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Original Paper

Hsa_Circ_0001275: A Potential Novel Diagnostic Biomarker for Postmenopausal Osteoporosis

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Key Words
Circular RNAs • Postmenopausal osteoporosis • CircRNA chip • Biomarker

Abstract
Background/Aims: Circular RNAs (circRNAs) serve as potential diagnostic biomarkers. In this study, we aimed to identify a potential biomarker from peripheral blood mononuclear cells (PBMCs) of patients with postmenopausal osteoporosis (PMOP). ***Methods:*** CircRNA expression in PBMCs from three pairs of samples from PMOP patients and controls was initially detected by circRNA microarray. The changes in selected circRNAs in PBMCs from 28 PMOP patients and 21 age- and sex-matched controls were confirmed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Next, samples from 30 PMOP patients and 20 controls were used for further verification. Pearson correlation test was performed to assess the correlation between circRNAs and clinical variables. The area under the receiver operator characteristic (ROC) curve was calculated to evaluate the diagnostic value. ***Results:*** Six differentially expressed circRNAs were identified by chip microarray analysis, of which only hsa_circ_0001275 showed consistency and statistical significance in qRT-PCR. The correlation analysis between age, body weight, height, WBC, lymphocyte and monocyte count, bone density, T-score, β-CROSSL, OSTEOC, and TP1NP showed that hsa_circ_0001275 was negatively correlated with T-score. ROC curves showed that hsa_circ_0001275 has significant diagnostic value in PMOP (AUC=0.759, P<0.001). ***Conclusion:*** This study suggests that hsa_circ_0001275 may serve as a potential diagnostic biomarker for PMOP.

Introduction

Postmenopausal osteoporosis (PMOP), one of the most common types of primary osteoporosis, is characterized by low back pain and pathological fracture caused by

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postmenopausal estrogen deficiency [1]. The gold standard for measurement of bone mineral density (BMD) for diagnosing osteoporosis is dual energy x-ray absorption (DXA) [2]. However, the clinical application of BMD measurement has serious limitations because changes in bone density occur slowly and may take six months to 2 years [3]. The biochemical markers of bone metabolism, which include products of bone metabolism or related hormones, can be detected from blood and urine [4-6]. The existing bone turnover markers **(**BTMs) that reflect human bone formation or bone resorption contribute to the determination of type of osteoporosis, differential diagnosis, and early evaluation of response to osteoporosis treatment. However, they cannot be used to diagnose osteoporosis [7]. Therefore, future research is focused on the identification of more specific and sensitive BTMs.

Circular RNAs (circRNAs) are a large class of non-coding RNAs that form a closed loop structure through covalent bond formation between the 3' and 5' ends of circRNA. They are more stable than linear RNA because of their closed loop structure, which prevents degradation by RNA exonuclease, providing richness to circRNAs [8-10]. Studies have reported that circRNAs play an important role in many diseases, including preeclampsia, Alzheimer's disease, diabetes, and tumors [11-14]. CircRNA can act as miRNA sponges [15-18], thereby adsorbing miRNA and removing the inhibitory effect of miRNA on its target genes, resulting in the upregulation of target genes.

In this study, in an effort to identify the expression of circRNAs in PMOP, we performed expression profiling of circRNAs by microarray and verified the results by using quantitative reverse transcription polymerase chain reaction (qRT-PCR). We performed receiver operator characteristic (ROC) curve analysis to determine the diagnostic value of significantly differentially expressed hsa_circ_0001275 as a biomarker of PMOP.

Materials and Methods

Case information

Over 99 reports of menopausal patients who received physical examination or BMD examination during their stay in the Third Affiliated Hospital of Guangzhou University of Chinese Medicine from October 2016 to August 2017 were collected. After ruling out other relevant metabolic diseases, 58 menopausal patients aged less than 70 years (inclusive) with T-scores lower than −2.5 SD (inclusive) at their femoral necks were included in the experiment group. Forty-one healthy menopausal women aged 70 years or lower with T-scores greater than −2.5 SD formed the control group of this study. The menopausal age, height, weight, WBC, lymphocyte and monocyte count, and bone markers (β-CROSSL, TPINP, and OSTEOC) of these subjects were recorded. Fresh venous blood was collected for follow-on experiments according to the requirements given below.

