Up-Regulation of Urinary Exosomal Hsa-microRNA-200b-3p and Hsa-microRNA-206 in Patients of Steroid-induced Osteonecrosis of Femoral Head

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Research

Keywords: Steroid-induced osteonecrosis of femoral head, urinary exosome, microRNAs, bioinformatics analysis

DOI: https://doi.org/10.21203/rs.3.rs-68462/v1
Abstract

Background:

Urine exosomal microRNAs (miRNAs) play vital roles in the occurrence and development of various diseases. However, less is known about miRNAs in steroid-induced osteonecrosis of the femoral head (SONFH). Here, we aimed to determine the value of miRNAs in urinary exosomes in the diagnosis of SONFH.

Methods:

RNA was extracted from urinary exosomes from 9 SONFH patients and 9 hip osteoarthritis (HOA) patients with age and gender matched and then miRNAs were analyzed by next generation sequencing. Next, the putative target genes of dysregulated miRNAs were predicted by miRNA databases. Furthermore, GO function, KEGG pathway, miRNAs-mRNAs network and protein-protein interaction (PPI) network were also constructed to analyze potentially pathological mechanisms. qRT-PCR and area under curve (AUC) analysis were performed to determine the diagnostic value of miRNAs. This study was approved by ethics review board in 1st Affiliated Hospital, Guangzhou University of Chinese Medicine.

Results:

Intriguingly, 15 miRNAs including hsa-miR-200b-3p and hsa-miR-206 were significantly upregulated in exosomes from SONFH patients. Furthermore, qRT-PCR and area under curve (AUC) analysis of an independent cohort of 30 SONFH patients, 10 HOA patients and 10 healthy donors confirmed that hsa-miR-200b-3p and hsa-miR-206 were upregulated in SONFH samples which AUC values were 0.938 (95 % CI: 0.828-1) and 0.926 (95 % CI: 0.806-1) respectively. The enriched functions and pathways included Hippo, PI3K-Akt, TGF-β and Wnt signaling pathways. The top five hub genes (MAPK1, EP300, RHOA, PIK3CA, and CBL) were selected from PPI network, which consisted of 180 nodes and 518 edges.

Conclusions:

Collectively, our results showed that hsa-miR-200b-3p and hsa-miR-206 in urinary exosomes which might serve as non-invasive biomarkers for SONFH.

Background

Steroid-induced osteonecrosis of femoral head (SONFH) is a refractory disease commonly affecting active adults between the third and fifth decade of life [1, 2]. SOFNH usually results in collapse of the femoral head and dysfunction of the hip joint which eventually results in the joint arthroplasty surgery [3, 4]. SONFH develops in 9% to 40% of patients receiving long-term steroid although it may also occur with short-term exposure to high doses of steroid [5]. The numbers of high-risk SONFH patients are pretty huge due to the large population with several diseases such as systemic lupus erythematosus, nephrotic syndrome as well as psoriasis who receive steroid therapy. Unfortunately, SONFH are commonly
asymptomatic at the initial occurrence which makes it very difficult to screen and manage in high-risk populations with conventional tools like MRI [6, 7]. Therefore, there is an urgent need for early diagnostic biomarkers for SONFH.

Biomarkers, referred to as liquid biopsies, can often be measured in many kinds of biofluids including urine for diseases diagnosis [8]. Remarkably, urinary exosomes contain molecules that reflect the disease status, and are considered to be a new type of liquid biopsies [9]. In particular, microRNAs (miRNAs) derived from urinary exosomes have been proved to be involved in the regulation of gene expression and play an essential role in the development of human diseases [10]. MiRNAs, as the main RNA in urinary exosomes, have been turned out to be more stable due to the protection of bilayer membrane which facilitates the urinary exosomal miRNA to be a noninvasive biomarker for early disease diagnosis [11, 12]. Recently, several studies have suggested that miRNAs play a significant role in the development of SONFH. What is more, Ye et al has reported differential expression urinary miRNAs in diagnosing idiopathic osteonecrosis of femoral head by analyzing miRNA PCR array [7]. However, the role of urinary exosomal miRNAs on the diagnosis of SONFH remains elusive.

In this study, we investigated the use of next generation sequencing to identify novel miRNA-based SONFH biomarkers in urinary exosomes and used quantitative real-time polymerase chain reaction (qRT-PCR) and area under curve (AUC) to validate the results. Furthermore, Gene Ontology (GO) function, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, miRNAs-mRNAs network and protein-protein interaction (PPI) network were also constructed to analyze potentially pathological mechanisms.

