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Analysis of altered microRNA expression profile in the reparative interface of the femoral head with osteonecrosis

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ABSTRACT

The reparative reaction is considered to be important during the occurrence of collapse in the femoral head with osteonecrosis (ONFH), but little is known about the long-term reparative process. The aim of this study was to determine and analyze the altered microRNA expression profile in the reparative interface of ONFH, and further validate the expression of the involved genes in the predicted pathways. Microarray analysis was performed comparing the reparative interface of patients with ONFH and normal tissue of patients with fresh femoral neck fracture (FNF) and partly validated by real-time PCR. Potential target genes of differentially expressed miRNAs were predicted by TargetScan and miRanda, and the target genes were used for further bioinformatics analysis such as Gene Ontology and Pathway assay. The filtered miRNAs and genes in the predict pathways were further examined by real-time PCR in another 6 independent ONFH patients. Among the 2578 miRNAs identified, 17 were consistently differentially expressed, 12 of which are up-regulated and 5 down-regulated. GO classification showed that the predicted target genes of these miRNAs are involved in signal transduction, cell differentiation, methylation, cell growth and apoptosis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) classification indicated that these genes play a role in angiogenesis and Wnt signaling pathways. The expression of miR-34a and miR-146a and genes in the predict pathways were significantly up-regulated. This study presented a global view of miRNA expression in the reparative interface of osteonecrosis. In addition, our data provided novel and robust information for further researches in the pathogenesis and molecular events of ONFH.

1. 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 (Zalavras and Lieberman, 2014; Fukushima et al., 2010; Assouline-Dayan, 2002). 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 (Hernigou et al., 2004; Johnson et al., 2014). While there are various theories on the pathogenesis of ONFH, such as extravascular accumulation of fat mediated vascular constriction, the intravascular blood coagulation theory, and the intravascular fat embolism concept (Schroer, 1994), the exact pathogenesis is still unclear.

The reparative reaction where osteoclasts mediate reabsorption of necrotic bone is believed to be initiated in early stages of ONFH. During the repair process, imbalanced osteoclast activity over osteoblast mediated bone reformation results in structural weakening, causing collapse of the femoral head in many patients (Hernigou et al., 2004; Nishii, 2002). The mechanism of collapse is still controversial, however it is generally considered that the reparative reaction rather than the osteonecrosis itself is the primary cause (Li et al., 2009; Kim et al., 2006). To our knowledge, previous studies have mainly focused on parameters of the necrotic zone, including the location, the size, and the pathological changes of this region (Ha et al., 2006; Steinberg et al., 2006; Mont et al., 2010). Currently, the molecular mechanisms contributing to the pathogenesis of ONFH at the reparative interface remain poorly understood.

MicroRNAs (miRNAs) belong to a family of non-protein-coding small RNAs that are involved in various physiological and pathological processes (Ambros, 2001; Bartel, 2004). They are approximately ~22 nucleotides in length and are expressed in a tissue- or cell-specific manner (Lagos-Quintana et al., 2002). Among them, a set of miRNAs have been confirmed to play fundamental roles in gene regulation of various orthopedic diseases, such as bone tumors (Kafchinski and Jones, 2014), osteoarthritis (Miyaki and Asahara, 2012), and rheumatoid arthritis (Furer et al., 2010). Although several miRNAs have been independently reported (Yamasaki et al., 2012; Sun et al., 2014; Jia et al., 2014), and one preliminary review discussing circulating microRNAs in ONFH has been written (Wang, X. et al., 2014; Wang, Y. et al., 2014), the miRNA expression profile of ONFH remains unclear. Therefore an in depth investigation

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on the role of miRNA in ONFH will help us better understand the molecular mechanisms of this debilitating disease.

The objective of our study is to provide a global view of miRNA expression in the reparative interface, and additionally present a novel and comprehensive molecular signature that contributes to the pathogenesis of ONFH that can be used to support future research efforts. We achieved this by (1) to determining the altered microRNA expression profile in the reparative interface of ONFH, (2) analyzing the results of our array using the Gene Ontology and Pathway assays, (3) and using the filtered miRNAs to predict pathways critical in ONFH.

2. Materials and methods

2.1. Ethical statement

The study was reviewed and approved by the Ethical Committee of Zhongshan Hospital, Fudan University and patients gave informed consent.

2.2. Patients

We selected 9 patients with ONFH who underwent total hip arthroplasty (THA) in our department. ONFH was diagnosed based on imaging examinations and the diagnosis was made according to the guidelines of the Chinese Medical Association for ONFH (Orthopedic Panel, 2012). We evaluated another 6 patients with fresh femoral neck fracture (FNF) who underwent THA in our department (excluding other types of bone and joint diseases) as the normal control group. All study characters were of Chinese Han nationality without any blood relationship to one another. The clinical characteristics of all patients are shown in Table 1.

