Identification of genes associated with osteoarthritis by microarray analysis

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Abstract. The aim of the present study was to investigate the mechanisms of osteoarthritis (OA). Raw microarray data (GSE51588) were downloaded from Gene Expression Omnibus, including samples from OA (n=20) and non-OA (n=5) knee lateral and medial tibial plateaus. Differentially expressed genes (DEGs) were identified using Student's t-test. Functional and pathway enrichment analyses were performed for the upregulated and downregulated DEGs. A protein-protein interaction network (PPI) was constructed according to the Search Tool for the Retrieval of Interacting Genes/Proteins database, and module analysis of the PPI network was performed using CFinder. The protein domain enrichment analysis for genes in modules was performed using the INTERPRO database. A total of 869 upregulated and 508 downregulated DEGs were identified. The enriched pathways of downregulated and upregulated DEGs were predominantly associated with the cell cycle (*BUB1*, *BUB1B*, *CCNA2*, *CCNB1* and *CCNE1*), and extracellular matrix (ECM)-receptor interaction (*CD36*, *COL11A2*, *COL1A1*, *COL2A1* and *COL3A1*). Functional enrichment analysis of the DEGs demonstrated that *FGF19*, *KIF11* and *KIF2C* were involved in the response to stress and that *ACAN*, *ADAMTS10* and *BGN* were associated with proteinaceous ECM. The top protein domain was IPR001752: Kinesin motor region involving three genes (*KIF2C*, *KIF11* and *KIF20A*). The identified DEGs, including *KIF2C*, *KIF11* and *KIF20A*, may be significant in the pathogenesis of OA.

Introduction

Osteoarthritis (OA) is the most prevalent chronic joint disease and results in a large economic burden due to the associated costs of medical care and lost earnings (1). OA is characterized by the disappearance of the cartilage, combined with sub-chondral bone sclerosis, formation of osteophytes and a mild inflammation of the synovial membrane (2).

The pathology of OA is complex and a large number of studies have focused on the pathogenesis of OA to identify a therapeutic method for patients with OA. The sub-chondral bone is involved in the pathophysiology of OA through biochemical and mechanical pathways (3). OA microarray analysis has predominantly focused on the articular cartilage, meniscus or synovium; however, only few studies on sub-chondral bone are available (4,5). Therefore, the present study assessed the sub-chondral bone in order to enhance the understanding of the pathology of OA. Zhen *et al* (6) reported that the pathological changes of OA may be caused by high concentrations of active transforming growth factor-β1 in the subchondral bone, and that inhibition of this process may represent a potential therapeutic method. According to a study by Valverde-Franco *et al* (7), bone-specific overexpression of *EphB4* on subchondral bone and cartilage has a protective effect on OA. With the number of studies increasing, further genes associated with OA are being identified.

Microarray analysis has been used to analyze the gene expression of thousands of transcripts in OA samples. Several novel candidate genes, including bone formation-associated genes (*CLEC3B*, *CDH11*, *GPNMB*, *CLEC3A*, *CHST11*, *MSX1*, *MSX2*) and genes encoding collagens (*COL13A1*, *COL14A1*, *COL15A1*, *COL8A2*), were identified by microarray analysis (8). Chou *et al* (4) only performed functional and pathway enrichment analyses of the identified differentially expressed genes (DEGs). In the present study, the microarray data (GSE51588) (4) were downloaded from Gene Expression Omnibus using additional bioinformatics analysis to gain further insight into the molecular mechanisms of OA. Gene Ontology (GO) functional terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the DEGs. In addition, a protein-protein interaction (PPI) network was constructed and protein domain enrichment of the genes in the modules of the PPI network were performed to screen the significant genes, which were involved in the pathogenesis of OA.

Materials and methods

Microarray data and data pre-processing. The microarray data (GSE51588) deposited by Chou *et al* (4) were downloaded

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from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/). The platform used was the GPL13497 Agilent-026652 Whole Human Genome Microarray 4x44 K v2 (Agilent Technologies, Palo Alto, CA, USA). A total of 50 samples were available, including 20 OA knee lateral tibial plateau samples, 20 OA knee medial plateau samples, 5 non-OA knee lateral tibial plateau samples and 5 non-OA knee medial plateau samples.

