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Genes relevant with osteoarthritis by comparison gene expression profiles of synovial membrane of osteoarthritis patients at different stages

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Abstract. – BACKGROUND: This study aimed to identify biological markers about osteoarthritis (OA) which is a polygenic disease by investigating the gene expression profiles of the synovium samples from early-stage and end-stage OA patients for the diagnosis and treatment of OA.

METHODS: The gene expression profile of GSE32317 was downloaded from Gene Expression Omnibus (GEO) database, including 10 samples from early-stage OA patients and 9 samples from end-stage OA patients. The differentially expressed genes (DEGs) were identified by Significance Analysis of Microarrays. The co-expression network of DEGs was constructed by Pearson correlation test. Then, modules in the constructed co-expression network were selected by MCODE Plugin. What's more, EASE (Expression Analysis Systematic Explorer) was used to define the significant functions and pathways in the identified modules.

RESULTS: Total 419 DEGs were identified, among which 112 were up-regulated and 307 down-regulated. We selected 7 statistically significant modules with gene number above 10 and phenotypic correlation test of modules showed that all the modules had significant correlation with OA ($p < 0.05$). The genes of module 1, module 2 and module 7 were significantly related to immune system functions, protein glycosylation functions, bone, chondrocytes and cartilage functions, respectively. The most significant pathway in module 3 and module 5 was Wnt signal pathway, and in module 4 was Toll-like receptor signal pathway.

CONCLUSIONS: DEGs related to immune response, cartilage development, protein glycosylation, muscle development, and DEGs participated in the Wnt signaling pathway and Toll-like receptor (TLR) signaling pathway might be the potential target genes for the OA treatment.

Key Words:
Osteoarthritis, Gene therapy, Differentially expressed genes.

Introduction

Osteoarthritis (OA), the most prevalent joint disease, is characterized by the progressive loss of articular cartilage that leads to chronic pain and functional restrictions in affected joints[1]. The clinical features of OA include pain, stiffness, reduced motion, swelling, crepitus and deformity. Although clinical research on OA has been extensively investigated, the etiology of this disease remains poorly elucidated.

Recent studies have demonstrated that biologic factors, such as matrix metalloproteinases (MMPs), cytokines, factors related to chondrocyte apoptosis and growth factors, play important roles in the degradation of OA cartilage[2]. Though researches about OA pathogenesis are mainly focused on articular cartilage, the studies that investigate the roles of synovial factors which have effects on the onset of OA and cartilage integrity are receiving increasing attention[3]. The synovial tissues lining the diarthrodial joints surfaces include various types of cells[4]. Synovial tissues are involved in the pathogenesis of arthritic joint disorders by producing tumor necrosis factor- , proinflammatory cytokines and MMPs is widely recongnized[4,5]. In OA, the inflammatory processes could be significantly be promoted by tissue-degrading MMPs and proinflammatory cytokines secreted by synovial fibroblasts and macrophages[3]. Therefore, OA synovium might be responsible for cartilage degradation. However, studies on OA synovium are mainly single gene expression analysis[6-8]. The genome-wide RNA expression profile of multiple genes could be useful in revealing gene functions in the etiology of such a complex disease. Especially pathway and network analysis would be more effective to provide clues for treatment, and unravel the pathogenesis of OA. Recently, the rela-

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tionship between the genes related with OA and rheumatoid arthritis has been studied[9]. Furthermore, the genes and pathways related with OA were also investigated by graph-clustering approach using the expression profiles of synovial tissues from OA patients and normal controls[10].

Therefore, the present study aimed to investigate the changes during the occurrence and development of OA synovium, which may be helpful to find out novel biological markers for the diagnosis, treatment, and drug development of OA. In the present study, we investigated the differences in gene expression profiles of early-stage and end-stage OA samples, and did function and pathway enrichment analysis to recognize the pathogenesis of OA and provide evidence for gene therapy of OA.

