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Original Article

Role of microRNAs in pathogenesis of osteonecrosis of the femoral head in BSO rat model

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Abstract: Osteonecrosis of the femoral head (ONFH), namely avascular necrosis, is caused by osteocyte death and collapse of the articular surface resulting from decreased vascular supply to the subchondral bone of the femoral head. Current, the pathogenesis and etiology of osteonecrosis of the femoral head have not been revealed completely. In this study, we build an ONFH rat model by buthionine sulphoximine (BSO) treatment, the microRNA profiling of ONFH were obtained by next-generation sequencing technology. Totally 24 microRNAs were identified dysregulated in ONFH models. MicroRNA target gene prediction shows that these miRNAs are associated with 763 downstream genes. Pathway enrichment analysis reveals that these target genes are related to GnRH signaling pathway, and MAPK signaling pathway. The coexpression analysis of target genes identified several PSMB genes and MAPK genes. Moreover, we observed the five relatively abundant microRNAs show strong correlation with serum Glutathione concentration. In summary, our study described the microRNA profile characteristic of ONFH, which provides new sights into understanding of the pathogenesis of ONFH. Also our results suggest that changes in the miRNA expression in ONFH may provide potential biomarkers for ONFH prognosis.

Keywords: microRNA, osteonecrosis, femoral head

Introduction

Osteonecrosis of the femoral head (ONFH) is the pathological process of ischemic changes in cellular constituents of the femoral head including bone, endothelial, adipose and hematopoietic cells under the action of one or more factors that cause cell necrosis and apoptosis [1-3]. It is a common disease that regularly affects patients aged 20-50 years and is characterized by destruction of the blood supply of the femoral head [4]. It is a progressive and devastating disease that if left untreated results in collapse of the femoral head, necessitating hip replacement in approximately 70% of patients [5]. While he causes of non-traumatic ON are complex. Several mechanisms have been implicated in the pathogenesis of this disease, including intraosseous hypertension, metabolic disturbance of fat, intravascular coagulation, damage of micrangium endothelial cells, apoptosis of osteoblasts and osteocytes, and disruption of the immune system [6]. However, the precise mechanism is not fully elucidated.

MicroRNAs (miRNAs) are a class of naturally occurring, small non-coding RNA molecules, about 21-25 nucleotides in length. Their main function is to downregulate gene expression in a variety of manners, including translational repression, mRNA cleavage, and deadenylation. It is estimated that miRNAs regulate more than 5300 human genes, which represent around 30% of the human gene set (Lewis et al., 2005). Accumulating evidence has demonstrated that miRNAs regulate diverse biological and pathological processes through regulating the target genes, including cell proliferation, differentiation and apoptosis, as well as tissue development [7]. Recent studies have demonstrated the involvement of miRNA in osteonecrosis regulation. Dysfunction of miR-17-5p is demonstrated to contribute to NOFH pathogenesis [8]. Adiponectin, considering as a serum biomarker for NOFH [9], is significantly associated with the presence of NOFH and also has been proved to regulate by mi-378, miR-221 and miR-423-5p [10]. Yuan et al. have identified thousands of upregulations such as miR-21, miR-17, and miR-

92a, whereas others tended to downregulation, such as miR-205 and miR-145, in reparative interface of the femoral head with osteonecrosis by using high-throughput techniques-gene chip [11]. Notably, evidence increasingly shows serum miRNAs differential expression might be a marker for disease diagnosis.

Glutathione (γ-glutamylcysteinylglycine, GSH), a ubiquitous sulfhydryl-containing tripeptide produced by most mammalian cells, is the cells principle mechanism of eliminating reactive oxygen species (ROS). Given that the approaches to prevent the development of steroid-induced osteonecrosis, significant inhibition has been reported in animal models with the use of lipid-lowering agents antioxidant reduced glutathione [12]. It has also been reported that, soon after steroid administration to the domestic rabbit, blood GSH levels significantly decrease [13]. In addition, buthionine sulphoximine is an inducer of oxidative stress, in particular interfering with the synthesis of GSH in vivo [14].