Materials And Methods

Patients and Samples Collection

The study was approved by the Ethical Committee of Guangzhou University of Chinese Medicine, and written informed consents were obtained from all patients (No. ZYYECK [2017] 033). As shown in Table 1, among the 50 people, 30 patients were diagnosed as SONFH, 10 patients were related to hip osteoarthritis (HOA), while others were defined as the healthy group. The average age of 30 patients in SONFH group was 45.37 years (range, 30–60 years), 46.70 years (range, 38–60 years) in HOA group and 44.40 years (range, 32–59 years) in healthy control. The diagnoses of SONFH and HOA were confirmed in all the patients [13, 14]. All urine samples were obtained from our orthopedic department from June 2017 to June 2019. The midstream urine of all the patients was collected in the 50 mL tube, then removed cell debris and other impurities using the centrifuge. Finally, the supernatant was extracted to a new tube and frozen at -80 °C for the following analysis.
Table 1
Patient demographics and clinical profiles.

| Factors          | SONFH (n = 30) | HOA (n = 10) | Healthy control (n = 10) |
|------------------|---------------|--------------|--------------------------|
| Gender, n (%)    |               |              |                          |
| Female           | 20 (66.67%)   | 7 (70%)      | 6 (60%)                  |
| Male             | 10 (33.33%)   | 3 (30%)      | 4 (40%)                  |
| Age (years)      | 45.37 ± 1.62  | 46.70 ± 2.43 | 44.40 ± 3.02             |
| ARCO stages, n (%)|             |              |                          |
| II               | 3 (10%)       | 0 (0%)       | 0 (0%)                   |
| III              | 16 (53.33%)   | 0 (0%)       | 0 (0%)                   |
| IV               | 11 (36.67%)   | 0 (0%)       | 0 (0%)                   |

Notes: SONFH: steroid-induced osteonecrosis of femoral head; HOA: hip osteoarthritis; ARCO: Association Research Circulation Osseous

Table 2
The miRNAs were selected to qRT-PCR validation.

| miRNA ID   | Forward 5’-3’ | Reverse 5’-3’ |
|------------|---------------|---------------|
| hsa-miR-200b-3p | GCTGCTGAATTCCATCTAATTCCAAAAG | TATTATGGATCCGCCCTGAGGCAATGGG |
| hsa-miR-206   | CGTCAGAAGGAATGATGCACAG   | ACCTGCATGTACATTTTTACATGT   |
| U6           | AGAGAAGATAGCAGGCCCCTG   | ATCCAGTGCAGGGGTCCGAGG     |

Urinary exosomes extraction

According to the instruction of manufacture, the urine samples were added 1/3 volume of RiboTM Exosome Isolation Reagent and upside down until completely mixed at 4 °C overnight. Next, 2 mL mixture was transferred to a new 2 mL tube and centrifuged at 1500 RCF for 30 s at 4 °C. After the supernatant discarded, a small portion of exosome was collected. Repeating the above step, the exosome pellet remained at the bottom of the tube until all the mixture was transferred.

Transmission electron microscopy (TEM)

Morphological examination was typically carried out using TEM which allowed investigators to appreciate the vesicular shape of exosome in addition to obtain an estimated measure of their diameter. The sample of exosome was dissolved in 0.01 mol/L phosphate-buffered saline (PBS). Subsequently,
10 µl of the suspension was spotted onto a glow-discharged copper grid for 2 s and then drained by the filter paper. Finally, exosome was stained with a drop of 3% aqueous solution of phosphotungstic acid for 2 s then was dried for several minutes. Exosome was observed using a TEM at 80 K electron volts.

**Nanoparticle-tracking analysis (NTA)**

We analyzed the size distribution of the exosome particles by a NanoSight NS300 Instrument (Malvern Instruments, UK) under the following conditions: cell temperature: 22 °C; Syringe speed: 40 µl/s. Exosomes were diluted with particle-free PBS, then injected into the NanoSight sample pool through the syringe. The particle concentrations and size distribution profiles were recorded and analyzed by NTA 3.2 software with the camera level of 15 and detection threshold of 4.