Methods

Samples

The microarray expression profile of GSE32317 was downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database[11]. In total, 19 samples of synovial membrane were available for further analysis including 10 samples from 10 early-stage knee OA patients (Kellgren Lawrence score ≤ 2) with documented cartilage degeneration but no loss of full-thickness cartilage who were undergoing arthroscopic procedures, and 9 samples from 9 end-stage knee OA patients with diffuse full thickness cartilage erosion who were undergoing the replacement of total knee joint. The platform information was GPL570 [HG-U133_Plus_2] Affymetrix U133 plus2.0 Array.

Data Preprocessing and Identification of Differentially Expressed Genes (DEGs)

Firstly, the probe-level data in CEL files were converted into expression measures. Then, background correction and quartile data normalization were performed by the robust multiarray average (RMA) algorithm[12]. The file named GPL570-tbl-1.txt in the platform annotation files provided by Affymetrix company was used to map the relationship between the probes and gene symbols. A probe was filtered when the probe did not have corresponding gene symbol. And the average value of gene symbol with multiple probes was obtained for further analysis. Finally, we got the expression profile dataset including 20,308 genes for the 19 samples.

The primary comparison of samples from early-stage OA patients to samples from end-stage OA patients was conducted by Significance Analysis of Microarrays (SAM) method[13]. Differentially expressed genes were identified by assimilating a set of gene-specific *t* tests with the threshold of false discovery rate (FDR) ≤ 0.05.

Co-Expression Network Construction for DEGs

Many genes together play important roles in the accomplishment of a biological function, and highly co-expressed genes participate in similar biological processes and pathways[14]. In order to construct the co-expression network, the expression values of DEGs were obtained and Pearson correlation test was performed. The r ≥ 0.7 was set as threshold.

Module Mining

Gene sets could approximately reflect biological modules. The MCODE Plugin of Cytoscape software (http://www.cytoscape.org/) was used to detect the modules in the constructed co-expression network[15]. MCODE could recognize the modules with specific functions by selecting the clusters of densely connected nodes from the network. We set Degree Cutoff = 2, K-core = 2, Max.depth = 100 as the parameters in MCODE for the detection of modules in co-expression network of DEGs.

Phenotypic Correlation Test of Modules

In order to test whether the excavated modules were correlated to the stages of OA, we calculated the average expression values of all the genes in each module. The differences in the average expression value of each module between the early-stage knee OA and end-stage knee OA samples were detected by *t*-test.

Functional Enrichment and Pathway Enrichment Analysis

The Gene Ontology (GO) functional enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis for modules were performed by using the online tool DAVID (Database for Annotation, Visualization, and Integrated Discovery)[16]. DAVID bioinformatics resources containing an integrated biological knowledgebase and analytic tools could systematically extract biological meaning from a large number of genes or proteins. GO terms and

Table I. EASE principle.

Background User/Genes	Hit	Not hit	*p*
Hit	(a'-1) a	b	$p = \frac{\binom{a+b}{a}\binom{c+d}{c}}{\binom{n}{a+c}}$
Not hit	c	d	

n = a'+b+c+d is the number of background genes; a'+b is the number of genes in the gene list including at least one gene set which was submitted by user; a'+c is the gene number of one gene list in the background genes; a' is the gene number of one gene set in the gene lists which was submitted by user. a' was replaced with a = a'-1 in EASE.

KEGG pathways with P value less than 0.1 were selected based on expression analysis systematic explorer (EASE) test implemented in the DAVID. The principle of EASE was shown in Table I and the following formula 1.

$$p = \frac{\binom{a+b}{a}\binom{c+d}{c}}{\binom{n}{a+c}} \quad (1)$$

Where n is the number of background genes; a' is the gene number of one gene set in the gene lists; a'+b is the number of genes in the gene list including at least one gene set; a'+c is the gene number of one gene list in the background genes; a' was replaced with a = a'-1 in EASE.

Results

Identification of DEGs

After normalization and preprocessing of the expression profile data of early-stage and end-stage OA samples with SAM4.0 software, we got 419 DEGs with the criterion of FDR ≤ 0.05, including 112 up-regulated genes and 307 down-regulated genes (Figure 1). The identified top 25 up-regulated DEGs and top 25 down-regulated DRGs were listed in Table II.