The Human Research Ethics Committee from the Third Affiliated Hospital of Guangzhou University of Chinese Medicine approved all aspects of this study (IRB No. 2016-001-01) and all patients signed an informed consent form.

Isolation of PBMCs

Fresh blood was collected from veins, and isolation of PBMCs was conducted within 6 h after blood collection to ensure the survival of lymphocytes. The density gradient separation technique was employed to isolate mononuclear cells from blood at 18–20°C using Ficoll-Paque PLUS reagent (GE Healthcare), according to the manufacturer's instructions.

Total RNA extraction from PBMCs and circRNA chip analysis

Total RNA was extracted from the above-mentioned isolated mononuclear cells by using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. CircRNA chip analysis (KangCheng, Biotech) was performed to identify the significantly changed circRNAs in PMOP patients (n=3) and controls (n=3). Sample preparation and microarray hybridization were performed based on the standard protocols of Arraystar (Arraystar Inc.). The extracted RNAs were purified by digestion with

Rnase R (Epicentre) to remove linear transcripts. Then, the enriched circular transcripts were labeled with fluorescent cRNA using Arraystar Super RNA Labeling Kit (Arraystar, Rockville, MD, USA). The labeled cRNAs were hybridized onto the Agilent Human CircRNA Array (V2.0, Arraystar). The arrays were scanned using the Agilent Scanner and analyzed by GenePix Pro 6.0 software (Axon, Foster City, CA, USA). CircRNAs having fold change ≥1.5 and $p \leq 0.05$ were selected as significantly differentially expressed. Hierarchical clustering was performed to show the distinguishable circRNA expression pattern among samples. All of the experimental results were saved as Microsoft Excel files.

RT-PCR assays of significantly differentially expressed circRNAs

The total RNA was separated from PBMCs according to the aforementioned method. The total RNA was transcribed into DNA according to the instructions in the PrimeScript RT Master Mix (Perfect Real Time) real-time RT-PCR kit (Takara-bio, China). PCR was performed with UltraSYBR Mixture (High ROX) (CWbiotech, China). The primers were designed and synthesized by Sangon (Realgene Company, Nanjing, China) (Table 3).

Statistical analysis

GraphPad Prism (version 7.0) was used to perform two-tailed independent *t*-test or ANOVA analysis for data processing. $P < 0.05$ indicated a significant statistical difference. The groups were compared for statistical significance using the Mann-Whitney test, Student's *t*-test, Wilcoxon signed-rank test, or chi-squared test, as appropriate. The associations between parameters were analyzed using the Pearson correlation. ROC curve analysis was performed to evaluate the diagnostic value of circRNAs that were dysregulated in PBMCs from PMOP patients compared to those from controls. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL).

Results

Characteristics of study subjects

As shown in Table 2, the average age, height, weight, WBC, and lymphocyte and monocyte count of the menopausal women showed no significant differences between the PMOP and control. In this study, the menopausal women were divided into two groups based on their T-score at the lumbar vertebrae or femurs, which was significantly different between the experiment and control groups (Table 1). The basic characteristics of all study subjects with PMOP and maintaining a low bone mass or healthy state were in line with the inclusion criteria, and they were eligible to participate in the follow-on studies.

CircRNA expression profiles

In total, 11, 246 circRNAs were analyzed by circRNA microarray in three pairs of PMOP and control samples. The fold changes (FC) of circRNA expression in both groups were measured to identify the differentially expressed circRNAs (Fig. 1A). The volcano plot identified 381 circRNAs whose levels changed substantially (FC ≥1.5), including 203 upregulated and 178 downregulated circRNAs, with statistical significance ($P < 0.05$; Fig. 1B,C). There are five types of circRNAs: exonic, intronic, antisense, sense overlapping, and intergenic circRNAs [19]. Most circRNAs were derived from exonic circRNAs (Fig. 1D),

