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Original Article

Identification of key genes and pathways associated with gender difference in osteonecrosis of the femoral head based on bioinformatics analysis

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Abstract

Objective: To identify different key genes and pathways between males and females by studying differentially expressed genes (DEGs). **Methods**: The gene expression data of GSE123568 were downloaded from GEO database, including osteonecrosis of the femoral head (ONFH) samples from 3 females and 7 males, and DEGs between different gender were identified with R software. Protein-protein interaction (PPI) network was constructed to further analyze the interactions between overlapping DEGs, and finally, GO, KEGG and gene set enrichment analysis (GSEA) were conducted for enrichment analysis. **Results**: 131 DEGs were identified between ONFH females and ONFH males, including 76 up-regulated genes and 55 down-regulated genes. And 10 hub genes were identified in PPI network, including SLC4A1, GYPA, CXCL8, IFIT1, GBP5, IFI44, IFI44L, IFIT3, KEL and AHSP. Functional enrichment analysis revealed that these genes were mainly enriched in cGMP-PKG signaling pathway, Fatty acid degradation, Non-alcoholic fatty liver disease, Systemic lupus erythematosus, Hematopoietic cell lineage and NO-cGMP-PKG signaling. **Conclusions**: NO-cGMP-PKG signaling may play an important role in the occurrence and development of ONFH. SLC4A1, GYPA, CXCL8, GBP5 and AHSP may be key genes associated with gender difference in the progression of ONFH, which may be ideal targets or prognostic markers for the treatment of ONFH.

Keywords: Differentially Expressed Genes, Gender Difference, Hub Genes, ONFH, Potential Therapeutic Drugs

Introduction

Osteonecrosis of the femoral head (ONFH) is a common hip disease with a high incidence in elderly patients, with the most common clinical symptom of severe pain[1]. ONFH can cause rapid hip destruction and dysfunction, and approximately 65-70% of patients with advanced ONFH require total hip arthroplasty[2]. There are various pathological mechanisms of ONFH, including abnormal lipid metabolism, microcirculation ischemia, apoptosis, etc. It is widely accepted that reduced blood perfusion leads to attenuation of normal repair function of cells and ultimately irreversible tissue destruction[3]. Most studies focused on bone tissues. However, cartilage is also an important factor in the development of this disease. In the early stage of the disease, radiographic changes, including rough cartilage surface, can be seen[4]. The cartilage degradation will be gradually worsened. And the mechanical stress changes resulting from cartilage degeneration may also further aggravate the progression of this disease.

In the Chinese National Representative Survey, the estimated number of nontraumatic osteonecrosis of the femoral head (NONFH) cases among Chinese people aged 15 years old and older was 8.12 million. The prevalence of NONFH was significantly higher in males than that in females, which was approximately 2 times in female patients[5]. Gender difference in their prevalence suggests its important role for gender hormones in the pathogenesis of ONFH, which has led to the hypothesis that androgens play a role in the development of ONFH. A study on the effect of gonadal

