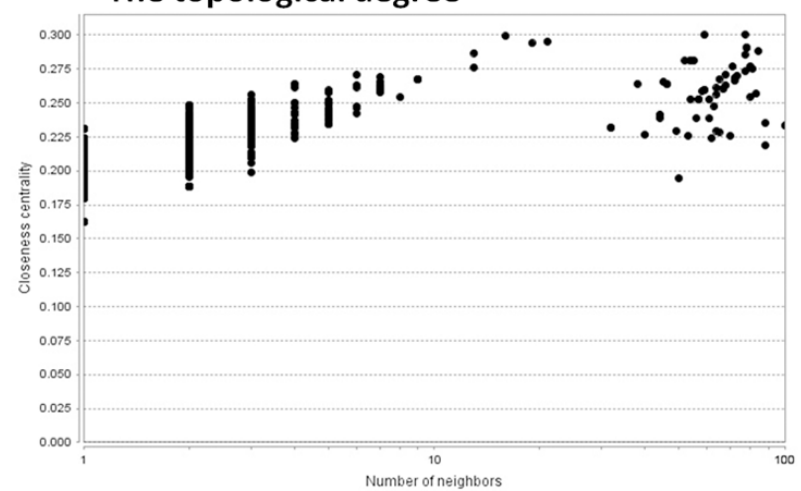


Figure 7. The topological structure analysis of network. A is the node degree distribution. B is the shortest length distribution. C is the closeness centrality. D is the topological degree.

Table 1. KEGG pathway analysis of modules ranging the top 3

	Term	Genes
Module 1	Pathway in cancer	NM_177435, NM_001017535, NM_000376, NM_004391, NM_000778, NM_002220, NM_003557
Module 2	Phosphatidylinositol signaling system	NM_001105540, NM_005185, NM_001135637, NM_001135638, NM_003646, NM_001135636, NM_019892, NM_201533, NM_201532, NM_001111125, NM_014043
Module 3	Endocytosis	NM_015470, NM_152284, NM_001135637, NM_001135638, NM_003557, NM_001135636, NM_018209, NM_024591, NM_019619, NM_175609, NM_015075

screening method, differentially expressed genes and corresponding functional identification were easier and more accurate. Unfortunately, the study of high-throughput screening is rare due to expensive equipment and annotation probe [16].

In this study, two databases of high-throughput data were analyzed to find the differentially expressed miRNAs in osteoporosis. As a result, a total of 799 differentially expressed miRNAs were identified out, among which six miRNAs, including up-regulated miR365, miR-10b, miR-129-3p and down-regulated miRNA-671-5p, miR-141 and miR-25, were figured as the most important network nodes, which may function as biomarkers of osteoporosis diagnosis. In our study, miR365, which was up-regulated, was reported to regulate cellular proliferation and modulate cell growth phenotypes, possibly causing the decreased bone mass and the imbalance of number between osteoblast and osteoclast in OS patients. Mir-10b, which was also up-regulated in the serum and bone tissue of osteoporotic patients, inhibited osteoblast differentiation by down-regulating cell proliferation. Mir-129-3p was also reported to regulate cell proliferation by down-regulating Cdk6 expression. As for down-regulated miRNAs, MiR-141, a member of the miR-200 family, has been reported to be related with various human malignancies. But there hasn't been any report on its involvement in osteoporosis. Hereinto, we could further explore its role on cell proliferation of OS patients to confirm its diagnosis value. MiR-25, another down-regulated miRNA in OS, was associated with the process of apoptosis, probably resulting in the decreasing of bone mass. However, the exact mechanism of the regulation effect of these miRNAs needs more researches, which can launched from their target genes and enriched pathways.

Early reports of miRNAs in osteoporosis may help us better understand the mechanism. MiRNA 21, was reported to be connected with the osteoporosis in cell proliferation and apoptosis, too [5, 21]. It had been reported that miRNA-21 was up-regulated in the serum of patients with osteoporosis and once inhibited, the proliferation of RBE cells was also prohibited and the speed of apoptosis increased. Let-7a were down-regulated in the osteoporosis samples, indicating that its target gene, NF-2, was anti-oncogene. At transcription level (or post-transcription level), the co-expression of one gene module probably occurs under the regulation of similar transcription factor. NF-2 exerted negative regulation on Stat-3, one of the signal transmitting and transportation activating factor, indicating its key role in the osteoporosis disease. In Chen's study, Mcl-1 was proved to be another target gene of miR-302 in osteoporosis and Bcl-2, another target gene of miR-204, happen to be another member of anti-apoptosis family [22]. The roles of the interested miRNAs we found in this study were consistent with the early anti-tumor research, which made our study more reliable. For further study, we analyzed the functional enrichments of the rest of differentially expressed miRNAs to explore molecular pathogenesis of this disease [23].

