SCIENTIFIC ARTICLE

Serum MicroRNA Differences Between Fracture in Postmenopausal Women with and without Diabetes

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Objective: To screen serum microRNAs (miRNAs) which could discriminate fracture status in postmenopausal women with or without diabetes.

Methods: The miRNA expression profile dataset GSE70318 was downloaded from Gene Expression Omnibus (GEO) database. This dataset composed of 74 samples, among these, 55 postmenopausal women was selected for bioinformatics analysis, including 19 osteoporotic fracture patients with type-2 diabetes, 19 osteoporotic fracture patients without type-2 diabetes, and 17 healthy control subjects. These samples were divided into two groups: fracture patients with diabetes *vs* healthy subjects (FH group) and fracture patients without diabetes *vs* healthy subjects (DFH group). Then, the differentially expressed miRNA (DEMs) in FH group and DFH group were respectively identified. The target genes of DEMs were predicted, followed by functional enrichment analysis. Furthermore, DEMs related to long non-coding RNAs (lncRNAs) were screened, and DEMs-lncRNA-target genes network was constructed. Subsequently, principal component analysis (PCA) of DEMs was performed to further explore the expression characteristics of the selected miRNAs in different types of fracture samples. Finally, the expression level of significant DEMs was calculated by quantitative real-time polymerase chain reaction (qPCR) to verify the accuracy of the results of bioinformatics analysis.

Results: A total of 18 and 23 DEMs were identified in FH and DFH groups, respectively. Gene ontology (GO) analysis showed that genes in FH group were significantly enriched in regulation of transcription (GO: 0045449) and genes in DFH group were mainly enriched in cellular response to hormone stimulus (GO: 0032870). Meanwhile, pathway analysis indicated that genes in FH group were primarily enriched in T cell receptor signaling pathway (hsa04660) and genes in DFH group were mainly implicated in neurotrophin-signaling pathway (hsa04722). Moreover, the miRNA-lncRNA analysis revealed that miR-155-5p regulated by lncRNA MIR155HG was up-regulated in FH group; in addition, the miR-181c was significantly up-regulated and miR-375 was observably down-regulated in DFH group. Furthermore, PCA analysis suggested that the screened miRNAs were able to differentiate these two types of fractures in postmenopausal women. The miR-181c and miR-375 might be regarded as potential predictors for fracture, while miR-155-5p might be a candidate diagnostic biomarker for diabetic fracture. Finally, the results of qPCR were consistent with that of microarray data.

Conclusions: Overall, these three miRNAs might be regarded as potential diagnostic biomarkers to discriminate fracture status in postmenopausal women with and or without diabetes, and they served a putative role in the pathogenesis of these two diseases. However, these findings were only observed in serum samples and further clinical trials are urgently demanded to validate our results.

Key words: Diabetic; Fracture; MicroRNA; Postmenopausal women; Principal component analysis

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Disclosure: The authors declare that they have no conflicts of interest.
Received 9 April 2020; accepted 18 October 2020

Introduction

Osteoporosis is a disease that affects the integrity and strength of bones for a variety of reasons. Specifically, it leads to low bone mass, skeletal fragility, and diminished bone strength, thereby increasing the risk of fractures[1]. Osteoporosis is a frequent disease in postmenopausal women. After menopause, estrogen deficiency exposes tissues to receptor activator of nuclear factor-kappa B (RANK) ligands, resulting in increased bone resorption and bone loss, which can lead to osteoporosis[2]. Thus, postmenopausal women can suffer osteoporosis due to decreased estrogen levels, which lead to a decrease in bone mineral density (BMD) and an increase in fractures[3]. In addition, osteoporotic fractures cause a large number of morbidities and mortalities and are regarded as one of public health problems. Meanwhile, osteoporosis is still underdiagnosed and undertreated[4]. Therefore, it is necessary for postmenopausal women to receive appropriate guidance in the prevention and treatment of osteoporosis. Apart from aging and hormonal changes directly related to osteoporosis, many studies have pointed out that certain underlying diseases are the culprits, such as diabetes[5]. Ten years ago, researchers had already found that postmenopausal women with diabetes experienced a higher incidence of fracture than women without diabetes[6]. Thus, exploring the effects of diabetes on postmenopausal fractures is helpful to distinguish the fracture types and provide a theoretical basis for clinical diagnosis.

Generally, computed tomography (CT) scan and magnetic resonance imaging (MRI) have shown better performance in fracture diagnosis[7] Despite the widespread availability of these measures, previous studies have demonstrated that neither MRI nor CT is 100% accurate in diagnosing fracture[8, 9]. In addition, the use of serum miRNAs has several major advantages for clinical diagnosis. For instance, serum-based biomarkers can be used directly for analysis; therefore, the diagnosis does not require exposure to radiation. Meanwhile, serum sample management, including sample collection and processing, is also easy[10]. Thus, the molecular causes of diabetic bone disease should be investigated to identify novel serum miRNAs. These markers may not only be applicable in routine clinical diagnoses, but also accurately assess the risk of fractures with or without diabetes.

Previous studies have investigated the mechanisms of fractures in postmenopausal women with diabetes. Compston[11] showed that bone formation and bone mass density in diabetes were less than normal, because the level of blood glucose was favorable control to make for decreasing bone resorption and bone mass losing. In addition, sex hormone-binding globulin (SHBG) levels are also associated with the risk of fracture in postmenopausal women. Jing *et al.*[12] indicated that higher serum SHBG levels were connected with lower BMDs, and it predicted an increased risk of fracture in postmenopausal women with diabetes. The above results suggest that serum markers are closely related to the occurrence and development of menopausal fractures. In addition, accumulating evidence demonstrates that molecular mechanism plays an important role in the pathogenesis of fracture with diabetes. For instance, microRNAs (miRNAs) are a class of small non-coding RNAs that can regulate gene expression at a post-transcriptional level, and are crucial to the etiology of bone homeostasis and type 2 diabetes. A previous study shows that several blood circulating miRNAs, such as miR-188-3p, miR-382-3p, miR-942, and miR-155-5p, are indicative of skeletal fractures in postmenopausal women with or without type 2 diabetes, and may be considered as biomarkers for predicting general fracture risk[13]. Moreover, Chen *et al.*[14] revealed that miR-1908 and miR-203a, regulated by stearoyl-coenzyme A desaturase-1 (SCD1), were associated with fracture risk and bone health in postmenopausal diabetic women. Furthermore, miR-148a-3p could be considered as a potential novel plasma-based biomarker for pathological changes associated with osteoporosis, which was increased in plasma of osteoporotic postmenopausal women[15]. In addition to miRNAs, long non-coding RNAs (lncRNAs) have also been identified to play critical roles in various biological processes, including transcription and post-transcriptional regulation, recruitment of epigenetic modifiers, and the control of mRNA decay[16]. Previous studies revealed the potential regulatory role of lncRNAs in diabetes and its complications. For instance, lncRNAs such as LRP2 binding protein (Lrp2bp) and perilipin 4 (Plin4) were regarded as potential biomarkers for diagnosis of osteoporosis[17]; lncRNA CRNDE is highly expressed in patients with postmenopausal osteoporosis and is associated with estrogen deficiency[18]. Despite the above research, the molecular mechanism of miRNAs or lncRNAs in fractures in postmenopausal women with or without diabetes has not been explored.

