Original Article
MicroRNA expression profiling in an ovariectomized rat model of postmenopausal osteoporosis before and after estrogen treatment

Xiao Xu1,2,3, Peng Zhang2, Xingfu Li1,3, Yujie Liang4, Kan Ouyang1, Jianyi Xiong1, Daping Wang1, Li Duan1

1Department of Orthopedics, Shenzhen Intelligent Orthopaedics and Biomedical Innovation Platform, Guangdong Artificial Intelligence Biomedical Innovation Platform, Shenzhen Second People’s Hospital, The First Affiliated Hospital of Shenzhen University Health Science Center, Shenzhen 518035, Guangdong, China; 2Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences, Shenzhen 518055, Guangdong, China; 3Guangzhou Medical University, Guangzhou 511436, Guangdong Province, China; 4Shenzhen Kangning Hospital, Shenzhen Mental Health Center, Shenzhen 518035, Guangdong Province, China

Received April 25, 2020; Accepted July 18, 2020; Epub August 15, 2020; Published August 30, 2020

Abstract: Postmenopausal osteoporosis (PMOP) is a common disease that seriously threatens human health. Estrogen deficiency plays an essential role in the pathogenesis of PMOP. MicroRNAs (miRNAs) are involved in the development and progression of PMOP. Therefore, identification of miRNAs in PMOP due to estrogen deficiency may contribute to earlier diagnosis and better treatment of this disease. The rat model of PMOP was established by ovariectomy. After one month of treatment, the knee joints were evaluated by microcomputed tomography and histological analysis. The plasma estrogen levels were quantified by enzyme-linked immunosorbent assays (ELISAs). MiRNA levels were analyzed by high-throughput sequencing and validated using quantitative real-time PCR (qRT-PCR). Two months after ovariectomy, osteoporosis occurred in the subchondral bone of the rats in the PMOP group, while fewer symptoms of osteoporosis occurred in the subchondral bone of the rats with estrogen replacement therapy. Cartilage degeneration was detected in the PMOP group. MiR-29a-3p, miR-93-5p, and miR-486 expression decreased in the PMOP group compared to the control group. After estrogen treatment for one month, the plasma levels of miR-29a-3p, miR-93-5p, and miR-486 recovered to the normal levels. Estrogen eliminated the expression changes in miR-29a-3p, miR-93-5p, and miR-486. The identification of these differentially expressed miRNAs will help elucidate the crucial role of miRNAs in the pathogenesis of PMOP. Our data could lead to the potential utilization of miRNAs in the diagnosis of PMOP and provide a possible therapeutic target for treatment of this disease.

Keywords: Postmenopause, osteoporosis, microRNAs, rats, estrogens

Introduction

Postmenopausal osteoporosis (PMOP) is often caused by different predisposing factors, such as aging and persistent calcium loss. PMOP, as a systemic skeletal disease, is often accompanied by an increase in bone fragility and fracture. Due to the increasing incidence of PMOP, it has become a major cause of disability worldwide [1]. Nevertheless, to date, the cause and mechanism of PMOP are still unclear [2]. The role of estrogen in PMOP has attracted increased attention. The increased prevalence of PMOP suggests that there is a link between estrogen and PMOP [3, 4]. Estrogen deficiency could disrupt bone remodeling mediated by the balance between bone resorption and bone formation, resulting in a low bone density and an increased risk of fracture [5-8].

MicroRNAs (miRNAs) are noncoding single-stranded RNA molecules of approximately 22 nucleotides in length that are encoded by endogenous genes. The primary function of miRNAs is to prevent the expression of target proteins through translational repression. Furthermore, miRNAs can degrade target mRNAs. One miRNA can target multiple protein-coding mRNAs, while many miRNAs function synergistically or competitively to target a single mRNA, thus creating a complex regulatory network in numerous biological processes [9]. MiRNAs are
MicroRNA expression profiling in postmenopausal osteoporosis

Linked with many diseases, such as cancer, viral infection, diabetes, immune-related diseases, aging, and neurodegenerative disorders [10-17]. Moreover, the role of miRNAs has been established in different aspects of disease, such as diagnosis, pathogenesis, and therapeutics [18, 19]. Many studies have identified various miRNAs as biomarkers for multiple diseases [20-22]. In the past decade, miRNAs have been recognized as important regulators of bone metabolism [23]. Studies on the relationship between miRNAs and bone metabolism have reported the roles of miRNAs in osteoporosis [24]. However, the connection between miRNAs and bone metabolism has not been fully elucidated.