In the functional analysis of the target genes of miRNA, we found that the occurrence of osteoporosis was mainly related to the ion-binding ability of proteins. As it is the phosphorylation of protein, once the location was affected, the misregulation of activation and inactivation of proteins would be triggered. What's more, the process can also affect the regulating interaction of protein and DNA. Protein phosphorylation was reported to be associated with various signaling pathways and plays essential roles in

regulating many biological processes. Abnormal regulation of protein phosphorylation was found in a serial of diseases, such as cancer, Alzheimer's disease, osteoporosis, etc. Our founding confirmed the above results. And what's more, clearly the relationship between protein phosphorylation and bone metabolism, providing evidence for its diagnostic role in clinical management.

In conclusion, six miRNAs were selected out in this study by miRNA-seq method, which may act as potential biomarkers for osteoporosis disease. What's more, phosphorylation pathways were found to be closely associated with osteoporosis, providing new insights into the pathogenesis of osteoporoses and feasible suggestions for treatment strategies. However, more researches are in need for further utilizations of the potential biomarkers and exploration of the molecular pathogenesis of this disease.

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Disclosure of conflict of interest

None.

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References

- [1] Cunningham J, Sprague SM, Group OW, Canata-Andia J, Coco M, Cohen-Solal M, Fitzpatrick L, Goltzmann D, Lafage-Proust MH, Leonard M, Ott S, Rodriguez M, Stehman-Breen C, Stern P, Weisinger J; Osteoporosis Work Group. Osteoporosis in chronic kidney disease. *Am J Kidney Dis* 2004; 43: 566-571.
- [2] Inui M, Martello G and Piccolo S. MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* 2010; 11: 252-263.

- [3] Bader AG, Brown D and Winkler M. The promise of microRNA replacement therapy. *Cancer Res* 2010; 70: 7027-7030.
- [4] Zhang B, Pan X, Cobb GP and Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol* 2007; 302: 1-12.
- [5] Seeliger C, Karpinski K, Haug AT, Vester H, Schmitt A, Bauer JS and van Griensven M. Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. *J Bone Miner Res* 2014; 29: 1718-1728.
- [6] Si M, Zhu S, Wu H, Lu Z, Wu F and Mo Y. miR-21-mediated tumor growth. *Oncogene* 2007; 26: 2799-2803.
- [7] Wang Y, Li L, Moore BT, Peng XH, Fang X, Lappe JM, Recker RR and Xiao P. MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis. *PLoS One* 2012; 7: e34641.
- [8] Kapinas K and Delany AM. MicroRNA biogenesis and regulation of bone remodeling. *Arthritis Res Ther* 2011; 13: 220.
- [9] Tusher VG, Tibshirani R and Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A* 2001; 98: 5116-5121.
- [10] Mangan ME, Williams JM, Lathe SM, Karolchik D and Lathe WC. UCSC genome browser: deep support for molecular biomedical research. *Biotechnol Annu Rev* 2008; 14: 63-108.
- [11] Hsu SD, Lin FM, Wu WY, Liang C, Huang WC, Chan WL, Tsai WT, Chen GZ, Lee CJ, Chiu CM, Chien CH, Wu MC, Huang CY, Tsou AP and Huang HD. miRTarBase: a database curates experimentally validated microRNA-target interactions. *Nucleic Acids Res* 2010; 39: D163-169.
- [12] Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM and Sherlock G. Gene Ontology: tool for the unification of biology. *Nat Genet* 2000; 25: 25-29.
- [13] Kanehisa M and Goto S. KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Res* 2000; 28: 27-30.
- [14] Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC and Lempicki RA. DAVID: database for annotation, visualization, and integrated discovery. *Genome Biol* 2003; 4: P3.
- [15] Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19: 92-105.
- [16] Brennecke J, Stark A, Russell RB and Cohen SM. Principles of microRNA-target recognition. *PLoS Biol* 2005; 3: e85.

- [17] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003; 13: 2498-2504.
- [18] Leaf-nosed bat. In: *Encyclopædia Britannica*: Encyclopædia Britannica Online; 2009.
- [19] van Wijnen AJ, van de Peppel J, van Leeuwen JP, Lian JB, Stein GS, Westendorf JJ, Oursler MJ, Im HJ, Taipaleenmäki H, Hesse E, Riester S, Kakar S. MicroRNA functions in osteogenesis and dysfunctions in osteoporosis. *Curr Osteoporos Rep* 2013; 11: 72-82.
- [20] Cao Z, Moore BT, Wang Y, Peng XH, Lappe JM, Recker RR and Xiao P. MiR-422a as a potential cellular microRNA biomarker for postmenopausal osteoporosis. *PLoS One* 2014; 9: e97098.
- [21] Garnero P. New developments in biological markers of bone metabolism in osteoporosis. *Bone* 2014; 66: 46-55.
- [22] Chen P, Satterwhite JH, Licata AA, Lewiecki EM, Sipos AA, Misurski DM and Wagman RB. Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. *J Bone Miner Res* 2005; 20: 962-970.
- [23] Lian JB, Stein GS, Van Wijnen AJ, Stein JL, Hassan MQ, Gaur T and Zhang Y. MicroRNA control of bone formation and homeostasis. *Nat Rev Endocrinol* 2012; 8: 212-227.