**Flow cytometry analysis of exosomes**

Exosomal surface proteins extracted from urine were identified by flow cytometry following a standard protocol. Specific primary antibodies were as follows: Anti-CD63(BD 557288) and Anti-CD81 (BD 551108). Briefly, urinary exosomes were coupled with Latex Beads (Invitrogen) and then blocked with 0.1% bovine serum albumin (BSA) (Sigma Aldrich). The samples were washed by PBS, stained with antibodies and tested according to the operation procedures of the instruments (BD AccuriTM C6 flow cytometer) equipped with a 488 nm, 50 mW solid-state laser.

**MiRNA-sequencing**

The urine exosomal miRNA-sequencing experiment was performed by RiboBio company (Guangzhou, China). Briefly, total RNA samples were fractionated on a 15% Tris-borate-EDTA polyacrylamide gel (Invitrogen) and only small RNAs ranging from 18 to 30 nucleotides were used for library preparation. After amplification by PCR, products were sequenced using the Illumina HiSeq 2500 platform. Sequencing data with P < 0.05 were considered as differentially expressed miRNAs.

**Analysis of miRNAs-mRNAs regulatory network**

The two groups selected the miRNAs according to the standard of area under the receiver operator characteristic (ROC) curve > 0.8. Dysregulated miRNA target genes were predicted by ENCORI (http://starbase.sysu.edu.cn/), miRDB (http://mirdb.org/), miRwalk (http://mirwalk.umm.uni-heidelberg.de/) and TargetScan7.2 (http://www.targetscan.org/vert_72/) databases. Using three or four databases jointly predicted results as candidate target genes of miRNAs. At last, the miRNAs-mRNAs regulatory network was constructed by the open-source software Cytoscape 3.7.1.
Go annotation and KEGG pathway analysis

DAVID (Database for Annotation, Visualization and Integrated Discovery) (http://david.abcc.ncifcrf.gov/) is a bioinformatics database that integrates biological data and analysis tools to provide systematic comprehensive annotation of biological function for large-scale gene or protein list. GO function and KEGG pathway were utilized to analyze based on DAVID. GO analysis includes three aspects: biological process (BP), cellular component (CC) and molecular function (MF).

PPI network construction and modules selection

The predicted mRNAs regulated by the candidate nine miRNAs were mapped to the STRING (http://string.embl.de/) database to establish the PPI network with the highest confidence > 0.9. Subsequently, the PPI network was evaluated by gene networking tool (Cytoscape 3.7.1). Then, Hub genes, the vital nodes in the PPI network, were selected by the degree algorithm of cytoHubba. Finally, the Molecular Complex Detection (MCODE) was carried out to identify crucial modules of PPI network with degree cutoff = 2, node score cutoff = 0.2, κ-core = 2, and max. depth = 100.

qRT-PCR analysis of miRNAs

Exosomal miRNA expression was examined using qRT-PCR in bone and urine samples (10 from healthy controls, 30 from SONFH patients, and 10 from HOA patients). Total RNA was extracted by an RNeasy Mini Kit (Introgen, Australia) according to the manufacturer's protocol. The cDNA was reverse transcribed and quantified by total RNA template using RIBOBIO miRNA primers specific for hsa-miR-200b-3p and hsa-miR-206 (Table. 2) and the Bulge-Loop™ miRNA qRT-PCR Starter Kit (RIBOBIO, Guangzhou, China) following the manufacturer's instructions. qRT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) on the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). PCR amplification was performed using the following parameters: 95 °C for 20 s, followed by 95 °C (10 s), 60 °C (20 s), and 70 °C (10 s) for 40 cycles, and a final extension step of 70 °C for 10 s. Relative level of miRNA expression was normalized to the housekeeping gene U6.

Statistical analysis

All data presented are acquired from at least three independent experiments. Data are displayed as means ± standard error of mean (SEM). Student's t test was performed to measure the significant difference between two groups. Comparisons of multiple groups were calculated using one-way ANOVA. \( P<0.05 \) was considered to be statistically significant.

Results

Characteristics of exosomes in urine
To quantify the exosomes isolated through RibotM Exosome Isolation Reagent, we detected exosome using TEM, NanoSight and flow cytometry analysis (Fig. 1). TEM indicated that the diameter of the serum exosomes ranged from 50 to 100 nm (Fig. 1A), whereas NTA showed the size distribution of the exosomes were abundant in 100–150 nm (Fig. 1B). Flow cytometry analysis of exosomal surface proteins represented high expression of CD63 and CD81 (96.9% and 98.8% respectively) (Fig. 1C).