Some studies have compared the miRNA expression in bone tissue between healthy individuals and PMOP patients and have identified some representative miRNAs [25]. The ovariectomized rat model is the most commonly used animal model for studies of PMOP [26]. However, few studies have been performed to compare the differences in miRNAs in the blood between rats with PMOP and healthy rats. To date, miRNA expression profiling in rats with PMOP caused by estrogen deficiency has not been reported. In this study, differentially expressed miRNAs were identified by miRNA sequencing. The sequencing results were further validated using qRT-PCR. The aim of this study was to identify some miRNAs as novel diagnostic biomarkers for PMOP. The results of this study will also provide valuable insights into the treatment of PMOP.

Materials and methods

Experimental animals and related methods

Six-month-old Sprague-Dawley (SD) female rats were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine. All experimental procedures in this study were performed in accordance with the appropriate ethical guidelines, and the use of animals in this study was approved by the Animal Care and Experiment Committee of Shenzhen Second Peoples’ Hospital. The rats were randomly assigned to the control group and the experimental group (PMOP model). The PMOP models were established by ovariectomy. The rats were anesthetized in a chamber that was saturated with 4% isoflurane. Two months after ovariectomy, microcomputed tomography (micro-CT) was performed to determine whether the PMOP models were successfully established. After the PMOP model was confirmed, the rats were assigned to the PMOP model control group and the estrogen replacement therapy group according to the principle of random distribution. Subsequently, the estrogen replacement therapy group was given subcutaneous injections of estrogen (0.1 mg/kg/day) for one month [27], and the PMOP model group and the control group were given the same amount of physiological saline for subcutaneous injection. One month later, all rats were euthanized under general anesthesia with carbon dioxide (CO₂), and plasma was collected. All the rat experiments adhered to the 3Rs of ethical animal use: replacement, reduction, and refinement. The estrogen level in the blood plasma was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer’s instructions. The knee joints were evaluated by micro-CT and histological analysis. The plasma was further processed for miRNA sequence analysis and qRT-PCR analysis.

Plasma collection and RNA isolation

The plasma was separated from rat heart blood by centrifugation. All plasma samples were immediately frozen in liquid nitrogen. TRIzol (Invitrogen, California, USA) was used to extract total RNA following the manufacturer’s protocol. The concentration and purity of the RNA were measured by a NanoDrop ND-1000 system (Agilent Technologies, Inc., Wilmington, DE, USA) with the A260/A280 ratio. All RNA samples were stored at -80°C until further use.

RNA sequencing

The miRNA sequencing library consisted of the total RNA for each sample. The preparatory process included the following steps: 1) 3’ adaptor ligation, 2) 5’ adaptor ligation, 3) cDNA synthesis, 4) PCR amplification, and 5) library selection. The library was denatured into single-stranded DNA molecules, captured on Illumina flow cells, amplified in situ as clusters, and subjected to 50-cycle sequencing on an Illumina HiSeq sequencer. After sequencing by an Illumina sequencer, image analysis, and base identification, the raw reads after quality con-
MicroRNA expression profiling in postmenopausal osteoporosis

trol were harvested. First, Q30 was used for quality control. CutAdapt software (v1.9.2) was used to de-join the original reads, remove the low-quality reads, and retain the reads with a length ≥15 nucleotides (nt) to obtain the unjoined reads (i.e., trimmed reads). Then, the trimmed reads of all samples were combined, and new miRNA predictions were obtained using miRDeep2 software (v2.0.0.5). The trimmed reads of each sample were aligned to the pooled human pre-miRNAs database [miRBase pre-miRNAs (v21) + newly predicted pre-miRNAs] using Novoalign software (v3.02.12), allowing up to 1 mismatch. The number of tags on each mature miRNA was statistically compared to the original expression level and normalized using the TPM (tag counts per million aligned miRNAs) method.

qRT-PCR analysis

qRT-PCR was used to confirm the expression levels of the selected miRNAs. In brief, the cDNAs were synthesized with SuperScript™ III Reverse Transcriptase (Invitrogen, California, USA) and small RNA-specific RT primers according to the manufacturer’s instructions. PCR was performed with the following parameters: 16°C for 30 min, 42°C for 30 min and 85°C for 5 min and chilled on ice for 5 min, after which the cDNA was stored at -20°C. The TaqMan Small RNA Assay (Applied Biosystems, Carlsbad, USA) was used to perform qRT-PCR in 20 µL reaction mixtures, according to the manufacturer’s instructions. The relative expression levels of the miRNAs were normalized against that of miR-16-5p and were calculated using the 2^(-ΔΔCt) method.