Co-expression Metwork of DEGs

It has been reported that genes with similar functions usually have similar expression patterns in the co-expression network[14]. The expression values of all screened DEGs were compared by Pearson correlation test to construct the co-expression network. Finally, we got a co-expression network with 918 nodes and 6,688 edges when r ≥ 0.7 was set as threshold. The co-expression network included two closely related subnetworks (Figure 2).

Module Mining

The MCODE plugin of the Cytoscape software was used to mine modules for co-expression network of DEGs. When we set Degree Cutoff = 2, K-core = 2, Max.depth = 100, we got 7 modules whose gene number is more than 10. The module 1 included 60 genes and 768 edges (Figure 3). Nodes represent biomolecules and edges between nodes indicate physical or functional interactions in a molecular network[17].

Phenotypic Correlation Test of Modules

In order to test whether the selected modules were related with the stages of OA, we calculated the average expression values of all the genes in each module. *t*-test analysis showed that the *p* values of all the modules are under 0.05 (Table III) which indicated that all the modules had close relationship with OA disease.

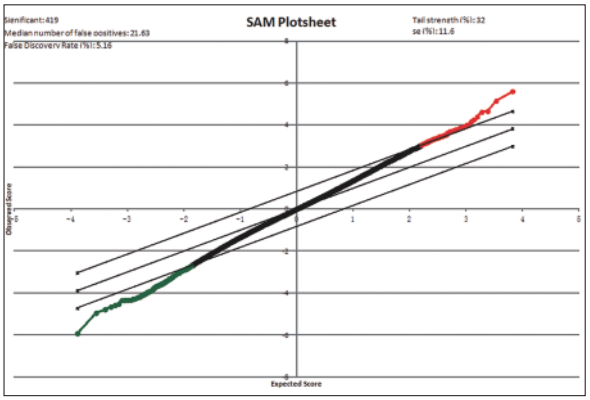

Figure 1. The identified 419 DEGs between early-stage and end-stage of OA including 112 up-regulated genes and 307 down-regulated genes with SAM method at FDR ≤ 0.05.

Table II. The identified top 25 up-regulated DEGs and top 25 down-regulated DRGs between early-stage and end-stage of OA with SAM method at FDR ≤ 0.05.

Gene ID	Gene name	Score (d)	Fold change	q-value (%)
2229	C20orf46	5.596170905	2.179872763	0
13189	OLFML2A	5.140839595	1.92912403	0
15064	RAI14	4.651435042	1.716643072	0
5112	EBF2	4.610661759	1.499832477	0
4076	CRIP2	4.391967381	1.593395895	1.17377362
1481	BMP5	4.256693046	2.713110988	1.728413902
14120	PLEKHG4	4.166425642	1.455693638	2.069548224
7360	HCG26	4.021625826	1.304638442	2.069548224
3971	COX2	3.982939715	1.120233646	2.069548224
10050	LOC100507560	3.930014419	1.580832257	2.069548224
7333	HAUS3	3.882039753	1.220742942	2.894828191
15500	RNF150	3.878898692	1.659261989	2.894828191
13547	PBLD	3.870877532	1.265176869	2.894828191
1095	ATF4	3.816536043	1.171190704	2.894828191
11334	LZTS2	3.784890178	1.181121884	2.894828191
824	APOLD1	3.784213014	1.583857071	2.894828191
14649	PRR14	3.769881013	1.183944879	2.894828191
4385	CYP2E1	3.744344786	1.211581373	2.894828191
13469	PAMR1	3.735013954	2.390403195	2.894828191
191	ACR	3.715431982	1.224955009	2.894828191
4388	CYP2R1	3.692689075	1.273911146	2.894828191
18597	TRIP10	3.6809289	1.308635507	2.894828191
5664	FAM129A	3.676544804	1.479610679	2.894828191
16772	SLN	3.676051366	2.746158957	2.894828191
4420	CYTH2	3.644618828	1.207632118	3.259806529
17387	STT3A	-5.9246976	0.696643257	0
19725	ZIC1	-4.951113533	0.606317856	0
5295	ELL2	-4.7813502	0.638005746	0
11409	MAN2A1	-4.658933226	0.7090414	0
7974	IGHM	-4.576413754	0.194170883	0
16050	SEC24A	-4.518802856	0.781052603	0
364	ADRB2	-4.355757173	0.711626759	0
13756	PDZRN4	-4.340806788	0.36489819	0
3637	CILP2	-4.337926793	0.446692391	0
18343	TNIK	-4.336687608	0.785064356	0
1006	ARSG	-4.33500647	0.6598244	0
7982	IGKV1-5	-4.329814863	0.309700372	0
1565	BTBD11	-4.285021733	0.426625788	0
9285	LOC100129034	-4.280761025	0.829986936	0
16131	SERPINA3	-4.268510406	0.436317134	0
17047	SPATA18	-4.244565522	0.667539421	0
7994	IGLV2-23	-4.217206207	0.189073914	0
6642	GBGT1	-4.198017871	0.737900515	0
15032	RAC2	-4.186349057	0.687506392	0
7985	IGKV4-1	-4.172872946	0.168020361	0
5172	EFEMP1	-4.118798474	0.792740801	0
15576	ROR2	-4.115068204	0.782353516	0
11268	LTBP2	-4.093319971	0.497011301	0
7992	IGLV1-36	-4.057897087	0.131100974	0
11969	MPI	-4.054468099	0.818489338	0