**Identifying differently expressed profile of the miRNAs in urine exosomes between patients with SONFH and HOA**

In contrast to patients with HOA, total of 28 differently expressed miRNAs (P < 0.01) in urinary exosomes of patients with SONFH were identified by miRNA sequencing analysis (Table 3). Hierarchical clustering and volcano plot (Fig. 2A, B) were performed to show statistically significant miRNAs, and up-regulated miRNAs were represented in red and down-regulated ones were in blue. To investigate the diagnostic value of miRNAs in our discovery sets, we constructed ROC curves using logistic regression models. AUC measures were > = 0.8 for SONFH groups compared to HOA controls (Table. 4).
### Table 3
Differentially abundant urinary exosomal miRNAs in SONFH compared with HOA patients, including 15 up-regulated miRNAs and 13 down-regulated miRNAs.

| MiRNA       | Log2(fold change) | P-value          | up/down |
|-------------|-------------------|------------------|---------|
| hsa-miR-1269a | 8.4259            | 7.07E-05         | up      |
| hsa-miR-486-5p | -3.5821           | 0.000192511      | down    |
| hsa-miR-155-5p | 7.5623            | 0.000207895      | up      |
| hsa-miR-331-3p | 7.4267            | 0.000226272      | up      |
| hsa-miR-451a | -3.9988           | 0.000276687      | down    |
| hsa-miR-122-5p | 3.1975            | 0.001284707      | up      |
| hsa-miR-7641 | 5.4938            | 0.001570851      | up      |
| hsa-miR-3180 | -5.9866           | 0.001829838      | down    |
| hsa-miR-320b | -1.3148           | 0.001923135      | down    |
| hsa-miR-142-3p | -1.3258           | 0.002052384      | down    |
| hsa-miR-200b-3p | 3.8559           | 0.00230958       | up      |
| hsa-miR-206  | 3.0967            | 0.002550576      | up      |
| hsa-miR-320c | -1.2339           | 0.002718105      | down    |
| hsa-miR-1293 | -5.2895           | 0.003312019      | down    |
| hsa-miR-3180-3p | -5.0648          | 0.003401133      | down    |
| hsa-miR-100-5p | 2.6931            | 0.004396865      | up      |
| hsa-miR-3591-3p | 3.4737           | 0.004652852      | up      |
| hsa-miR-10a-3p | 4.3681            | 0.005251878      | up      |
| hsa-miR-335-3p | 7.2219            | 0.006135322      | up      |
| hsa-miR-506-3p | 6.8737            | 0.006460523      | up      |
| hsa-miR-4539 | -4.5135           | 0.007192054      | down    |
| hsa-miR-3168 | -4.9686           | 0.007707735      | down    |
| hsa-miR-381-3p | 3.8833            | 0.007720655      | up      |
| hsa-miR-486-3p | -3.9845           | 0.009076666      | down    |
| hsa-miR-181c-5p | 5.8787            | 0.009378071      | up      |
| hsa-miR-483-5p | -4.4283           | 0.009557514      | down    |
### Table 4
Area under the curve (AUC) for urinary exosomal miRNAs level in the diagnosis of SONFH.

| MiRNA        | AUC   | 95%CI     | specificity | sensitivity |
|--------------|-------|-----------|-------------|-------------|
| hsa-miR-200b-3p | 0.938 | 0.828-1   | 0.778       | 1           |
| hsa-miR-206   | 0.926 | 0.806-1   | 0.778       | 1           |
| hsa-miR-3591-3p | 0.926 | 0.806-1   | 0.889       | 0.889       |
| hsa-miR-122-5p | 0.913 | 0.775-1   | 0.889       | 0.889       |
| hsa-miR-100-5p | 0.877 | 0.692-1   | 0.778       | 1           |
| hsa-miR-7641  | 0.827 | 0.638-1   | 0.889       | 0.778       |
| hsa-miR-381-3p | 0.815 | 0.630-1   | 1           | 0.667       |
| hsa-miR-1269a | 0.833 | 0.670–0.997 | 1           | 0.667       |
| hsa-miR-155-5p | 0.833 | 0.670–0.997 | 1           | 0.667       |
| hsa-miR-331-3p | 0.833 | 0.670–0.997 | 1           | 0.667       |
| hsa-miR-10a-3p | 0.827 | 0.638-1   | 0.889       | 0.778       |