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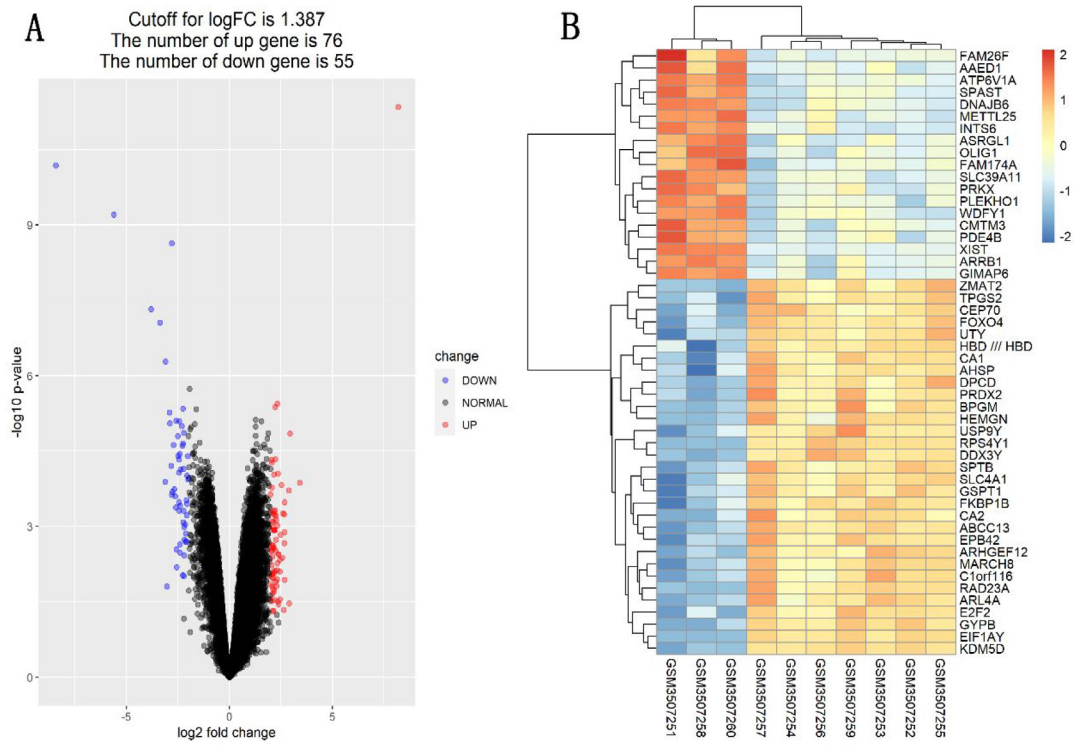

Figure 1. (A) Volcano plots of DEGs. Red nodes indicate DEGs up-regulated with P<0.05 and logFC>2, blue nodes indicate DEGs down-regulated at P<0.05 and logFC<-2. (B) Heat maps of the top 50 DEGs (tissue samples in bar charts, individual genes in rows). Red represents up-regulated genes, blue indicates down-regulated genes in ONFH patients.

glands and testosterone on the incidence of femoral head lesions in spontaneously hypertensive rats (SHR) found a difference in the incidence of femoral head lesions between the two genders because of the effects of testosterone on femoral head growth plates[6].

Recently, microarray techniques based on high-throughput platforms have been widely used in gene expression profiling. Extensive effort has been spent in identifying or evaluating specific genes and pathways for ONFH progression. However, most studies have focused on gene expression data of patients compared with healthy subjects, and there is less literature focusing on gene expression data of patients compared with gender difference.

To this end, a comprehensive bioinformatics analysis identified DEGs in males and females with ONFH. Gene expression profiles of males and females with ONFH were obtained from the gene expression Omnibus (GEO) database, and then the hub genes of DEGs were identified through protein interaction analysis. This study helps to further understand the gender-specific mechanisms of ONFH and provides potential biomarkers and therapeutic targets for the treatment of ONFH.

Materials and Methods

Data source

In this study, the dataset GSE123568 of ONFH was selected in the GEO database, by searching with “femur head” [MeSH Terms] or “femoral head” [All Fields], “necrosis” [MeSH Terms] or necrosis [All Fields], and “Homo sapiens” [porgn] as keywords, including non-steroid-administered peripheral blood samples from 3 females and 7 males with ONFH. Differential genes were screened according to the samples from female ONFH patient group and male ONFH patient group.

Data preprocessing and identification of DEGs

Differentially expressed genes (DEGs) between the female ONFH patient group and the male ONFH patient group were compared with R software (Version 4.1.1) and related R packages in GSE123568. The datasets were first background-adjusted and normalized by log2 transformation. Background correction, quantile normalization and probe summarization of raw microarray data were performed by RMA algorithm[7] in the linear models of microarray data (limma) package[8]. P values