Table 1. Basic characteristics of menopausal women in both groups. Note: The data above are expressed as the mean ± standard deviation

Item	Control group (n = 41)	PMOP (n = 58)	*P-value*
Age (years)	64.32 ± 3.15	68.88 ± 3.97	*0.094*
Height (cm)	156.98 ± 1.69	153.14 ± 3.70	*0.100*
Weight (kg)	58.85 ± 2.58	55.84 ± 2.40	*0.095*
WBC (10^9/L)	6.97 ± 0.46	6.80 ± 0.46	*0.606*
Lymphocyte (10^9/L)	2.19 ± 0.21	2.18 ± 0.15	*0.970*
Monocyte (10^9/L)	0.50 ± 0.04	0.53 ± 0.05	*0.400*
BMD (g/cm^2)	0.57 ± 0.12	0.40 ± 0.06	*0.010*
T-score	-1.27± 0.32	-3.57 ± 0.25	*0.000*
CROSSL (ng/mL)	0.55 ± 0.08	0.78 ± 0.11	*0.002*
TPINP (ng/mL)	51.13 ± 6.52	61.85 ± 8.13	*0.056*
OSTEOC (ng/mL)	16.78 ± 2.52	21.80 ± 2.56	*0.008*

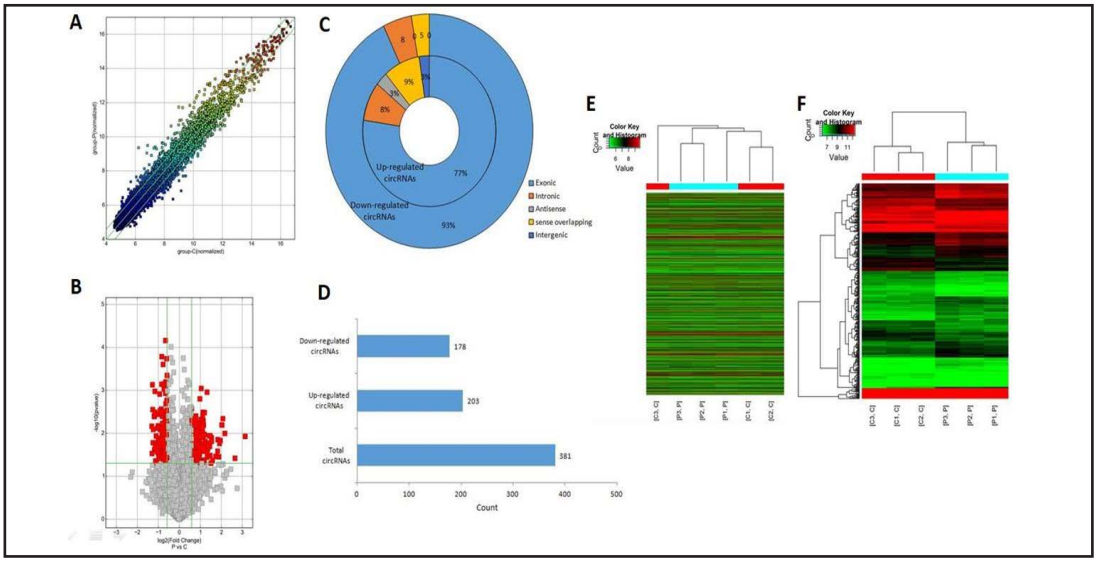

Fig. 1. Differences and characteristics of circRNA expression profiles in PBMCs of the PMOP group and control group. (A) The scatterplot figuratively expresses the changes in circRNA expression. The green lines represent fold change (FC) lines. The circRNAs above the top green line and below the bottom green line demonstrated a change in expression of at least 1.5 fold between the two compared samples. (B) The volcano plot was prepared according to the FC values and p-values. The vertical lines correspond to 1.5-fold up- and downregulation, respectively, and the horizontal line represents p = 0.05. The red points in the plot represent differentially expressed circRNAs with statistical significance. (C) The histogram reveals significantly differentially expressed circRNAs, including upregulated and downregulated circRNAs, obtained from the results of chip microarray (FC ≥ 1.5, P<0.05). (D) The Fig. illustrates the five types of differentially expressed circRNAs, most of which originate from exonic circRNAs. (E) Each row represents a sample, whereas each line represents a circRNA, with the red part indicating a relatively high level of expression and the green part denoting a relatively low level of expression; P is the PMOP group and C is the control group, with each containing three different samples. (F) Cluster analysis of significantly upregulated or downregulated circRNAs.

