Original Research

Identification of miR-708-5p in peripheral blood monocytes: Potential marker for postmenopausal osteoporosis in Mexican-Mestizo population

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Impact statement

This is the first study in which hsa-miR-708-5p has been identified in peripheral blood monocytes (osteoclast precursors) and associated with postmenopausal osteoporosis through small RNA-Sequencing, in an Admixed Mexican Mestizo population. By conducting *in silico* and bioinformatic analyzes, we identified target genes and important signaling pathways involved in bone metabolism pointing hsa-miR-708-5p as a candidate marker for osteoporosis in Mexican population. These approaches provide a landscape of the post-transcriptional regulation, which can be useful for the management of postmenopausal osteoporosis along with the potential use of microRNAs as markers for its early detection.

Abstract

Osteoporosis is the most frequent disorder of bone metabolism, owing to an alteration between osteoclast and osteoblast activity, which results from the interaction of genetic, environmental, and epigenetic factors. microRNAs, small non-coding RNAs of around 22 nucleotides with important regulatory roles in gene regulation, that target mRNAs for post-transcriptional destabilization have been suggested as biomarkers in several disorders. In this work, we used small RNA sequencing to identify microRNAs from peripheral blood monocytes that were differentially expressed between non-osteoporotic and osteoporotic Mexican postmenopausal women, to elucidate the potential role of microRNAs as non-invasive marker candidates in osteoporosis. We identified six candidate microRNAs: four were up-regulated (miR-708-5p, miR-34b-5p, miR-3161, miR-328-5p), while two were down-regulated (miR-4422 and miR-939-3p) in osteoporotic women. Differential expression was validated by quantitative RT-PCR and only the upregulation of miR-708-5p was found to be statistically significant. Bioinformatic analysis of target genes for miR-708-5p showed 15 signaling pathways related to bone metabolism. Since monocytes are osteoclast precursors, 10 potential target genes present in these pathways and related to osteoclastogenesis were identified (*AKT1*, *AKT2*, *CCND1*, *PARP1*, *SMAD3*, *CXCL5*, *FKBP5*, *MAP2K3*, *MMP2,* and *IKBKG*). Five of them were found to be down-regulated according to microarray expression data. This is the first time that miR-708-5p has been identified in peripheral blood monocytes and associated with postmenopausal osteoporosis. Our results suggest that miR-708-5p reduces the expression of *AKT1, AKT2, PARP1 FKBP5,* and *MP2K3* in peripheral blood monocytes contributing to an osteoporotic phenotype and could be a candidate marker for postmenopausal osteoporosis in Mexican population.

Keywords: microRNAs, monocyte, osteoporosis, small RNA sequencing, biomarkers, genomics

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Introduction

Bone is considered a dynamic tissue that goes through constant turnover to maintain size, shape, and structural integrity to adequately regulate mineral homeostasis. This highly coordinated process is controlled by the action of specialized cells during bone remodeling: osteocytes, osteoblasts, and osteoclasts. A disequilibrium between osteoclast (resorption) and osteoblast (formation) activity can lead to different skeletal disorders and one of the most studied is osteoporosis (OP).[1] OP is a very frequent bone remodeling-related disorder "characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk."[2] Predisposition to OP is known to be a genetic, epigenetic, and environmental interaction. Some genetic factors involved in OP are evidenced by the familial aggregation of this disease.[3] Nonetheless, candidate gene and genome-wide association studies have been somehow discouraging, mainly because they just detect a proportion of the population under risk. Further investigations with different approaches are required to improve the detection rate of these studies.[4]

Recently, post-transcriptional regulatory mechanisms of gene expression including those modulated by microRNAs (miRNAs) have been reported to be outstanding in the pathogenesis of OP.[5] miRNAs are small non-coding RNAs of around 22 nucleotides with key roles as regulators of gene expression by the specific binding to mRNAs for post-transcriptional destabilization. miRNAs are highly conserved across biological evolution and have been estimated to regulate the expression of about 60% human mRNAs so far.[6] Numerous miRNAs have been identified to have essential roles during osteoclast and osteoblast differentiation, mainly in animal models and cell lines.[7] The understanding of how osteoclastogenesis is regulated at the post-transcriptional level is crucial. It is known that osteoclasts and their precursors have important effects on different processes during normal and pathologic bone remodeling.[8] Some reports have proposed miRNAs as promising biomarkers and therapeutic targets in many pathologies such as diabetes, cancer, cardiovascular, and bone diseases.[9,10] Given that human peripheral blood monocytes (HPBMs) are osteoclast precursor cells and can secret osteoclast-specific cytokines, including: "Tumor necrosis factor alpha" (TNF-α), "Interleukin 1" (IL-1), and "Interleukin 6" (IL-6), they are considered a suitable model for bone-related studies.[11,12] Some studies have been focused on the identification of miRNAs in HPBM and their association with human OP, but most of them have been done with DNA microarray technology. However, microarrays have some limitations such as cross-hybridization and background noise which restricts the preciseness assessment of expression, especially for molecules with low abundance. Moreover, probes vary substantially in their hybridization features and microarrays are limited to study the molecules for which probes have been designed.[13,14] Few studies have started to use next-generation sequencing technologies to profile miRNAs in HPBM.[15] Within RNA sequencing techniques, small RNA sequencing (sRNA-Seq) has arisen as the novel standard for analysis of expression profiles of microRNAs. sRNA-Seq provides advantages over microarrays such as increased sensitivity and specificity, identification of polymorphisms as well as the edition of miRNA sequences and the detection of known and novel miRNAs.[16,17] This work aimed to analyze the expression profile of miRNAs in HPBM from both osteoporotic and non-osteoporotic Mexican postmenopausal women, using sRNA-Seq to elucidate the possible role of miRNAs as non-invasive markers in OP.