Histological staining

Rats were euthanized, and knee joints were fixed in 4% paraformaldehyde for 48 hours. After micro-CT scanning, the joints were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for two months. Hematoxylin-eosin (HE) staining was performed for histological identification.

Statistical analysis

All numerical data are presented as the mean ± standard deviation. The differences among the three groups were determined by one-way ANOVA. P<0.05 was considered statistically significant. All results were analyzed by SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Characterization of the rat models of PMOP

To determine whether ovariectomy could successfully imitate estrogen deficiency conditions in rats, we compared the E2 levels between healthy control rats and postmenopausal rats. The ELISA results showed that the average plasma level of E2 was 35.9 ± 4.25 pg/mL in female rats at the age of 8 months. However, it was lower than 5.8 pg/mL in the postmenopausal rats, which was the minimally detectable value by ELISA (Figure 1A). Two months after ovariectomy, the healthy anatomic structure of the knee joint was lost, and signs of osteoporosis were detected in the PMOP model (Figure 1C). An increase in trabecular space and a decrease in bone mass were detected in the micro-CT image. Subchondral bone destruction and osteoporosis were clearly present in the three-dimensional (3D) reconstruction (Figure 1E). These data suggested that the rat model of PMOP was successfully established.

Distinct miRNA expression profile in the rats with PMOP compared to the healthy controls

We performed miRNA sequencing using plasma obtained from the control group, the PMOP model group and the estrogen replacement therapy group to investigate miRNA expression. We analyzed the miRNA expression data by calculating the fold changes (≥1.5 or ≤-1.5) and P-values (P<0.05) based on the expression ratios. In the comparison between the PMOP model group and the control group, 28 differentially expressed miRNAs were found; 25 were downregulated and the rest were upregulated (Figure 2). Detailed information on the differentially expressed miRNAs is listed in Table 1.

qRT-PCR analysis of differentially expressed miRNAs

The microarray data were validated by qRT-PCR. Nine selected miRNAs were quantified by qRT-PCR using the miRNA SYBR Green assay. Nine miRNAs were differentially expressed between the control group and the PMOP group (Figure 3A-I). The expression values (-delta CT)
Figure 1. Estrogen deficiency leads to bone loss. A. The estrogen expression level significantly decreased in the PMOP group compared with the control group. B. Morphometric analysis showed that the tibia/fibula in the control group was normal. C. The tibia/fibula exhibited osteoporosis in the PMOP model group. D. Subchondral bone showed a healthy bone mass in the control group. E. Bone loss of subchondral bone in the PMOP model group was detected. All these results revealed that estrogen deficiency could induce osteoporosis, and the PMOP model was successfully established.
from the qRT-PCR experiment validated the microarray data.

**Estrogen could upregulate the decreased miR-29a-3p, miR-93-5p and miR-486 levels in PMOP and reverse osteoporosis in subchondral bone of the knee**

To verify whether estrogen can protect against the reduction of miRNA expression induced by PMOP, we selected miR-29a-3p, miR-93-5p, and miR-486, which were significantly reduced in the PMOP model group compared with the control group. Then, miRNA expression was detected using qRT-PCR to verify the findings of miRNA sequencing. Estrogen treatment significantly inhibited the decreased miR-29a-3p, miR-93-5p, and miR-486 levels in the PMOP group (Figure 4).

Our results showed that rats with estrogen replacement therapy had less severe osteoporosis of the knee subchondral bone than the untreated rats. Notably, micro-CT analysis showed that the PMOP model group, compared to the control group, had a reduced bone volume fraction (BV/TV) (P<0.01). The results suggest that the bone mass was reduced in the rats with PMOP compared to the control rats. Although no significant difference was observed in the BV/TV values between the PMOP model group and the estrogen replacement therapy group, estrogen replacement therapy could partly reverse osteoporosis (Figure 5).