2.3. Sample preparation

When THA was performed, the isolated femoral head was obtained and cut along the coronal plane to check the appearance of the reparative interface zone. The reparative interface is the tissue on the edge of the necrotic zone (Fig. 1). The reparative interface was then cut into smalls pieces of approximately $5 \times 5 \times 5$ mm^3, and rapidly immersed in a portable liquid nitrogen tank. The samples from the tank were stored in a −80 °C freezer after leaving the operating room.

2.4. RNA isolation and microRNA microarray

The samples were rapidly immersed the liquid nitrogen for 1 h and crushed by a pincer. Total RNA in the samples was isolated using Trizol Reagent (Invitrogen) according to the manufacture's protocol. The integrity and concentration of all RNA samples were quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific). All samples met the quality control standards. The total RNA of 3 patients diagnosed with ONFH (Patients 1–3) and 3 patients diagnosed with FNF (Patients 10–12) were hybridized to an Affymetrix GeneChip miRNA 4.0 Array containing 2578 human miRNA sequences. RVM *t*-test was applied to filter the differentially expressed miRNAs for the ONFH and normal control group because the RVM *t*-test can raise degrees of freedom effectively in the cases of small samples. After the significant analysis and FDR analysis, we selected the differentially expressed miRNAs according to the p value threshold. P value of <0.05 was considered as significant difference.

Table 1
Clinical characteristics of the ONFH patients and controls.

Patient	Age	Gender	Diagnosis	ONFH classification	Other illness
1	22	Female	Glucocorticoid-related ONFH	IIIB	SLE
2	31	Female	Glucocorticoid-related ONFH	IIIC	SLE
3	33	Female	Glucocorticoid-related ONFH	IIIB	SLE
4	61	Female	Idiopathic ONFH	IIIC	/
5	46	Female	Glucocorticoid-related ONFH	IIIC	RA
6	43	Female	Glucocorticoid-related ONFH	IIIC	SLE
7	65	Male	Alcohol-related ONFH	IIIC	/
8	63	Male	Alcohol-related ONFH	IV	/
9	56	Male	Idiopathic ONFH	IIIC	/
10	78	Female	FNF	/	/
11	84	Female	FNF	/	/
12	76	Female	FNF	/	/
13	69	Female	FNF	/	/
14	88	Male	FNF	/	/
15	82	Female	FNF	/	

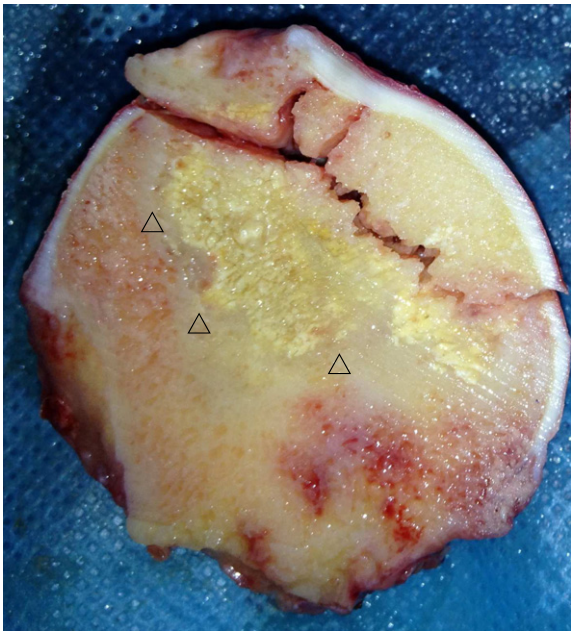

Fig. 1. Samples acquired in the reparative interface of the femoral head in ONFH patients (△).

2.5. Bioinformatics analysis

Bioinformatics analysis (Genminix Informatics Ltd., Shanghai, China) was performed for miRNAs expressed in significant amounts. We used the TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) to predict the intersection of target genes of miRNAs. GO analysis was applied to analyze the main function of the differential expression target genes according to the Gene Ontology (GO, http://www.geneontology.org/) which is the key functional classification of NCBI. Pathway analysis was used to find out the significant pathway of the differential genes according to Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/). GO terms and KEGG Pathway annotation of the miRNA target genes were found using the web-based tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). Fisher's exact test and χ^2 test were used to classify the GO category and select the significant pathway, and the false discovery rate (FDR) was used to correct the p value. We chose only GO terms with p value of <0.01 and FDR value of <0.01, and Pathways with p value of <0.05 and FDR value of <0.05.

2.6. Quantitative real-time PCR (RT-PCR)

To validate the microarray data, we used the RNA-tailing and primer-extension RT-PCR method to detect the expression of the miRNA. RNA samples used in RT-PCR validation experiments were the