In this study, to demonstrate the molecular mechanism of osteonecrosis and the involvement of GSH in ONFH, we use buthionine sulphoximine to build an ONFH model. High-thorough put micRNA sequencing approach is employed to characterizing the alteration miRNA profiles. Functional annotation of miRNA expression and their target gene protein-protein net-work analysis is performed to reveal the molecular mechanism of progress of osteonecrosis. Moreover, we estimated the potential miRNA function as GSH regulator which may contribute to osteonecrosis. Our study might provide insights into the molecular mechanisms of osteonecrosis.

Materials and methods

ONFH animal model construction

Male Wistar rats aged 24 weeks (body weight 400-450 gm) were purchased. Rats were subcutaneously injection of pro-oxidant DL-buthionine-(S,R)-sulfoximine (BSO) (500 mg/kg) for 14 consecutive days. 14 days after the start of the ON induction, about 70% BSO-treated animals developed ONFH, of which six were randomly selected for further analysis.

Glutathione (GSH) assay

12 days after the start of the ON induction, blood samples from tail vein were collected for glutathione (GSH) determination assay (QuantiChrom Glutathione (GSH) Assay Kit, Cat# DIGT-250, BioAssay System), following manufacturer's instruction. Raw readings were calculated with microplate spectrophotometer (uQuant Biotech, USA).

MicroRNA isolation and deep sequencing

MicroRNA was isolated from rat femoral tissue using TriReagent (Ambion Inc, TX) according to the manufacturer's protocol the quality was assessed by the Agilent 2100 Bioanalyzer and samples with a RIN value of 7 and above were used for further analysis. Small RNA sequencing libraries were created following the Illumina®TruSeq™ Small RNA Sample Preparation protocol. In brief, 3' and 5'RNA adapter, specifically modified to target the ends of small RNA molecules, were ligated to 1 μg of high quality total RNA. Reverse transcription was performed to generate cDNA libraries and PCR was used to amplify and add unique index sequences to each library. Small RNA libraries were pooled and 32 bases were sequenced for each cDNA molecule using an Illumina® Genome Analyzer IIx. Indexes were sequenced in order to identify the source of each read.

Sequencing data analysis and normalization

Real-time analysis, base calling and filtering of low quality reads were done by Illumina's software packages (SCS2.9/RTA1.9 and Off-line Basecaller v1.9). Fastx was used to cut remaining adapter sequence and remove PCR primer. Reads map to the reference human genome (hg19) by using miRDeep2. All reads mapping to 10 or more genomic regions were excluded from further analysis. The mapped reads were annotated using known databases. The miRBase database release 21 was used to identify miRNAs, using BEDTools Version-2.16.2.

To calculate the read count for miRNAs, reads that mapped uniquely within a mature miRNA sequence with a maximum of one mismatch were considered hits. Reads mapping to more than one mature miRNA sequence were assigned according to the frequency of uniquely mapped reads found for these miRNAs. Than the read counts were normalized by dividing the total reads of one sample, and log2 transformed. The normalized expression values for each miRNA were generated by dividing the read count of the miRNA with the according

Table 1. Differentially expressed miRNAs in osteoarthritis

	miRNA	FC
Up-regulated	rno-miR-351-3p	3.84
	rno-miR-224-5p	3.84
	rno-miR-471-5p	3.62
	rno-miR-3068-3p	3.23
	rno-miR-342-5p	3.05
	rno-miR-324-3p	2.68
	rno-miR-3561-3p	2.66
	rno-miR-152-3p	2.42
	rno-miR-743a-5p	2.42
	rno-miR-3084d	2.42
	rno-miR-24-2-5p	2.11
	rno-miR-141-5p	2.11
	rno-miR-144-5p	2.11
	rno-miR-300-5p	2.11
	rno-miR-181d-5p	2.11
	rno-miR-434-5p	2.11
	rno-miR-434-3p	2.11
	rno-miR-471-3p	2.11
	rno-miR-758-3p	2.11
	rno-miR-294	2.11
	rno-miR-449c-5p	2.11
	rno-miR-3549	2.11
	rno-miR-3568	2.11
	rno-miR-133c	2.11
Down-regulated	rno-miR-150-5p	0.56
	rno-let-7c-5p	0.55
	rno-miR-760-5p	0.54
	rno-miR-218a-2-3p	0.53
	rno-let-7g-5p	0.51
	rno-miR-466b-3p	0.30
	rno-miR-23a-5p	0.25
	rno-miR-759	0.13

normalization factor. The data set normalized against annotated mature miRNAs was chosen for the remaining analyses.