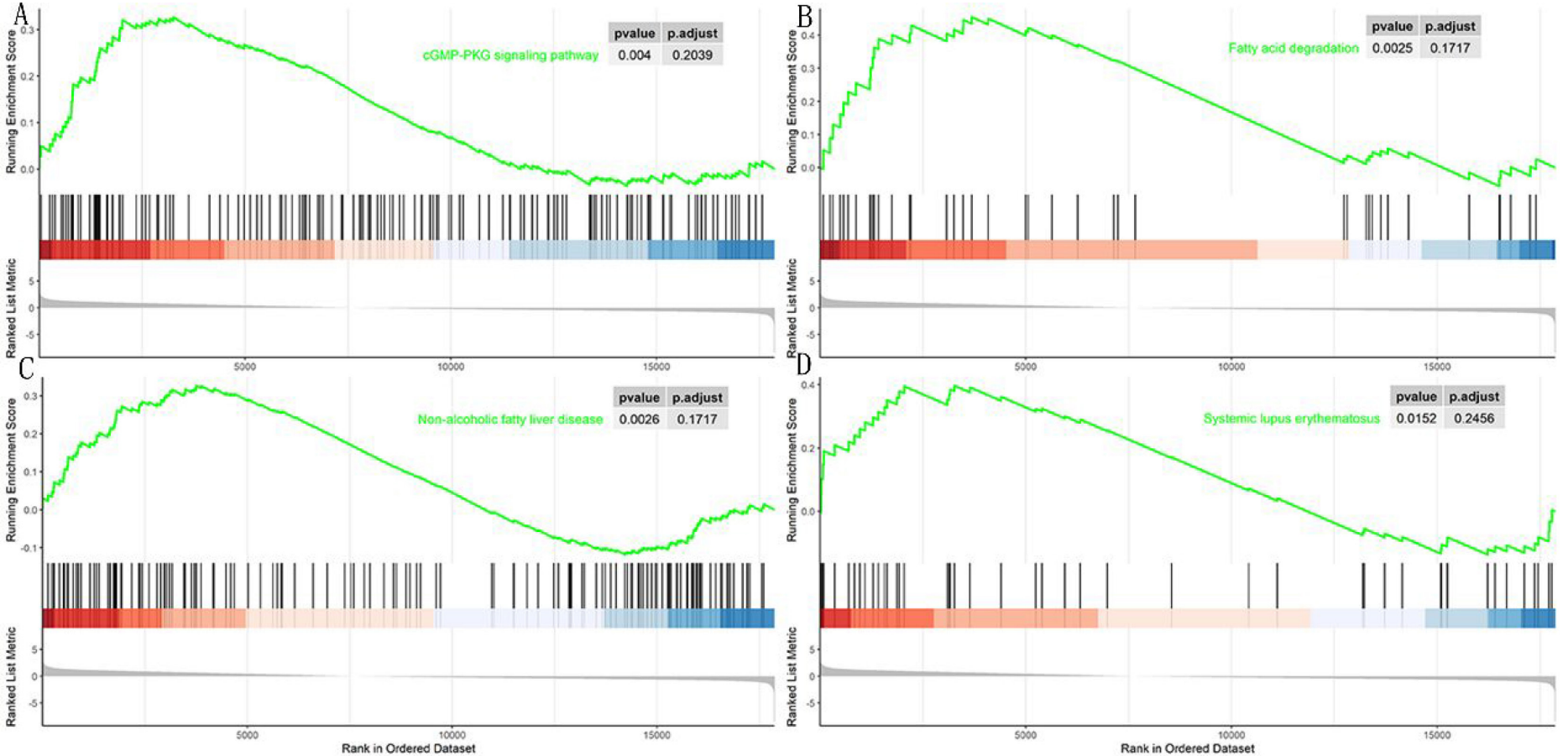

Figure 2. Results of GSEA pathway enrichment analysis on DEGs by the Kegg gene set database.

were adjusted for comparison with the false discovery rate (FDR) of Benjamini and Hochberg (BH) test[9] in the limma package[8]. DEGs were selected with P<0.05 and |log2 fold change (FC) | > 2 as the commonly used thresholds[10,11]. Volcano maps and heatmaps of DEGs were generated with the ggplot2 software package in the R software.