In the present study, a bioinformatics analysis was performed based on the serum miRNAs expression profile of postmenopausal women. The differentially expressed miRNAs (DEMs) between different groups were identified, followed by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Subsequently, DEMs-gene network and DEMs-lncRNA-target genes network were constructed. This study aimed to investigate the specific serum miRNAs that can discriminate fracture status in postmenopausal women with or without diabetes. The purpose of this study was to investigate: (i) the molecular mechanism of fracture in postmenopausal women; (ii) the serum miRNA signature for different fracture types; and (iii) the potential diagnostic biomarkers to discriminate fracture status in postmenopausal women with and or without diabetes.

Material and Methods

Ethics Statement

All participating patients have signed informed consent prior to participation in this study. Every experimental procedure was approved by the Ethics Committee of Honghui Hospital.

The clinical characteristics of these patients are listed in Table S1.

Data Resource

The miRNA expression profile dataset GSE70318 deposited by Heilmeier *et al.*[13] was downloaded from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), and was produced on the platform of GPL20631, Custom LNA™ Universal RT microRNA PCR panels. This dataset composed of 74 samples; among these, we selected 55 postmenopausal women (range, 50 to 75 years) to perform bioinformatics analysis, including 19 osteoporotic fracture patients with type-2 diabetes (mean age, 63.3 ± 6.1 years), 19 osteoporotic fracture patients without type-2 diabetes (mean age, 64.7 ± 5.8 years), and 17 healthy control subjects (mean age, 58.1 ± 5.0 years). Patients with distal fractures that occurred at the start of menopause and after the start of T2D were included, while patients with high-energy trauma such as car accidents, malignant tumors, or tumor-like lesions were excluded. Samples were divided into two groups: fracture patients with type-2 diabetes *vs* healthy control subjects (FH group), and fracture patients without type-2 diabetes *vs* healthy control subjects (DFH group).

Data Preprocessing and Differential Expression Analysis

The normalization of gene expression profile data was performed by the Linear Models for Microarray Data (limma, http://www.bioconductor.org/packages/release/bioc/html/limma.html) package[19] in R (version: 3.4.1) software. The DEMs in FH and DFH groups were identified using limma package, respectively. *P*-value <0.05 and |log fold change (logFC)| > 1 was selected as the cut-off criteria of DEMs.

Venn Diagram and Bidirectional Hierarchical Clustering Analysis of DEMs

VENNY (Version2.1, http://bioinfogp.cnb.csic.es/tools/venny/index.html) is an online software that applied to the Venn diagram analysis based on gene expression value. In this study, VENNY was utilized to identify common and different DEMs between FH and DFH groups. Furthermore, bidirectional hierarchical clustering based on Euclidean distance was performed on the expression value of DEMs in two groups. The heat map was visualized by pheatmap[20] package in R software.

Target Genes Prediction and MiRNA-gene Network Construction

The potential target genes for DEMs in the two groups were obtained using three databases, including miRanda (http://www.microrna.org/microrna/home.do)[21], mirTarbase 2016 (http://mirtarbase.mbc.nctu.edu.tw/)[22], and TargetScan release 7.1 (http://www.targetscan.org/)[23]. Then, the predicted target genes that existed in all three databases were selected for further analysis. The miRNA-gene regulatory networks were established by the Cytoscape (version: 3.4.0, http://www.cytoscape.org/) software[24].

Gene Ontology Annotation and Pathway Analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID version: 6.8, http://david.ncifcrf.gov/) is a gene functional classification tool that systematically extracts biological meaning from large gene or protein lists[25] GO (http: //www. Geneontology. org/) and KEGG (http://www. genome.jp/kegg/pathway.html) pathways enrichment analyses of target genes were performed using DAVID. The *P*-value <0.05 was considered statistically significant.

Construction of mRNA-miRNA-lncRNA Network

The starBase v2.0 is a database to provide the most comprehensive CLIP-Seq experimentally supported miRNA-lncRNA interaction networks[26]. In the present study, miRNA-lncRNA interactions were revealed using starBase database. Then, the competing endogenous RNA (ceRNA) network was obtained *via* integrating the miRNA-gene pairs and miRNA-lncRNA pairs. Additionally, functional enrichment analysis of mRNA in the ceRNA network was also performed and visualized using DAVID and Cytoscape software, respectively.

Principal Component Analysis for miRNA

Principal component analysis (PCA) is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components[27]. PCA is widely used in data processing and dimensionality reduction[28] The contribution rate of principal component >80% was considered as the meaningful component. The PCA for miRNA in the FH and DFH groups was performed using psych package in R software.

RNA Extraction and Real-time Quantitative Polymerase Chain Reaction (qPCR)

To verify the reliability of the results of bioinformatics analysis, qPCR experiment of several DEMs was performed. The blood samples from fracture patients with type-2 diabetes, fracture patients without type-2 diabetes, and healthy subjects were collected. Subsequently, blood was clotted in an upright position for 40 min, and then centrifuged at 2000 × g for 15 min. Serum supernatant was obtained for further study. Total RNA from the serum was extracted using RNAiso Plus reagent (9109, TaKaRa, Dalian, China) according to the instructions of the manufacturer. The quality and concentration of the RNA were detected using a NanoDrop 1,000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, the RNA was reverse transcribed into complementary DNA (cDNA) using PrimeScript™RT Master Mix kit (RR036A, TaKaRa, Dalian, China), and qPCR was performed by utilizing a Power SYBR Green PCR Master Mix (A25742, Thermo Scientific, MA, USA) on an ABI 7900HT Fast RT-PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The process of qPCR amplification is as follow: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. Relative

gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method, and U6 small-nuclear RNA (U6 snRNA) was used as a reference gene to normalize the expression of miRNA. The primer sequences are listed in Table S2.

