Microarray-based screening of differentially expressed genes in glucocorticoid-induced avascular necrosis

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Abstract. The underlying mechanisms of glucocorticoid (GC)-induced avascular necrosis of the femoral head (ANFH) have yet to be fully understood, in particular the mechanisms associated with the change of gene expression pattern. The present study aimed to identify key genes with a differential expression pattern in GC-induced ANFH. E-MEXP-2751 microarray data were downloaded from the ArrayExpress database. Differentially expressed genes (DEGs) were identified in 5 femoral head samples of steroid-induced ANFH rats compared with 5 placebo-treated rat samples. Gene Ontology (GO) and pathway enrichment analyses were performed upon these DEGs. A total 93 DEGs (46 upregulated and 47 downregulated genes) were identified in GC-induced ANFH samples. These DEGs were enriched in different GO terms and pathways, including chondrocyte differentiation and detection of chemical stimuli. The enrichment map revealed that skeletal system development was interconnected with several other GO terms by gene overlap. The literature mined network analysis revealed that 5 upregulated genes were associated with femoral necrosis, including parathyroid hormone receptor 1 (*PTHR1*), vitamin D (1,25-Dihydroxyvitamin D3) receptor (*VDR*), collagen, type II, α1, proprotein convertase subtilisin/kexin type 6 and zinc finger protein 354C (*ZFP354C*). In addition, *ZFP354C* and *VDR* were identified to transcription factors. Furthermore, *PTHR1* was revealed to interact with *VDR*, and α-2-macroglobulin (*A2M*) interacted with fibronectin 1 (*FN1*) in the PPI network. *PTHR1* may be involved in GC-induced ANFH via interacting with *VDR*. *A2M* may also be involved in the development of GC-induced ANFH through interacting with *FN1*. An improved understanding of the molecular mechanisms underlying GC-induced ANFH may provide novel targets for diagnostics and therapeutic treatment.

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Introduction

Avascular necrosis of the femoral head (ANFH) is a progressive pathological process that leads to avascular necrosis and collapse of the femoral head due to an obstructed blood supply, resulting in impaired hip joint function and permanent disability (1,2). ANFH causes significant morbidity as well as impairment of daily function to patients (3). In addition, ANFH is considered irreversible, therefore any diagnostic strategy or therapeutic intervention for ANFH is best introduced at an early stage (4).

Glucocorticoid (GC) administration is known to be the most common non-traumatic cause of ANFH (5). GCs are widely used to treat most inflammatory disorders and are also included in most chemotherapy protocols (6). Therefore, ANFH due to GC use is a potential major complication for a large patient group (6). However, the exact pathogenesis of AFNH associated with GC remains to be fully elucidated. There are several alternative mechanisms including fat embolization, modified artery constriction, intramedullary pressure changes, coagulation disorders, circulatory impairment and cell dysfunction. There is evidence that apoptosis of osteoblasts and osteocytes, not compromised vascularity, is the direct effect and primary etiology of GC-induced ANFH (7,8). Kerachian *et al* (9). suggested that GCs may contribute to differential gene expression in ANFH in rats, and revealed that GC-induced ANFH in rats may be mediated by α-2-macroglobulin (*A2M*). In addition, Samara *et al* (10). demonstrated that osteoprotegerin, receptor activator of nuclear factor-κB (RANK) and RANK ligand mRNA levels were higher in the necrotic part of the femoral head of patients with avascular necrosis compared with the normal site (9). However, despite the strong association of GC with ANFH, the underlying mechanisms are yet to be fully understood, in particular the mechanisms associated with the change of gene expression pattern.

Using the same microarray data as Kerachian *et al* (9), the present study performed bioinformatic analysis with the aim of identifying additional genes associated with GC-induced ANFH. In the present study, differentially expressed genes (DEGs) were

Table I. The enriched GO terms of upregulated genes and downregulated genes.