The knee joints of the rats in all groups underwent H&E staining to facilitate the observation of histological changes. In the control group, the cartilage of the knee joint was smooth on
the surface, with regularly arranged chondrocytes. A well-developed tubular compact substance and well-developed spongious substance could be observed in the subchondral bone structure. In the PMOP model group, an irregular superficial layer of cartilage and decreases in chondrocytes and cartilage matrix were observed. The bone trabecular arrangement, the number of trabeculae and the trabecular connections in the rats of the PMOP group were decreased compared with those of the control group (Figure 6).

Discussion

Osteoporosis is a systemic disease characterized by reduced bone mass and degeneration of bone tissue. The incidence of osteoporosis increases with age, and approximately 30% of postmenopausal women show symptoms of osteoporosis [28, 29]. An imbalance between the rates of bone formation and resorption results in abnormal bone remodeling [30]. There are many causes of PMOP, but the exact molecular mechanism of PMOP has not yet been elucidated. Multiple factors have been associated with the occurrence of PMOP. Among them, changes in miRNA expression profiles may indicate the progression of PMOP [31]. At present, no sensitive diagnostic biomarkers for PMOP are available. In this study, miR-93-5p, miR-486 and miR-29a-3p were shown to be specific, simple and rapid biomarkers for the early diagnosis of PMOP.

The animal model of PMOP can mimic the physiologic characteristics of postmenopausal women. In this study, ovariectomy was used to
efficiently and quickly establish a rat model of PMOP. The bone loss features of the PMOP model are similar to bone changes in postmenopausal patients [32]. The data from micro-CT scanning are the gold standard for assessing the risk of osteoporosis [33, 34]. Therefore, in this study, the changes in subchondral bone were evaluated by micro-CT, directly reflecting the degree of osteoporosis. Two months after the operation, the rats in the PMOP model group showed significant osteoporosis in the subchondral bone, suggesting that the PMOP model was successfully established. After one month of estrogen treatment, the rats in the estrogen replacement therapy group showed a lower degree of osteoporosis in the subchondral bone than the untreated rats. Our investigation suggests that estrogen plays a role in the treatment of PMOP. Other studies have also found that estrogen has a therapeutic effect on PMOP [35, 36].

Ovariectomized rats are standard animal models for PMOP studies. Bone density by X-ray or micro-CT scanning under anesthesia is a routine marker to indicate whether the animal model was established successfully, which is costly and time-consuming. Compared to bone density detection, measurement of the miRNA level of animal plasma is economical and straightforward. Based on the miRNA results of this study, the expression levels of miR-29a-3p and miR-93-5p in the plasma could be potential diagnostic markers for PMOP in rat models.

MiRNAs are small noncoding RNAs that regulate a wide range of physiological cell processes. The abnormal expression of miRNAs is associated with the pathogenesis and status of many diseases, such as PMOP [37-42]. In this experiment, we found that the miR-29a-3p level in rat plasma of the PMOP model group was

Figure 4. MiRNA levels were evaluated by qRT-PCR after estrogen treatment. Estrogen treatment significantly reversed the decline in the miR-29a-3p, miR-93-5p, and miR-486 levels in the PMOP model group.
Studies have shown that miR-29a-3p expression in PMOP patients is significantly lower than that in healthy patients [43]. MiR-29a-3p showed a protective effect in a glucocorticoid-induced mouse model of osteoporosis by repressing glucocorticoid-induced osteoporosis. 

Figure 5. Estrogen replacement therapy prevents estrogen deficiency-induced osteoporosis. A. The morphometric analysis of the tibia/fibula in the control group was normal. B. The tibia/fibula exhibited osteoporosis in the PMOP model group. C. Estrogen replacement therapy ameliorated osteoporosis in the tibia/fibula. D. A healthy bone mass of subchondral bone was observed in the control group. E. Bone loss of subchondral bone was observed in the PMOP model group. F. Estrogen replacement therapy increased the bone mass in the subchondral bone. G. Bone volume/tissue volume (BV/TV) of subchondral bone shows that estrogen replacement therapy significantly increased the bone mass.

Figure 6. Histological analysis of the protective role of estrogen in cartilage and subchondral bone. A. The smooth surfaces and the healthy bone mass in the control group. B. The rough surfaces and bone loss in the postmenopausal osteoporosis (PMOP) model group. Magnification 40×.
MicroRNA expression profiling in postmenopausal osteoporosis

coid-induced excessive osteoclast formation and bone resorption [44]. Other studies also found that the expression of miR-29a-3p was advantageous for delaying the progression of glucocorticoid-mediated osteoporosis [45]. MiR-29a-3p contributed to bone mass in mouse models by positively regulating osteoblast differentiation [46, 47]. Data from animal studies and clinical investigations suggest that the therapeutic effect of estrogen on PMOP could be due to increased miR-29a-3p expression levels.