For data pre-processing, the expression profile chip was pre-processed using the Affy package in Bioconductor (http://www.bioconductor.org/packages/release/bioc/html/affy.html) (9) and Affymetrix annotation files from Brain Array Lab (Affymetrix, Santa Clara, CA, USA; http://www.affymetrix.com/analysis/). The background correction, quartile data normalization and probe summarization were performed using the robust multiarray average algorithm (http://www.bioconductor.org) (10) to obtain a gene expression matrix.

Identification of DEGs. The expression values for the normalized data were calculated using the Limma package in R (Affymetrix; http://www.affymetrix.com/analysis/) (11). DEGs were identified by Student's t-test. The raw P-value was adjusted into false discovery rate (FDR) using the Benjamini & Hochberg method (12). An FDR <0.01 and |log2fold change|>1 were selected as cut-off criteria.

Enrichment analysis for DEGs. Functional enrichment of the DEGs in the biological process, molecular function and cellular component categories was performed using the GO database (http://geneontology.org/) (13). Based on the KEGG database (http://www.genome.jp/kegg/pathway.html) (14), pathway enrichment of the DEGs was performed. P<0.01 was selected as a cut-off criterion.

Construction of the PPI network and module analysis. The identified DEGs were mapped to the Search Tool for the Retrieval of Interacting Genes version 9.1 database (http://www.string-db.org/) (15) to search for the interaction associations between the proteins, and a confidence score >0.4 was selected as cut-off criterion. Cytoscape software (http://www.cytoscape.org/) (16) was subsequently used to visualize the PPI network.

Module analysis of the PPI network was performed by CFinder (http://www.cfinder.org/) (17). The search algorithm using the Clique Percolation Method was used to identify the k-clique percolation clusters of the network (18). Larger k-clique values represented higher stringency during the identification of dense groups and provided smaller groups with a higher density of links inside them. A k-cliques value of 5 was selected as the cut-off criterion. The protein domain enrichment analysis of genes in the module was analyzed using the INTERPRO database (http://www.ebi.ac.uk/interpro/) (17,19) and P<0.05 was selected as the cut-off criterion.

Results

DEG analysis. For microarray analysis, 1,377 DEGs were identified between the OA group and the non-OA group. Of these genes, 869 DEGs were upregulated and 508 DEGs were downregulated.

Functional and pathway enrichment analysis of the DEGs. The functional enrichment analysis of the downregulated DEGs revealed that the top five enriched pathways were associated with systemic lupus erythematosus ($P=1.17x10^{-13}$), the cell cycle ($P=1.47x10^{-6}$), complement and coagulation cascades (P=0.000685), nitrogen metabolism (P=0.001086) and amoebiasis (P=0.001278) (Table I). *BUB1*, *BUB1B*, *CCNA2*, *CCNB1* and *CCNE1* were the genes involved in the cell cycle. The top five enriched GO terms included response to stress ($P=4.91x10^{-14}$), involving *CCNA2*, *CCNB1*, *FGF19*, *KIF11* and *KIF2C*, immune system-associated processes ($P=1.87x10^{-12}$) involving *FGF19*, *KIF11* and *KIF2C*, response to wounding ($P=2.04x10^{-12}$), including *CCNB1*, *KIF11* and *KIF2C*, defense response ($P=2.37x10^{-12}$), involving *FGF19*, and mitotic cell cycle ($P=2.60x10^{-12}$) associated with *BUB1*, *BUB1B*, *CCNA2*, *CCNB1*, *CCNE1*, *KIF20*, *KIF11* and *KIF2C* (Table II).