Materials and methods

Subjects and study design

Fourteen unrelated postmenopausal women, 7 non-osteoporotic and 7 with OP at the hip from the "Mexican Health Worker Cohort Study" (MHWCS) were recruited for this study. The inclusion/exclusion criteria, BMD quantification, informed consent forms and the complete characteristics of the participants and the MHWCS have been previously described.[18–21]

Monocyte isolation

Fifteen milliliters of peripheral blood were collected from each participant in CPT tubes (BD Biosciences, New Jersey, United States). Peripheral blood mononuclear cells (PBMCs) were obtained and $CD14^+$ cells were enriched performing a negative selection using an EasySep kit (Stemcell Tec., Vancouver, Canada). Purity of monocytes was evaluated by fluorescent staining with phycoerythrin (PE) and fluorescein isothiocyanate-(FITC) conjugated antibodies against CD14 and CD45, respectively (BD Biosciences, New Jersey, United States). The stained cell samples were examined in a FACSAria I Sorter (BD Biosciences, New Jersey, United States). Purity >85% was assessed to proceed to RNA isolation (Data not shown).

RNA isolation, cDNA libraries construction, and sequencing

Total RNA from HPBM $CD14^+$ was purified employing the "miRNEasy Mini Kit" (QIAGEN, Hilden, Germany). Quantity and integrity of RNA were evaluated employing a Qubit 3.0 Fluorometer and a 2100 Bioanalyzer, respectively. The construction of cDNA libraries was carried out from 30 ng of RNA employing the "SMARTer small RNA-Seq Kit for Illumina" (Clontech, California, United States). Briefly, input total RNA was first polyadenylated, cDNA synthesis was carried out followed by incorporation of adapter sequences, and Illumina adapters were added during the PCR amplification. Finally, cDNA was size-selected using Agencourt AMPure XP beads. Purified cDNA was sequenced for 50 cycles on a NextSeq 500 (Illumina, California, United States).

sRNA-Seq data processing

Files were exported in FASTQ format and visualized for quality control employing FastQC "http://www.bioinformatics.babraham.ac.uk/projects/fastqc". Seqtk tool

"https://github.com/lh3/seqtk" was employed to remove the first three bases of each read (part of the template-switching oligo). Stretches of more than 15 adenines and Illumina sRNA 3′ adapter sequence (AGATCGGAAGAG) were removed with reaper.[22] sRNA-Seq reads were processed using the exceRpt pipeline (https://rkitchen.github.io/exceRpt/), as previously described.[23] Briefly, the pipeline filters out reads of poor sequence quality or likely being contaminant sequences (UniVec) or likely rRNAs. Surviving reads are then aligned without gaps to pre-miRNA sequences from miRbase (v21)[24] and to the transcriptome and genome (GRCh38) with STAR.[25] The alignment requires a seed length of 16 and allows zero mismatches if the read length is less than 20 or one mismatch if longer.

Differential expression analysis

We estimated effective library size with the TMM method (edgeR v3.18.1) finding the scaling factors that minimize fold-changes for most genes.[26] We down-weighted outlier samples using the voomWithQualityWeights function.[27] Finally, we adjusted a linear model and estimated a moderated t-statistic with limma v3.32.7[28] assessing the differences in gene expression between groups. False discovery rate for multiple testing was used to correct *P*-values.[29] Library weights were estimated using the voom package[27] through limma v3.32.7. Differential expression analysis was performed only for miRNAs with at least one count-per-million in at least three libraries.

RT-qPCR analysis

cDNA was prepared from 100 ng of RNA employing a "TaqMan microRNA Reverse Transcription kit" (Applied Biosystems, Massachusetts, United States). The expression of hsa-miR-708-5p, hsa-miR-3161, hsa-miR-939-3p, and hsa-miR-4422 was measured employing TaqMan microRNA assays (ID: 002341, 243808, 471661 and 464021, respectively; Applied Biosystems, Massachusetts, United States) by qPCR on a "QuantStudio 7 Flex Real-Time PCR system" (Applied Biosystems, Massachusetts, United States). RNU44 (ID: 001094) and RNU48 (ID: 001006) were used as normalizers (Applied Biosystems, Massachusetts, United States). The delta–delta Ct method was performed for relative quantification.

