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Bioinformatics Analyses of Regulatory Network of Biomarkers in Chondrocytes from Patients with Osteoarthritis

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HIGHLIGHTS

- BP term related to neuron differentiation was the most significant GO terms.
- Regulation of actin cytoskeleton may play an important role in OA progression.
- *GNAO1* and *POU3F4* may be biomarkers of OA.

Abstract: This study aimed to explore the biomarkers associated with osteoarthritis (OA). Gene expression profile GSE16464 was downloaded from the Gene Expression Omnibus database. The differentially expressed genes (DEGs) in chondrocytes between OA patients and normal donors were analyzed, which were then subjected to Gene Ontology (GO) and pathway enrichment analyses, followed by microRNA (miRNA) and transcription factor (TF) prediction, and regulatory network construction. Finally, the copy number variants (CNVs) of target genes were searched. Total 79 up- and 147 down-regulated DEGs were identified. Nine GO terms were obtained and the biological process (BP) term related to neuron differentiation was enriched by 13 DEGs, such as *GNAO1*, *POU3F4* and*RPS27A*. Pathway of regulation of actin cytoskeleton was enriched by six DEGs such as *FGF18*. Six miRNAs such as miRNA-524 and seven TFs, such as FOXO4 were detected. In the regulatory network, *GNAO1*, *POU3F4* and *RPS27A* were key target genes and their CNVs were identified. Pathway of regulation of actin cytoskeleton and BP related to neuron differentiation may play important roles in OA progression. DEGs of *FGF18*, *GNAO1* and *POU3F4* as well as their regulatory factors such as FOXO4 and miRNA-524 may be potential biomarkers associated with OA.

Keywords: osteoarthritis; differentially expressed genes; functional enrichment analysis; microRNA; transcription factor; regulatory network

INTRODUCTION

Osteoarthritis (OA) is a chronic, incurable and costly disease, causing significant pain and disability [1]. It possesses joint symptoms and signs of changes in the underlying bone and at the joint margins as well as defective integrity of articular cartilage [2]. The regenerative and self-repair capacity of articular cartilage is very limited in adults [3], so OA is already the leading cause of disability among the elderly [4]. This has also resulted in significant direct health-care costs related associated with joint replacement requirements for patients with advanced disease [5]. Now, the exact mechanism behind cartilage degradation remains unclear, thus, numerous studies have intended to explore the mechanism and therapeutic method for OA.

It has been reported that genetic factors have been found to play critical roles in the OA progression. Insulinlike growth factor 1, for instance, is an important growth factor for cartilage homeostasis and has been found to reduce expression in OA patients [6]. Cytokines, including interleukin 1 and tumor necrosis factor alpha, are secreted by OA cartilage, which have been suggested to induce cartilage degradation [7]. Additionally, Zhong and coauthors [8] reported that SMAD family member 3 (*SMAD3*) mutations, a copy number variant (CNV), were linked to OA . SMAD3, the downstream mediator of the transforming growth factor beta signaling pathway, could inhibit terminal hypertrophic differentiation of chondrocytes and is essential for maintaining the integrity of articular cartilage [9]. Although progresses have been achieved about the pathogenesis of OA, the genetic mechanisms of OA are far from being understood.

In the present study, we downloaded the microarray data of GSE16464 and identified the differentially expressed genes (DEGs) between OA and normal donors (ND) chondrocytes to explore the biomarkers of OA. Dehne and coauthors [3] have used the dataset GSE16464 to study the chondrogenic differentiation potential of osteoarthritic chondrocytes. However, the biomarkers and regulatory factors of OA are still needed to be explored. Therefore, we performed functional and pathway enrichment analyses, as well as microRNA (miRNA) and transcription factor (TF) prediction to identify the biomarkers and regulatory factors related to OA. The regulatory network was constructed followed by CNV searching. We aimed to explore the underlying biomarkers and regulatory factors associated with OA. Findings of this study may help to understand the OA genesis and may potentially serve as biomarkers of OA.

MATERIAL AND METHODS

Affymetrix microarray data

The gene expression profile data (GSE16464, deposited by Dehne and coauthors [3]) were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database with " osteoarthritis" and "normal" as the key words, which were obtained based on the platform of GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The dataset included 12 samples, 6 of which were monolayer chondrocytes (from 3 normal donors and 3 OA donors) and the remaining 6 were 3D-cultured chondrocytes (from the same 3 normal donors and 3 OA donors). The OA specimens were obtained from patients with primary OA who had undergone total knee arthroplasty, and the normal specimens from healthy patients who had undergone amputation following a road traffic accident.