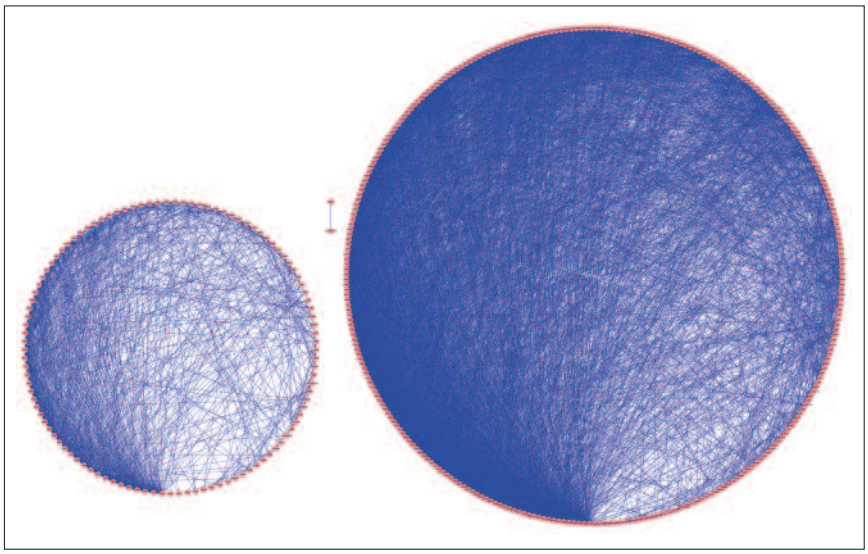

Figure 2. The two closely related subnetworks of the co-expression network with 918 nodes and 6,688 edges at the criterion of r ≥ 0.7.

Functional Enrichment and Pathway Enrichment Analysis

We performed GO functional enrichment and KEGG pathway enrichment analysis for the identified 7 modules of DEGs.

The genes of module 1 have closely relationship with immune system, such as immune response (p = 1.07E-11), antigen receptor-mediated signaling pathway (p = 0.005), immune response-activating cell surface receptor signaling pathway (p = 0.007), immune response-regulating cell surface receptor signaling pathway (p = 0.008), positive regulation of immune response (p = 0.011), immune response-activating signal