Statistical Analysis

Data were reported as the mean ± SD for triplicate republication. Statistical analysis was conducted using GraphPad Prism 5 (San Diego, CA). Student's *t*-test was applied to compare the differences between the qPCR results of two groups. $P < 0.05$ was considered statistically significant.

Results

Identification of DEMs in FH Group and DFH Group

A total of 18 and 23 DEMs were obtained in FH and DFH groups, respectively. The Venn diagram showed that a total of five common DEMs (co-DEMs group), including miR-188-3p, miR-369-3p, miR-382-3p, miR-495-3p, and miR-550a-5p, existed in two groups. Meanwhile, the results of bidirectional hierarchical clustering analysis in FH and DFH groups are shown in Fig. 1, the results indicated that the screened DEMs could separate different types of samples.

MiRNA-gene Regulation Network Construction and Analysis

To investigate the function of DEMs, the target genes of miRNAs (DEMs in the FH group, DFH group, as well as co-DEMs group) were identified using three databases including imiRanda, mirTarbase, and TargetScan release 7.1. The Venn diagram showed that a total of 175, 167, and 34 miRNA-gene interactions were obtained in the FH group, DFH group, and co- DEMs group (Fig. 2). Then, these interactions were further used for structuring the miRNA-gene regulation network. The results showed that miR-155-5p and miR-377-3p were up-regulated in the FH group (Fig. 3A); and miR-382-3p as well as miR-495-3p were up-regulated in the co-DEMs group (Fig. 3C). Furthermore, we observed that miR-154-5p, miR-376a-3p, miR-376c-3p, and miR-136-5p were up-regulated, while miR-32-3p, miR-32-3p, miR-375, and miR-7-5p were down-regulated in DFH group (Fig. 3B).

Function and Pathway Analysis

GO function and KEGG pathway analysis were performed to explore the biological function of target genes. In the FH group, genes were significantly enriched in regulation of transcription (GO: 0045449); in the DFH group, genes were mainly related to cellular response to hormone stimulus (GO: 0032870); and genes in co-DEMs group were primarily connected with transcription (GO: 0006350). According to the *P*-value, the top five GO terms in each group are listed in Table 1. Furthermore, the KEGG pathways like T cell receptor signaling pathway (hsa04660), Neurotrophin signaling pathway (hsa04722), and Antigen processing and presentation (hsa04612) were the most significant pathways enriched by target genes in FH, DFH, and co-DEMs groups, respectively. Additionally, the top five pathways in each group are listed in Table 2.

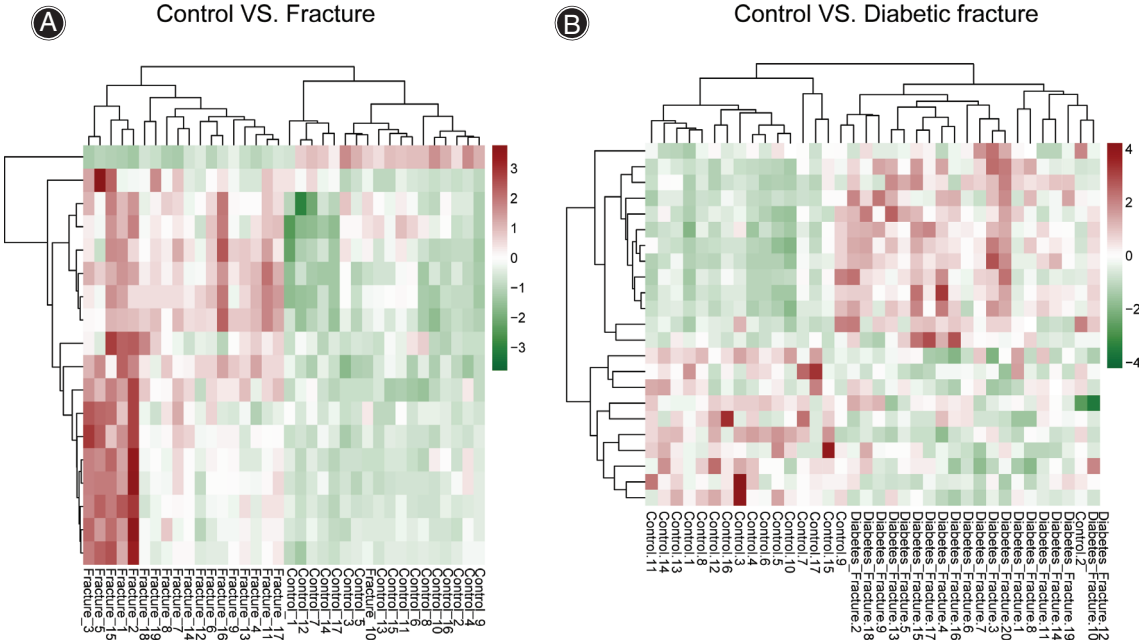

Fig. 1 The results of bidirectional hierarchical clustering analysis of differentially expressed miRNA in FH (A) and DFH (B) groups. Red presents the up-regulated miRNA; green represents the down-regulated miRNA.