**The biomolecular information and pathways of miRNAs involved in SONFH.**

Between SONFH and HOA groups, we selected the 9 miRNAs (hsa-miR-200b-3p, hsa-miR-206, hsa-miR-122-5p, hsa-miR-100-5p, hsa-miR-381-3p, hsa-miR-1269a, hsa-miR-155-5p, hsa-miR-331-3p and hsa-miR-10a-3p) according to AUC ranking, their target genes were predicted by ENCORI, miRDB, miRWalk and TargetScan, which jointly predicted results as candidate target genes for miRNAs. The functions and pathways enrichment of candidate genes were analyzed using two online databases, including KEGG pathway (http://www.genome.jp/kegg) and Gene Ontology website (http://www.geneontology.org/). As shown in Fig. 3A-C, candidate target genes involved in GO terms (biological process, molecular function and cellular component) primarily focused on gland development \( (p = 4.85 \times 10^{-15}) \), cell-cell junction \( (p = 6.25 \times 10^{-11}) \), DNA-binding transcription activator activity \( (p = 8.69 \times 10^{-15}) \). SONFH molecular pathogenic pathway in KEGG primarily were Hippo signaling pathway \( (p = 1.73 \times 10^{-7}) \), PI3K-Akt...
signaling pathway \( (p = 1.14 \times 10^{-6}) \), TGF-β signaling pathway \( (p = 6.04 \times 10^{-6}) \), and Wnt signaling pathway \( (p = 2.68 \times 10^{-5}) \) (Fig. 3D).

**PPI network construction and analysis of modules**

In order to analyze the pathogenesis of different miRNAs in the development of femoral head necrosis, we constructed a protein-protein interaction network of 9 miRNAs and the predicted target mRNAs between SONFH and HOA group. Using the STRING database (http://string-db.org) and Cytoscape software, total of 1418 mRNAs were filtered into PPI network complex, containing 180 nodes and 518 edges (Fig. 4A). Top 5 hub genes of the PPI network through the degree algorithm of cytoHubba were identified as mitogen-activated protein kinase 1 (MAPK1), E1A binding protein p300 (EP300), ras homolog family member A (RHOA), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA), Cbl proto-oncogene (CBL) as so on. Top 20 hub genes of the overall PPI network were displayed in Table 5.

| Rank | Gene       | Score | Rank | Gene       | Score |
|------|------------|-------|------|------------|-------|
| 1    | MAPK1      | 67    | 11   | ESR1       | 34    |
| 2    | EP300      | 66    | 12   | WASL       | 33    |
| 3    | RHOA       | 54    | 12   | SMAD3      | 33    |
| 4    | PIK3CA     | 50    | 14   | MAPK8      | 32    |
| 5    | CBL        | 48    | 14   | ACTR3      | 32    |
| 6    | BTRC       | 39    | 16   | NCOA3      | 31    |
| 7    | VAMP2      | 38    | 16   | CLTC       | 31    |
| 7    | KRAS       | 38    | 16   | AP2A1      | 31    |
| 9    | ARRB1      | 37    | 16   | KEAP1      | 31    |
| 10   | DVL2       | 35    | 20   | WNT5A      | 30    |

According to the degree of importance, top 2 significant network modules were obtained from the PPI network for further analysis using Cytotype MCODE. Pathway enrichment analysis showed that module 1 with MCODE score of 24.49 (nodes = 50) (Fig. 4B), which were chiefly connected with Hippo and Wnt signaling pathway. Module 2 with MCODE score of 17 (nodes = 17) (Fig. 4C) were mainly related to spliceosome. Hub genes, namely, CBL, beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC), vesicle associated membrane protein 2 (VAMP2), arrestin beta 1 (ARRB1), dishevelled segment polarity protein 2 (DVL2), WASP like actin nucleation promoting factor (WASL), actin related protein 3
(ACTR3), adaptor related protein complex 2 subunit alpha 1 (AP2A1), kelch like ECH associated protein 1 (KEAP1) and Wnt family member 5A (WNT5A) were present in module 1.

