Identification of key mRNAs and microRNAs in the pathogenesis and progression of osteoarthritis using microarray analysis

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Abstract. Osteoarthritis (OA) is a common type of disease affecting the joints that results from the breakdown of joint cartilage and the underlying bone; currently, its pathogenesis is still unclear. The aim of the present study was to identify key mRNAs and miRNAs involved in the pathogenesis and progression of OA using microarray analysis. The gene expression profile of GSE27492 was downloaded from the Gene Expressed Omnibus database, and included 49 arthritic mouse ankle samples collected at 6 time points (0, 1, 3, 7, 12 and 18 days) following the induction of arthritis via serum transfer. Differentially expressed genes (DEGs) were identified in ankle samples taken on days 1, 3, 7, 12 and 18 following serum transfer compared with day 0 samples, and overlapping DEGs in day 3, 7, 12 and 18 samples were identified. The Database for Annotation, Visualization and Integrated Discovery online tool was used to perform functional and pathway enrichment analyses of the overlapping DEGs. The miRWalk database was used to identify potential micro (mi) RNAs regulating the selected overlapping DEGs, and regulatory miRNA-target mRNA pairs were obtained. The Cytoscape platform was used to establish and visualize the miRNA-mRNA regulatory network. The present results revealed that 35, 103, 62 and 75 DEGs were identified in day 3, 7, 12 and 18 samples, respectively. A total of 17 overlapping DEGs were identified among the 4 sample sets, and revealed to be enriched in 14 gene ontology terms and 3 Kyoto Encyclopedia of Genes and Genomes pathways. miRWalk analysis identified 242 potential miRNA-mRNA regulatory pairs and 211 nodes were revealed to be involved in the miRNA-mRNA regulatory network. The present study identified potential genes, including C-type lectin domain family 4 member D, chemokine (C-X-C motif) ligand 1 and C-C motif chemokine ligand, and pathways, including chemokine signaling pathways, cytokine-cytokine receptor interactions and nucleotide-binding oligomerization domain-like receptor signaling pathways, which may be involved in the pathogenesis and progression of OA. These findings may help elucidate the molecular mechanisms underlying OA pathophysiology, and may be useful for the development of novel therapeutic targets for the treatment of patients with OA.

Introduction

Osteoarthritis (OA), also known as wear-and-tear arthritis or osteoarthrosis, is a degenerative joint disease and the most common form of arthritis (1). The incidence of OA increases with age, and obesity is also a risk factor for the disease (2-4). In the USA, ~27 million patients with OA have been reported, and the prevalence of the disease is significantly enhanced at ages >50 years for males and >40 years for females (5,6). According to the American College of Rheumatology, ~70% of people >70 years of age exhibit X-ray evidence of OA (7). As the population ages, it has been estimated that ~20% of Americans will be over the age 65 years by 2030 (8), thus raising the risk for developing OA.

OA is caused by the breakdown of cartilage in one or more joints, and its symptoms include joint pain, stiffness, swelling, tenderness and inflexibility (9). Currently available therapeutic strategies for the treatment of OA include lifestyle modifications (e.g. exercise and weight loss), analgesics and sometimes surgical intervention (10); the primary focus of OA treatment is the reduction of pain and improving the function of the affected joints. OA is a chronic condition and its pathogenesis involves the interaction of multiple factors including genetic, metabolic, biochemical and biomechanical factors (11). Berenbaum *et al* (12) suggested that OA may be caused by mechanical stress on the joint and an ongoing low-grade inflammatory response. Histone deacetylase 4 has been demonstrated to contribute, at least in part, to the mechanisms underlying cartilage degeneration during OA

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pathogenesis (13). Several genes have been associated with the development and progression of OA (14,15); however, the mechanisms underlying the pathophysiology of the disease have yet to be fully elucidated, whereas the need for more effective and highly specific therapeutic strategies for the treatment of patients with OA is urgent.

In the present study, a set of bioinformatics approaches were used to comprehensively analyze the microarray data from mice with serum-transferred arthritis, publicly available at the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) were identified, and functional and pathway enrichment analyses were performed, in order to identify novel biomarkers and suggest putative mechanisms that may be involved in the pathogenesis and progression of OA.