Differential expression analysis and target gene annotation

Differential expression of genes between ONFH and healthy rat samples were performed by calculating fold changes using the normalized value of each miRNA, and statistical significance of differentially expressed genes was presented by calculating a t test *p*-value. Then, significance of a differentially expressed miRNA between two samples was determined according the threshold of |log2 (fold change)| larger than 2 and *p*-value was less than 0.01.

Target genes of differentially expressed miRNA were predicted with miRDB. All the target genes were used to query the KEGG pathway database to determine the biological function of these DEGs. Enriched pathway was determined by both significant fisher exact test (*p*-value < 0.05), and at least 3 differentially expressed genes were involved in the pathway. The pathway enrichment analysis was performed by using "KEGG.db" and "KEGGprofile" packages in R project. GO enrichment analysis were performed with DAVID tools.

Protein-protein interaction and GSH association analysis

The interaction network of target was constructed using STRING database which is a database of known and predicted protein interactions. The interactions were filter according to the high confidence score and experimental evidence.

The correlation coefficient between GSH concentration in blood serum and miRNA profiles were calculated. The correlation coefficient cutoff was set as larger than 0.85, and 5 miRNAs meet the criteria.

Results

miRNA expression profiling and differentially expression analysis

The total sequencing reads were filtered with low quality reads, and reads about 15~40nt in length were remained. All the clean were align to the Rat mature miRNA in mirbase with at least 16nt overlap allowing offsets. About 30% of total sequences were successfully aligned to the reference miRNA. 588 genes were detected with at least 1 sequence read. Differentially expression analysis was performed to identify miRNA expression alteration in osteoarthritis and control subjects. We identified 24 up-regulated and 8 down-regulated miRNAs listed in **Table 1** by applying the criteria fold change > 2 and *p* value cutoff < 0.05.

miRNA target gene prediction and functional annotation

Identification of genes whose expression is regulated by miRNAs provides a lead for the func-

Table 2. Pathway enrichment analysis of miRNA target genes

Term	Count	%	*P* value
GnRH signaling pathway	11	1.8	5.41E-04
Long-term depression	9	1.5	9.11E-04
Aldosterone-regulated sodium reabsorption	7	1.1	1.79E-03
MAPK signaling pathway	18	2.9	2.97E-03
Gap junction	9	1.5	3.12E-03
Fc gamma R-mediated phagocytosis	9	1.5	5.21E-03

tional roles of miRNAs, predicting target genes of the miRNAs identified in our differentially expressed analysis would greatly facilitate understanding the miRNA-regulated biological correlates of osteoarthritis. We predict target genes of miRNA with miRDB. The profiles of differentially expressed miRNA are related to 763 genes according to miRDB. To further characterizing the function those miRNA, all the target genes were used to query the KEGG database. The significantly enriched pathways were listed in **Table 2**. GnRH signaling pathway is the top enriched pathway, and 18 target genes were involved in MAPK signaling pathway. Gene Ontology was performed to reveal the biological process of target genes. In **Table 3**, the top significant GO term of 763 target genes is related regulation of transcription, the positive regulation of biosynthetic process was also enriched.

Protein-protein interaction network of target genes

To identify the association between the target genes and to further understand the function of miRNAs, we performed protein-protein interaction (PPI) analysis to construct the interaction network. STRING is a protein-protein interaction database which provided the association of genes with experimental evidence. All the 763 target genes were used to construct the interaction network. We observed several hub genes, the Mapk3 and Oas3 were two hub genes in our PPI network in **Figure 1**. Furthermore, 11 Psmb family members were interacted.