**MiRNAs-mRNAs interaction network**

To determine the regulatory connection between miRNAs and the predicted genes, miRNA-mRNA interaction network has been evaluated by gene networking tool (Cytoscape 3.7.1). As shown in Fig. 5, 1221 selected genes were identified as targets of top 9 miRNAs. The miRNAs are presented in yellow triangles while mRNAs were showing in dark blue, sky blue, green, red and pink triangles based on the number of their regulating miRNAs. Dark blue color represents one interaction while sky blue color represents two; green color, three; red color, four; and pink color, five interactions. As demonstrated in Fig. 5, genes, namely clock circadian regulator (CLOCK), zinc finger protein 652 (ZNF652) and nuclear factor I A (NFIA), were the most mutually cross-linked. In addition, hsa-miR-200b-3p and hsa-miR-206 were connected with 361 and 289 target genes, making them the most conspicuous miRNAs that could be involved in the pathogenesis of SONFH.

**Hsa-miR-200b-3p and hsa-miR-206 are highly expressed in urinary exosomes from patients with SONFH.**

To determine whether exosomal miRNAs could be detected in urine from patients, we collected 10 urine samples from healthy control, 30 urine samples from SONFH group and 10 urine samples from HOA group. Total RNA was isolated from the urine exosome and the expression of hsa-miR-200b-3p and hsa-miR-206 was analyzed by qRT-PCR. As shown in Fig. 6A-B, the expression of urine exosomal hsa-miR-200b-3p and hsa-miR-206 was significantly higher in the group of SONFH patients compared to healthy control and HOA group. ROC curves were constructed to compare the diagnostic value of hsa-miR-200b-3p (Fig. 6C) and hsa-miR-206 (Fig. 6D) to predict SONFH. The AUC for hsa-miR-200b-3p was 0.938 (95% CI: 0.828-1) and for hsa-miR-206 was 0.926 (95% CI: 0.806-1), indicating strong predictive power. We further verified the expression of miRNAs in the femoral heads which were harvested from SONFH patients underwent total hip arthroplasty (Fig. 6E). Compared with the normal tissue of the femoral head, the levels of hsa-miR-200b-3p (Fig. 6F) and hsa-miR-206 (Fig. 6G) were prominently increased in the necrotic area. Therefore, both hsa-miR-200b-3p and hsa-miR-206 could serve as promising biomarkers to detect the SONFH.

**Discussion**

Early detection and early intervention are essential to the hip preservation of SONFH which could delay or avoid the hip arthroplasty [15, 16]. However, SONFH is a latent disease and usually develops several years even after intensive steroid treatment. Additionally, early stage SONFH is usually asymptomatic. Although magnetic resonance imaging (MRI) is still believed to be the most conclusive method to definitively diagnose the early stage SONFH, the cost of MRI is pretty expensive posing a heavy burden on health care system and very inconvenient to be fully utilized in screening SONH high risk population [17, 18]. Therefore, it is extremely important to find an economical and convenient laboratory test for detecting SONFH development in patients who are receiving steroid therapy. To our knowledge, the present study
first evaluated the urinary exosomal miRNAs level in patients with SONFH. And we demonstrated urinary exosomal hsa-miR-200b-3p and hsa-miR-206 were significantly higher in patients with SONH compared to the HOA patients. ROC analysis illustrated that the urinary exosome level had high diagnostic accuracy for the detection of SONFH. Apparently, the urological examination is more convenient and economical than MRI scanning.

The overuse of glucocorticoid, which is one of the main pathogenic factors of SONFH, can result in the damage and dysfunction of vascular endothelial cells [19]. Accumulating studies have indicated that miR-200b represses angiogenesis by targeting vascular endothelial growth receptor 2 (VEGFR2) [20] and C-X-C motif chemokine ligand 1 (CXCL1) [21]. Moreover, glucocorticoid could inhibit osteogenesis, leading to the breakdown of bone trabecula and the reduction of bone mineral density [22, 23]. Similarly, miR-206 was reported to be downregulated in osteoblast cells during osteogenesis, suggesting that miR-206 has a regulatory function during the differentiation of mesenchymal stem cells [24]. In our study, has-miR-200b-3p and has-miR-206, which may participate in the pathogenic mechanism of SONFH, were significantly upregulated in SONFH patients compared with HOA patients.