Materials and methods

Microarray data. The microarray data of GSE27492 was downloaded from the GEO database (www.ncbi.nlm.nih. gov/geo/), which was provided by Jacobs *et al* (16). The dataset included 49 arthritic mouse ankle samples collected at 6 time points (0, 1, 3, 7, 12 and 18 days, including 7, 5, 9, 11, 9 and 8 samples, respectively) following the induction of arthritis via serum transfer. Microarray data obtained from GSE27492 were sequenced on the platform of GPL81 [MG_U74Av2] Affymetrix Murine Genome U74A 2.0 Array (Affymetrix; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Data preprocessing. The original raw data were converted into a format recognizable by the R statistical software program and scripting language, and the *Affy* v1.40.0 package (http://www.bioconductor.org/packages/2.13/bioc/html/affy. html) (17) was used for background correction and quartile normalization. This was followed by the conversion of probe symbols to gene symbols; if multiple probes corresponded to a single gene symbol, the average expression value of the probes was used as the expression value of the gene.

Screening of DEGs. The comparison of DEGs was performed using the Limma v3.18.13 package (http://www.bioconductor. org/packages/2.13/bioc/html/limma.html) (18) on R; $P<0.05$ and $|\log_2(\text{fold change})|>1$ were defined as the cut-off values for screening. A total of 5 sets of DEGs were detected, including DEGs compared between ankle samples obtained on days 1, 3, 7, 12 and 18 post-serum injection and ankle samples obtained on day 0; these were denoted as DEGs-1d, DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d, respectively. The 5 DEG sets were compared and the overlapping DEGs were identified and used for subsequent analyses.

Functional and pathway enrichment analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (https://david.ncifcrf.gov/) (19) is used for systematically associating the functional terms with gene or protein lists. In the present study, DAVID was used to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the previously identified overlapping DEGs. $P<0.05$ was considered the cut-off value.

micro (mi) RNA target prediction. The miRWalk database version 2.0 (http://www.umm.uni-heidelberg. de/apps/zmf/mirwalk/) (20) is a powerful and accurate database that displays miRNAs and their corresponding target genes and binding sites in mice, rats and humans. In the present study, miRNAs regulating the identified overlapping DEGs were predicted based on the information on miRWalk. Putative targets were predicted by >5 bioinformatics algorithms among the 10 algorithms in the miRWalk database: DIANA-microT v4.0 (http://diana.imis. athena-innovation.gr/DianaTools/index.php?r=microT_CDS/ index), miRanda-rel2010 (http://www.microrna.org/ microrna/getDownloads.do), miRDB v4.0 (http://mirdb. org/miRDB/download.html), miRWalk v2.0 (http://zmf. umm.uni-heidelberg.de/apps/zmf/mirwalk2/), RNAhybrid v2.1 (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/ dl_pre-page.html), TargetScan v6.2 (http://www.targetscan. org/cgi-bin/targetscan/data_download.cgi?db=vert_61),RNA22 v2.0 (https://cm.jefferson.edu/rna22/), PITA (https://genie. weizmann.ac.il/pubs/mir07/mir07_exe.html), PICTAR5 and PICTAR4 (http://pictar.mdc-berlin.de/).

Construction and analysis of the miRNA-mRNA regulatory network. After the miRNA-mRNA regulatory pairs were identified, the miRNA-mRNA regulatory networks were constructed and visualized using Cytoscape v3.5.1 software (http://www.cytoscape.org/download.php) (21). In addition, nodes were analyzed according to the intimate connections with other nodes. The important nodes in the network where identified when the degree of node attributes was ≥1, where 'degree' represented the connections with other nodes. In the regulatory network, a circular node represented the miRNA and a hexagonal node represented the mRNA.

Results

DEGs in OA. A total of 35 (28 up- and 7 downregulated), 103 (81 up- and 22 downregulated), 62 (53 up- and 9 downregulated) and 75 (67 up- and 8 downregulated) DEGs were identified among the DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d sets, respectively; no DEGs were identified in the DEGs-1d set. The numbers of the DEGs within the DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d sets are presented in Fig. 1. The number of DEGs appeared to reach a maximum on day 7 following the initial serum injection. In addition, a Venn diagram was constructed for the 4 DEG sets to display the overlapping genes, and is presented in Fig. 2. A total of 17 overlapping DEGs were detected among the aforementioned 4 DEG sets, and are listed in Table I. Among those, the expression of 16 genes was upregulated and the expression of 1 gene was downregulated.