Target gene prediction and pathway enrichment analysis

We used MiRNet and miRTarBase to search validated hsa-miR-708-5p target genes. Genes with validation reports were submitted to the web tools STRING and KEGG for pathway analysis. All genes represented in bone metabolism-related pathways with a false discovery rate- < 0.05 were selected for further analyzes. Ingenuity pathways analysis (IPA) software was used to build an interaction network based on both, validated target genes and miRNA data (QIAGEN, Hilden, Germany).

Microarray data analysis

The raw data obtained from a "GeneChip Human Genome U133 Plus 2.0 Array" (Affymetrix, California, United States), which contained data of HPBM belonging to twelve independent samples of postmenopausal Mexican-Mestizo women, were re-processed to compare these expression data with the predicted target genes. The raw data were processed as described in a previous study.[18]

Statistical analysis

Results were analyzed employing the "Mann-Whitney U test". Data are shown as mean ± standard deviation. Results are the average of three independent assays. All analyzes were done employing Prism V 6.0 (GraphPad Software, California, United States). Statistical significance was settled on a *P*-value ≤ 0.05.

Results

Characteristics of the study subjects

Anthropometric features, including: age, weight, height, BMI, and blood glucose level were not found to be statistically significant ($P > 0.05$), while bone-associated features such as hip BMD and T-score were statistically significant across groups ($P < 0.001$) as shown in Table 1.

Small RNA-sequencing data analysis

All samples (seven non-osteoporotic and seven osteoporotic) were sequenced as described in Methods. FASTQ files were visualized with FastQC for quality control reports such as basic statistics, quality scores, GC content, sequence length distribution, and adapter content. Because of the cDNA synthesis protocol, the reads ended with two special features: three nucleotides in every read at the 5′ end, derived from the template-switching oligo and a significant proportion of stretches of adenines at the 3′ end. Seqtk was used for processing FASTQ files, removing the first three nucleotides of every read and the stretches of adenines. Illumina sRNA 3′ adapter sequence (AGATCGGAAGAG) was removed with reaper and only clean reads between 18 and 24 nucleotides, the typical length range of miRNAs, were kept. After these filtering steps 22,575,505 clean reads were selected for further analysis. The highest frequency of read length was observed at 21 nucleotides

Table 1. Anthropometric parameters of the study population.

	Non-osteoporotic $n = 7$	Osteoporotic $n = 7$	*P*
Age (years)	68.86 ± 0.89	70.43± 3.20	0.2692
Height (cm)	154.7 ± 2.028	150.6 ± 1.806	0.1684
Weight (kg)	56.53 ± 2.055	50.96 ± 3.317	0.1972
BMI (kg/m^2)	23.77 ± 1.19	23.47 ± 3.38	0.8718
BGL (mg/dL)	123.3 ± 34.96	100.9 ± 13.85	0.2203
Hip BMD (g/cm^2)	0.9811 ± 0.06	0.6781 ± 0.01	**0.0006**
Hip t-Score (SD)	−0.2110 ± 0.50	−2.616 ± 0.1142	**0.0006**

BMI: body mass index; BGL: blood glucose level; BMD: bone mineral density; SD: standard deviation.
Note: Data are mean ± SD.

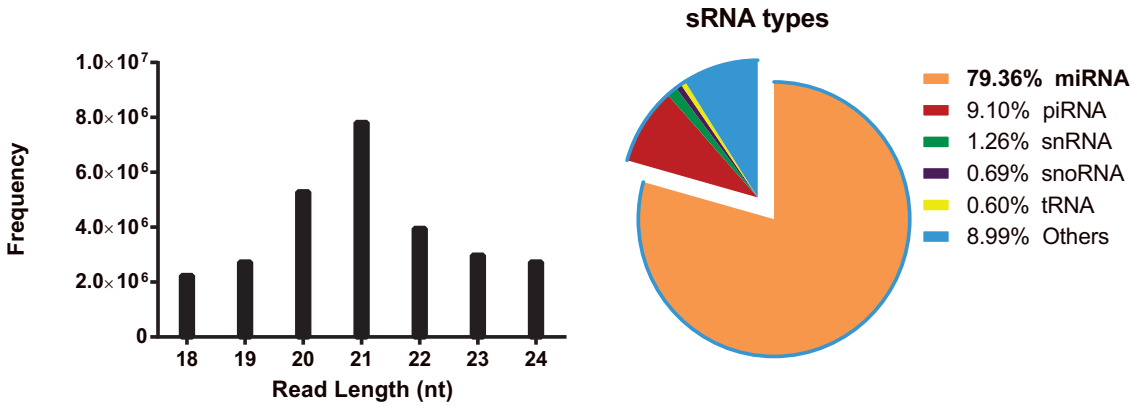

Figure 1. Overview of sRNA-Seq results. Frequency of sRNA-Seq reads according to their length is shown (a). Percentage of 18–24 nt clean reads mapping to each sRNA type (b). (A color version of this figure is available in the online journal.)