Notably, increasing numbers of research have suggested miRNAs are involved in the pathogenesis of numerous diseases and could act as biomarkers and potential therapeutic targets [25]. In the present study, GO function, KEGG pathway, miRNAs-mRNAs network and PPI network were constructed to analyze potentially pathological mechanisms in the development of SONFH. Hippo, PI3K-Akt, TGF-β and Wnt signaling pathway were identified to be involved in SONFH. Hub genes mainly include MAPK1, EP300, RHOA, PIK3CA and CBL. The Hippo/YAP pathway is involved in the growth, death and migration of vascular smooth muscle cells and endothelial cells, which may contribute to the vascular remodeling of SONFH [26]. The PI3K/Akt signaling pathway plays an essential role in stem cell-regulated cell survival, proliferation, migration and angiogenesis [27]. Besides that, several studies have also demonstrated that activation of the PI3K/Akt signaling pathway can significantly enhance the functions of endothelial cells [28]. The TGF-β signaling pathway plays a fundamental role in embryonic skeletal development and postnatal bone homeostasis [29]. Similar to other TGF-β family proteins, bone morphogenetic protein 2 (BMP-2) has been implicated in the pathogenesis of SONFH and has therapeutic benefits for SONFH by inducing bone cells and cartilage [30]. The coupling of angiogenesis, dead bone absorption and new bone formation is a crucial link in the repair of femoral head necrosis. The Wnt signaling pathway plays an important role in promoting bone formation and inhibiting adipose tissue formation. Wnt signaling can increase the level of osteoprotegerin (OPG), reduce the expression of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), promote osteoblast production and inhibit bone resorption [31]. β-catenin, the key regulator of Wnt signaling, selectively inhibits the expression of peroxisome proliferator activated receptor gamma (PPAR-γ) and inhibits adipose formation [32].

Furthermore, Ep300 is an important transcriptional co-activator and histone acetyltransferase which undergoes down-regulation with aging [33]. It was reported that Ep300-activitated RUNX family transcription factor 2 (Runx2) activity and acetylation could be apparently declined by up-regulation of miR-132-3p, resulting in the suppression of osteoblast differentiation [34]. Accumulating studies have
demonstrated that repression of RHOA preserved the stability of podosome and sealing zone, which is possibly correlated with increased microtubule acetylation and stabilization in osteoclasts [35–37]. Besides, in the skeletal system, CBL plays an essential role in bone remodeling by regulating bone resorption of osteoclasts through interacting with phosphatidylinositol 3-kinase (PI3K) [38]. Therefore, hub genes are involved in the coupling of osteogenesis and osteoclastogenesis, which may be key factors in the progression of femoral head necrosis.

**Conclusion**

In conclusion, our study identified that specific urinary exosomal hsa-miR-200b-3p and hsa-miR-206 were differentially expressed in SONFH which could be served as biomarkers in SONFH early diagnosis. What is more, the angiogenesis and osteogenesis may be impaired in the femoral head by steroid which could result in the dysfunction of Hippo, PI3K-Akt, TGF-β and Wnt signaling pathway. Our study for the first time identified urinary exosomal miRNAs through integrated bioinformatics analysis as promising biomarkers for SONFH and provided a new strategy for the early diagnosis of SONFH. However, large-scale validation samples and further molecular mechanism are required to clarify the role of miRNAs in SONFH.

**Abbreviations**

ACTR3: actin related protein 3; AP2A1: adaptor related protein complex 2 subunit alpha; ARRB1: arrestin beta 1; AUC: area under curve; BMP-2: bone morphogenetic protein 2; BP: biological proces; BSA: bovine serum albumin; BTRC: beta-transducin repeat containing E3 ubiquitin protein ligase; CBL: Cbl proto-oncogene; CC: cellular component; CLOCK: clock circadian regulator; CXCL1: C-X-C motif chemokine ligand 1; DVL2: dishevelled segment polarity protein 2; EP300: E1A binding protein p300; GO: Gene Ontology; HOA: hip osteoarthritis; KEAP1: kelch like ECH associated protein 1; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK1: mitogen-activated protein kinase 1; MCODE: molecular complex detection; MF: molecular function; miRNAs: microRNAs; MRI: magnetic resonance imaging; NFIA: nuclear factor I A; NF-κB: nuclear factor κB; NTA: Nanoparticle-tracking analysis; OPG: osteoprotegerin; PBS: phosphate-buffered saline; PI3K: phosphatidylinositol 3-kinase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PPAR-γ: peroxisome proliferator activated receptor gamma; PPI: protein-protein interaction; qRT-PCR: quantitative real-time polymerase chain reaction; RANKL: receptor activator of nuclear factor-κB ligand; RHOA: ras homolog family member A; ROC: receiver operator characteristic; Runx2: RUNX family transcription factor 2; SEM: standard error of mean; SONFH: steroid-induced osteonecrosis of femoral head; TEM: Transmission electron microscopy; VAMP2: vesicle associated membrane protein 2; VEGFR2: vascular endothelial growth receptor 2; WASL: WASP like actin nucleation promoting factor; WNT5A: Wnt family member 5A; ZNF652: zinc finger protein 652.