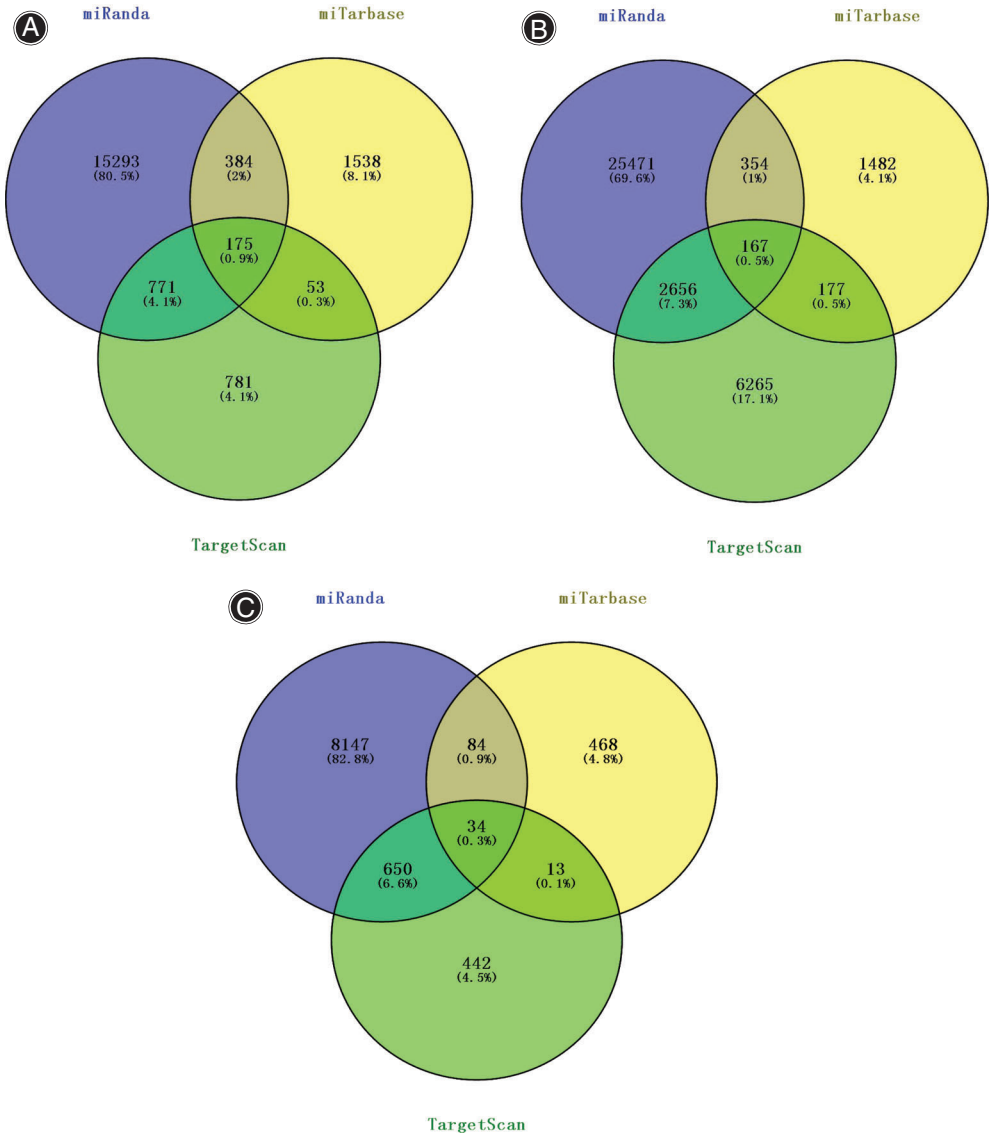

Fig. 2 The results of Venn diagram analysis of differentially expressed miRNA revealed in three groups by using three databases. A, FH group; B, DFH group; C, co-DEMs group; blue represents the miRanda database; yellow represents the mirTarbase 2016 database; and green represents the TargetScan release 7.1 database.

mRNA-lncRNA-miRNA ceRNA Network Investigation

The starBase database was used to explore the miRNA associated lncRNAs in three miRNA-target gene interaction networks. The results identified that four lncRNAs, including maternally expressed 3 (MEG3), BCL6 corepressor pseudogene 1 (BCORP1), brain cytoplasmic RNA 1 (BCYRN1), and MIR155 host gene (MIR155HG), were associated with miR-155-5p in FH group. As for DFH group, we noticed miR-181c could target lncRNA X inactive specific transcript (XIST); the expression of miR-375 was regulated by ATP binding cassette subfamily C member 13 (ABCC13); zinc finger protein 271 and pseudogene (ZNF271) might regulate the expression of miR-375; moreover, miR-7-5p was targeted by several lncRNAs, such as ATP binding cassette subfamily A member 17, pseudogene (ABCA17P), ABCC13, and zinc finger and BTB domain containing 12B, pseudogene (ZBTB12B). However, no miRNA-lncRNA relation was observed in co-DEMs group. Then, the interactions of miRNA-lncRNA and miRNA-target gene were integrated to construct the ceRNA network (Fig. 4). The functional enrichment analysis of genes in ceRNA network was performed. Results showed that genes in FH group were closely connected with regulation of transcription function (GO: 0045449) and significantly enriched in T cell receptor signaling pathway (hsa04660) (Fig. 5A). Meanwhile, genes in DFH group were primarily involved in the positive regulation of cell migration function (GO: 0030335) and adipocytokine signaling pathway (hsa04920) (Fig. 5B).

Results of PCA for MiRNAs in Two Groups

In order to examine overall miRNAs expression pattern of FH and DFH, miRNAs in FH group and DFH group were selected for the PCA investigation. As presented in Fig. 6,