GSEA

To better understand the biological mechanisms associated with ONFH, candidate molecular pathways were explored with GSEA software (Version 4.1.0) for DEGs. The gene sets, Hallmark and Canonical Pathways, were downloaded from the molecular features database (http://www.gsea-msigdb.org/gsea/msigdb/index.jsp).

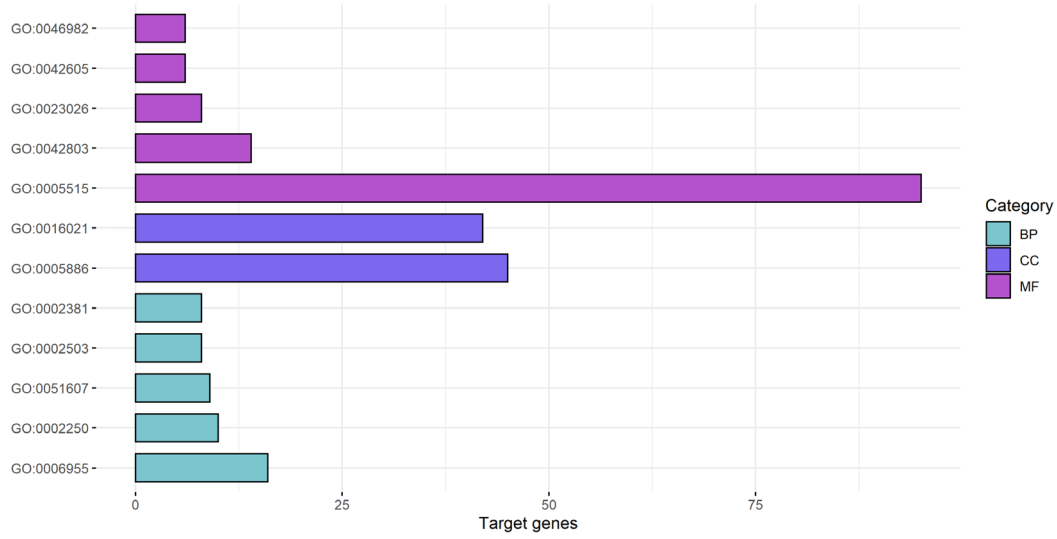

Figure 3. GO enrichment analysis on overlapping DEGs with females and males in ONFH.

Table 1. Significant terms identified by GO enrichment analysis for down-regulated DEGs in ONFH samples (p<0.05).

Category	Term	Biological function	Count	P
BP	GO:0006955	Immune response	16	0.000000
	GO:0002250	Adaptive immune response	10	0.001707
	GO:0051607	Defense response to virus	9	0.000088
	GO:0002503	Peptide antigen assembly with MHC class II protein complex	8	0.000000
	GO:0002381	Immunoglobulin production involved in immunoglobulin mediated immune response	8	0.000000
	GO:0002504	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	8	0.000000
	GO:0019886	Antigen processing and presentation of exogenous peptide antigen via MHC class II	8	0.000000
	GO:0050870	Positive regulation of T cell activation	8	0.000000
	GO:0019882	Antigen processing and presentation	8	0.000000
	GO:0071222	Cellular response to lipopolysaccharide	8	0.000176
CC	GO:0005886	Plasma membrane	45	0.002669
	GO:0016021	Integral component of membrane	42	0.039437
	GO:0070062	Extracellular exosome	33	0.000001
	GO:0016020	Membrane	24	0.023415
	GO:0005887	integral component of plasma membrane	21	0.000495
	GO:0005765	Lysosomal membrane	11	0.000078
	GO:0000139	Golgi membrane	10	0.014823
	GO:0010008	Endosome membrane	9	0.000154
	GO:0009986	Cell surface	9	0.035184
	GO:0042613	MHC class II protein complex	8	0.000000
MF	GO:0005515	Protein binding	95	0.004893
	GO:0042803	Protein homodimerization activity	14	0.000688
	GO:0023026	MHC class II protein complex binding	8	0.000000
	GO:0042605	Peptide antigen binding	6	0.000004
	GO:0032395	MHC class II receptor activity	5	0.000001