Data preprocessing and differential expression analysis

The original CEL data were performed background correction, quartile data normalization, and probe summarization by the robust multiarray average (RMA) [10] algorithm in R affy [11] package.

The paired t-test based on the limma [12] package in R language was used to identify genes that were significantly differentially expressed between OA and ND samples. The log2-fold change (log2FC) was calculated. $|\log_2 FC| > 1$ and p-value < 0.01 were considered as the cutoff values for DEGs screening.

Gene ontology and pathway enrichment analyses

In order to analyze the DEGs in functional level, we performed Gene ontology (GO) [13] functional enrichment analysis using the DAVID [14] online tool to obtain the enriched biological process (BP) terms. The p-value < 0.05 was set as the threshold value. Additionally, KEGG Orthology Based Annotation System (KOBAS) [15] software was used to analyze the Kyoto Encyclopedia of Genes and Genomes (KEGG) [16] pathway based on the hypergeometric test with threshold of p-value < 0.05.

miRNA and TF searching

Web-based Gene Set Enrichment Analysis Toolkit (WebGestalt) is an integrated system used for

exploring gene sets in various biological contexts [17]. In this study, the miRNAs and TFs associated with OA were predicted using the WebGestalt online tool with threshold of adjusted p-value < 0.05.

Protein-Protein interaction (PPI) pairs prediction and regulatory network construction

The Search Tool for the Retrieval of Interacting Genes (STRING) database [18] online tool was applied to search the PPI pairs of DEGs and only those experimentally validated interaction pairs with a combined degree > 0.4 were selected as significant. Then, based on the obtained PPI pairs, and interactions between DEGs and miRNAs/TFs, the regulatory network was constructed and visualized using Cytoscape [19] software. In the regulatory network, each node stands for a gene or miRNA or TF and the edges represent the interactions between nodes. Finally, the miRNAs and TFs of DEGs that were enriched in the most significant BP terms and pathways were searched.

Genomic variants searching

CNV [20] is a segment of DNA ranging from one kilobase to several megabases in size, which represents an imbalance between two genomes from one species. Database of Genomic Variants (DGV) [21] is a collection of structural variation in the genomes of control individuals from worldwide populations. In the present study, CNVs of the important DEGs were searched based on the database of DGV.

RESULTS

Identification of DEGs

Based on the thresholds of p-value < 0.01 and |log2FC| > 1, a total of 226 DEGs were obtained between OA and ND samples. Thereinto, 79 were up-regulated and 147 were down-regulated.

GO and pathway enrichment analyses

After GO enrichment analysis for the DEGs, nine GO BP terms were obtained (Table 1). Among them, BP term related to neuron differentiation was the most significant, which was enriched by the largest number of DEGs, such as guanine nucleotide binding protein (G protein), alpha activating activity polypeptide O (*GNAO1*), POU Class 3 Homeobox 4 (*POU3F4*) and ribosomal protein S27a (*RPS27A*).

Pathway enrichment analysis only identified two pathways, including pathways of regulation of actin cytoskeleton and focal adhesion (Table 1). The pathway of regulation of actin cytoskeleton was the most significant and it was enriched by six DEGs such as fibroblast growth factor 18 (*FGF18*) and Rho-associated, coiled-coil containing protein kinase 1.

Table 1. Gene Ontology (GO) and pathway enrichment analyses for the differentially expressed genes (DEGs).