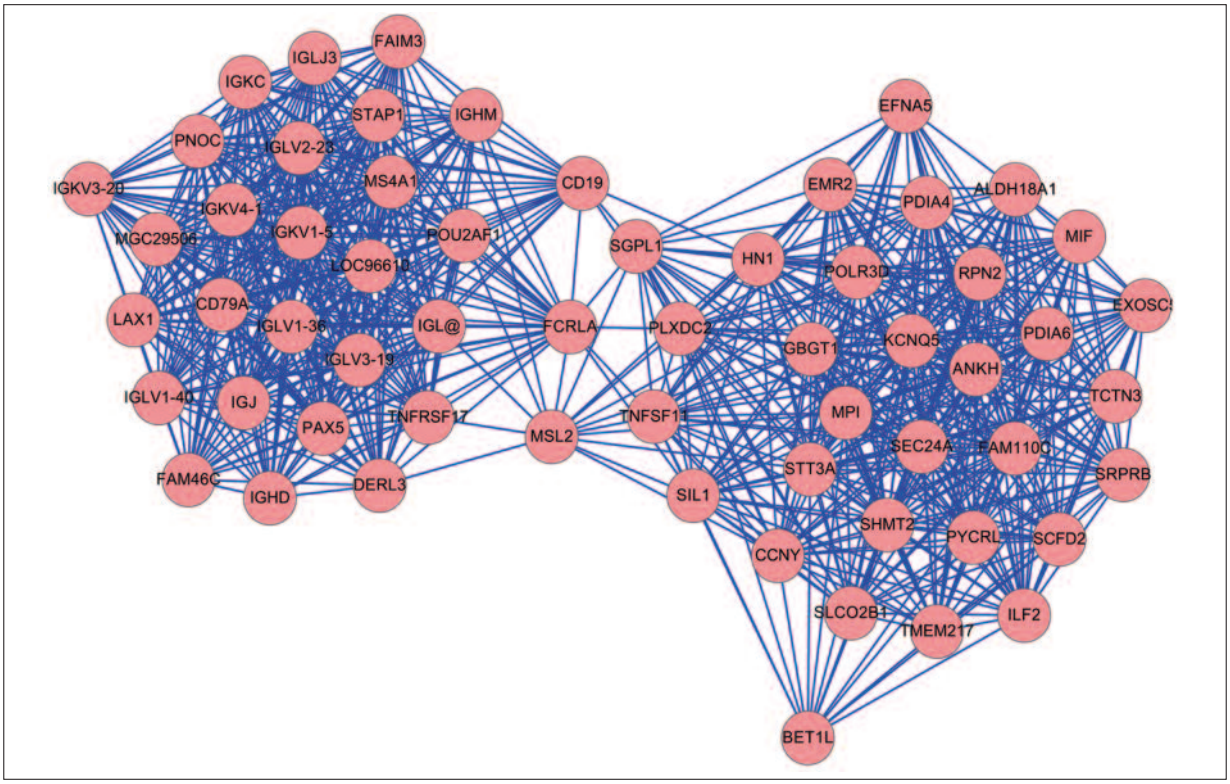

Figure 3. The statistically significant module 1 of DEGs includes 60 genes and 768 edges at the criteria of Degree Cutoff = 2, K-core = 2, Max.depth = 100.

Table III. Correlation of modules with phenotype.

Module	Node_number	p
Module_1	60	0.000166728383933108
Module_2	54	5.99572494339073e-05
Module_3	17	0.000184848729303361
Module_4	43	2.49108756159443e-05
Module_5	24	8.90072321341422e-05
Module_6	13	1.12720235959767e-05
Module_7	21	2.80029482024044e-06

transduction ($p = 0.012$), and activation of immune response ($p = 0.036$). The genes in module 2 were significantly related to the protein glycosylation functions, such as protein amino acid glycosylation ($p = 0.005$), glycosylation ($p = 0.005$), biopolymer glycosylation ($p = 0.005$), glycoprotein biosynthetic process ($p = 0.009$), glycoprotein metabolic process ($p = 0.018$). Module 3 was related with functions about morphogenesis of epithelium. Module 5 was related with cell proliferation and muscle development, such as regulation of cell proliferation ($p = 0.018$), negative and positive regulation of endothelial cell proliferation ($p = 0.024$), muscle organ development ($p = 0.031$), actin filament-based process ($p = 0.040$), regulation of endothelial cell proliferation ($p = 0.042$), skeletal muscle tissue development ($p = 0.084$). The function of module 6 had a relationship with transcription ($p = 0.052$). The genes in module 7 were related with bone, chondrocytes and cartilage, such as positive regulation of chondrocyte differentiation ($p = 0.006$), endochondral bone morphogenesis ($p = 0.013$), bone morphogenesis ($p = 0.016$), regulation of osteoblast differentiation ($p = 0.034$), cartilage development ($p = 0.059$), regulation of ossification ($p = 0.062$), and skeletal system morphogenesis ($p = 0.087$).