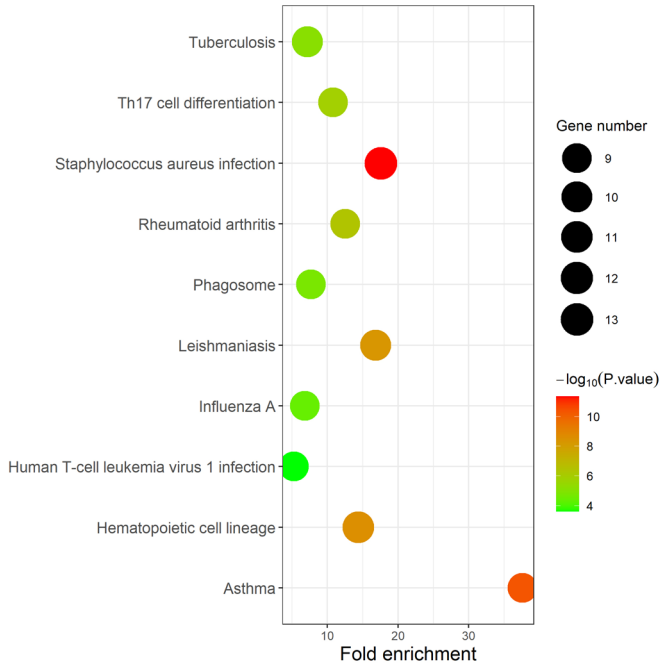

Figure 4. Results of KEGG pathway enrichment analysis on DEGs.

GO and KEGG pathway enrichment analysis

KEGG pathway and GO term enrichment of DEGs were analyzed with Database for Annotation, Visualization and Integrated Discovery (DAVID), to explain the biological process of DEGs between males and females[12,13].

PPI network construction and Hub gene identification

To understand the mutual function of DEGs, DEGs were imported into the interaction gene retrieval tool (STRING, https://www. string-db.org/), a PPI network was established with the default threshold of the composite score >0.4. The PPI network was imported into the Cytoscape Software v.3.8.2 for PPI network visualization. The Hub gene (method) was screened and visualized with the plug-in software cytohubba[14]. Protein expression profiles of hub genes at tissue level (low, medium, high) and gene expression level (normalized expression, NX) were obtained from the human protein atlas (HPA) database.

Results

Identification of DE-miRNAs

A total of 131 DEGs were identified in the synovium of postmenopausal ONFH females as compared to ONFH males in GSE123568. Among them, 76 were regulated and 55 were down-regulated. Volcano maps and heat maps are shown in Figure 1.

GSEA

The expression information of 131 DEGs were uploaded to GSEA software, and genes were analyzed at the overall level of OS gene expression profiles using Hallmark and KEGG gene set databases. The results showed that most DEGs were involved in cGMP-PKG signaling pathway, Fatty acid degradation, Non-alcoholic fatty liver disease and Systemic lupus erythematosus (Figure 2).

GO and KEGG enrichment analysis on target genes

GO term enrichment analysis showed that, in BP, DEGs were enriched in immunoglobulin production involved in immunoglobulin-mediated immune response, peptide antigen assembly with MHC class II protein complex, defense response to virus and immune response. In CC, DEGs were enriched in both integral components of membrane and plasma membrane. In MF, DEGs were enriched in protein heterodimerization activity, peptide antigen binding, MHC class II protein complex binding, protein homodimerization activity and protein binding (Figure 3, Table 1).

Target genes were highly expressed in multiple pathways, such as NO-cGMP-PKG signaling, through KEGG pathway analysis (Figure 4).