Table 2. The 10 circRNAs with the most significant upregulation or downregulation

circRNA	Regulation	chrom	type	P-value	FDR	FC (abs)
hsa_circ_0028882	up	chr12	exonic	0.038050619	0.582919329	6.2583507
hsa_circ_0033628	up	chr14	exonic	0.010009192	0.526378433	4.490164
hsa_circ_0007788	up	chr16	exonic	0.004537529	0.526378433	4.2088212
hsa_circ_0090446	up	chrX	exonic	0.006400725	0.526378433	4.0975288
hsa_circ_0006766	up	chr7	sense overlapping	0.006368491	0.526378433	4.0534075
hsa_circ_0037798	up	chr16	sense overlapping	0.014691147	0.536099088	3.8751497
hsa_circ_0002131	up	chr8	exonic	0.035209786	0.581786998	3.6941614
hsa_circ_0001275	up	chr3	antisense	0.034438204	0.581786998	3.3053839
hsa_circ_0008802	up	chr12	exonic	0.04252221	0.588697961	2.9871181
hsa_circ_0003391	up	chr11	exonic	0.015341845	0.536099088	2.9024062
hsa_circ_0084021	down	chr8	exonic	0.029377538	0.565846946	2.4678755
hsa_circ_0002082	down	chr11	sense overlapping	0.00073674	0.382780266	2.4298076
hsa_circ_0031235	down	chr14	exonic	0.016528688	0.5403153	2.3902739
hsa_circ_0000524	down	chr14	exonic	0.008104243	0.526378433	2.3659075
hsa_circ_0064555	down	chr3	exonic	0.003956361	0.526378433	2.3054268
hsa_circ_0008604	down	chr17	exonic	0.024048273	0.552782626	2.277248
hsa_circ_0057748	down	chr2	exonic	0.004052203	0.526378433	2.2694271
hsa_circ_0031241	down	chr14	exonic	0.001051266	0.39758787	2.2417359
hsa_circ_0006801	down	chr3	exonic	0.003213766	0.526378433	2.2399374
hsa_circ_0074371	down	chr5	exonic	0.0204961	0.548602794	2.2196464

with no significant difference between upregulation and downregulation. To determine the expression of differentially expressed circRNAs in the PMOP and control groups clearly, ther-

mography was employed to perform cluster analysis (Fig. 1E–F). According to the FC values of differential expression, we focused on the analysis of 20 circRNAs having the greatest magnitude of upregulation and downregulation (Table 2).

RT-PCR assay results of significantly differentially expressed circRNAs

Six circRNAs were selected based on the FC of differential expression and initial expression quantity, including five upregulated circRNAs (hsa_circ_0028882, hsa_circ_0001275, hsa_circ_0006766, hsa_circ_0007788, and hsa_circ_0003391) and one downregulated circRNA (hsa_circ_0006801). To determine the expression levels of these circRNAs (Table 1), we performed RT-PCR (PMOP: n=28, Control: n=21). The trends for five of the six circRNAs were consistent with the results of chip analysis. However, only the trend shown by hsa_circ_0001275 was statistically significant (P<0.01; Fig. 2). We selected 50 samples again (PMOP: n=30, Control: n=20) for verification of hsa_circ_0001275. We found that the results were consistent with the previous results (P<0.01; Fig. 3A).