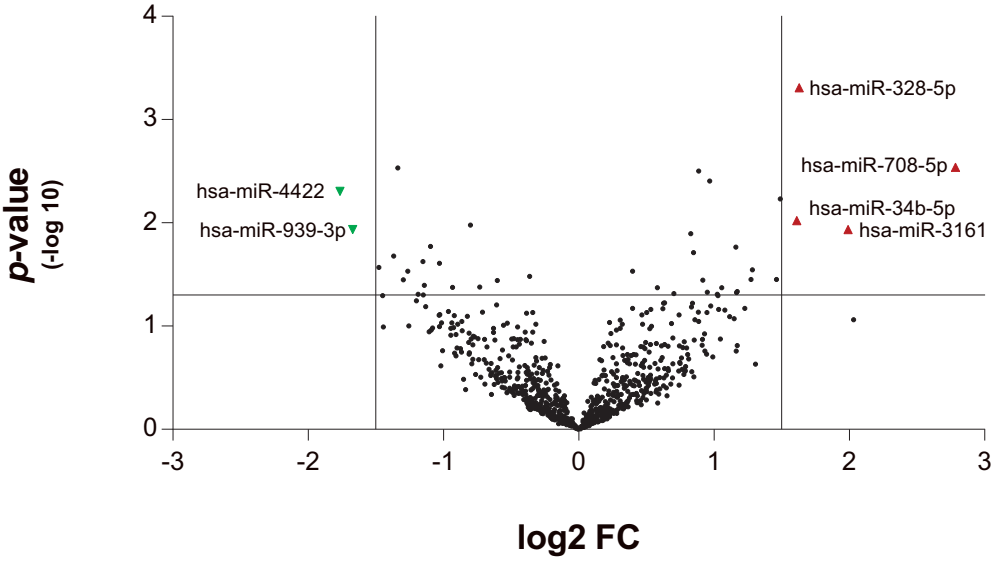

Figure 2. Volcano plot. Differentially expressed miRNAs in HPBM from postmenopausal osteoporotic women. Upright triangles represent up-regulated miRNAs and upside-down triangles represent down-regulated miRNAs. (A color version of this figure is available in the online journal.)

corresponding to the average length of miRNAs (Figure 1 (a)). Classification of small RNA shows that the major fraction of them (17,916,938 reads) mapped to mature miRNAs (79.36%), followed by 2,053,536 reads mapping to piRNAs (9.10%), as illustrated in Figure 1(b).

Differentially expressed (DE) miRNAs in osteoporotic postmenopausal women

Our analysis allowed us to study 885 microRNAs for differential expression between groups (osteoporotic versus non-osteoporotic). Comparison of the miRNA expression levels between groups is depicted in Figure 2 as a volcano plot. Before applying a false-discovery correction step, 46 miRNAs showed significant differences ($P \leq 0.05$). Top ten up-regulated and top ten down-regulated miRNAs in the osteoporotic group are shown in Table 2. For validation analysis, hsa-miR-708-5p and hsa-miR-3161 (up-regulated) and hsa-miR-4422 and hsa-miR-939-3p (down-regulated) were selected. These miRNAs were selected based on a raw P-value ≤ 0.05, a log2 Fold Change > 1.5, and their potential role in the regulation of osteoclastogenesis-related genes, according to literature.

RT-qPCR validation

Differential expression of the selected miRNAs (miR-708-5p, miR-3161, miR-939-3p and miR-4422) was validated by RT-qPCR analysis. Only the up-regulation of miR-708-5p in the osteoporotic group was statistically significant ($P = 0.01$). Changes in the expression of miR-3161, miR-4422, and miR-939-3p between groups were not statistically significant; however, a trend in the expression of all three miRNAs was observed (Figure 3).

microRNA target prediction and pathways analysis

In the first step, to investigate a potential functional association, the validated targets of miR-708-5p reported in miRNet and miRTarBase were retrieved. All 104 target genes reported as validated were submitted for functional protein association to the web tool STRING. The Pathway enrichment analysis in STRING revealed 15 KEGG

pathways with 17 overrepresented genes related to bone metabolism. The most miR-708-5p enriched pathways were: osteoclast differentiation, TNF, JAK-STAT, MAPK, NF-Kappa B, and Wnt signaling pathways. Other pathways were identified including PI3K-Akt, Estrogen, HIF-1, FoxO, and Cytokine-cytokine signaling pathways (Table 3). In the second step, the 17 overrepresented bone metabolism-related target genes of miR-708-5p contained in the 17 KEGG pathways were processed on the IPA functional tool to build an interactive network (Figure 4).

Table 2. Top 10 up-regulated and top 10 down-regulated miRNAs in postmenopausal osteoporotic women.

miRNA	Log2 fold change	*P*
hsa-miR-708-5p	**2.78**	**0.0029**
hsa-miR-3161	**1.98**	**0.0116**
hsa-miR-328-5p	**1.62**	**0.0005**
hsa-miR-34b-5p	**1.61**	**0.010**
hsa-miR-6741-5p	1.49	0.0059
hsa-miR-6791-5p	1.16	0.0171
hsa-miR-181a-3p	0.96	0.0039
hsa-miR-501-3p	0.88	0.0032
hsa-miR-20b-3p	0.84	0.0194
hsa-miR-181b-3p	0.82	0.0127
hsa-miR-556-5p	−0.79	0.0105
hsa-miR-2278	−1.02	0.0246
hsa-miR-4433b-3p	−1.09	0.017
hsa-miR-1278	−1.15	0.0237
hsa-miR-3127-3p	−1.26	0.0295
hsa-miR-7848-3p	−1.33	0.0029
hsa-miR-4677-5p	−1.36	0.0211
hsa-miR-133a-3p	−1.47	0.0269
hsa-miR-939-3p	**−1.66**	**0.0116**
hsa-miR-4422	**−1.76**	**0.005**

Note: Bold letters indicate DE miRNAs.