Category	Term	Count	P-value
Upregulated			
BP	GO:0051216~cartilage development	6	2.27×10^{-06}
	GO:0001501~skeletal system development	8	1.06×10^{-05}
	GO:0006953~acute-phase response	4	1.24×10^{-04}
	GO:0060348~bone development	5	4.81×10^{-04}
	GO:0002062~chondrocyte differentiation	3	0.00175
	GO:0030198~extracellular matrix organization	4	0.0022
	GO:0002526~acute inflammatory response	4	0.0024
	GO:0006954~inflammatory response	5	0.0038
CC	GO:0005576~extracellular region	17	7.61×10^{-08}
	GO:0044421~extracellular region part	13	2.06×10^{-07}
	GO:0031012~extracellular matrix	8	5.73×10^{-06}
	GO:0005615~extracellular space	9	5.55×10^{-05}
	GO:0005578~proteinaceous extracellular matrix	6	3.73×10^{-04}
	GO:0005604~basement membrane	3	0.0181
	GO:0044420~extracellular matrix part	3	0.0315
MF	GO:0005539~glycosaminoglycan binding	5	1.49×10^{-04}
	GO:0001871~pattern binding	5	2.45×10^{-04}
	GO:0030247~polysaccharide binding	5	2.45×10^{-04}
	GO:0030246~carbohydrate binding	6	0.0019
	GO:0005201~extracellular matrix structural constituent	3	0.0037
	GO:0008201~heparin binding	3	0.0158
Downregulated			
BP	GO:0007186~G-protein coupled receptor protein signaling pathway	33	8.15×10^{-19}
	GO:0007166~cell surface receptor linked signal transduction	35	2.64×10^{-18}
	GO:0007606~sensory perception of chemical stimulus	26	1.89×10^{-15}
	GO:0007600~sensory perception	27	3.35×10^{-15}
	GO:0050911~detection of chemical stimulus involved in sensory perception of smell	25	6.47×10^{-15}
	GO:0050907~detection of chemical stimulus involved in sensory perception	25	7.79×10^{-15}
	GO:0009593~detection of chemical stimulus	25	9.98×10^{-15}
	GO:0007608~sensory perception of smell	25	1.13×10^{-14}
CC	GO:0016021~integral to membrane	39	8.59×10^{-16}
	GO:0031224~intrinsic to membrane	39	2.67×10^{-15}
	GO:0042612~MHC class I protein complex	3	0.010435218
	GO:0042611~MHC protein complex	3	0.015440507
MF	GO:0004984~olfactory receptor activity	26	9.54×10^{-17}
	GO:0016503~pheromone receptor activity	3	0.0349

GO, Gene Ontology; BP, biological processes; CC, cellular component; MF, molecular function.

identified in femoral head samples of steroid-induced ANFH rats compared with placebo-treated rat samples. Gene Ontology (GO) and pathway enrichment analyses were performed upon these DEGs. An enrichment map was generated to identify over-represented themes with regard to the enriched GO terms, and a text-mining gene association network associated with femoral necrosis was generated, followed by the construction of a transcriptional regulation network and a protein-protein interaction (PPI) network. An improved understanding of the molecular mechanisms underlying GC-induced ANFH will likely provide novel targets for diagnostic and therapeutic treatment.

Materials and methods

Microarray data. The ArrayExpress Archive of Functional Genomics Data (http://www.ebi.ac.uk/arrayexpress) is one

Table II. The enriched KEGG pathways of upregulated and downregulated genes.

Term	Count	P-value
Upregulated		
rno04740:Olfactory transduction	9	0.0073
Downregulated		
rno04740:Olfactory transduction	19	2.09×10^{-08}
rno05332:Graft-versus-host disease	4	0.0018
rno05330:Allograft rejection	4	0.0020
rno04940:Type I diabetes mellitus	4	0.0029
rno05320:Autoimmune thyroid disease	4	0.0030
rno04514:Cell adhesion molecules (CAMs)	5	0.0052
rno05416:Viral myocarditis	4	0.0081
rno04612:Antigen processing and presentation	4	0.0084

of three major international repositories for functional genomics public data, alongside the Gene Expression Omnibus at NCBI and the DDBJ Omics Archive (11). The mRNA expression profile deposited by Kerachian *et al* (9), was downloaded from the ArrayExpress database (accession number, E-MEXP-2751; http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-2751/). The platform used was A-AFFY-99-Affymetrix GeneChip Rat Exon 1.0 ST Array [RaEx-1_0-st-v1]. As the original study described (9), glucocorticoid pellets or placebo pellets were administered to age-matched Wistar Kyoto rats for 6 months. Following the 6 month period of the experiment, rats were sacrificed and tissue samples were obtained from the proximal femur, containing the femoral head. In the present study, a dataset consisting of 5 steroid-induced ANFH rat samples (named Steroid_ANFH) and 5 placebo-treated rat samples (named Placebo_no_ANFH) were used in the follow-up analysis.