Pearson correlation test of clinical variables and hsa_circ_0001275 in PBMCs from PMOP patients

To determine whether the significantly and differentially expressed circRNAs in PBMCs from PMOP patients were relevant biomarkers for the severity of PMOP, we performed the Pearson correlation test to assess the correlation between PMOP-related clinical features and hsa_circ_0001275 in PBMCs from PMOP patients. As shown in Table 4, hsa_

Table 3. Internal reference and annular primer sequence

	5′-3′ (Forward)	5′-3′ (Reverse)
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG
hsa_circ_0001275	TCTTCTTCTC CACTCCTGAA	GAGCAAGGGC CCTAGCTCAA
hsa_circ_0006766	CCCTATCCCT TTTCCATATC	CTAACTTACC CCTGTAATGG
hsa_circ_0007788	CTCCCACTTCTTGCCCCAGA	CCCTGGGCAC TCAGCAAGTA
hsa_circ_0003391	CACAACATCT TCCCCCACAT	GATGCCAAGG CTTTTTGTCA
hsa_circ_0006801	TTCAAGAAAG GTTTTAAATG	ACATTGTGAA CTATGGCTGC
hsa_circ_0028882	ACTTCTGCCCACGTTGTTTTC	AGGCTGCAGTCCTTGTTTTTG

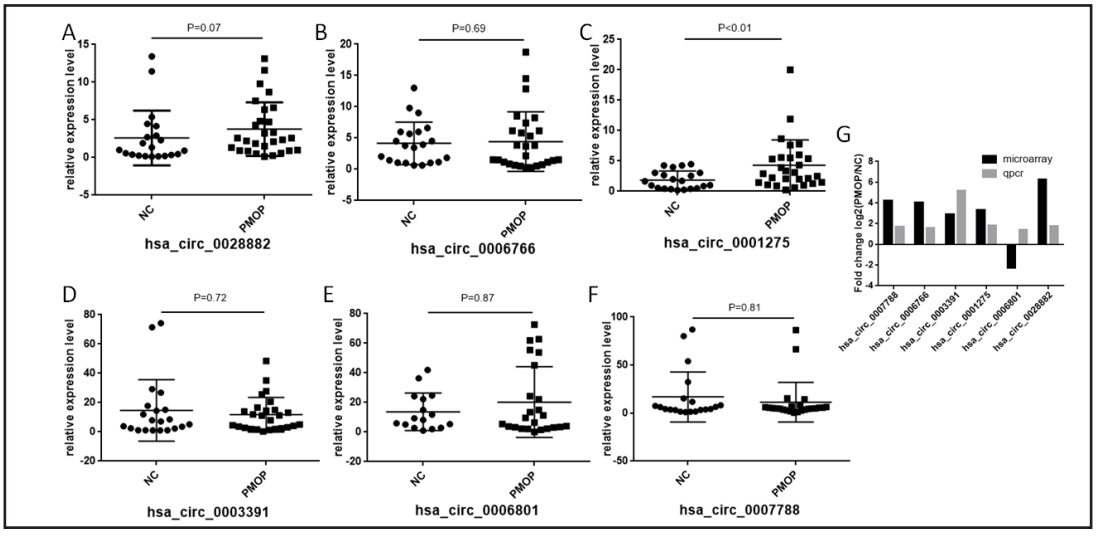

Fig. 2. RT-PCR assay results of six circRNAs. (A-F) Forest scatterplot: The RT-PCR assay was performed to verify the expression levels of six circRNAs in the PMOP group, which included PBMCs derived from approximately 28 PMOP patients, and in the control group, which included PBMCs from 21 menopausal women. Hsa_circ_0001275 showed statistical significance (P<0.01). (G) The histogram presents the comparison between RT-PCR assay results and chip microarray data. Except for hsa_circ_0006766, the trends for the other five circRNAs, as given by the PCR results, were in compliance with the chip analysis results. However, only hsa_circ_0001275 exhibited statistical significance (P<0.01).