Pathological alteration in osteoarthritis of rat model

The pathological progress of osteoarthritis was observed that inflammation and deterioration occurred in the BSO group. In **Figure 2**, H.E-stained specimens (100×) also showed the presence of osteonecrosis in the femoral head in BSO group compared to the control. Both left and right femoral heads show inflammation and osteonecrosis while control group show normal staining. We further calculated the expression correlation of GSH level with miRNA expression for each sample. Correlation coefficient were calculated between serum GSH level and miR expression level and scatter plots showed five miRNAs were negatively correlated with serum GSH presenting coefficients above 0.85. Scatter plot in **Figure 3** shows the correlation between miRNA abundance and GSH concentration in serum.

Discussions

In this study, we build an osteonecrosis of the femoral head rat model with the treatment of buthionine sulphoximine (BSO) which function as an inhibitor of gamma glutamine synthetase which is an important enzyme in GSH biosynthesis. The serum GSH level was evaluated during the period of BSO treatment. We observed the pathological change of rat femoral head after BSO treatment and increasing of GSH in osteonecrosis rat. miRNA expression profiles were evaluated by using the next-generation sequencing technology. Our results demonstrated 24 up-regulated and 7 down-regulated miRNAs. Among these miRNAs, let-7c has been identified as a key regulator of ONFH [15]. As one of the most-studied miRNAs to date, Let-7 is known to play an important role in the regulation of programmed cell death [15]. Let-7c is reported to negatively regulate the expression of Bcl-xl (B-cell lymphoma-extra large) [16], a short isoform of anti-apoptotic member of the Bcl-2 family [17]. Down-regulation of let-7c might cause non-traumatic ONFH by promoting osteoblast apoptosis [18].

In order to get insights into the function of miRNAs, we predict the target genes of differentially expressed miRNAs, and 763 target genes are obtained. The KEGG pathway enrichment analysis was performed to characterizing the molecular function of target genes. The results show that GnRH signaling pathway is top enriched pathway which involved 11 target

Table 3. GO term of miRNA target genes

Term	Discription	Count	%	*P* value
GO:0045449	regulation of transcription	89	14.6	1.54E-07
GO:0010557	positive regulation of macromolecule biosynthetic process	42	6.9	9.97E-06
GO:0009891	positive regulation of biosynthetic process	43	7.0	2.43E-05
GO:0031328	positive regulation of cellular biosynthetic process	42	6.9	3.57E-05
GO:0045941	positive regulation of transcription	36	5.9	4.25E-05
GO:0010628	positive regulation of gene expression	36	5.9	6.86E-05
GO:0045935	positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	38	6.2	1.22E-04
GO:0010604	positive regulation of macromolecule metabolic process	47	7.7	1.44E-04
GO:0051173	positive regulation of nitrogen compound metabolic process	38	6.2	2.18E-04
GO:0045893	positive regulation of transcription, DNA-dependent	30	4.9	3.23E-04