The functional enrichment analysis of the upregulated DEGs demonstrated that the top five enriched pathways were associated with extracellular matrix (ECM)-receptor interaction ($P=3.01x10^{-5}$), axon guidance (P=0.000472), amoebiasis (P=0.003458), focal adhesion (P=0.003709) and cancer-associated pathways (P=0.00407) (Table III). Certain genes, including *CD36*, *COL11A2*, *COL1A1*, *COL2A1* and *COL3A1*, were involved in the ECM-receptor interaction pathway. In addition, the GO functional enrichment analysis demonstrated that *ACAN*, *ADAMTS10* and *BGN* were involved in proteinaceous ECM ($P=1.11x10^{-15}$) and ECM ($P=2.22x10^{-15}$) terms, *A2M*, *ABCC6* and *ACAN* were involved in single-multicellular organism process ($P=4.66x10^{-15}$), and multicellular organism process ($P=8.27x10^{-14}$) terms, while *ADAMTS10*, *COL10A1* and *COL11A2* were involved in the ECM part ($P=5.17x10^{-14}$) (Table IV).

Analysis of PPI network and modules. Two modules, including module 2 (Fig. 1) and module 5 (Fig. 2) were extracted from the constructed PPI network. Protein domain enrichment analysis revealed that the genes in module 5 were not significantly enriched in any protein domain. A total of 10 protein domains were enriched for the genes in module 2 (Table V), including IPR001752: Kinesin, motor region (P=0.001518) involving three genes (*KIF2C*, *KIF11* and *KIF20A*), IPR013212: Mad3/BUB1 homology region 1 (P=0.002759) associated with two genes (*BUB1* and *BUB1B*) and IPR014400: Cyclin A/B/D/E (P=0.016448) involving two genes (*CCNB1* and *CCNA2*).

Discussion

In the present study, the gene expression profiles of the OA group and the non-OA group were analyzed and 1,377 DEGs were identified, including 869 upregulated DEGs and 508 downregulated DEGs. The functional enrichment for the downregulated DEGs revealed that the *FGF19* gene was involved in the response to stress as well as the defense response. According to Tew *et al* (20), apoptosis and proliferation are involved in the progression of cartilage wounding during the development of OA. Costouros and Kim (21)

Table I. Top five enriched KEGG pathways for downregulated differentially expressed genes.

KEGG pathway	Gene count	P-value	Genes
Systemic lupus erythematosus	27	$1.17x10^{-13}$	*C9*, *CTSG*, *ELANE*, *H2AFX*, *HIST1H2AC*
Cell cycle	17	$1.47x10^{-6}$	*BUB1*, *BUB1B*, *CCNA2*, *CCNB1*, *CCNE1*
Complement and coagulation cascades	9	0.000685	*C5AR1*, *C9*, *CR1*, *F12*, *F3*
Nitrogen metabolism	5	0.001086	*CA1*, *CA2*, *CA8*, *GLUL*, *HAL*
Amoebiasis	11	0.001278	*ARG1*, *ARG2*, *C9*, *COL4A6*, *CTSG*

KEGG, Kyoto Encyclopedia of Genes and Genomes.

Table II. Top five enriched GO terms for the downregulated differentially expressed genes.

GO ID	Term	Gene count	P-value	Genes
0006950	Response to stress	155	$4.91x10^{-14}$	*KIF11*, *KIF2C*, *FGF19*, *ADORA3*, *AEN*
0002376	Immune system process	111	$1.87x10^{-12}$	*KIF11*, *KIF2C*, *FGF19*, *AHCY*, *APOBEC3B*
0009611	Response to wounding	75	$2.04x10^{-12}$	*KIF11*, *KIF2C*, *ADM*, *ADORA3*, *AHCY*
0006952	Defense response	85	$2.37x10^{-12}$	*FGF19*, *AHCY*, *ALOX5*, *ALOX5AP*, *APOBEC3B*
0000278	Mitotic cell cycle	61	$2.60x10^{-12}$	*KIF11*, *KIF2C*, *KIF20A*, *AURKA*, *AURKB*

GO, gene ontology.

Table III. Top five enriched KEGG pathways for the upregulated differentially expressed genes.