GO terms annotation and KEGG signaling pathway enrichment of the overlapping DEGs. Functional and pathway enrichment analyses were performed for the 17 overlapping DEGs. As presented in Table II, the overlapping DEGs were significantly enriched in 14 GO terms. The majority of the GO terms were associated with immune processes, including immune responses, response to injury and inflammatory responses.

KEGG enrichment analysis was performed to understand the signaling pathways of DEGs involved in OA. The 17

Table I. Overlapping differentially expressed genes in arthritic ankle samples obtained on days 3, 7, 12 and 18 following the initial serum injection, compared with ankle samples isolated on day 0.

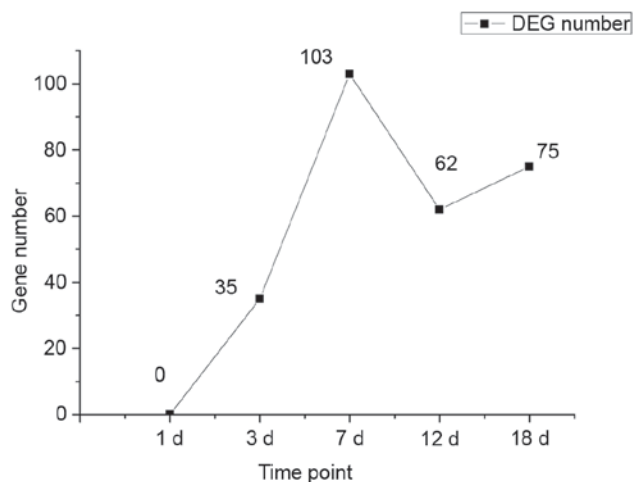| Gene	logFC_3d	logFC_7d	logFC_12d	logFC_18d	Regulation type
AF251705	1.305238	1.457843	1.423637	1.534935	up
Adam8	1.397521	1.545198	1.64087	1.397731	up
Arg1	3.216408	3.470759	3.253301	3.500893	up
Basp1	1.183961	1.402966	1.330427	1.417313	up
Ccl2	3.136399	2.754264	2.608425	2.605791	up
Ccl7	2.379615	1.747449	1.676018	1.364921	up
Ccl9	1.592837	1.25133	1.348077	1.222959	up
Ccr2	1.395741	1.265384	1.07037	1.021065	up
Clec4a2	1.344762	1.371277	1.486593	1.286852	up
Clec4d	2.328534	3.102078	3.12611	3.014611	up
Cxcl1	1.152963	2.378911	2.050006	2.15751	up
Ephx1	-1.12827	-1.5318	-1.36148	-1.05361	down
Fabp5	1.300109	1.492187	1.360936	1.174031	up
Fcgr1	1.565833	1.337319	1.266228	1.255231	up
Gp49a	1.08629	1.48513	1.472467	1.684876	up
Il1rn	2.356432	2.933974	2.905693	3.029609	up
Saa3	2.536273	3.692907	3.451593	4.212274	up

FC, fold change.

Figure 1. DEG numbers within the DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d sets. DEGs were compared between ankle samples obtained on days 1, 3, 7, 12 and 18 post-serum injection and ankle samples obtained on day 0, and denoted as DEGs-1d, DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d, respectively. No DEGs were identified between samples obtained on days 1 and 0. DEG, differentially expressed gene; d, day.

Figure 2. Venn diagram for the 4 DEG sets: DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d. A total of 17 overlapping DEGs were detected among the 4 aforementioned DEG sets. DEG, differentially expressed gene; d, day.

overlapping DEGs were revealed to be enriched in 3 KEGG signaling pathways, including chemokine signaling pathway, cytokine-cytokine receptor interaction and nucleotide-binding oligomerization domain (NOD)-like receptor signaling pathway (Table III).

Construction of the miRNA-mRNA regulatory network. A total of 242 miRNA-mRNA regulatory pairs were predicted using the miRWalk database, and, according to the predicted database number, the top 40 most significant pairs are listed in Table IV. The miRNA-mRNA regulatory network is presented in Fig. 3, as constructed using Cytoscape software, and the 40 most significant nodes, listed in Table V, all had higher degrees, which reflects their intimate connections with other nodes. A total of 211 nodes composed the miRNA-mRNA regulatory network, including 9 mRNAs and 202 miRNAs (Fig. 3).