PPI network construction and selection and analysis on Hub genes

To help understand the interactions between overlapping DEGs, a PPI network was constructed with the STRING

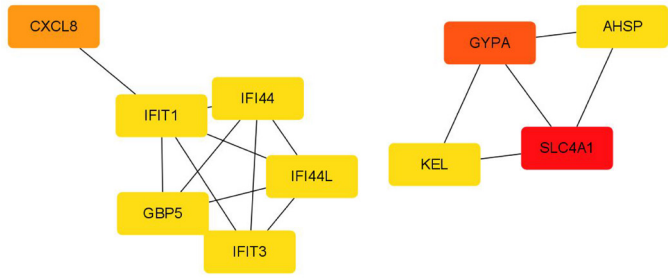

Figure 5. PPI network of top 10 Hub genes.

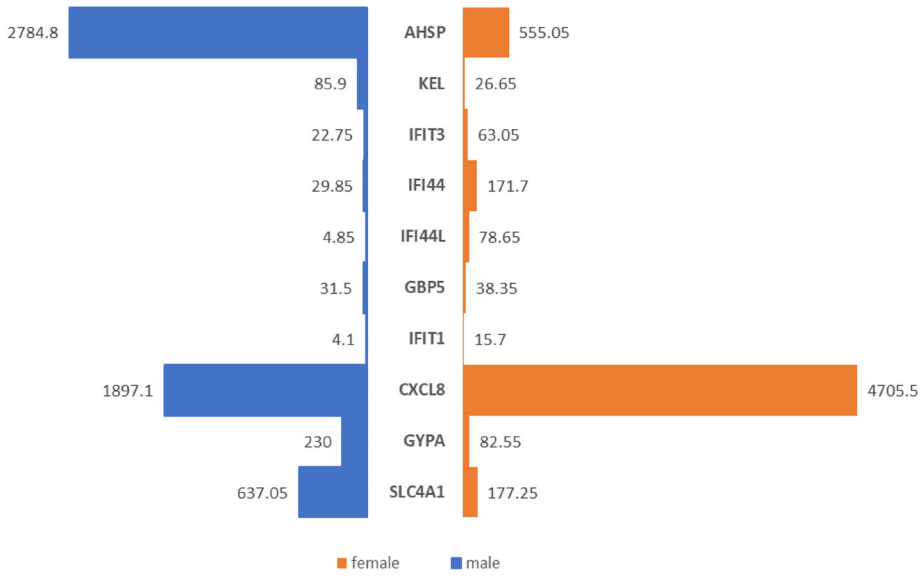

Figure 6. Analysis of hub gene protein expression in bone marrow of different genders in human tissues from HPA database. Yellow represents females with ONFH, and blue represents males with ONFH.

database. The PPI network was imported into the Cytoscape software V3.8.2 for PPI network visualization. The degree values of DEGs were calculated and sorted with the plug-in software cytohubba. Those with a higher degree value were Hub genes, which were more likely to be associated with ONFH. As shown in Figure 5, there were 10 Hub genes with the largest degree value, including SLC4A1, GYPA, CXCL8, IFIT1, GBP5, IFI44, IFI44L, IFIT3, KEL and AHSP. Among them, GBP5, IFI44, IFIT1, IFIT3 and IFI44L have higher levels in ONFH females than in ONFH males. SLC4A1, GYPA, CXCL8, AHSP and KEL were down-regulated in ONFH females as compared with ONFH males.

To identify the specificity of Hub genes during the course of ONFH, proteins (Figure 6) and gene expression levels of Hub genes in human bone marrow were compared in HPA database. And it was found that at the protein level, SLC4A1, GYPA, CXCL8, GBP5, IFI44, KEL and AHSP were specifically expressed in the bone marrow. Therefore, it was thought that these Hub genes may represent key genes in the process of ONFH by gender difference.

Discussion

ONFH is a common disease on hip with a gender specificity in the prevalence and incidence. Male patients develop ONFH 3.4 years earlier than female patients. Male patients outnumber female patients, with an overall gender ratio of 7:3. Autoimmune diseases, alcohol consumption, glucocorticoids,

etc., are risk factors for inducing ONFH. Studies have shown that male patients with ONFH have a greater incidence than female patients with ONFH even if steroid hormones are also used[15]. In addition, another study showed that females are less prone to ONFH compared with males even with greater alcohol consumption and longer duration[16]. This proves that the underlying mechanism of gender difference leading to ONFH is unclear. It is of great importance to investigate the molecular mechanisms responsible for gender differences in ONFH.