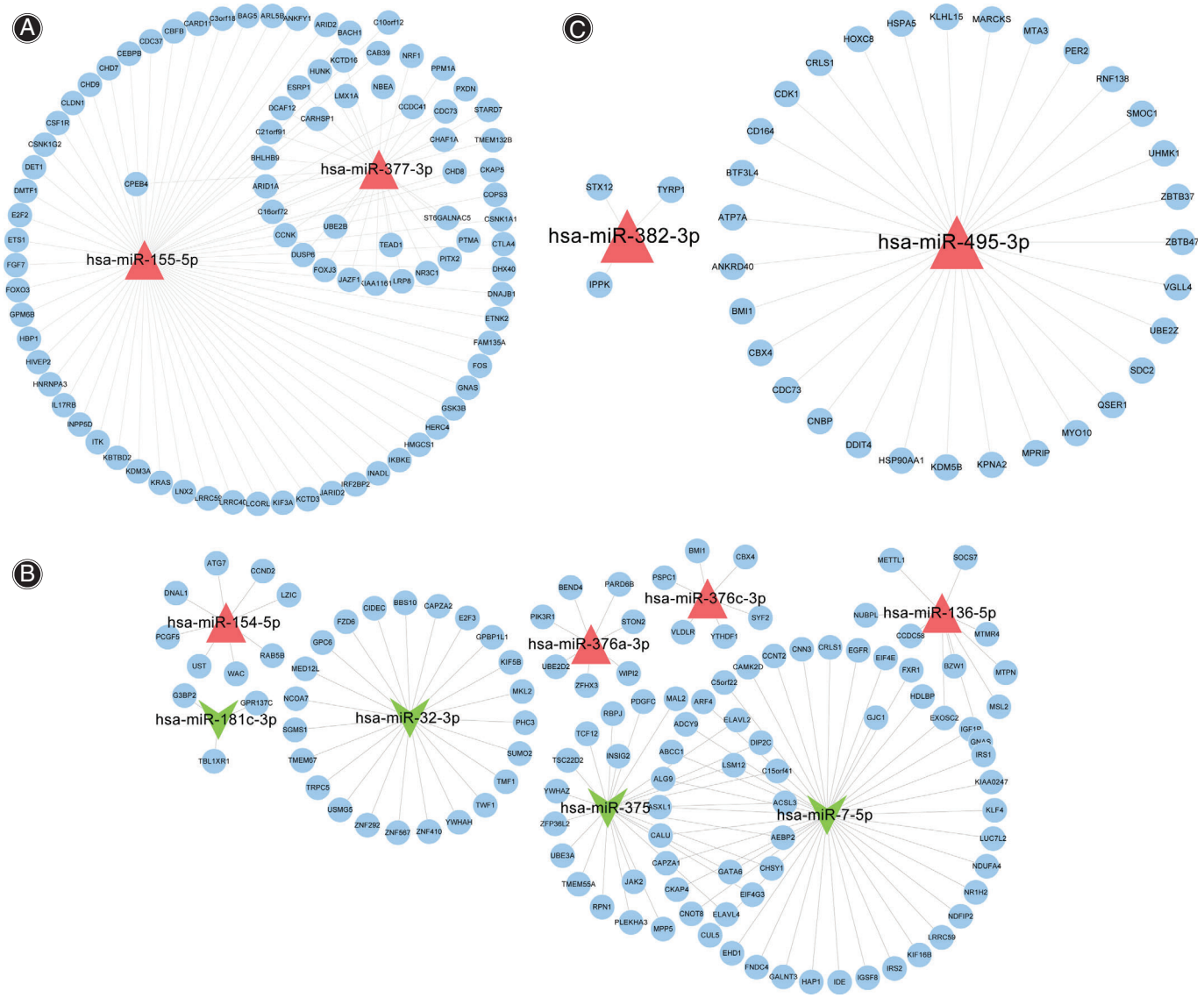

Fig. 3 The results of miRNA-target gene interaction network investigation in FH (A), DFH (B), and co-DEMs (C) groups. Red triangle represents the up-regulated miRNA; blue inverted triangle represents the down-regulated miRNA; and blue circle represents the target gene.

FH and NC samples were separated by the two miRNAs (miR-155-5p and miR-377-3p); DFH and NC samples were distinguished by eight miRNAs, such as miR-7-5p, miR-375, and miR-181c. The results indicated that the different types of samples were clearly distinguished by the DEMs obtained in the above analysis.

qPCR Validation of DEMs

To verify the reliability of microarray data, a qPCR was performed to examine the expression level of miR-155-5p, miR-181c, and miR-375 in the three groups (Fig. 7). Results revealed that the miR-155-5p was significantly up-regulated in the fracture patients with diabetes group compared with NC and fracture patients without diabetes groups. Additionally, compared with NC or fracture patients with diabetes group, miR-181c was highly expressed in fracture without diabetes group, while miR-375 was significantly decreased in fracture without diabetes group. These results were consistent with the above analysis.

Discussion

The risk of fractures in postmenopausal women with diabetes is higher than that in women without diabetes[29]. Although miRNAs or lncRNAs play important roles in the fracture process, the details of molecular mechanism on diabetic postmenopausal fracture remains poorly understood. In the current bioinformatics study, the differences of miRNA expression between normal fracture and diabetes fracture

TABLE 1 The results of GO function analysis for DEMs in three groups (Top 5 listed)

Groups	Term	Gene	Count	*P*-value
FH	GO:0045449 ~ regulation of transcription	BACH1, E2F2, MEF2A, SPI1, RORA…	68	5.24E-11
	GO:0006350 ~ transcription	BACH1, E2F2, MEF2A, SPI1, RORA…	57	6.41E-09
	GO:0051252 ~ regulation of RNA metabolic process	BACH1, E2F2, MEF2A, SPI1, FOXO3…	50	1.72E-07
	GO:0006355 ~ regulation of transcription, DNA-dependent	BACH1, E2F2, MEF2A, SPI1, FOXO3…	49	2.75E-07
	GO:0006357 ~ regulation of transcription from RNA polymerase II promoter	SPI1, FOXO3, RORA, CBFB, ZIC3…	27	5.32E-05
DFH	GO:0032870 ~ cellular response to hormone stimulus	IGF1R, IRS2, ADCY9, CCND2, SOCS7…	10	1.94E-07
	GO:0032869 ~ cellular response to insulin stimulus	IGF1R, IRS2, CCND2, SOCS7, IRS1…	7	5.05E-06
	GO:0032868 ~ response to insulin stimulus	IGF1R, IRS2, CCND2, SOCS7, IRS1…	7	4.66E-05
	GO:0043434 ~ response to peptide hormone stimulus	IGF1R, IRS2, CCND2, SOCS7, JAK2…	8	6.34E-05
	GO:0007169 ~ transmembrane receptor protein tyrosine kinase signaling pathway	EGFR, IGF1R, IRS2, ARF4, SOCS7…	9	1.03E-04
co-DEMs	GO:0006350 ~ transcription	EGFR, IGF1R, IRS2, ARF4, SOCS7…	10	1.32E-02
	GO:0016568 ~ chromatin modification	BMI1, CBX4, CDC73, KDM5B	4	1.51E-02
	GO:0019941 ~ modification-dependent protein catabolic process	CDK1, UBE2Z, KLHL15, CBX4, RNF138	5	2.28E-02
	GO:0043632 ~ modification-dependent macromolecule catabolic process	CDK1, UBE2Z, KLHL15, CBX4, RNF138	5	2.28E-02
	GO:0051603 ~ proteolysis involved in cellular protein catabolic process	CDK1, UBE2Z, KLHL15, CBX4, RNF138	5	2.64E-02

co-DEMs, a total of 5 common DEMs in both FH group and DFH group; DEMs, differentially expressed miRNA; DFH group, fracture patients without type-2 diabetes *vs* healthy control subjects; FH group, fracture patients with type-2 diabetes *vs* healthy control subjects; GO, Gene Ontology; miRNA, microRAN.; *P*-value < 0.05 was considered as cut-off value of the significant difference.