KEGG pathway	Gene count	P-value	Genes
ECM-receptor interaction	12	$3.01x10^{-5}$	*CD36*, *COL11A2*, *COL1A1*, *COL2A1*, *COL3A1*
Axon guidance	13	0.000472	*EPHB3*, *LRRC4C*, *NTN1*, *NTN4*, *PLXNA3*
Amoebiasis	10	0.003458	*COL11A2*, *COL1A1*, *COL2A1*, *COL3A1*, *COL4A5*
Focal adhesion	15	0.003709	*AKT2*, *COL11A2*, *COL1A1*, *COL2A1*, *COL3A1*
Pathways in cancer	21	0.00407	*AKT2*, *ARNT2*, *COL4A5*, *CSF1R*, *DAPK2*

KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix.

Table IV. Top five enriched GO terms for the upregulated differentially expressed genes.

GO ID	Term	Gene count	P-value	Genes
0005578	Proteinaceous extracellular matrix	51	$1.11x10^{-15}$	*ACAN*, *ADAMTS10*, *BGN*, *C1QTNF8*, *C1QT-NF9*
0031012	Extracellular matrix	55	$2.22x10^{-15}$	*ACAN*, *ADAMTS10*, *BGN*, *C1QTNF8*, *C1QTNF9*
0044707	Single-multicellular organism process	305	$4.66x10^{-15}$	*A2M*, *ABCC6*, *ACAN*, *ADAM20*, *ADAP2*
0044420	Extracellular matrix part	33	$5.17x10^{-14}$	*ADAMTS10*, *C1QTNF8*, *C1QTNF9*, *COL10A1/2*
0032501	Multicellular organismal process	309	$8.27x10^{-14}$	*A2M*, *ABCC6*, *ACAN*, *ADAM20*, *ADAP2*

GO, gene ontology.

reported that the use of programmed cell death inhibitors can improve the results of cartilage repair surgeries. fibroblast growth factor (FGF)2 is a mitogen that induces chondrocyte proliferation in wounded cartilage (22). *FGF18* induces the increase of cartilage thickness of the tibial plateau and also increases remodeling of the subchondral bone by repairing