Table 4. Pearson correlation coefficients of clinical variables and hsa_circ_0001275. Note: *P < 0.05, **P < 0.01

	Height	Weight	WBC	Lymphocyte	Monocyte	BMD	β-CROSSL	TP1NP	OSTEOC	T-score	hsa_circ_0001275
Age	-0.715**	-0.052	0.025	-0.064	0.147	0.102	0.085	0.052	0.038	-0.192	-0.081
Height	-	0.145	-0.015	0.073	-0.103	-0.108	-0.008	-0.011	-0.082	0.125	0.094
Weight		-	-0.108	-0.148	-0.008	0.063	-0.147	-0.240	-0.279**	0.305**	0.052
WBC			-	0.420**	0.235*	0.106	0.003	-0.090	-0.085	0.039	0.035
Lymphocyte				-	0.081	-0.182	-0.024	-0.113	-0.191	-0.025	0.152
Monocyte					-	0.081	0.084	-0.113	-0.036	-0.038	-0.031
BMD						-	-0.040	-0.019	0.151	0.236*	-0.121
β-CROSSL							-	0.546**	0.638**	-0.231*	0.192
TP1NP								-	0.720**	-0.184	0.160
OSTEOC									-	-0290**	0.153
T-score										-	-0.257*

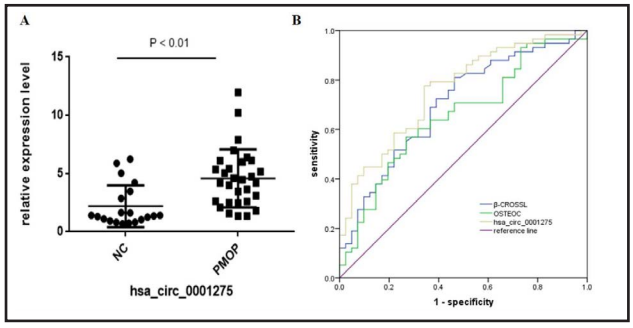

Fig. 3. (A) Forest scatterplot: RT-PCR assay was performed to determine the expression levels of hsa_circ_0001275 in the PMOP group, which included PBMCs derived from approximately 30 PMOP patients, and in control group, which includes PBMCs from 20 menopausal women. The results of this validation were consistent with previous results (P<0.01). (B) ROC analysis of hsa_circ_0001275, β-CROSSL, and OSTEOC in PBMCs of PMOP patients. The largest AUC was found for hsa_circ_0001275 (AUC: 0.759, P<0.001), followed by β-CROSSL (AUC: 0.700, P<0.01) and OSTEOC (AUC: 0.655, P<0.01).

circ_0001275 was correlated with T-score (r = −0.257, $P < 0.05$). However, the levels of hsa_circ_0001275 in PBMCs from PMOP patients did not correlate with age, height, weight, WBC, lymphocyte and monocyte count, BMD, β-CROSSL, TP1NP, or OSTEOC.

ROC curve analysis of hsa_circ_0001275 among PMOP patients

We performed ROC curve analysis to assess the diagnostic value of significantly differentially expressed hsa_circ_0001275 for PMOP. ROC curves showed that the level of hsa_circ_0001275 in PBMCs could distinguish patients with PMOP from the controls [area under the curve (AUC): 0.759, 95% CI: 0.664–0.853, P< 0.01] (Fig. 3B). Therefore, hsa_circ_0001275 may be a potential diagnostic biomarker for PMOP. When compared with bone metabolism biomarkers, the largest AUC was found for hsa_circ_0001275, followed by β-CROSSL (AUC: 0.700, P< 0.01) and OSTEOC (AUC: 0.655, P< 0.01) (Fig. 3B).

Discussion

In this study, we aimed to identify a potential biomarker in PBMCs of patients with PMOP. We found that the expression of hsa_circ_0001275 was significantly increased in PMOP patients, and therefore it can serve as a potential diagnostic biomarker for PMOP. Thousands of circRNAs in the genome repositories of different species have been identified by applying RNA microarray technology. Over 2400 circRNA candidates have been identified in human serum. In mice and *Caenorhabditis elegans,* 1903 circRNAs and 724 circRNAs have been identified, respectively. Owing to its closed loop structure, circRNA is not degraded by RNA exonuclease, making it highly conserved and remarkably stable. Therefore, circRNA can be a potential diagnostic biomarker [20-22]. A previous study on hepatocellular carcinoma reported that ciRS-7 could be considered a biomarker for microvascular invasion in hepatic cell carcinoma and a sponge for microRNA-7 [23]. Another study reported that hsa_

circ_0001649 could be utilized as a novel diagnostic biomarker for hepatocellular carcinoma [24]. In addition, clinical study of samples showed that circRNA is not restricted to clinical blood samples and can be found in cerebrospinal fluid, urine, and other body fluids. Thus, studies on other body fluids may help to identify circRNA as a novel biomarker for diagnosis, prognosis, and prediction of therapeutic efficacy.