Data preprocessing and screening of DEGs. The raw data (CEL. format files) were preprocessed in R using the Bioconductor package, oligo (12). Data were subjected to background correction, quantile normalization, and calculation of expression values using the robust multi-array average algorithm (13). The probe symbols were then transformed into the corresponding gene symbols. When several probes were mapped to one gene, the average value was calculated and represented as the expression levels of this gene. Finally, the gene expression matrix was obtained.

Upregulated and downregulated DEGs were screened in Steroid_ANFH against Placebo_no_ANFH using the linear models for microarray data (limma) package version 2.14.1 (14) in Bioconductor. In addition, P-values from unpaired t-tests (15) in the limma package were calculated to determine the significance of differences between samples. In the present study, genes with $\log_2$fold change (FC) ≥0.4 and P<0.05 were selected as DEGs. Furthermore, the DEGs identified between Steroid_ANFH and Placebo_no_ANFH were clustered using gplots package (version 2.12.1; https://cran.r-project.org/web/packages/gplots/index.html) (16) in R to evaluate whether the identified DEGs were sample-specific. The clustering results were visualized as heat maps.

Functional and pathway enrichment analysis. The database for annotation, visualization and integrated discovery (DAVID) is a web-accessible program that provides functional interpretation of large gene lists through facilitating the transition from data collection to biological meaning (17). In the present study, in order to analyze the identified upregulated genes and downregulated genes in Steroid_ANFH on the functional level, GO (18) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (19) pathway enrichment analyses were performed using DAVID (17). The hypergeometric test (20) was applied to calculate P-values. In particular, gene count ≥2 and P<0.05 were selected as the threshold for identifying significant GO terms and KEGG pathways.

Visualization of GO enrichment analysis. To gain a full view of the probable functions of the upregulated and downregulated genes, an enrichment map was constructed with the Enrichment Map plugin (21) in Cytoscape version 2.8 (22) (http://www.baderlab.org/Software/EnrichmentMap), using the GO enrichment results as the input. The parameters were set as follows: Similarity cutoff, overlap coefficient; cutoff, 0.5; P-value cutoff, 0.005; false discovery rate Q value cutoff, 0.1. In the Enrichment Map, each node represented a gene set corresponding to an enriched GO term. Node size corresponded to the total number of genes in each GO term. Each weighted line indicated the overlap between two gene sets and the line thickness indicated the number of overlapping genes between nodes.

Generation of association network through text-mining. The Agilent Literature Search plugin (23) was used in conjunction with Cytoscape (22) for text-mining literature with structured queries and for obtaining gene-association networks according to the text-mining results (24). In the present study, in order to further ensure that the upregulated and downregulated genes were biologically associated with ANFH, the Agilent Literature Search tool (23) was used to query published literature associated with the context, and meanwhile construct an association network according to the text-mining results. 'Femoral necrosis' was provided as context terms while the genes from the identified DEG (upregulated and downregulated genes) gene-set were used as search terms. In addition, the species was limited to 'rat'.

Computational searches for transcription factors (TFs). The standard integrated analysis package Genomatix Software Suite version 2.1 (Genomatix GmbH, Munich, Germany;