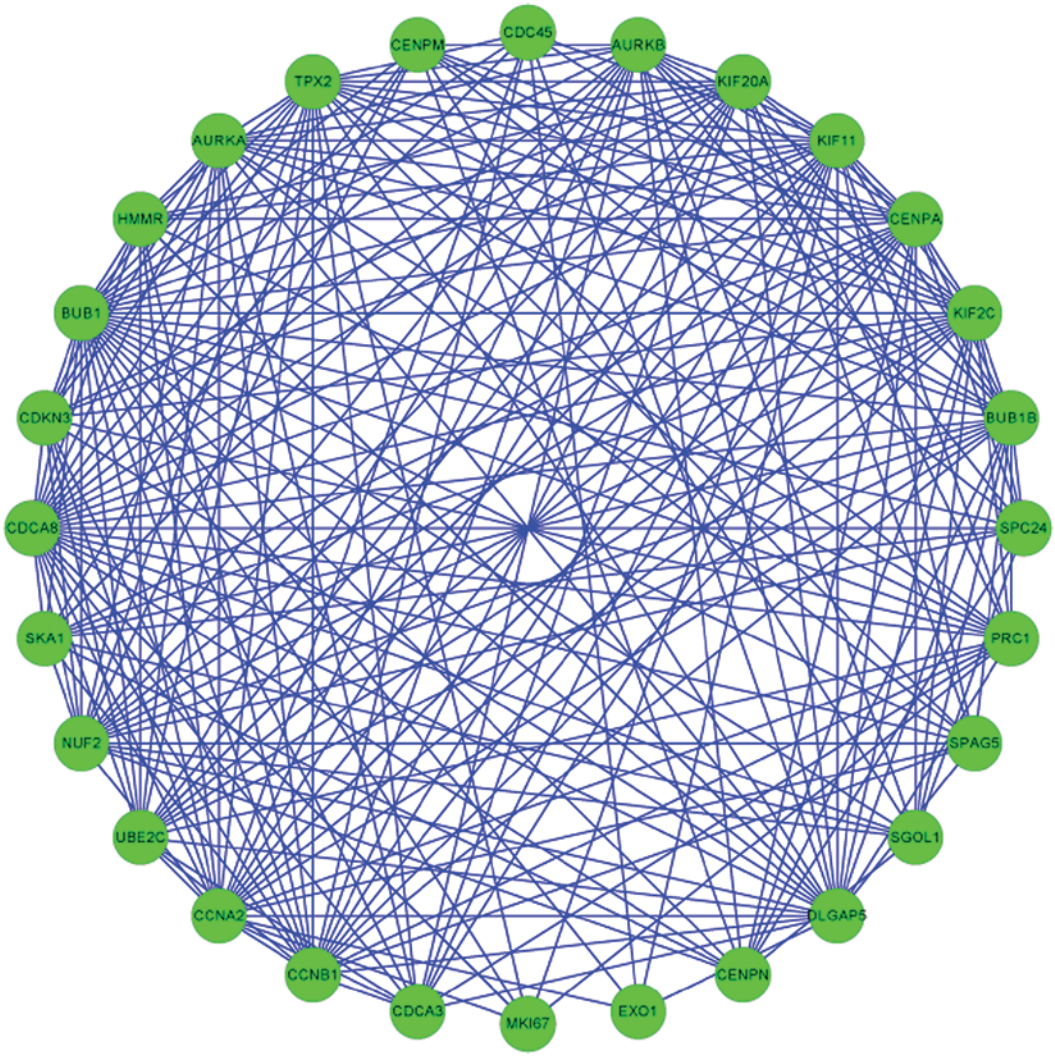

Figure 1. Module 2 of protein-protein interaction network of the downregulated differentially expressed genes.

damaged cartilage in OA rats (23). *FGF2* and *FGF18* are involved in cartilage wounding, which may be considered as one of the responses to stress. Therefore, the present study hypothesized that *FGF19* may be involved in the repair of damaged cartilage in OA.

Functional enrichment analysis of the downregulated DEGs further revealed that *KIF11* and *KIF2C* were involved in the response to stress, immune system process, response to wounding and mitotic cell cycle. In addition, the protein domain enrichment analysis of the genes in module 2 showed that *KIF2C*, *KIF11* and *KIF20A*, were significantly enriched in the protein domain of IPR001752: Kinesin, motor region. *KIF2C*, *KIF11* and *KIF20A* belong to the Kinesin family of proteins. Kinesins are important in cell division and at least 12 kinesins are involved in mitosis and cytokinesis (24). *KIF22* is a member of the kinesin-like protein family and KIF22 mRNA was detected in human bone, cartilage, joint capsules, ligaments, skin and primary cultured chondrocytes (25). Mutations in *KIF22* may be the cause of spondyloepimetaphyseal dysplasia with joint laxity, which is an autosomal dominant skeletal disorder (26). Phosphocitrate is known to downregulate multiple genes responsible for cell proliferation. *KIF23* and *KIFC1* were shown to be differentially expressed between phosphocitrate-treated and -untreated telomerase-transduced OA 13A fibroblast-like synoviocytes (27). *KIF2C* was also identified as a DEG in phosphocitrate-treated vs. untreated

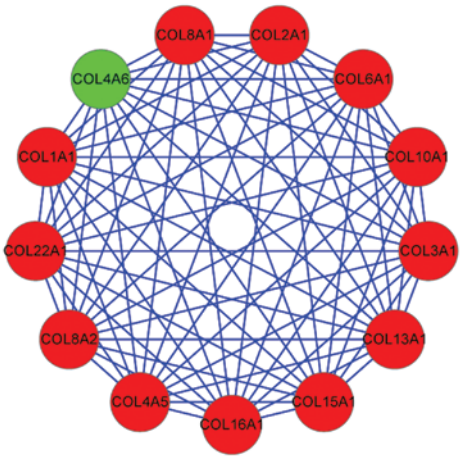

Figure 2. Module 5 of protein-protein interaction network. The red nodes illustrate upregulated differentially expressed genes and the green nodes illustrate downregulated differentially expressed genes.

Table V. Enriched protein domains for genes in module 2 of the protein-protein interaction network (category, Interpro).