The KEGG pathway enrichment results showed that the genes (DAAM2, FZD6) in module 3 and genes (CSNK1E, CAMK2G, TCF7L1) in module 5 were significantly participated in Wnt signal pathway ($p = 0.086$, $p = 0.027$), and the most significant pathway in module 4 was Toll-like receptor signal pathway ($p = 0.023$) involving PIK3CG, MAPK13, TLR5.

Discussion

The pathogenesis of OA is still unclear, and there are currently no treatments that could effectively prevent the development of OA. cDNA array technology is increasingly being used to generate gene profiles for screening gene targets for disease and to investigate the basis of disease[18]. In order to investigate the changes during the occurrence and development of OA, we compared the gene expression profiles of early-stage and end-stage OA samples. Total 419 DEGs were selected which indicated that there are significant differences between early-stage and end-stage OA patients. Furthermore, we identified 7 statistically significant modules in the constructed co-expression network. And phenotypic correlation test of modules showed that all the modules of DEGs have significant correlation with OA which indicated that the genes in these modules play important roles in the development of OA.

In our study, a cluster of genes is related with immune response, such as POU2AF1, IGL@, IGJ, PAX5, TNFRSF17, IGHM, MIF, IGKV1-5, TNFSF11 (RANKL), ILF2 (NFAT), LAX1, IGHD, MS4A1 (CD20), IGKV4-1, FAIM3, IGKV3-20, CD79A, CD19 and IGKC. And we found that these genes related with immune response were down-regulated in end-stage of OA samples. Therefore, increased expression of these genes in the early-stage of OA might have a potential role for innate immune system activation in the pathogenesis of OA. Cellular and molecular interactions could benefit for the correlation between skeletal and immune systems[19]. Hematopoietic stem cells in bone marrow which interacted with bone cells could be differentiated into immune cells[20]. Several regulatory molecules including receptors, transcription factors, signaling molecules, and cytokines are shared by the skeletal and immune system[19,20]. POU2AF1, a B-cell-specific transcriptional co-activator, could bind to the 5' region of TNFRSF17 which is a B-cell maturation factor to enhance the transcription of TNFRSF17. When POU2AF1 is activated by amplification or other mechanisms, POU2AF1 could directly activate its target gene TNFRSF17 to promote the progression of multiple myeloma (MM) through accelerating growth of MM cells[21]. Receptor activator of nuclear factor B ligand (RANKL; also known as TNFSF11) has a role in immune cell differentiation and T cell-dendritic cell interactions[22,23]. Activated fibroblast-like synoviocytes and T lymphocytes in the synovial membrane could express RANKL[24]. Takayanagi et al[25] have found that the expression of RANKL mRNA was highly increased in all tissues from patients with rheumatoid arthritic

(RA), but not from patients with OA. Activation of the RANK-RANKL signaling pathway is directly responsible for dramatic focal erosions that are observed in inflammatory arthritis[26]. Therefore, our study results were consistent with previous reports.

B cells and antibodies together participate in the body's humoral immune response. With the support of stromal cells and osteoblast lineage cells, B cells could develop in bone marrow and could be released into the blood and lymphatic systems. MS4A1 (CD20), CD19 and CD79a are mainly expressed in B cells. Paired box transcription factor 5 (PAX5) is strongly implicated in B cell neoplasms. PAX5 controls the differentiation of B cells and is mainly responsible for the expression of B cell receptor complex by direct transcriptional activation of genes encoding CD79a (Ig-α)[27]. Massive decrease could be occurred in trabecular bone, tibia and femur of 15-d-old mice due to loss of PAX5[20]. B cell-mediated immune responses could contribute to the pathogenesis of RA according to the clinical benefits of rituximab for the treatment of anti-CD20 antibody[28].