Target gene prediction and validation

The 17 target genes contained in bone-related signaling pathways were submitted to a bibliography research by searching relevant references in PubMed. Ten potential target genes for miR-708-5p have been previously associated with osteoclastogenesis: "AKT Serine/Threonine Kinase 1" (*AKT1*), "AKT Serine/Threonine Kinase 2" (*AKT2*), "Cyclin D1" (*CCND1*), "Poly(ADP-Ribose) Polymerase 1" (*PARP1*), "SMAD Family Member 3" (*SMAD3*), "C-X-C Motif Chemokine Ligand 5" (*CXCL5*), "FK506 Binding Protein 5" (*FKBP5*), "Mitogen-Activated Protein Kinase Kinase 3" (*MAP2K3*), "Matrix Metallopeptidase 2" (*MMP2*), and "Inhibitor of Nuclear Factor Kappa B Kinase Subunit Gamma" (*IKBKG*). To validate these genes, we evaluated the expression of the potential target genes through an array, previously reported.[18] The assumption is that a higher expression level of a given miRNA should correspond to a lower expression level of the target gene. Only five potential target genes for miR-708-5p and associated with osteoclastogenesis: *AKT1*,[30,31] *AKT2*,[32] *PARP1*,[33] FKBP5,[34] and *MAP2K3*[35] were validated. The analysis showed a diminution in mRNA expression levels of these genes between study samples ($P = 0.008$, $P = 0.004$, $P = 0.01$, $P = 0.01$ and $P = 0.01$, respectively) (Figure 5).

Discussion

In this study, we analyzed differentially expressed microRNAs in HPBM from osteoporotic Mexican

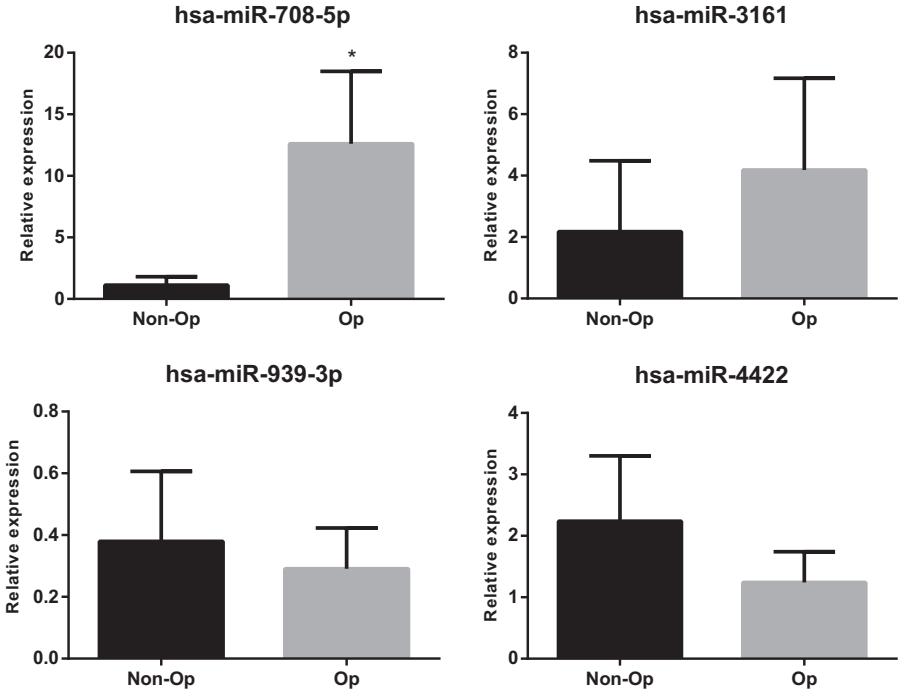

Figure 3. Relative expression of selected miRNAs in the osteoporotic group compared to non-osteoporotic. Significant differences were found only for hsa-miR-708–5 (*$P = 0.01$) by Mann–Whitney U test.

Table 3. KEGG pathway analysis.