TABLE 2 The results of KEGG pahtway analysis for DEMs in three groups (Top 5 listed)

Group	Term	Gene	Count	*P*-value
FH	hsa04660:T cell receptor signaling pathway	ITK, CARD11, FOS, KRAS, GSK3B…	8	2.45E-04
	hsa04662:B cell receptor signaling pathway	CARD11, FOS, KRAS, GSK3B, PIK3C…	7	2.24E-04
	hsa04910:Insulin signaling pathway	KRAS, GSK3B, SOCS1, PRKAR1A, RHEB…	9	1.60E-04
	hsa04722:Neurotrophin signaling pathway	YWHAZ, KRAS, GSK3B, PIK3CA, RAP1B…	7	3.18E-03
	hsa04150:mTOR signaling pathway	YWHAZ, KRAS, GSK3B, PIK3CA, RAP1B…	5	3.11E-03
DFH	hsa04722:Neurotrophin signaling pathway	IRS2, YWHAZ, YWHAH, CAMK2D, IRS1…	6	3.65E-03
	hsa04920:Adipocytokine signaling pathway	IRS2, JAK2, ACSL3, IRS1	4	1.85E-02
	hsa04540:Gap junction	EGFR, ADCY9, PDGFC, GNAS	4	3.87E-02
	hsa04960:Aldosterone-regulated sodium reabsorption	EGFR, ADCY9, PDGFC, GNAS	3	4.65E-02
	hsa04912:GnRH signaling pathway	EGFR, ADCY9, CAMK2D, GNAS	4	4.92E-02
co-DEMs	hsa04612:Antigen processing and presentation	HSP90AA1, HSPA5	2	1.93E-03
	hsa04914:Progesterone-mediated oocyte maturation	CDK1, HSP90AA1	2	1.99E-02
	hsa04666:Fc gamma R-mediated phagocytosis	MYO10, MARCKS	2	2.18E-02

co-DEMs, a total of five common DEMs in both FH group and DFH group; DEMs, differentially expressed miRNA; DFH group, fracture patients without type-2 diabetes *vs* healthy control subjects; FH group, fracture patients with type-2 diabetes *vs* healthy control subjects; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA.; *P*-value < 0.05 was considered as cut-off value of the significant difference.

were investigated. The functional enrichment analysis of miRNA target genes showed that genes in FH group were primarily implicated in regulation of transcription, and genes in DFH group were associated with cellular response to hormone and insulin stimulus functions. Particularly, genes in FH group were enriched in T cell receptor signaling pathway, while genes in DFH group were involved in neurotrophin-signaling pathway. Moreover, we found that specific miRNAs had differential expression in samples from FH and DFH groups. Specifically, miR-155-5p (regulated by lncRNA MIR155HG) was significantly up-regulated in FH group compared with DFH and NC groups. Meanwhile, compared to both FH and NC groups, miR-181c (connected to lncRNA XIST) was up-regulated in DFH group; whereas miR-375 was down-regulated in DFH group. Furthermore, PCA

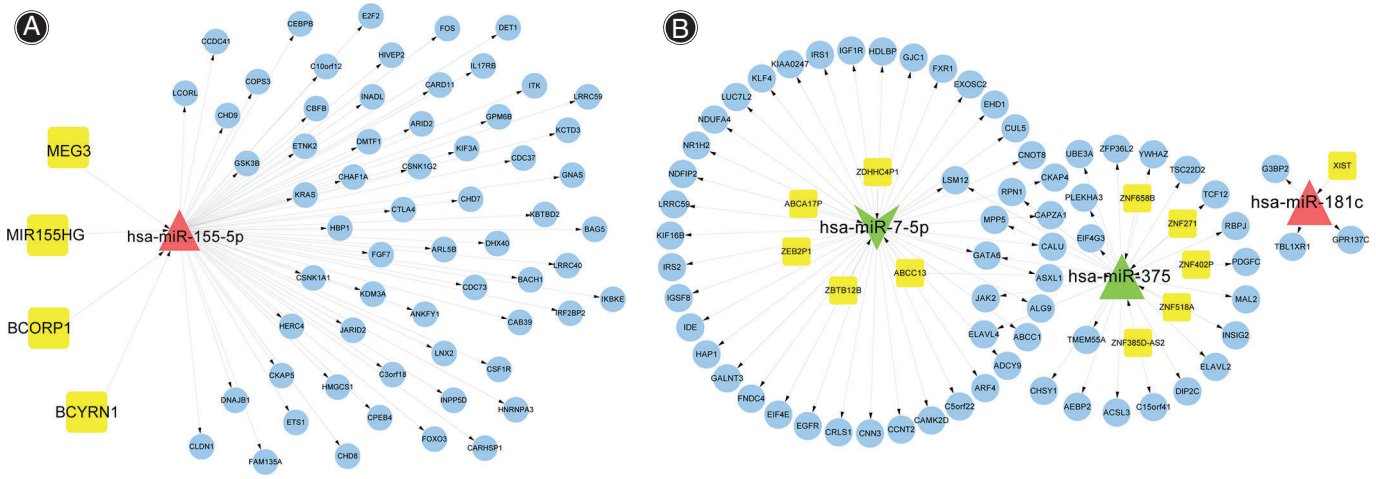

Fig. 4 The ceRNA interaction network in FH (A) and DFH (B) groups. Red triangle represents the up-regulated miRNA; green triangle represents the down-regulated miRNA; blue circle represents the target gene associated with miRNA; and yellow block represents the lncRNAs.