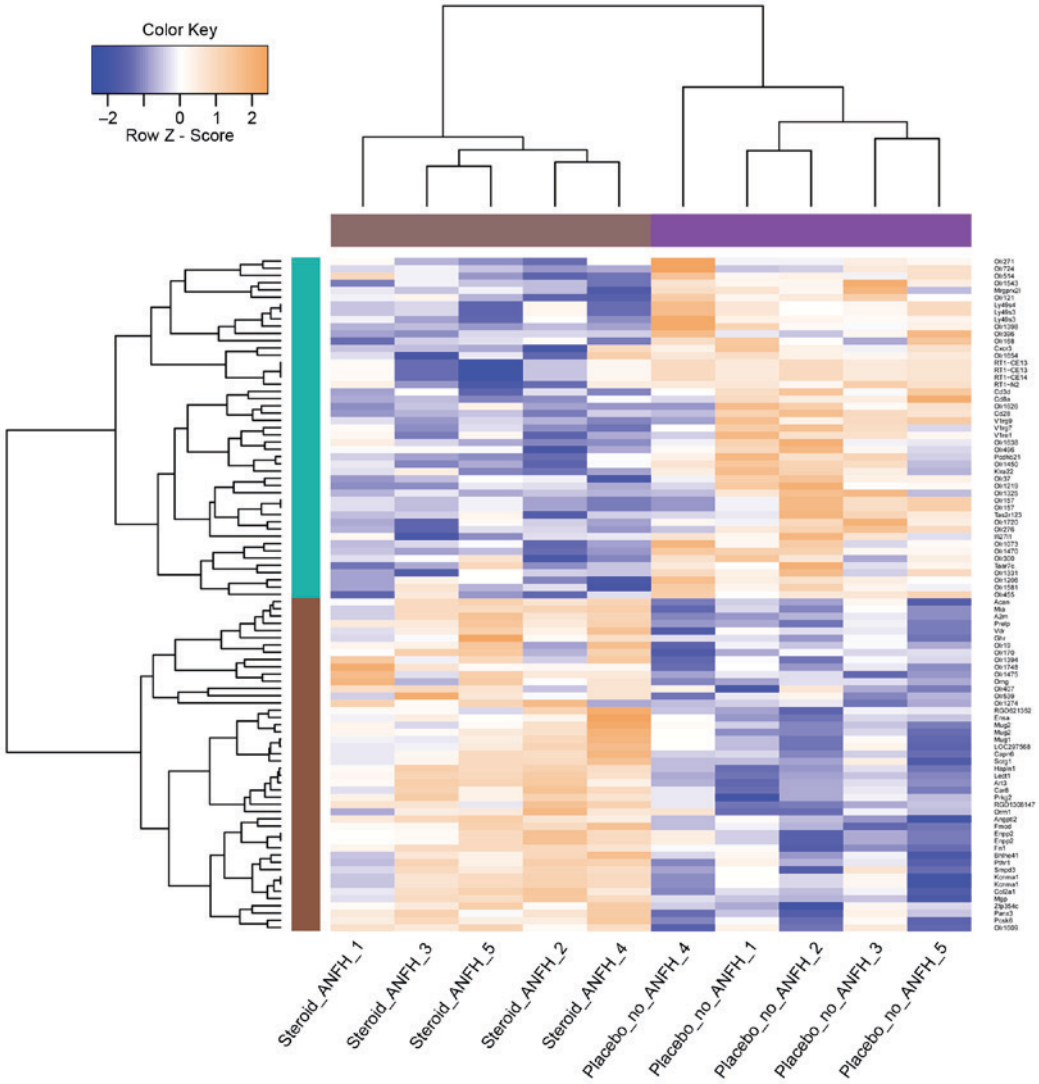

Figure 1. Heat map of differentially expressed genes. Blue or brown indicates higher or lower expression levels of genes, respectively. White indicates genes without altered expression. The brown and purple bars along the top indicate steroid-induced ANFH rat samples and placebo-treated rat samples, respectively. The brown and green bars on the left-hand side indicate the distant boundary of upregulated and downregulated genes.

http://www.genomatix.de) provides large amounts of functional information about genes and their interactions, including TFs (25). In the present study, to gain insights into the transcriptional regulation of the identified DEGs, a software tool within the Genomatix software suite (http://www.genomatix.de/cgi-bin//GePS/gene-tf_analysis.pl?) (25) was applied to search for IFs that regulated the DEGs and to evaluate the number of TFs with altered expression in the samples. This analysis was based on the literature-derived gene-TF annotation. The input gene-set was the identified upregulated and downregulated genes. The parameter of evidence level was set as the sentence level.

Construction of PPI network. The online database resource Search Tool for the Retrieval of Interacting Genes (STRING) is a comprehensive database providing functional links of proteins, including experimental as well as predicted interaction information by text mining or comparative genomics (26). Interactions of proteins in the STRING database were provided with a confidence score (26). In the present study, a PPI network was constructed using data derived from the STRING database. Genes included in the PPI network were all identified DEGs and a confidence score ≥0.4 was set as the threshold. Proteins encoded by upregulated and downregulated genes were represented by nodes in the network, and lines indicated interactions among proteins. Hub proteins in the PPI network were deemed as those that interacted with the most proteins and that had a higher degree. Cytoscape software version 2.8 (22) was applied for network visualization.