Discussion

OA is a polygenic disorder, and a genetic contribution serves a critical role in the development and progression of the

Table II. GO enrichment pathways of the overlapping differentially expressed genes.

Category	GO term	Gene number	P-value	Genes
BP	Immune response	11	5.86×10^{-12}	CXCL1, CCL2, GP49A, CCR2, IL1RN, CCL9, CLEC4A2, AF251705, CLEC4D, FCGR1, CCL7
BP	Response to injury	7	1.09×10^{-6}	CXCL1, ARG1, CCL2, CCR2, SAA3, FCGR1, CCL7
BP	Inflammatory response	6	3.12×10^{-6}	CXCL1, CCL2, CCR2, SAA3, FCGR1, CCL7
MF	Chemokine activity	4	4.66×10^{-6}	CXCL1, CCL2, CCL9, CCL7
MF	Chemokine receptor binding	4	5.05×10^{-6}	CXCL1, CCL2, CCL9, CCL7
CC	Extracellular space	7	1.65×10^{-5}	CXCL1, ARG1, CCL2, IL1RN, SAA3, CCL9, CCL7
BP	Defense response	6	8.70×10^{-5}	CXCL1, CCL2, CCR2, SAA3, FCGR1, CCL7
CC	Extracellular region part	7	1.70×10^{-4}	CXCL1, ARG1, CCL2, IL1RN, SAA3, CCL9, CCL7
MF	Cytokine activity	4	4.92×10^{-4}	CXCL1, CCL2, CCL9, CCL7
BP	Chemotaxis	3	0.006254	CCL2, CCL9, CCL7
BP	Taxis	3	0.006254	CCL2, CCL9, CCL7
CC	Extracellular region	7	0.009876	CXCL1, ARG1, CCL2, IL1RN, SAA3, CCL9, CCL7
BP	Locomotor behavior	3	0.027823	CCL2, CCL9, CCL7
MF	Carbohydrate binding	3	0.031976	CLEC4A2, CLEC4D, CCL7

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

Table III. Enriched Kyoto Encyclopedia of Genes and Genomes pathways of the overlapping differentially expressed genes.

Category	Pathway Name	Count	P value
KEGG_PATHWAY	Chemokine signaling pathway	4	8.21×10^{-4}
KEGG_PATHWAY	Cytokine-cytokine receptor interaction	4	1.57×10^{-3}
KEGG_PATHWAY	NOD-like receptor signaling pathway	2	8.19×10^{-2}

KEGG, Kyoto Encyclopedia of Genes and Genomes; NOD, nucleotide-binding oligomerization domain.

disease (22,23). Loughlin (23) suggested that the genetic risk for OA may primarily result from alterations in gene expression modulation, an effect typically mediated though the regulation of transcription. In the present study, no DEGs were detected in ankle samples obtained on day 1 following the initial serum injection, compared with in day 0 samples. These findings suggested that serum-transferred arthritis may be developed at time points later than 1 day post-injection, or that the induced OA may not alter gene expression compared with normal ankle tissue at this early time point. Conversely, the number of DEGs reached its maximum value on day 7 following the initial serum injection, thus indicating that 7 days following serum transfer, the development of OA was ongoing. Subsequently, the number of DEGs appeared to be decreased on days 12 and 18 post-injection.

Previous research has demonstrated that various types of cells, cytokines and chemokines, the complement cascade, and other immune processes are involved in the pathogenesis of OA (24-26). Wang *et al* (27) reported that complement expression and activation were significantly enhanced in OA synovial tissue, particularly during the early stages of OA pathogenesis. Kandahari *et al* (28) have reviewed and summarized the roles of the innate and adaptive immune

response in early OA pathogenesis. There were less genes in the DEG sets, DEGs-12d and DEGs-18d, when compared with DEGs-7d, which may be associated with the suppression of the autoimmune system. In addition, similar alterations in gene expression were detected among the 17 overlapping DEGs (16 up- and 1 downregulated) in the 4 DEG sets (DEGs-3d, DEGs-7d, DEGs-12d and DEGs-18d) that were examined. These findings suggested the overlapping DEGs may serve critical roles in the pathogenesis and progression of OA.