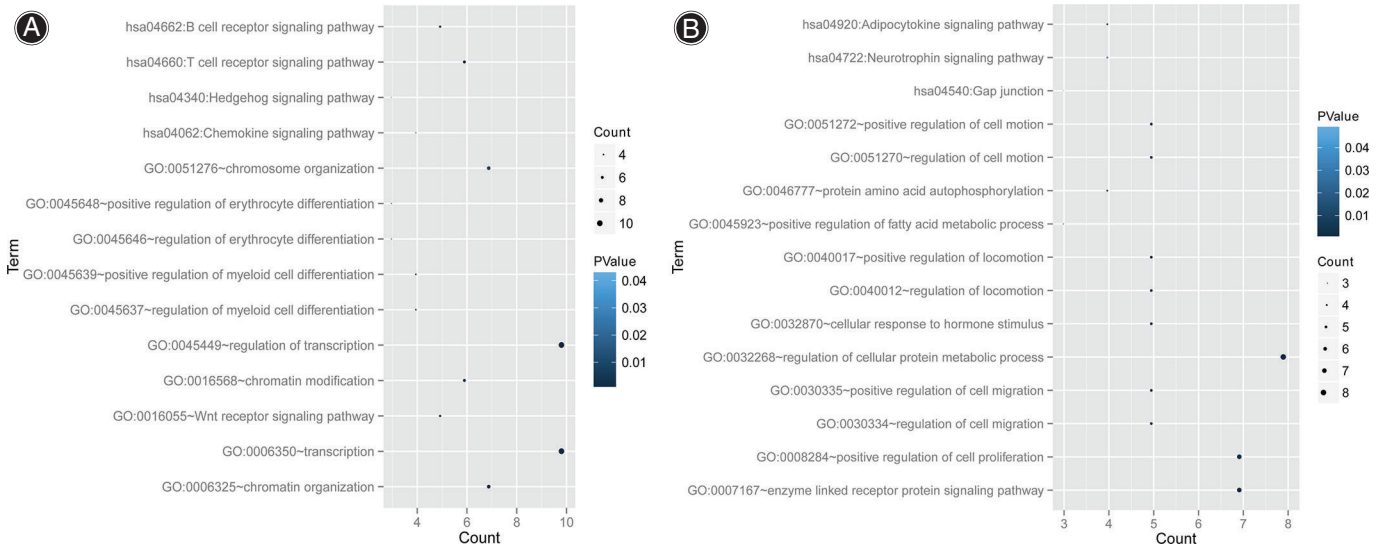

Fig. 5 The results of GO function and KEGG pathway analysis of differentially expressed miRNA in ceRNA network. A, the functional enrichment analysis of DEMs in FH group; B, the functional enrichment analysis of DEMs in DFH group; the larger the ink point, the more genes assembled; the deeper the ink point color, the bigger the *P*-value.

analysis indicated that screened miRNAs could distinguish disease and normal groups.

Biological Function of Specific MiRNAs

In DFH group, GO function analysis showed that DEMs were closely associated with the cellular response to hormone stimulus and cellular response to insulin stimulus. At the physiological level, ovarian senescence results in a changing hormonal milieu that causes estrogen levels to decrease, leading to menopause[30]. In addition, insulin is the principal hormone that regulates the uptake of glucose from the blood into most cells of the body[31]. Therefore, deficiency of insulin or the insensitivity of its receptors plays a central role in diabetes[32]. Wallander *et al.*[33] showed that the risk of fractures differed substantially among type 2 diabetes patients who with or without insulin treatment. This phenomenon might be due to the accelerated loss of cartilage caused by diabetes during fracture repair[34]. Actually, the decreased systemic insulin levels in the diabetic state lead to the reduction of localized insulin levels at fracture site with concomitant increases in diabetic fracture healing[35]. Thus, we speculated that the dysregulation of cellular response to hormone stimulus and cellular response to insulin stimulus functions in

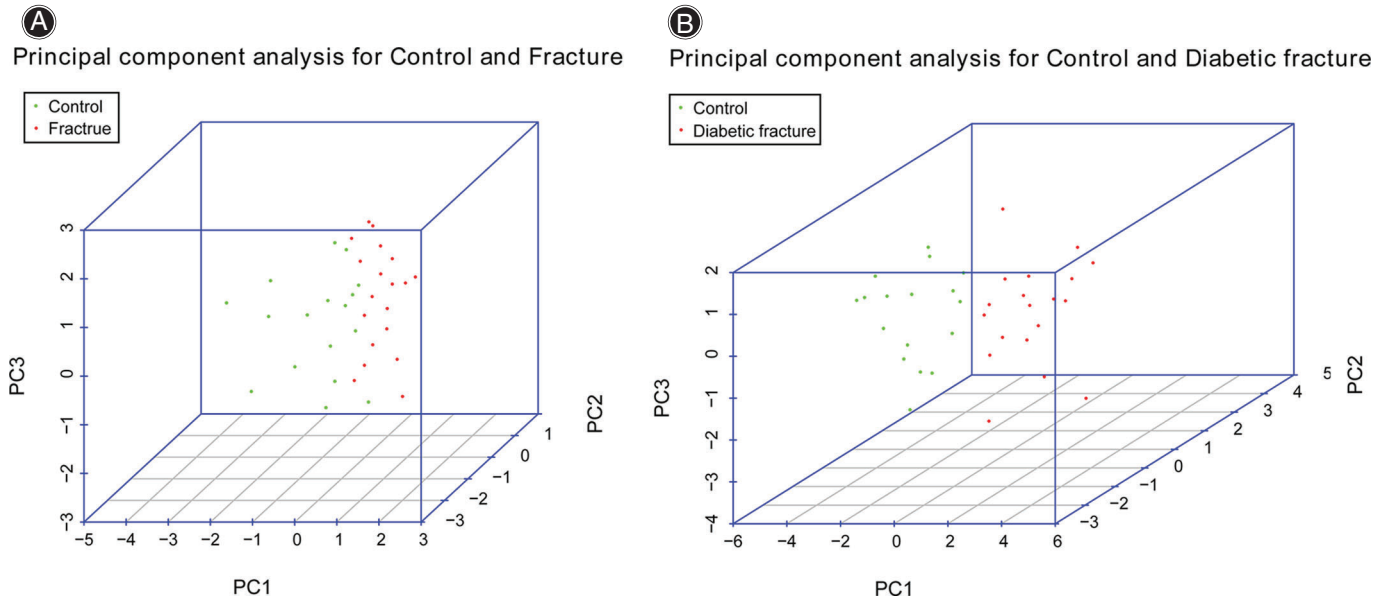

Fig. 6 The 3D result of principal component analysis for miRNA in two groups. A, the DEMs in fracture patients with type-2 diabetes *vs* control group; B, the DEMs in fracture patients without type-2 diabetes *vs* control group; green circle represents the control samples; and the red circle represents the fracture or diabetic fracture samples.