Results

DEG screening. Compared with Placebo_no_ANFH controls, a total of 46 upregulated genes and 47 downregulated genes were identified in Steroid_ANFH samples. The mean $\log_2$FC value of upregulated genes was 0.5655 while the average $\log_2$FC value of downregulated genes was -0.5832. Furthermore, the expression pattern of identified DEGs correctly distinguished the Steroid_ANFH samples

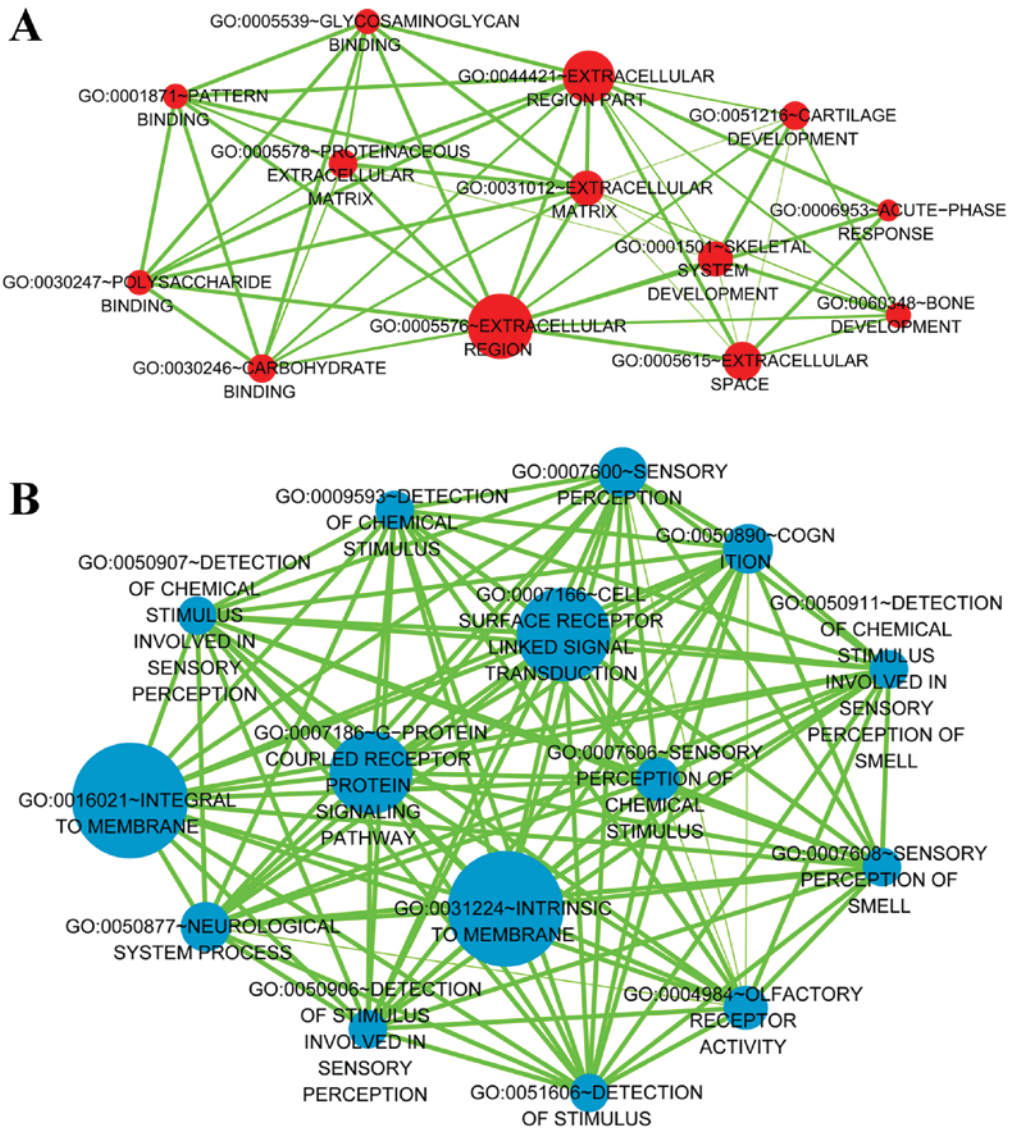

Figure 2. Enrichment map of GO terms of up and downregulated genes. (A) Enrichment map of GO terms of upregulated genes. Red nodes indicate GO terms of upregulated genes. The node size represents the number of genes that belong to this GO term. The width of the line (green color) is proportional to the size of the overlapping genes. (B) Enrichment map of GO terms of downregulated genes. Blue nodes represent a GO term enriched by downregulated genes and the node size is representative of the number of enriched genes in this GO term. Each weighted line (green color) represents the overlapping genes between the two GO terms.

from Placebo_no_ANFH control samples, as visualized in Fig. 1.