The overlapping DEGs were enriched in 14 GO terms, including immune, injury and inflammatory response pathways, chemokine activation pathways and chemokine receptor binding. The majority of the identified pathways were associated with the processes of immunity and inflammation. OA is considered an inflammatory disease (11), and the implication of immune responses in the pathogenesis of OA has previously been studied (29). Synovial inflammation, immune cell activation and proinflammatory cytokine production have been demonstrated to participate in the pathogenesis and progression of OA (23), whereas anti-cytokine therapy has been suggested as an effective therapeutic approach for the treatment of patients with OA (30). Therefore, the GO terms that

Figure 3. miRNA-mRNA regulatory network for the overlapping DEGs. In the regulatory network, a circular node represents miRNAs and a hexagonal node represents mRNAs. A total of 211 nodes composed the miRNA-mRNA regulatory network, including 9 mRNAs and 202 miRNAs. mi, micro; DEG, differentially expressed gene.

were identified in the present study may be closely associated with the pathogenesis and progression of OA, and may provide useful indicators for the development of treatment strategies for patients with OA.

Chemokine (C-X-C motif) ligand 1 (*CXCL1*) and C-C motif chemokine ligand 2 (*CCL2*) were revealed to be involved in most of the GO terms that were investigated. *CXCL1* has been reported to induce hypertrophic differentiation, apoptosis and calcification in chondrocytes (31); *CCL2* has been implicated in the lung recruitment of leukocytes, natural killer cells and T cells (32), and has been demonstrated to enhance nociception (33). Amin and Islam (34) reported that in human OA chondrocytes, the expression of

CXCL1 is increased by 38-fold, and the expression of *CCL2* is increased by 53-fold. Stone *et al* (35) reported that the increased expression of *CXCL1* is associated with discoid meniscus injuries during OA development. In addition, 3 KEGG pathways were revealed to be enriched in the 17 overlapping DEGs, including the chemokine signaling pathway, cytokine-cytokine receptor interaction and the NOD-like receptor signaling pathway. Chemokines are critical for the perpetuation of the inflammatory response, via attracting proinflammatory cells to the inflamed joints (36). Several chemokines have exhibited proinflammatory and angiogenic properties in OA and rheumatoid arthritis (RA) studies (37). Zhang *et al* (38) demonstrated that the
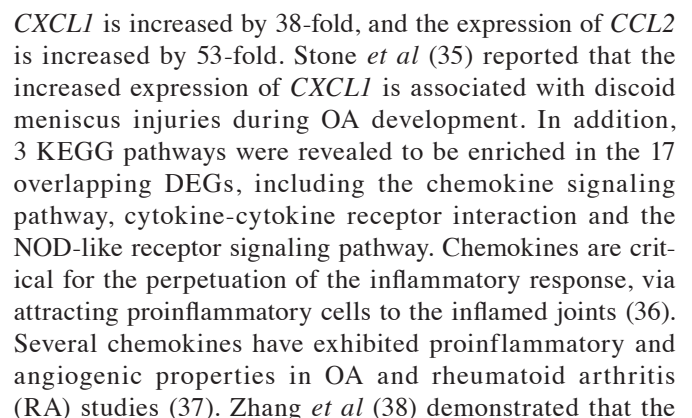
Table IV. Top 40 most significant miRNA-mRNA regulatory pairs for the overlapping differentially expressed genes, according to the predicted database number.