Term	Name/function	Count	P-value	Genes
IPR001752	Kinesin, motor region	3	0.001518	*KIF2C*, *KIF11*, *KIF20A*
IPR019821	Kinesin, motor region, conserved site	3	0.001518	*KIF2C*, *KIF11*, *KIF20A*
IPR013212	Mad3/BUB1 homology region 1	2	0.002759	*BUB1*, *BUB1B*
IPR015661	Mitotic checkpoint, serine/threonine protein kinase, BUB1	2	0.002759	*BUB1*, *BUB1B*
IPR008271	Serine/threonine protein kinase, active site	4	0.012294	*BUB1*, *BUB1B*, *AURKA*, *AURKB*
IPR014400	Cyclin A/B/D/E	2	0.016448	*CCNB1*, *CCNA2*
IPR004367	Cyclin, C-terminus	2	0.019164	*CCNB1*, *CCNA2*
IPR017441	Protein kinase, ATP binding site	4	0.023893	*BUB1*, *BUB1B*, *AURKA*, *AURKB*
IPR006671	Cyclin, N-terminus	2	0.044611	*CCNB1*, *CCNA2*
IPR013763	Cyclin-associated	2	0.049887	*CCNB1*, *CCNA2*

IPR, interpro; ATP, adenosine triphosphate.

OA meniscal cells (28). These results suggested that *KIF2C*, *KIF11* and *KIF20A* may be involved in the mitotic cell cycle and contribute to the development of OA.

In addition, functional enrichment analysis of the downregulated DEGs revealed that *BUB1*, *BUB1B*, *CCNB1* and *CCNA2* were involved in the mitotic cell cycle process. Furthermore, the protein domain enrichment analysis of the genes in module 2, including *BUB1* and *BUB1B*, revealed that these genes were significantly enriched in the protein domain of IPR013212: Mad3/BUB1 homology region (1), and the genes in module 2, including *CCNB1* and *CCNA2*, were significantly enriched in the protein domain of IPR014400: Cyclin A/B/D/E. Several previous studies have reported that *BUB1*, *BUB1B*, *CCNB1* and *CCNA2* had roles in the regulation of the cell cycle (29-32). According to Tew *et al* (20), apoptosis and proliferation were involved in processes of cartilage wounding. Based on these results, the present study hypothesized that *BUB1*, *BUB1B*, *CCNB1* and *CCNA2* affected the development of OA through the cell cycle.

In addition, upregulated DEGs, including *CD36*, *COL11A2*, *COL1A1*, *COL2A1* and *COL3A1*, were revealed to be involved in the ECM and ECM-receptor interaction pathway. The structural matrix macromolecules in the ECM and growth factors regulate the chondrocyte function via specific membrane receptors (33). *TSP-1* is involved in the cell-matrix interactions of various tissues in cartilage. The number of *CD36*-positive chondrocytes, which is considered to be the receptor of *TSP-1*, is significantly increased in severely osteoarthritic cartilage (34). The expression of *CD36* patterning receptor has attenuating effects on the inflammatory, pro-catabolic responses to *S100A11* and tumor necrosis factor α in chondrocytes (35). The α1(X) collagen gene *COL10A1* is a hypertrophic chondrocytes-specific molecular marker and a transcriptional target of *RUNX2* during chondrogenesis (36,37). *RUNX2* also regulates the expression of *COL10A1* in hypertrophic chondrocytes and matrix metallopeptidase 13 in terminal hypertrophic chondrocytes (38). The collagen genes (*COL11A2*, *COL1A1* and *COL2A1*) are involved in the progression of OA through the mediation of sensitivity to OA (39). The upregulated expression of *COL1A1* and *COL2A1* reflects the metabolic activation of OA chondrocytes, and the expression of *COL11A2* is also upregulated with the progression of OA (40). The results suggested that the DEGs associated with the ECM are important in the regulation of chondrocyte function.

In conclusion, in the present study, gene expression analysis was performed and 1,377 DEGs were identified, among which 869 DEGs were upregulated and 508 DEGs were downregulated. The identified DEGs may be involved in the pathogenesis of OA, particularly *FGF19*, *BUB1*, *KIF2C*, *CD36* and *COL11A2*, which are involved in the cellular stress response, cell cycle and the ECM. However, the results of the present study require confirmation by further studies.

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