Pathway	# of genes	False discovery rate	Genes
HTLV-I infection	9	7.08E-09	*AKT1,AKT2,CCND1,CCND3,CDKN1A,CRTC3,IKBKG,SRF,SMAD3*
PI3K-Akt signaling pathway	7	6.37E-07	*AKT1,AKT2,BCL2,CCND1,CCND3,CDKN1A,IKBKG*
TNF signaling pathway	5	1.42E-06	*AKT1,AKT2,CXCL5,IKBKG,MAP2K3*
Jak-STAT signaling pathway	5	6.40E-06	*AKT1,AKT2,CCND1,CCND3,CNTFR*
Estrogen signaling pathway	4	2.51E-05	*AKT1,AKT2,FKBP5,MMP2*
HIF-1 signaling pathway	4	3.44E-05	*AKT1,AKT2,BCL2,CDKN1A*
MAPK signaling pathway	5	4.26E-05	*AKT1,AKT2,IKBKG,MAP2K3,SRF*
FoxO signaling pathway	5	5.82E-05	*AKT1,AKT2,CCND1,CDKN1A, SMAD3*
p53 signaling pathway	3	0.000288	*CCND1,CCND3,CDKN1A*
NF-kappa B signaling pathway	3	0.000597	*BCL2,IKBKG,PARP1*
Osteoclast differentiation	3	0.00134	*AKT1,AKT2,IKBKG*
Rap1 signaling pathway	3	0.00522	*AKT1,AKT2,MAP2K3*
VEGF signaling pathway	2	0.00744	*AKT1,AKT2*
Cytokine-cytokine receptor interaction	3	0.00943	*CNTFR,CXCL5,EDA2R*
Wnt signaling pathway	3	0.0321	*SMAD3,CCND1,CCND3*

Note: Bone-related signaling pathways from hsa-miR-708-5p validated target genes.

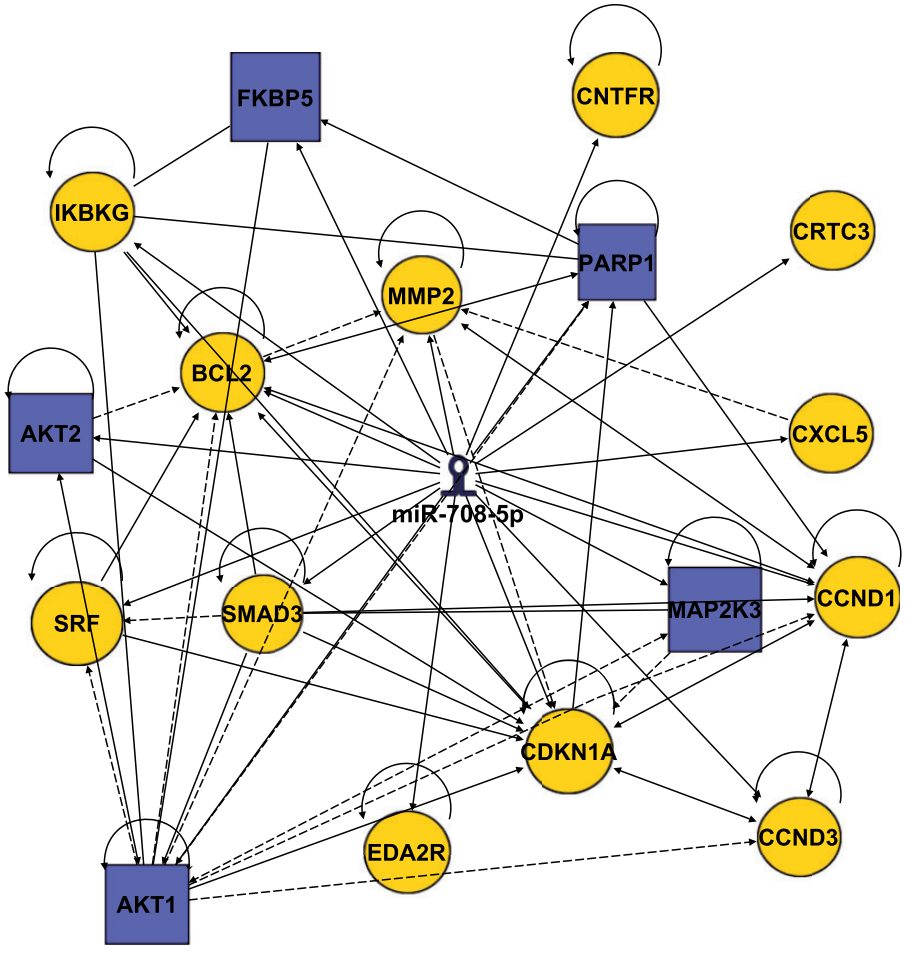

Figure 4. Interaction pathway analysis between hsa-miR-708-5p and its target genes. Circles represent bone-related target genes of hsa-miR-708-5p and squares represent validated target genes of hsa-miR-708-5p from an Affymetrix Gene Chip Human U133 Plus 2.0 Array. Solid lines mean direct interactions, dotted lines mean indirect interactions. Curved lines mean autoregulation. (A color version of this figure is available in the online journal.)

postmenopausal women performing sRNA-Seq, validation by RT-qPCR, and gene set enrichment analysis. The major statement of this work highlights miR-708-5p as a promising novel marker for postmenopausal OP like those reported before (miR-133a, miR-1270, miR-422a, and miR-194-5p).[18,36,37] The microRNA miR-708-5p has been previously identified in cervical cancer and involved in some other disorders, such as neurodegenerative and