The activity between osteoblasts and osteoclasts influences bone remodeling. One study found that miR-93-5p was reduced during osteoblast differentiation and mineralization [48, 49]. Another study revealed that miR-93-5p was significantly upregulated in the serum of male and female osteoporotic patients [50, 51], which could be explained by the inhibitory effect of miR-93-5p on osteoblast differentiation. The role of miR-93-5p in osteoclasts remains unclear. In this study, we found a low expression of miR-93-5p in the rat plasma of PMOP. Different expression levels of miR-93-5p in rats with PMOP and patients with osteoporosis suggest that miR-93-5p may play an essential role in osteoclasts.

The expression of miR-486 was significantly decreased in the plasma of the rats with PMOP. The expression levels of miR-486 changed with the level of estrogen, suggesting a close relationship between miR-486 and estrogen. Few studies have been carried out to study the role of miR-486 in the pathological process of PMOP. Further studies are needed to demonstrate the specific mechanism of miR-486.

The effects of estrogen on the expression of miRNAs in osteoporosis are still largely unclear. In osteoporosis models induced by estrogen deficiency, inhibition of miR-21 may impair bone formation, and miR-21 also plays an essential role in estrogen-induced osteoclast apoptosis [52, 53]. During osteoclast formation, estrogen has a negative effect on bone degradation [54, 55]. In this study, three new biomarkers were identified. However, the mechanisms between estrogen and miR-29a-3p, miR-93-5p, and miR-486 require further investigation.

MiR-101a-3p and miR-374-3p were upregulated miRNAs. To our knowledge, miR-101a-3p is mainly related to mental diseases and heart disease [56-58], and miR-374 has a protective effect on chondrocyte in OA [59]. The functions of miR-101a-3p and miR-374-3p in PMOP need further study.

Although our efforts were mainly focused on osteoporosis, accumulating evidence supports a relationship between osteoporosis and osteoarthritis (OA). Osteoporosis can result in cartilage degeneration [60]. Patients with osteoporosis are more likely to develop OA [61]. Bone and cartilage are considered functional units in the pathogenesis of OA [62]. The changes in subchondral bone could reflect the degree of cartilage degeneration. Cartilage degeneration accelerates the loss of subchondral bone. Some studies have found that bone mass plays a protective role against cartilage degeneration in knee OA [63]. Bone mineral density (BMD), a main risk factor for osteoporosis, can reflect the severity of OA to some extent [64]. Prior induction of osteoporosis can exacerbate the severity of cartilage lesions in experimental animal models of OA, indicating that the pathological changes in the knee joints of individuals with osteoporosis can accelerate the progression of OA [65, 66]. Meanwhile, anti-osteoporosis drugs have shown beneficial effects on OA by improving subchondral bone quality by regulating the metabolic activity of bone and thus maintaining stability between subchondral bone and cartilage [67, 68]. In this study, we found that miR-29a-3p, miR-93-5p, and miR-486 could be promising candidates for biomarkers in the early diagnosis of osteoporosis. Interestingly, these miRNAs also have a connection with OA [69-71]. The results of this study provide more evidence to support a close relationship between osteoporosis and OA.

Conclusions

The results of this study demonstrated that miRNA dysregulation might participate in the pathogenesis of PMOP. MiR-29a-3p, miR-93-5p, and miR-486 may provide novel biomarkers for the diagnosis and treatment of osteoporosis.

Acknowledgements

National Natural Science Foundation of China (No. 81772394; No. 81972116; No. 819720-85); Key Program of Natural Science Founda-
MicroRNA expression profiling in postmenopausal osteoporosis


[40] Chen Z, Bemben MG and Bemben DA. Bone and muscle specific circulating microRNAs in postmenopausal women based on osteoporosis and sarcopenia status. Bone 2019; 120: 271-278.


[59] Shi FL and Ren LX. Up-regulated miR-374a-3p relieves lipopolysaccharides induced injury in CHON-001 cells via regulating Wingless-type MMTV integration site family member 5B. Mol Cell Probes 2020; 51: 101541.


[68] Karsdal MA, Bay-Jensen AC, Lories RJ, Abramson S, Spector T, Pastoureau P, Chris-