In our work, some genes were found to be related with cartilage development, such as HOXA11, RUNX2 (runt-related transcription factor 2) which were down-regulated in end-stage of OA. RUNX2, a member of the runt homology domain transcription factor family, is a key transcription factor associated with osteoblast differentiation[29,30]. While, it has been reported that the RUNX2 immunoreactivity is enhanced in fibrillated OA cartilage and increased RUNX2 expression in OA bone has been observed by Blair et al[31,32]. What's more, the research on expression change of RUNX2 in synovial membrane from patients with OA is few. Therefore, the result that the expression of RUNX2 in synovial membrane of end-stage OA is decreased needs to be confirmed by further studies.

It has been reported that there is abnormal glycobiology in OA development. Matsuhashi et al[33] recently have demonstrated that there are alterations in cartilage N-glycans at the early phases of a rabbit OA model. Atsushi U et al[34] have found that the release of MMP-13 and ADAMTS-5 is related to the changes about *N*-glycans and *N*-glycogenesduring cartilage degradation of OA. Therefore, the results of these studies suggest that N-glycans play important roles in the initiation and progression of OA. Glycans of glycoproteins usually are found in the extracellular membrane (ECM), serum and on the cell surface. Our results showed that a cluster of genes related with protein glycosylation, such as B3GNT9, MAN2A1, ALG8, SERP1 was down-regulated in end-stage OA which indicated that the decrease of these genes expression may contribute to the degradation of cartilage. Meanwhile, muscle weakness has been shown to be a predictor of the onset of knee OA and is associated with the progression of OA[35]. Recent data have demonstrated that quadriceps weakness precedes the onset of knee OA[36,37]. Our GO enrichment analysis showed that the expression levels of some genes (MYL6, CAV2, LAMA5) related with muscle development were decreased in end-stage of OA which were consist with the previous studies.

The KEGG pathway enrichment analysis showed that the significant pathways in modules were Wnt signaling pathway and Toll-like receptor (TLR) signaling pathway. It has been demonstrated that Wnt signal pathway involved in cartilage distortion and subchondral bone changes[38]. The analysis of Wnt signaling pathway has revealed that the Wnt-16 and several Wnt target genes were up-regulated, while FRZB is down-regulated in injured cartilage[39]. The increased expression of Wnt-induced signaling protein 1 (WISP-1) could be found in experimental and human OA[40]. In our study, we found that DAAM2, FZD6 in module 3 and CSNK1E, CAMK2G, TCF7L1 in module 5 were involved in the Wnt signaling pathway. Therefore, these genes have the potential to be used as target genes for OA treatment. Meanwhile, PIK3CG, MAPK13, TLR5 in module 4 could participate in the Toll-like receptor signaling pathway. The early-onset inflammation and cartilage destruction in immune complex-mediated arthritis could be affected by TLR4 via increased production of cytokine and enhanced expression of Fc receptor which were mediated by IL-10[41]. Recent studies have suggested that the Toll-like receptor (TLR) family is involved in development and progression of OA. In human OA chondrocytes, TLR ligands could activate catabolic pathways. TLR-induced collagenase expression appears to contribute to cartilage catabolism in OA which suggesting that modulation of chondrocyte TLR expression or activation may be a route by which cartilage breakdown can be blocked[42]. On the other hand, the expression of Toll-like receptors, such as TLR-4, TLR-5 and TLR-9 could be

found in osteoblasts and the secretion of pro-inflammatory cytokines could be induced by the exposure of these cells to pathogen-associated molecular patterns (PAMP). Therefore, Toll-like receptors could modulate the functions of osteoblasts and osteoclasts to promote the regulation of osteoclastogenesis.

Conclusions

Our study have identified the pathogenic genes of OA development by analyse the expression profiles of early-stage and end-stage OA samples. The genes related immune response, cartilage development, protein glycosylation, muscle development, and genes participate in the Wnt signaling pathway and Toll-like receptor (TLR) signaling pathway might be the potential target genes for the OA treatment. However, the results of our study need to be confirmed by further experiments.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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