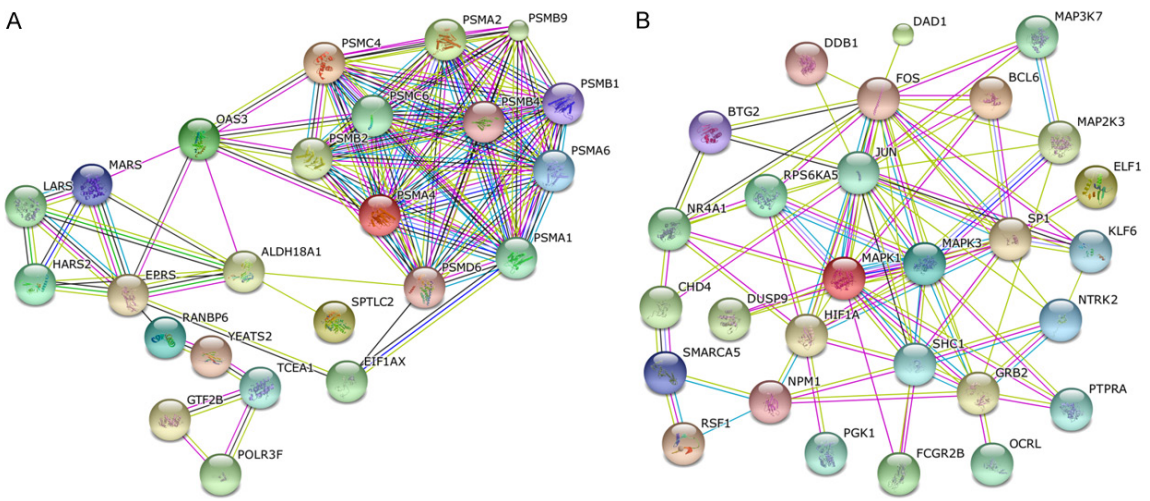

Figure 1. Protein-protein interaction network of DEGs.

genes, and another aldosterone-regulated sodium reabsorption pathway is also enriched. GnRH is risk factors for osteoporosis patients and is related to bone remodeling, and gradual bone loss [19]. The aldosterone system plays a key role in osteoporosis women [20], and one of the major contributing factors to osteoporosis is withdrawal of estrogen during menopause in women. Therefore, previous study demonstrated that hormone regulate system are involved in the progression of osteoporosis [21]. Our study further elucidates the association of hormone regulation and osteonecrosis of the femoral head.

In addition, 18 genes are involved in MAPK signaling pathway. MAPK signaling pathway involved in regulation of crucial inflammation mediators [22]. Recently, growing understanding of the osteoporosis suggests that factors involved in inflammation are linked with osteoporosis [23]. Chronic inflammation and the immune system remodeling and other pathological conditions are commonly associated with osteoporosis [24, 25]. We believe the MAPK pathway could be responsible for some of the pathological changes observed in the osteonecrosis. Furthermore, the long-term depression, Gap junction and Fc gamma R-mediated phagocytosis pathways are also enriched.

The GO analysis of predict target genes is also performed to identify the biological process. The result revealed several GOs related to regulation of transcription and positive regulation of macromolecule biosynthetic process. These groups represent the most of the significantly enriched GO terms.

The protein-protein interaction network of target genes is performed to characterizing the function cluster of genes. Several MAPK genes are hub genes in the network, such as Mapk1, Mapk3, Mapk4, Map2k3. The Fos and Jun

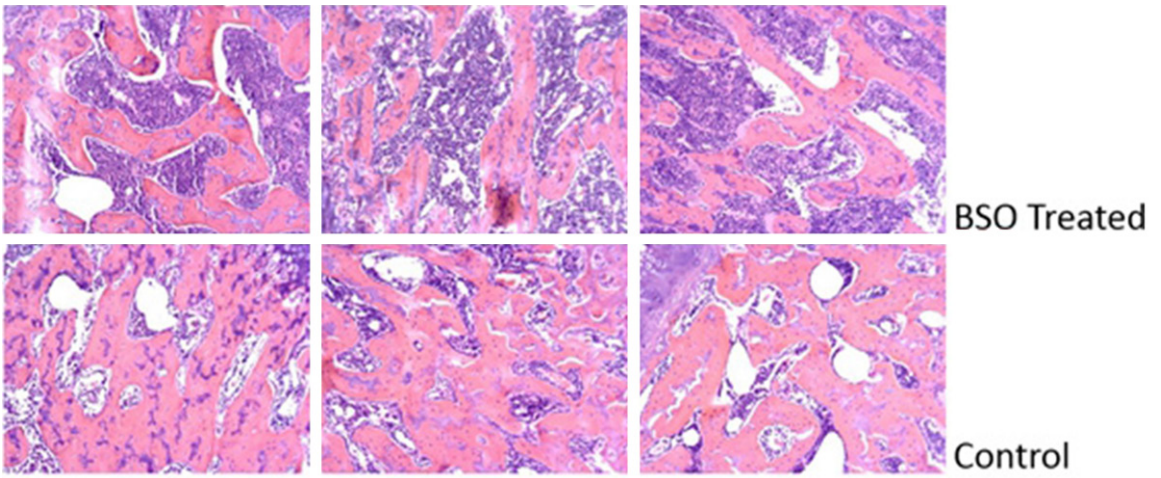

Figure 2. H.E-stained specimens (100×) showed the presence of osteonecrosis in the femoral head in BSO group compared to the control.