**Declarations**

**Acknowledgements**
Author Contributions

DLC and GYZ conducted studies and wrote the paper cooperatively. DLC, YL and MZ took part in the experiments in vitro. GYZ, QH, JZY and XTZ acquired the samples. LY, LFW and ZQL analyzed the data. PC and HBW designed and supervised the research. PC revised the manuscript.

Funding

The authors acknowledge the support from the National Natural Science Foundation of China (NSFC) [No. 81603641 and No.81774339], Guangdong Provincial Science and Technology Project [No. 2017A020213030] and 2019 Key Research of Guangzhou University of Chinese Medicine [No.XK201912].

Availability of data and materials

The obtained results of the research are available on reasonable request.

Ethics approval and consent to participate

The research was approved by ethics review board in 1st Affiliated Hospital, Guangzhou University of Chinese Medicine.

Consent for publication

All authors have read and approved the content and agree to submit for consideration for publication in the journal.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1
Characterization of exosomes isolated from human urine. (A) The transmission electron microscopy image shows the representative morphology of urine-derived exosome with sizes ranging from 100-150 nm in diameter. Scale bar: 100 nm. (B) Size distribution and concentration of urinary exosomes was analyzed using NanoSight instrument, which were most abundant in 120-150nm. (C) Flow cytometry analysis for exosome-associated proteins CD63 and CD81 in the patient samples.

Figure 2

MicroRNA expression profiling in urinary exosomes using microRNA sequencing in the discovery set. The hierarchical clustering (A) and volcano plot (B) based on the 2588 miRNAs which were differentially expressed (67 up-regulated and 46 down-regulated) in SONFH compared with HOA groups. A red strip indicates up-regulated microRNA expression, a blue strip represents down-regulated microRNA expression, and deeper color means a larger fold change (p < 0.05).
Figure 3

Gene ontology and pathway enrichment analysis of top 9 differentially microRNAs between SONFH and HOA groups. (A-C) Top 10 statistically enriched gene ontology analysis (biological processes, molecular functions and cellular components). (D) The top 20 KEGG pathways enriched in the target genes of these 9 differentially miRNAs.
Figure 4

Protein-protein interaction network complex among predicted target genes of top 9 miRNAs and modular analysis. (A) Using the STRING online database, total of 636 predicted target genes of top 9 miRNAs were filtered into the PPI network complex. Network nodes represent proteins and edges indicate interaction between proteins. (B-C) The top 2 prominent modules selected from the PPI network.
Figure 5

Regulatory network of top 9 miRNAs and their predicted target mRNAs. Yellow triangles represented top 9 miRNAs (hsa-miR-200b-3p, hsa-miR-206, hsa-miR-122-5p, hsa-miR-100-5p, hsa-miR-1269a, hsa-miR-155-5p, hsa-miR-331-3p, hsa-miR-10a-3p, hsa-miR-381-3p). Dark blue and sky blue triangles on behalf of one or two miRNAs interact with genes. Green, red and pink triangles represented more than three miRNAs predicted mRNAs.
Figure 6

Urinary exosome hsa-miRNA-200b-3p and hsa-miRNA-206 were good diagnostic markers for SONFH and HOA. Scatter plots of urine exosomal levels of hsa-miRNA-200b-3p (A) and hsa-miRNA-206 (B) in healthy controls (n = 10), SONFH group (n = 30) and HOA group (n = 10). Expression levels of microRNAs were normalized to U6. ROC curves of exosomal hsa-miRNA-200b-3p (C) and hsa-miRNA-206 (D) for SONFH versus HOA. The areas under the curve (AUC) were 0.938 (95% CI: 0.828-1) and 0.926 (95% CI: 0.806-1), separately. (E) Schematic diagram of coronal incision of femoral head in the patient diagnosed as SONFH. (F-G) qRT-PCR validation of the expression levels of hsa-miRNA-200b-3p and hsa-miRNA-206 in the necrotic area and normal tissue of femoral heads in SONFH. All bar graphs are expressed as mean ± SEM. Significant differences between the two groups are displayed as *p < 0.05 and ***p < 0.001.