Term	Name	P-value	DEGs
GO biological process terms			
GO:0030182	neuron differentiation	2.36E-03	*SCLT1*, *KLK8*, *GNAO1*, *MCF2*, *MYO7A*, *CTF1*, *PTPRR*, *SLITRK4*, *CLIC5*, *MNX1*, *POU3F4*, *APBB2*, *RPS27A*
GO:0048666	neuron development	9.93E-03	*SCLT1*, *KLK8*, *SLITRK4*, *GNAO1*, *MCF2*, *CLIC5*, *CTF1*, *MYO7A*, *APBB2*, *RPS27A*
GO:0008203	cholesterol metabolic process	1.63E-02	*EBP*, *HNF1A*, *CYP11A1*, *APOF*, *NPC1L1*
GO:0016125	sterol metabolic process	2.22E-02	*EBP*, *HNF1A*, *CYP11A1*, *APOF*, *NPC1L1*
GO:0002712	regulation of B cell mediated immunity	2.63E-02	*GIMAP5*, *C3*, *TNFSF12*
GO:0002889	regulation of immunoglobulin mediated immune response	2.63E-02	*GIMAP5*, *C3*, *TNFSF12*
GO:0008624	induction of apoptosis by extracellular signals	3.09E-02	*MCF2*, *PSEN2*, *ITSN1*, *ECT2*, *RPS27A*
GO:0060548	negative regulation of cell death	3.74E-02	*GIMAP5*, *SLC25A4*, *ROCK1*, *SNCB*, *PSEN2*, *ESR1*, *ITSN1*, *APBB2*, *RPS27A*

Cont Table 1

GO:0030030	cell projection organization	4.16E-02	*KLK8*, *SLITRK4*, *GNAO1*, *ROCK1*, *MCF2*, *CLIC5*, *MYO7A*, *APBB2*, *RPS27A*
Pathway			
hsa04810	Regulation of actin cytoskeleton	3.67E-02	*FGF18*, *ROCK1*, *CHRM3*, *MYLK3*, *DIAPH3*, *ITGA2B*
hsa04510	Focal adhesion	4.43E-02	*ARHGAP5*, *ROCK1*, *MYLK3*, *RAP1A*, *ITGA2B*

MiRNA and TF searching

After miRNAs and TFs prediction, six miRNAs including miRNA-19, miRNA-330, miRNA-218, miRNA-524, miRNA-493 and miRNA-124A were obtained. In addition, seven TFs were detected, such as forkhead box O4 (FOXO4) and paired box 4 (PAX4) (Table 2).

Table 2. List of microRNA (miRNA) and transcription factor (TF).

miRNA/TF	ID	Parameters
miRNA-19	DB_ID:848	O = 10; rawP = 3.24E-05; adjP = 3.00E-04
miRNA-330	DB_ID:866	O = 7; rawP = 3.00E-04; adjP = 1.4E-03
miRNA-218	DB_ID:813	O = 6; rawP = 4.00E-03; adjP = 1.20E-02
miRNA-524	DB_ID:726	O = 6; rawP = 7.10E-03; adjP = 1.28E-02
miRNA-493	DB_ID:750	O = 5; rawP = 6.40E-03; adjP = 1.28E-02
miRNA-124A	DB_ID:775	O = 6; rawP = 1.99E-02; adjP = 2.93E-02
E12	DB_ID:1976	O = 22; rawP = 1.80E-05; adjP = 9.90E-05
MAZ	DB_ID:2017	O = 20; rawP = 7.12E-05; adjP = 3.00E-04
AP1	DB_ID:2015	O = 11; rawP = 1.50E-03; adjP = 2.70E-03
FAT	DB_ID:1898	O = 15; rawP = 1.40E-03; adjP = 2.70 E-03
AP4	DB_ID:1850	O = 13; rawP = 1.50E-03; adjP = 2.70 E-03
FOXO4	DB_ID:1962	O = 14; rawP = 7.00E-03; adjP = 1.10E-02
PAX4	DB_ID:1965	O = 10; rawP = 1.33E-02; adjP = 1.63E-02

O: number of genes in the gene set and also in the category; rawP: p-value from hypergeometric test ; adjP: p-value adjusted by the multiple test adjustment.

PPI pairs prediction and regulatory network construction

In this study, 46 PPI pairs of DEGs were obtained. Then, combining the interactions between DEGs and miRNAs/TFs, the regulatory network was constructed. The network consisted of 113 edges and 67 nodes including five miRNAs and six TFs (Figure 1). Among these nodes, *GNAO1* that participated in the BP terms associated with neuron differentiation was regulated by three miRNAs including miRNA-218, miRNA-330 and miRNA-524 and six TFs such as FOXO4 and PAX4. *POU3F4* that was also involved in neuron differentiation related BP term, was regulated by six TFs. Additionally, *RPS27A* was regulated by E12.