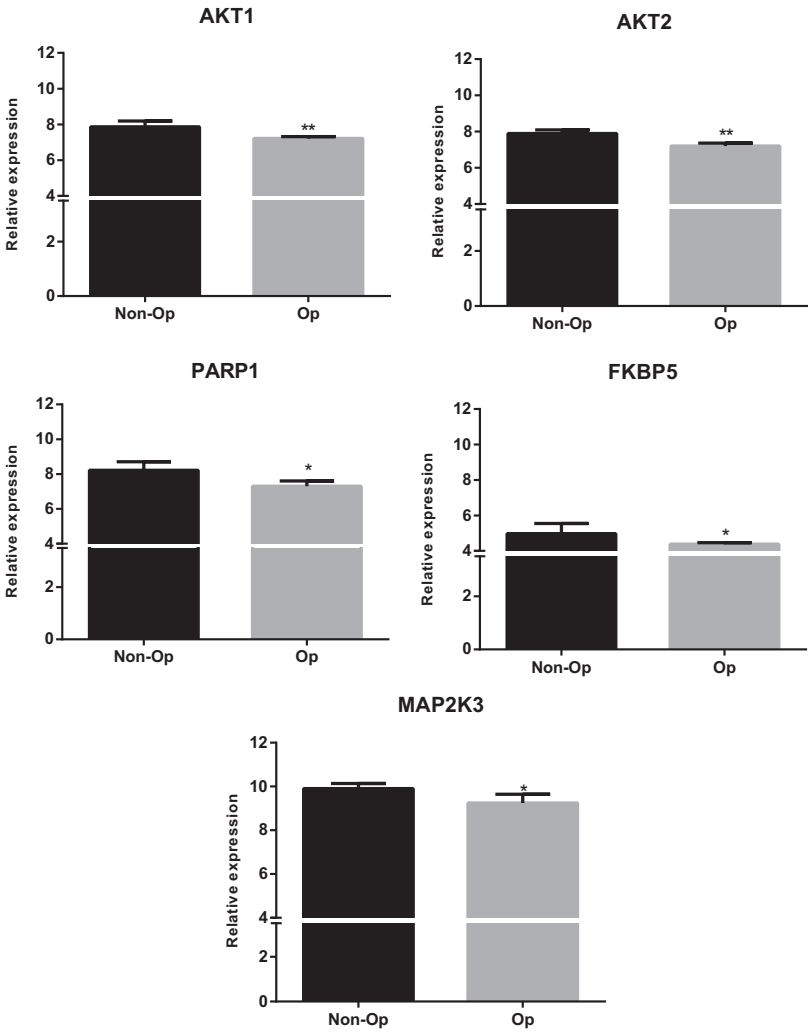

Figure 5. Relative expression of hsa-miR-708-5p target genes from an Affymetrix GeneChip Human U133 Plus 2.0 Array in osteoporotic group compared to non-osteoporotic. (*$P < 0.05$, **$P < 0.005$) by Mann–Whitney U test.

cardiovascular pathologies as well as in the immune response.[38,39]

Possible cellular sources of miR-708-5p were examined. According to the Human miRNA Expression Database (miRmine), miR-708-5p was identified in osteosarcoma cells (143B cell line), and it is not present in human serum from postmenopausal women. Normalized data from the public database "The Human miRNA Tissue Atlas (https://ccb-web.cs.uni-saarland.de/tissueatlas/)" showed low levels of expression for miR-708-5p in bone in contrast to other tissues, including: brain, lung, and colon. However, recent reports showed miR-708 as suppressor of the differentiation of mesenchymal stem cells at a both adipogenic and osteogenic level.[40] To our knowledge, miR-708-5p expression has not been identified in human osteoblasts or osteoclasts nor related with an osteoporotic phenotype. Functional studies in the future will be helpful to comprehend the likely function of miR-708-5p in osteoclastogenesis.

By performing bioinformatic analyzes, we identified and classified target genes for miR-708-5p. Gene enrichment analysis showed 15 KEGG pathways significantly associated with categories involved in bone metabolism. We were especially interested in osteoclast differentiation and Wnt signaling pathways, which comprise and drive the biogenesis of the two most important cells involved in bone remodeling: osteoclasts and osteoblasts, respectively. Through bioinformatic analyzes and literature search, we found key genes for miR-708-5p related to monocyte-osteoclast differentiation and OP: *AKT1, AKT2, CCND1, PARP1, SMAD3, CXCL5, FKBP5, MAP2K3, MMP2,* and *IKBKG*. Further, we found that five of these target genes *(AKT1, AKT2, PARP1 FKBP5, and MP2K3*) showed expected expression changes (lower expression in samples where miR-708-5p was higher) according to an expression analysis employing microarrays on HPBM from postmenopausal women.[18]

Previous reports have highlighted key roles in bone metabolism for the five genes found as targets for miR-708-5p. The Ser and Thr kinase AKT, is exceptionally essential given the effects of AKT dysfunction in different diseases.[41] From the three members of the Akt family, AKT1 is the most common isoform in osteoblasts and osteoclasts. A study in mice showed Akt1 as a key modulator of both

osteoclasts and osteoblasts because it promotes their survival and differentiation maintaining bone mass and turnover.[42] Moreover, Mukherjee *et al.*[43] identified that Akt1 can modulate osteoclast and osteoblast differentiation. Our results confirm previous findings which suggest that *AKT1* gene may have an important role in OP.[44]

Previous studies identified Akt2 as essential regulator of osteogenesis mediated by IGF.[45] Akt2 deficiency inhibits BMP2-regulated osteoblast differentiation from mesenchymal stem cells. Restitution of Akt2 or the up-regulation of the key transcription factor Runx2 suppresses osteoblast differentiation. These observations show important roles for Akt2 during osteogenic differentiation indicating that Akt1 and Akt2 could have different consequences on mesenchymal precursors differentiation. Taken together, the evidence suggests that AKT1 and AKT2 might have an essential role in OP. Because functional studies of AKTs in human are scarce, other analyzes are necessary to explain the function of AKT1 and AKT2 in individuals with OP.