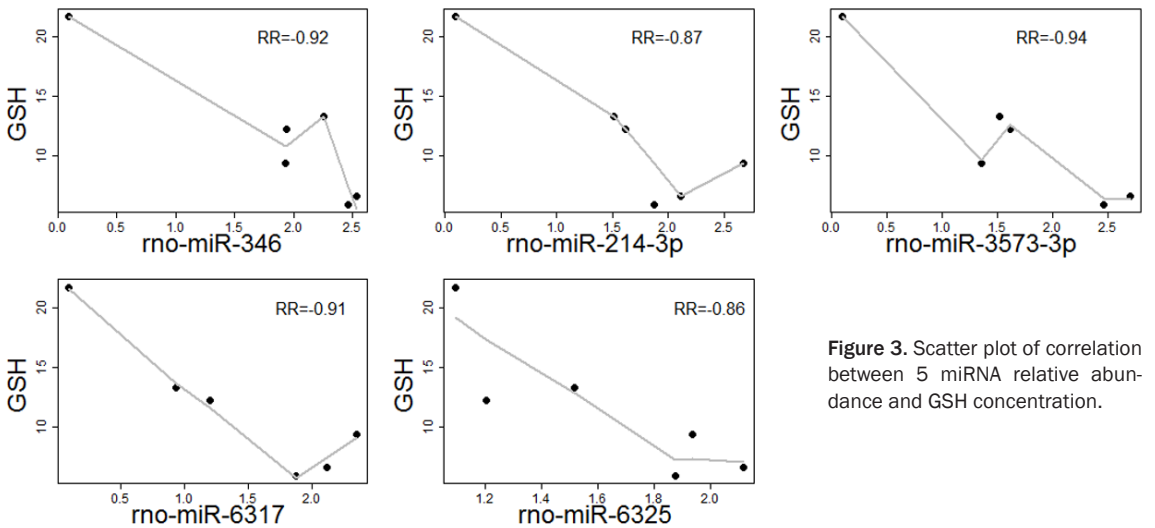

Figure 3. Scatter plot of correlation between 5 miRNA relative abundance and GSH concentration.

genes are involved in the network which are important factor of immune. In addition, SP1 is a key transcription factor. Several members of Psm gene family are involved in this network; those genes provide instructions for making parts of cell structures called proteasomes, such as Immunoproteasomes and thymoproteasomes. Proteasomes are ubiquitous in cells throughout the body, however at least two specialized types of proteasomes have been identified specific to certain tissues. Immunoproteasomes are mainly located in immune cells, where they function in regulating the immune system's response against foreign antigens [26]. Another example is thymoproteasomes, which are found only in the thymus, where T lymphocytes were mainly trained and selected [27].

Finally, we try to demonstrate the association between miRNA and GSH. GSH is an antioxidant enzyme functioning as a factor related to maintenance of membrane integrity, cell structure, metabolism of foreign bodies and many other functions. Research shows that if GSH levels decrease, oxidative stress is also induced, causing tissue and vascular injury. Our results indicate that expression profiles of 5 miRNA are inversely correlated with GSH serum level.

In conclusion, our study reveals the dysregulation of miRNA in the ONFH. Bioinformatics-

based analysis of these changes in expression provides a useful tool for understanding the molecular mechanisms responsible for collapse and necrosis of the femoral head as well as relationship between GSH and miRNAs. Further investigations by transgenic models are needed to clarify the roles of identified miRNA in osteogenesis and osteonecrosis.

Disclosure of conflict of interest

None.

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