Biological interpretation of ANFH gene expression signature. GO terms, including biological processes, cellular component, and molecular function categories of the significantly up and downregulated genes were summarized in Table I. The results demonstrated that the upregulated genes were significantly concerned with bone development processes, including cartilage development, skeletal system development, bone development and chondrocyte differentiation. The downregulated genes were enriched in different GO terms, including cell surface receptor-linked signal transduction and detection of chemical stimulus. The results of KEGG pathway enrichment analysis were summarized in Table II. From the results, upregulated genes were revealed to be involved in one pathway; olfactory transduction. The downregulated genes were enriched in distinct pathways, including antigen processing and presentation.

Overview of enrichment map profiling. In the present study, the enrichment map was generated to unravel overrepresented themes in ANFH through enrichment analysis with regard to the enriched GO terms. The enrichment maps of upregulated genes and downregulated genes were visualized in Fig. 2A and B, respectively. The enrichment network algorithmically clustered GO terms with highly similar genes and the GO term of GO:0001501~SKELETAL SYSTEM DEVELOPMENT was interconnected with several other GO terms by gene overlap. The degree of this node (GO:0001501) was 7. The enrichment map of downregulated genes revealed that GO terms in this enrichment network were tightly linked and the degree of each GO term in this network was 14.

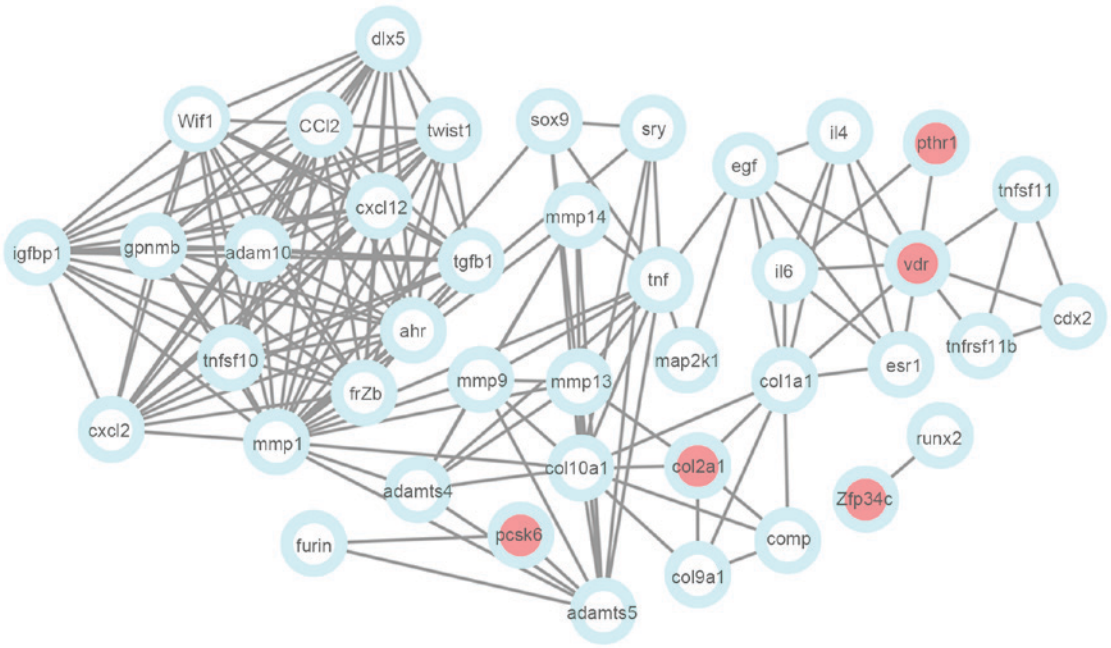

Figure 3. A text-mining association network, associated with femoral necrosis. Red nodes indicate upregulated genes. Each line indicates the interaction between two genes, identified by mining the literature.

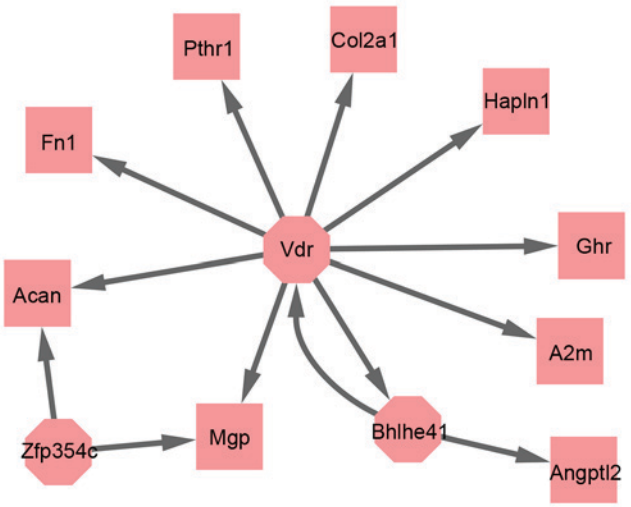

Figure 4. Transcriptional regulation network. Octagonal nodes represent transcription factors. Square nodes represent the target genes.