In this study, a comprehensive bioinformatics analysis was performed with two GEO Data gene expression profile datasets to identify the biological mechanisms involved in the pathogenesis of gender difference in ONFH. A total of 64 differential genes were identified in postmenopausal ONFH females and ONFH males, including 10 up-regulated genes and 54 down-regulated genes. Among them, SLC4A1, GYPA, CXCL8, IFIT1, GBP5, IFI44, IFI44L, IFIT3, KEL and AHSP were Hub genes. GO enrichment analysis on DEGs revealed that DEGs were mainly enriched in immunoglobulin production involved in immunoglobulin-mediated immune response, peptide antigen assembly with MHC class II protein complex, defense response to virus, and immune response integral component of membrane, plasma membrane, protein heterodimerization activity, peptide antigen binding, MHC class II protein complex binding, protein homodimerization activity, and protein binding. These functional enrichment analyses indicated that DEGs play a role in autoimmunity, assembly and binding of peptide antigens in complexes with MHC class II proteins, demonstrating that autoimmunity plays an important role in the pathogenesis of ONFH. Furthermore, GSEA and KEGG pathways with overlapping DEGs showed that these genes were mainly enriched in cGMP-PKG signaling pathway, Fatty acid degradation, Non-alcoholic fatty liver disease, Systemic lupus erythematosus and Hematopoietic cell lineage. According to the previous studies on ONFH, cGMP-PKG signaling pathway[17], Fatty acid degradation[18,19], Non-alcoholic fatty liver disease[20], Systemic lupus erythematosus[21,22] and Hematopoietic cell lineage[23] are involved in the pathogenesis of ONFH.

In the constructed PPI network, 10 key genes were identified, including SLC4A1, GYPA, CXCL8, IFIT1, GBP5, IFI44, IFI44L, IFIT3, KEL and AHSP, which may play an important role in the occurrence and development of ONFH. More evidence in previous studies has proved that SLC4A1, GYPA, CXCL8 and GBP5 play an important role in the pathogenesis of ONFH. The etiology and pathology of ONFH are not fully defined, with multiple pathogenic factors, including hemoglobinopathies, autoimmune diseases, inflammatory cytokines, angiogenesis and osteogenic differentiation. In previous studies, SLC4A1 was found to be associated with inflammation, cell death and transcriptional regulation, which is associated with chondrocyte differentiation and cartilage development. SLC4A1 can be retained in the nucleus and recruited the transcription factor NF-κB to bind to CXCL8, resulting in increased H3K27ac in the CXCL8 promoter, and subsequently, CXCL8 expression is elevated. The activation of CXCL8 can exacerbate the inflammatory response in trophoblast cells by inducing both TNF-α and IL-1β[24]. Guanylate-binding proteins (GBPs) are negatively induced by FN to regulate age-related bone loss and osteolysis. Moreover, some studies have proved that both GBP2 and GBP5 negatively regulate osteoclast differentiation *in vitro*, and the loss of GBP5 can lead to a greater age-related bone loss in mice[25]. GYPA is the main intrinsic membrane protein of erythrocytes, and GYPB becomes the antigen-ic-determining cluster of MN and Ss blood groups. According to a retrospective study, there is a significant difference in disease progression between patients with blood group A and those with blood group O. Among these, ONFH patients with blood group A had the most advanced progression[26]. As different IFN inducible proteins, how IFIT1, ifi44, ifi44l, IFIT3 play a role in the development and progression of ONFH needs to be further explored. However, in previous studies, IFI44L was found to be induced by estrogen, showing different expression patterns[27]. Another study found that age and gender differences play an important role in proteomic response differences, most notably in immune responses, oxidative stress, and apoptotic cell death. Gender difference has distinct effects on DNA damage and repair pathways and associated cellular outcomes (pro-survival versus Pro apoptotic)[28]. While AHSP was identified as a potential general biomarker candidate across age and gender. All the above may suggest a different pathogenesis between female patients with ONFH compared with male patients with ONFH. In addition to the genes already discussed, there is a lack of data to prove whether IFIT1, IFI44, IFI44L, IFIT3, KEL and AHSP are strongly associated with the occurrence and progression of ONFH. Given the specific pathophysiological roles of SLC4A1, GYPA, CXCL8 and GBP5 in ONFH, and the specific expression of IFIT44, IFI44L, IFIT1, IFIT3, KEL and AHSP in bone marrow, mainly involving in neutrophil-humoral immune response, it is also of a certain value to investigate the role of IFIT1, IFI44, IFI44L, IFIT3, KEL and AHSP in the inflammation, immune response, oxidative stress and apoptosis of ONFH.