As precursor cells of osteoclasts, PBMCs produce cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, which have direct effects on osteoclast formation [25, 26]. However, there are few studies on the effect of PBMCs on the pathogenesis of osteoporosis. In this study, we performed a microarray analysis of dysregulated circRNAs by comparing the transcriptome profiles of PBMCs from patients with PMOP with those from controls. In total, 381 significantly and differentially expressed circRNAs were detected, of which 203 were upregulated and 178 were downregulated. These observations may facilitate future pathophysiology research on PMOP and help to determine whether circRNAs in PBMCs could be used as novel, non-invasive biomarkers for diagnosis and treatment of PMOP.

CircRNA microarray profiling and qRT-PCR validation identified five upregulated circRNAs (hsa_circ_0028882,hsa_circ_0001275, hsa_circ_0006766, hsa_circ_0007788 and hsa_circ_0003391) and one downregulated circRNA (hsa_circ_0006801). Only hsa_circ_0001275 was significantly differentially expressed in PBMCs of PMOP patients compared with the controls. These results indicate that the altered expression of circRNAs may be related to their involvement in the pathogenesis of PMOP. Furthermore, our study found that the expression of hsa_circ_0001275 in PBMCs of PMOP patients was not correlated with age, height, weight, WBC, lymphocyte and monocyte count, BMD, β-CROSSL, TP1NP, or OSTEOC, indicating that this circRNA may not be a relevant biomarker for disease severity or systemic inflammation in PMOP. However, we found that the levels of hsa_circ_0001275 were correlated with the T-score. The T-score was used to compare the bone density value of patients to the average bone density of normal young people. T-score = (subject's BMD value − mean BMD of young people)/standard deviation of BMD of young people, and it indicates whether the subject's BMD is lower or higher than the mean BMD of young people by a few standard deviations. According to the World Health Organization, T-score below −2.5 SD (inclusive) can be diagnosed as osteoporosis. In addition, in this study, the levels of hsa_circ_0001275 in PBMCs showed potential diagnostic value for PMOP and a high ROC AUC value (AUC: 0.759, 95% CI: 0.664–0.853, $P< 0.01$) compared to traditional bone metabolism indices, indicating its high potential as a diagnostic biomarker. Studies in multiple centers with a large number of samples are needed in the near future. Additionally, studying the functions of hsa_circ_0001275 could improve the current understanding of the mechanisms underlying PMOP occurrence and progression.

There are some limitations to this study. First, the sample size was relatively small, and the data from this study should be replicated in large-scale studies and in other populations with different races or from different regions. Second, to determine whether hsa_circ_0001275 can be a diagnostic biomarker, its ability to distinguish PMOP from senile osteoporosis, secondary osteoporosis, and other orthopedic diseases, such as rheumatoid arthritis, ankylosing spondylitis, and osteoarthritis, which are characterized by joint damage, should be evaluated. Further studies on other orthopedic diseases are warranted to strengthen the contention that hsa_circ_0001275 may serve as a diagnostic biomarker for PMOP. Third, we did not analyze circRNAs in the plasma, serum, or exosomes. Studies have shown that circRNAs in exosomes are more stable than those in serum or plasma, making them more reliable as potential biomarkers.

To the best of our knowledge, this is the first study to use circRNA microarray to determine the expression of circRNA in PBMCs of PMOP patients and controls. The findings of this study will help to determine the role and function of circRNA in the occurrence and development of PMOP. In addition, we found that circRNAs have applied value in PMOP diagnosis. These results suggest that circRNAs have potential clinical significance and may

help to explain the molecular mechanisms and biological functions of PMOP, and therefore deserve further study.

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Disclosure Statement

The authors declare that no conflict of interests exists.

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