Extracting significant associations and the construction of the literature mined network of femoral necrosis. With the available data in regard to interactions of different proteins, protein partners for the genes associated with femoral necrosis were searched for using the Agilent Literature Search tool, and a text-mining association network associated with femoral necrosis was generated (Fig. 3). The literature mined network analysis revealed that a total of 5 upregulated genes identified in the present study were involved in the network, including parathyroid hormone receptor 1 (*PTHR1*), vitamin D (1,25-Dihydroxyvitamin D3) receptor (*VDR*), collagen, type II, α1 (*COL2A1*), proprotein convertase subtilisin/kexin type 6 (*PCSK60*), and zinc finger protein 354C (*ZFP354C*). These 5 upregulated genes were identified to be associated with femoral necrosis, and interacted with other disease-related genes to generate the network.

The construction of transcriptional regulation network and PPI network. The transcriptional regulation network was visualized in Fig. 4. The results revealed that out of all the DEGs 3 TFs were identified, namely *ZFP354C*, *VDR*, and basic helix-loop-helix family, member E41 (*BHLHE41*). These 3 TFs were all upregulated and their target genes were also upregulated (Fig. 4). In addition, *ZFP354C* and *VDR* were also identified as associated with femoral necrosis, as mentioned above.

Furthermore, a PPI network was constructed (Fig. 5). The PPI network consisted of 31 nodes and 67 lines (interactions). The results revealed that the interactive relationships of proteins were relatively simple. However, olfactory receptor genes (including *Olr168* and *Olr1654*) were revealed to make up an interaction module. Furthermore, the PPI network revealed that *PTHR1* interacted with *VDR*. *A2M* was also revealed to interact with fibronectin 1 (*FN1*).

Discussion

There is a considerable negative effect of ANFH on the workforce and, subsequently, on economics. Major efforts are being made to understand GC-induced ANFH at a molecular level. In the present study, a total of 93 DEGs (46 up and 47 downregulated genes) were identified in GC-induced ANFH samples compared with controls. These DEGs were enriched in different GO terms and pathways, including chondrocyte differentiation and detection of chemical stimuli. The enrichment map revealed that the GO term of skeletal system development was interconnected with several other GO terms by gene overlap. The literature mined network analysis revealed that 5 upregulated genes were associated with femoral necrosis, including *PTHR1*, *VDR*, *COL2A1*,