GSEA and KEGG pathways showed that Hub genes were mainly enriched in cGMP-PKG signaling pathway, Fatty acid degradation, Non-alcoholic fatty liver disease, Systemic lupus erythematosus and Hematopoietic cell lineage. NO-cGMP-PKG signaling plays a key role in skeletal homeostasis, and NO is required for bone remodeling, and partially mediates the mechanical stimulation and the anabolic effects of estrogen[17]. Short-chain fatty acid (SCFA) is the modulator of osteoclast metabolism and bone mass in the body. Treating mice with SCFA and feeding them with a high-fiber diet significantly increased bone mass and prevented bone loss caused by menopause and inflammation. The protective effect of SCFA on bone mass is associated with the inhibition of osteoclast differentiation and bone resorption *in vitro* and *in vivo*, while bone formation is not affected. Mechanistically, propionate (C3) and butyrate (C4) induce metabolic reprogramming of osteoclasts, leading to enhanced glycolysis at the

cost of oxidative phosphorylation, resulting in the down-regulation of basic osteoclast genes, such as TRAF6 and NFATc1[29]. Non-alcoholic fatty liver disease (NAFLD) is non-autoimmune hepatitis associated with the presence of steatosis, oxidative stress and inflammatory mediators (such as TNF-α and IL-6) and altered nuclear factors in NAFLD (e.g., CAR, PXR, PPAR-α)[30], which is also a risk factor for ONFH, with one case report indicating increasing corticosteroid doses. Higher levels of liver enzymes started up and ONFH occurred successively, which indicates that the liver enzymes may be involved in the steroid induction of ONFH[20]. Systemic lupus erythematosus (SLE) is a common autoimmune disease. Current studies show that clinical factors associated with SLE, including disease activity, genetic polymorphisms, vasculitis, Raynaud's phenomenon, hypertriglyceridemia, antiphospholipid syndrome (APS) and autoimmune antibodies, are also associated with the risk of osteonecrosis[31-35]. NOD-like receptor contains a family pyrin domain of 3 (NLRP3) that assembles protein complexes called NLRP3 inflammasome when certain pathogen products or sterile danger signals are detected. NLRP3 inflammasome was shown to be involved in the absorption and dissolution mechanisms of bone[36,37].

Conclusions

In conclusion, the results of this study suggest that cGMP-PKG signaling pathway, Fatty acid degradation, Non-alcoholic fatty liver disease, Systemic lupus erythematosus, Hematopoietic cell lineage and NO-cGMP-PKG signaling may play an important role in the occurrence and development of gender-differential ONFH between males and females. SLC4A1, GYPA, CXCL8, GBP5 and AHSP may be key genes associated with the progression of gender-differential ONFH and may be ideal targets for ONFH. In addition, the roles of IFIT1, IFI44, IFI44L, IFIT3 and KEL in inflammation, immune response, oxidative stress and apoptosis of ONFH are also valuable and may be associated with the treatment of ONFH.

Authors' contributions

ZY designed the study and drafted the manuscript. JX and GW were responsible for the collection and analysis of the experimental data. YZ, XL and FZ revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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