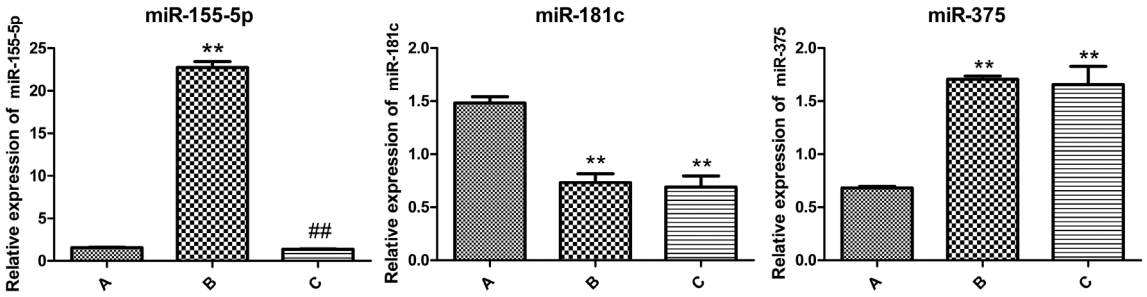

Fig. 7 The expression level of miR-155-5p, miR-181c, and miR-375. A: fracture patients without type-2 diabetes, B: fracture patients with type-2 diabetes, C: healthy subjects. (** $P < 0.01$ *vs* A group, ## $P < 0.01$ B *vs* C).

the process of diabetes leaded to poor fracture-healing process.

Role of MiR-181c and MiR-375 in Fractures in Postmenopausal Women

Furthermore, a previous study has indicated that the miRNA dysfunction in regulating insulin synthesis results in overt diabetes[36]. In the present study, the results implicated that miR-181c was up-regulated in DFH group. Evidence suggests that the miR-181 family members (including miR-181a, miR-181b, miR-181c, and miR-181d) play central roles in the differentiation stages of chondrocyte and chondrocyte formation[37]. Ma *et al.*[38] revealed that miR-181c-5p was linked to the progression of bone loss in osteoporosis, and was also involved in bone homeostasis mediated by osteoclasts and osteoblasts. Kocijan *et al.*[39] indicated that miR-181c-5p was regulated in postmenopausal osteoporosis cohort. A previous study demonstrated that miR-181 was significantly more highly expressed in standard healing fractures than unhealing fractures[40]. In addition, the relationship between miR-181c and aging has been recognized[41]. These findings supported our view that miR-181c might serve a role in fractures in postmenopausal women. Additionally, we also found miR-375 was significantly down-regulated in DFH group. MiR-375, a short RNA molecule, was one of the first miRNAs identified in the pancreas[42]. It has been found to play a vital role in osteogenic differentiation. Chen *et al.*[43] confirmed that miR-375 as a positive regulator in the osteogenic differentiation of mesenchymal stem cells (MSCs) and overexpression of miR-375 could significantly enhance the alkaline phosphatase activity and calcium deposition in human adipose MSCs, indicating overexpression of

miR-375-promoted bone regeneration. Meanwhile, Sun *et al.*[44] found that miR-375-3p could affect bone-formation-related pathways, suggesting that miR-375-3p and its targets might be used as diagnostic biomarkers for osteoporosis and also as novel therapeutic agents in osteoporosis treatment. Furthermore, specific circulating miRNAs, such as miR-375, reflected the presence of osteoporotic vertebral fractures in postmenopausal women[45]. These studies emphasized the role of miR-375 in the development of postmenopausal women with fractures. Therefore, we speculated that miR-181c and miR-375 might exert key functions in fractures, which could be considered as novel diagnosis marks of postmenopausal women with fracture.

Role of MiR-155-5p in Fractures in Postmenopausal Women with Diabetes

As for FH group, lncRNA-miRNA interaction network showed that miR-155-5p was up-regulated and regulated by lncRNAs such as MIR155HG. miR-155 (encoded by MIR155HG gene) is one of the best characterized miRNAs associated with various physiological and pathological processes including hematopoietic lineage differentiation, immunity, and inflammation[46]. Wu *et al.*[47] observed that miR-155 modulated tumor necrosis factor-regulated osteogenic differentiation *via* targeting the suppressor of cytokine signaling 1 protein. The significant relationship between miR-155 expression level and type 1 diabetes was also explored.[48] Assmann *et al.*[49] reported that miR-155 involved in numerous biological processes, including myeloid cell differentiation, immunity, and inflammation; meanwhile, miR-155 dysregulation was associated with cardiovascular diseases, diabetic kidney disease, and other diabetic chronic complications. Furthermore, Hu *et al.*[50] proved that MIR155HG played a functional role in hematopoiesis, which further lead to the miR-155-5p being up-regulated in bone marrow. Kobayashi *et al.*[51] demonstrated that the miR-155 was dysregulated in postmenopausal women with ovarian cancer. These genes were not only related to fractures but also to diabetes. Taken together, we speculated that the up-regulation of miR-155-5p might induce the diabetic fractures in postmenopausal women by affecting the process of bone marrow hematopoiesis. Moreover, the result of PCA indicated that the different types of samples were clearly distinguished by these miRNAs, suggesting miR-155-5p, miR-375, and miR-181c might be regarded as biomarkers to differentiate fracture with or without diabetes in postmenopausal women. Meanwhile, the expression of these miRNAs was verified by qPCR, the results were consistent with microarray data.

Limitations

There were some limitations in this study. Firstly, the study was performed based only on the serum samples. Secondly, the sample sizes used in verification were small and functional studies of these genes in different types of fracture were not conducted. Thirdly, whether the differences in miRNAs were caused by physiological reactions after fracture was unclear. Finally, due to a lack of clinical research, whether miRNAs affect fracture healing has not been studied. Thus, the collection of a large clinical sample is needed to confirm our results and further investigate the mechanism of fracture in postmenopausal women in more detail.

Conclusions

In conclusion, miR-181c and miR-375 might be regarded as potential predictors for fracture in postmenopausal women, while miR-155-5p might be a candidate diagnostic biomarker in diabetic fracture in postmenopausal women. These results may provide further insight into fracture in postmenopausal women with and without diabetes.

Acknowledgments

This work was supported by Key Social Development Projects of Shaanxi Science and Technology Administration (grant number 2015SF116, 2015SF110 and 2016SF340).

Supporting Information

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Table S1. The clinical characteristics of patients used in qPCR verification

Table S2. The primer sequences of validated miRNAs

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