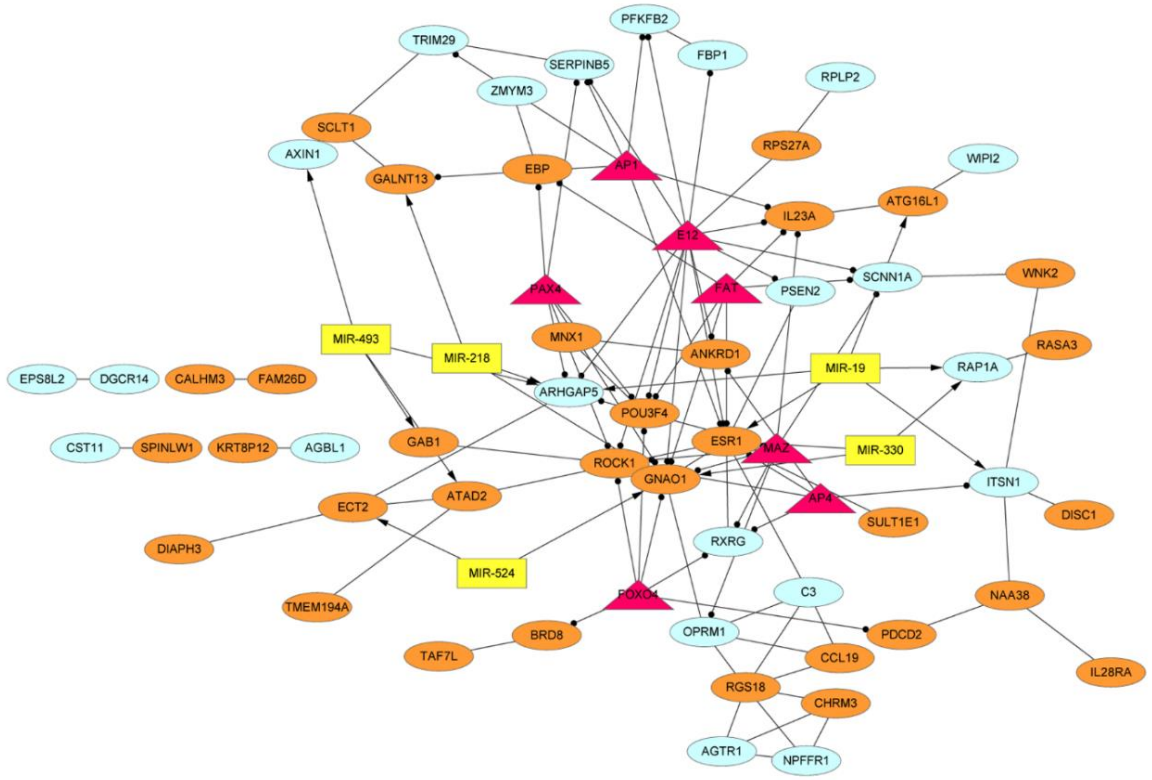

Figure 1. The regulatory network of differentially expressed genes (DEGs). Oval represents DEGs (orange: up-regulated DEGs; blue: down-regulated DEGs); yellow rectangle represents microRNA; pink triangle represents transcription factor.

Based on the BP terms and pathways, as well as the regulatory network, *GNAO1*, *POU3F4* and *RPS27A* were identified as the important DEGs. CNVs of the three DEGs were shown in Table 3.

Table 3. Copy number variation (CNV) of the important differentially expressed genes.

Variant ID	Type	Subtype	PubMed ID
GNAO1			
esv2714509	CNV	Deletion	23290073
esv272874	CNV	Insertion	20981092
nsv525929	CNV	Loss	19592680
nsv906714	CNV	Loss	21882294
dgv2870n71	CNV	Loss	21882294
nsv521726	CNV	Loss	19592680
nsv523649	CNV	Loss	19592680
nsv519346	CNV	Loss	19592680
nsv906715	CNV	Loss	21882294
nsv833242	CNV	Loss	17160897
POU3F4			
nsv510842	CNV	Loss	20534489
RPS27A			
esv2720078	CNV	Deletion	23290073
nsv522016	CNV	Gain	19592680

DISCUSSION

OA is a slowly progressive rheumatic disease observed mainly in elderly people [22]. The identification of biomarkers in OA is critical for the development of novel therapeutic strategies. In this study, a total of 226 DEGs were identified between OA and ND samples. Neuron differentiation was the most significant BP term, and regulation of actin cytoskeleton was the most significant pathway. In addition, six miRNAs and seven TFs that regulated the DEGs were predicted. In the regulatory network, *GNAO1* was regulated by three miRNAs and six TFs, *POU3F4* was regulated by four TFs, and *RPS27A* was regulated by E12. Importantly, the CNVs of the three DEGs above were searched. The results suggested that these genes and regulatory factors may play important roles in the progression of OA.