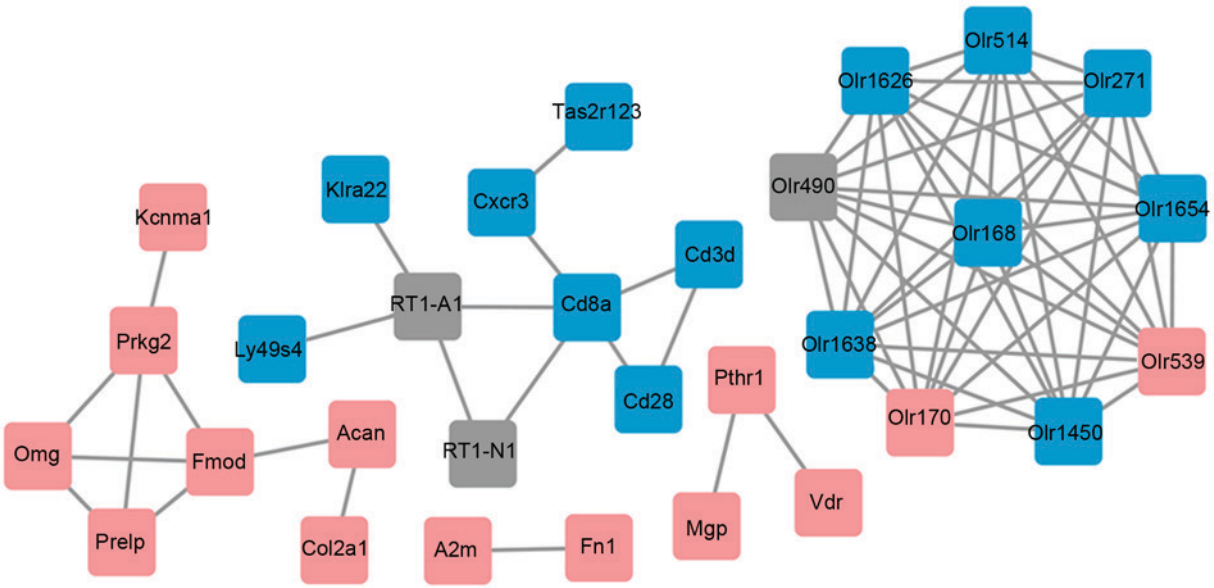

Figure 5. Protein-protein interaction network. Red nodes indicate upregulated genes. Blue nodes represent downregulated genes. Gray nodes indicate genes without altered expression.

PCSK60, and *ZFP354C*. In addition, ZFP354C and *VDR* were also identified as TFs. Furthermore, *PTHR1* was identified to interact with *VDR* and *A2M* was revealed to interact with *FN1* in the PPI network.

The protein encoded by *PTHR1* is a member of the G-protein coupled receptor family 2, and this protein is a receptor for parathyroid hormone (PTH) and for parathyroid hormone-like hormone (PTHLH) (27). Previous studies have demonstrated that injected PTH may be used to restore bone loss resulting from excessive glucocorticoid use, and PTH-associated protein peptides may be useful as putative bone regenerative therapies in GC-related bone diseases (28,29). On the other hand, *VDR* encodes the nuclear hormone receptor for vitamin D3, and VDR may act as a TF that mediates the action of vitamin D3 through controlling the expression of hormone sensitive genes (30). Previous evidence demonstrates that *VDR* polymorphisms are putatively associated with the risk of osteonecrosis and are significantly associated with the development of osteonecrosis (31). In addition, the work of Goltzman *et al* (32) revealed that the development of the cartilaginous growth plate and parathyroid gland size were each coordinately regulated by calcium and by the active form of vitamin D, 1,25-dihydroxyvitamin D [1,25 (OH)2D], and that an intact 1,25(OH)2D/VDR system was required for an appropriate osteoclastic response to increased PTH. Consistent with this notion, the present study revealed that the upregulated gene *PTHR1* may interact with another upregulated gene, *VDR*, in the PPI network. Collectively, this suggests that *PTHR1* may be involved in the pathogenesis of GC-induced ANFH via interacting with *VDR*.

The protein encoded by *A2M* is a protease inhibitor and cytokine transporter (33). A2M inhibits multiple proteases, including trypsin, thrombin and collagenase (33). Kerachian *et al* (9) reported that A2M shared vascular, cartilaginous and osteogenic functions and may be involved in the development of early GC-induced ANFH (9). *FN1* encodes fibronectin, a glycoprotein present on the cell surface and in the extracellular matrix, and this protein is involved in cell adhesion and migration processes (34). Previous data has revealed that fibronectin-aggrecan complex may be useful as a marker for cartilage degradation in patients with non-arthritic hip pain (35). ANFH is characterized by pain in the hip, bone collapse and functional loss (6). In addition, a close connection was identified between *FN1* and *A2M* genes in the study of Zhang *et al* (36). Furthermore, A2M was revealed to interact with *FH1* in the PPI network in the present study. In this context, it is possible to suggest that *A2M* has an interaction with *FN1* and is involved in the development of GC-induced ANFH. However, further studies and experiments are required to confirm this finding.

However, the present study has several limitations. First, the sample size is small, so further investigations based on larger samples may be required. Second, this is a computational study based on bioinformatics approaches, which gives simulated results. The lack of cross-checking and further experimental verification were also a limitation of the present investigation. Statistical validation using other datasets may be used to cross-check these results. Furthermore, experimental verifications will be performed to determine the findings in future studies using different approaches, including reverse transcription-quantitative polymerase chain reaction and western blot analysis.

In conclusion, the present study demonstrated that several potential key genes (*PTHR1*, *VDR*, *A2M*, and *FN1*) were associated with the development of GC-induced ANFH. *PTHR1* may be involved in the pathogenesis of GC-induced ANFH via interacting with *VDR*. *A2M* may also have a significant function in the development of GC-induced ANFH through interacting with *FN1*. Further investigations with more samples as well as further experimental validation may provide novel insights into the mechanism of GC-induced ANFH and will aid the discovery of novel diagnostic and therapeutic approaches.

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