Gene	miRNA	Database number	Gene	miRNA	Database number
BASP1	hsa-miR-150	9	CLEC4D	hsa-miR-502-3p	6
BASP1	hsa-miR-7	8	BASP1	hsa-miR-7	6
BASP1	hsa-miR-200b	8	CCL2	hsa-miR-33b	6
BASP1	hsa-miR-200c	7	CLEC4D	hsa-miR-607	6
CCL7	hsa-miR-23a	7	BASP1	hsa-miR-298	6
CLEC4D	hsa-miR-20a	7	BASP1	hsa-miR-429	6
CLEC4D	hsa-miR-106a	7	CLEC4D	hsa-miR-18b	6
CLEC4D	hsa-miR-106b	7	CLEC4D	hsa-miR-520e	6
CCL7	hsa-miR-23b	7	BASP1	hsa-miR-767-5p	6
CCL2	hsa-miR-1	7	CLEC4D	hsa-miR-548d-3p	6
BASP1	hsa-miR-635	7	BASP1	hsa-miR-96	6
CCL2	hsa-miR-527	6	BASP1	hsa-miR-623	6
TINAGL1	hsa-miR-665	6	CCL2	hsa-miR-506	6
CLEC4D	hsa-miR-502-5p	6	CCL2	hsa-miR-33a	6
BASP1	hsa-miR-7	6	CLEC4D	hsa-miR-20b	6
CLEC4D	hsa-miR-568	6	BASP1	hsa-miR-552	6
BASP1	hsa-miR-142-5p	6	CLEC4D	hsa-miR-520f	6
ARG1	hsa-miR-648	6	ARG1	hsa-miR-23b	6
BASP1	hsa-miR-765	6	CCL2	hsa-miR-495	6
CLEC4D	hsa-miR-450b-5p	6	TINAGL1	hsa-miR-9	6

mi, micro.

Table V. Top 40 most significant nodes, according to the intimate connections with other nodes in the miRNA-mRNA regulatory network.

Node	Degree	Node	Degree
CLEC4D	78	hsa-miR-548d-3p	2
BASP1	38	hsa-miR-495	2
CCL7	35	hsa-miR-494	2
TINAGL1	29	hsa-miR-613	2
CCL2	22	hsa-miR-206	2
ARG1	19	hsa-miR-637	2
CXCL1	10	hsa-miR-374a	2
IL1RN	7	hsa-miR-374b	2
EPHX1	4	hsa-miR-944	2
hsa-miR-7	4	hsa-miR-576-5p	2
hsa-miR-380	3	hsa-miR-485-3p	2
hsa-miR-586	3	hsa-miR-186	2
hsa-miR-587	3	hsa-miR-590-3p	2
hsa-miR-650	3	hsa-miR-570	2
hsa-miR-23a	2	hsa-miR-432	2
hsa-miR-23b	2	hsa-miR-539	2
hsa-miR-1	2	hsa-miR-493	2
hsa-miR-765	2	hsa-miR-938	2
hsa-miR-607	2	hsa-miR-300	2
hsa-miR-767-5p	2	hsa-miR-548c-3p	2

miR, microRNA.

chemokine signaling pathway was involved in *CCL2* expression in tissues isolated from patients with RA, which may contribute to the chronic inflammation associated with RA. Cytokine-cytokine receptor interactions have been reported to serve critical roles in OA pathophysiology (39). The NOD-like receptor has been demonstrated to participate in inflammatory responses in various diseases and conditions, including cholesteatoma, wound healing and autoimmune encephalomyelitis (40-42). The results of the present study suggested that the enriched GO terms and KEGG pathways may be closely associated with the pathogenesis and progression of OA, and thus they may have potential as novel therapeutic targets for the treatment of patients with OA.

Following the construction of the miRNA-mRNA regulatory network, C-type lectin domain family 4, member D (*CLEC4D*) was identified as a major miRNA target, and had the highest node degree, thus suggesting that it may serve a critical role during OA pathogenesis. *CLEC4D* codes for a C-type lectin receptor, which recognizes trehalose 6,6'-dimycolate, a mycobacterial cell wall component, and induces potent innate immune responses (43). Since OA is a type of autoimmune disorder, *CLEC4D* may be implicated in its development and progression. Steichen *et al* (44) reported that *CLEC4D* serves a protective role during the resolution of Gram-negative-induced pneumonia. In the present study, hsa-miR-7 was identified as the miRNA regulating the most mRNAs in the constructed network, thus suggesting that hsa-miR-7 may be involved in the pathogenesis and progression of OA; however further

studies are required to investigate the roles of hsa-miR-7, and other regulatory miRNAs, in the mechanisms underlying the development of OA.

In conclusion, the present study identified potential genes, including *CLEC4D*, *CXCL1* and *CCL2*, and pathways, including chemokine signaling pathway, cytokine-cytokine receptor interaction and NOD-like receptor signaling pathway, which may be implicated in the pathogenesis and progression of OA. These findings may help elucidate the molecular mechanisms underlying OA pathophysiology, and may be useful for the development of novel therapeutic targets for the treatment of patients with OA.

Acknowledgements

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