In this study, the pathway of regulation of actin cytoskeleton was the most significant pathway. The actin cytoskeletal is believed to be an important regulator of chondrocyte phenotype [23]. It has been suggested that actin cytoskeleton contributes to proper bone development and growth plate function [24]. Several human chondrodysplasias have been found to be linked to mutations which affect the actin cytoskeleton [25]. Benya and coauthors [26] have reported that disruption of the actin cytoskeleton by dihydrocytochalasin B can promote redifferentiation of chondrocytes. OA is characterized by the apoptosis of chondrocytes, therefore, pathway of regulation of actin cytoskeleton may play an important role in the progression of OA. In addition, this pathway was enriched by six DEGs, such as *FGF18* which was down-regulated in our study. FGF18 is a member of the fibroblast growth factor (FGF) family which possess broad activities about mitogenic and cell survival, and participate in a variety of biological processes such as cell growth, tissue repair and tumor growth [27]. Importantly, FGF18 has been shown to regulate the bone development and has significant anabolic effects on cartilage [28]. Study has reported that FGF18 is required for chondrogenesis in the skeletal development of the mouse [29]. Taken together, pathway of regulation of actin cytoskeleton and its enriched DEGs, *FGF18*, were closely associated with OA, thus, this pathway and gene may be used as potential targets for OA treatment.

From the result of regulatory network construction, we could found that *GNAO1* and *POU3F4* were up-regulated in the network. Additionally, the two DEGs was also associated with the function of neuron differentiation, the most significant GO term. GNAO1 is a member of the subunit family of Gα proteins, and is involved in various transmembrane signaling systems as modulators or transducers [30, 31]. *GNAO1* has been reported to be enriched obviously in the growth cones of neuron [32]. *POU3F4* encodes a member of the POU-III class of neural transcription factors, which is expressed widely in the developing of central nervous system. There is hardly any report about the association between *GNAO1*/*POU3F4* and OA. Specially, TF of FOXO4, which regulated the two DEGs in the regulatory network, has been found to play an essential role in the pathogenesis of age-related diseases including OA [33]. As it was reported that chondrocytes could produce reactive oxygen species (ROS) in answer to cytokines and mechanical stress [34]. ROS induces cell death to increase vulnerability of aging articular cartilage [35]. FOXO4 belonging to FOXO family can control oxidative stress resistance through regulating antioxidants [36, 37]. Importantly, Ludikhuize and coauthors [38] suggested that FOXO4 were expressed and phosphorylated in synovial tissue from OA patients. Additionally, *GNAO1* was also the target gene of miRNA-524 in the regulatory network. MiRNA-524 has been found to behave as a tumor suppressor to suppress cell proliferation [39], so we speculated that miRNA-524 might inhibit the cartilage cell proliferation. Additionally, Zhu and coauthors [39] indicated that primary chondrogenesis could be modulated by miRNA-524. Importantly, the CNVs of *GNAO1* and *POU3F4* were obtained in this study. There were limited reports about the relationship between CNVs of the two DEGs and OA, so we speculated that the two CNV-driven DEGs may be biomarkers of OA. From the aforementioned, *GNAO1*, *POU3F4* and its regulatory factors as well as the BP terms related to neuron differentiation may play important roles in OA progression.

Although bioinformatics technologies have the potential to indentify and validate the biomarkers of serious diseases, some limitations still remain in this study. On one hand, the sample size for microarray analysis was small. On the other hand, this study was lack of experimental verification. Therefore, we will collect OA tissue samples as much as possible to confirm the results in the future.

CONCLUSION

Pathway of regulation of actin cytoskeleton and BP term related to neuron differentiation have the potential to be used as targets for OA treatment. In addition, *FGF18*, CNV-driven DEGs of *GNAO1* and *POU3F4* as well as their regulatory factors such as TF of FOXO4 and miRNA-524 may be potential biomarkers associated with OA progression and treatment.

Conflicts of Interest: The authors declare no conflict of interest.

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