PARP1 also called "ADP-ribosyltransferase diphtheria toxin-like 1" is an ADP-ribosyltransferases family member[46] that has been shown to repress the expression of osteoclastic factors, including: *Tracp* and *Tcirg1* in osteoclastogenesis induced by RANKL.[47] This suggests that PARP1 may also modulate osteoclast differentiation and consequently bone remodeling. Recent studies demonstrated that a PARP1 mutant inhibits osteoclastogenesis, although silencing of PARP1 or the suppression of its enzymatic activity promotes the function and differentiation of osteoclasts, indicating that a stable PARP1 mutant negatively regulates osteoclast differentiation.[48,49] However, additional studies are needed for a better understanding of PARP1 role in bone homeostasis.

The phosphorylation of the "Inhibitor of NF-κB alpha" (IKBKB)[50] is mediated by the association of the "Inhibitor of kappaB kinase alpha" (IKKα) and its cofactor FKBP5 causing proteasome-mediated degradation of IKBKB, translocation to the nucleus of NF-κB and its further activation.[51] FKBP5 is known to have a direct effect on RAW 264.7 osteoclast differentiation by an alternative mechanism besides NF-κB activation. These observations support that the overexpression of FKBP5 partially regulates OP induced by glucocorticoids.[52] More recently, the c.163G>C mutation in *FKBP5* was related to enhanced osteoclast differentiation and activity in Paget's disease of bone.[52,53] Additional functional studies of FKBP5 are necessary to understand its role in the development of OP.

The MAPK p38 family are essential regulators of osteoclastogenesis mediated by RANKL, this has made them candidate therapeutic targets for OP.[54] MAPK kinases, MKK3 also known as MAP2K3, and MKK6, can differentially regulate p38 function.[55] Studies in ovariectomy-mice showed that deficiency of MAP2K3 is related with a phenotype of high bone mass and the down-regulation of NFATc1, a key regulator in osteoclast differentiation. The MAP2K3-deficiency alters bone mass by the regulation of osteoclast numbers in vivo.[56] Targeting MAP2K3 might modulate bone loss or could have positive effects on bone homeostasis by modulating osteoclasts' function or differentiation.

OP is an age-related disease; the mean age of the non-osteoporotic (68.8) and osteoporotic women (70.4) was very similar, excluding the possibility that the discovered microRNA miR-708-5p was influenced by the age of the participants. Moreover, the similar BMI values of both groups show that body weight has minimum or no effect on the differences in the identified microRNA in HPBM from postmenopausal osteoporotic women. The average T-score and hip BMD between both groups showed important differences, making possible to accurate distinguish the osteoporotic and non-osteoporotic women. Therefore, the miRNA identified in this study is more likely to be related with the postmenopausal osteoporotic status. It is expected that the changes in miRNA expression between non-osteoporotic and osteoporotic women should be subtle, as reported by other groups,[57,58] but these small differences could increase exponentially in the bone microenvironment.[59]

This is one of the few studies directed to analyze miRNAs using next-generation sequencing in circulating monocytes. However, our results must be interpreted with caution knowing their limitations. To begin with, the sample size used in this work was somewhat little although comparable to other publications using next-generation sequencing (RNA-seq) in other diseases and circulating monocytes.[15,60,61] Consequently, we were only able to identify one miRNA showing clear changes between groups. Therefore, other miRNAs with slighter changes in their expression might have not been identified. More miRNAs associated with OP could be identified with larger samples. Second, we used raw *P* values instead of adjusting for multiple testing to prevent the loss of power, in agreement with other studies. In this context, false positive results were controlled by confirming the differential expression of miR-708-5p in the sRNA-Seq analysis by RT-qPCR and the possible target genes by the *in silico* microarray analysis.

In conclusion, our results together with the reported roles of *AKT1, AKT2, PARP1 FKBP5*, and *MP2K3* in monocyte differentiation highlight miR-708-5p as a candidate marker for postmenopausal OP in Mexican population. Nonetheless, these findings require validation in independent studies and with bigger sample sizes.

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Authors' contributions: AHCM, EGRS, and RVC designed the study, MQ and JS collected the samples, AHCM and MMMA performed the experiments, AHCM, EGRS, RVC, PGR, and CAG analyzed and interpreted the data, AHCM,

EGRS and RVC wrote the document, PGR and CAG reviewed and revised the document. All authors have reviewed and approved the final version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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