Significantly dysregulated genes in osteoarthritic labrum cells identified through gene expression profiling

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Abstract. The aim of the present study was to explore the molecular basis and identify significant genetic alterations in acetabular labrum cells associated with osteoarthritis (OA). Gene expression data of osteoarthritic and normal human labrum cells were downloaded from a public database and reanalyzed. Significant differentially expressed genes (DEGs) were acquired by performing a thorough analysis of microarray data between the OA acetabular labrum cells and control cells. Key genes in OA labrum cells were revealed by a combination of weighted gene co-expression network analysis (WGCNA) and protein-protein interaction (PPI) analysis. Literature mining and drug screening were further performed for these key genes. In total, 141 DEGs between OA and normal labrum cells were identified. In addition, WGCNA and PPI analysis identified 23 DEGs as key genes in the OA labrum. All the key genes were significantly downregulated in OA labrum cells and were grouped into two different WGCNA-PPI common subnetworks. Kinase insert domain receptor (*KDR*), *CD34*, cadherin 5 (*CDH5*), Fms related tyrosine kinase 1 (*FLT1*) and *asporin* were hub nodes in the PPI network of DEGs. These key genes were significantly enriched in functional clusters of transforming growth factor, alkaline phosphatase, bone morphogenic protein and extracellular matrix. Drug screening analysis identified several drugs targeting the key genes, including arachidonic acid, yohimbic acid and mimosine. The results of the present study indicate that the changes of *FLT1*, *KDR*, *CD34* and *CDH5* in acetabular labrum cells may be involved in the pathogenesis of OA and could serve as biomarkers and therapeutic targets of OA. Additionally, arachidonic acid, yohimbic acid and mimosine may act as potential drugs for OA.

Introduction

Osteoarthritis (OA) is one of the most common types of arthritis worldwide and affects ~15% of the population (1). General symptoms of OA include pain and stiffness in joints and physical disability (2). Although loss of articular cartilage is considered to be the primary cause of OA, other joint tissues including subchondral bone, synovium and acetabular labrum are also involved in the development and progression of OA (2). Moreover, increasing evidence supports a role for genetic factors in OA (1), which may provide clues for the diagnosis and treatment of OA.

A series of genes have been identified to be associated with OA, such as growth differentiation factor 5 (3), *DOI2* (4,5) and SMAD family member 3 (6). Previously, bioinformatics methods have been increasingly used and have facilitated the identification of genetic variations in OA (7). Inflammatory genes such as *CD55*, prostaglandin E synthase (*PTGES*) and TNF-α induced protein 6 (*TNFAIP6*) have been shown to be significantly upregulated in articular cartilage in a genome-wide study, indicating that inflammation may be involved in OA progression (7). Dysregulation of genes related to anti-oxidative defense mechanism has also been identified in OA. Another study using expression profiles from OA and normal articular cartilage showed that superoxide dismutase 2 (*SOD2*), *SOD3*, and glutathione peroxidase 3 were significantly downregulated in OA articular cartilage, suggesting a close correlation between the dysregulation of anti-oxidative defense and cartilage matrix damage (8).

Extensive studies have been performed to investigate the molecular mechanisms of OA development and progression (9-12). Studies have reported that chitinases, including chitinase 3 like 1 and chitotriosidase, are upregulated while lubricin is downregulated in OA cartilage and they are considered to be potential markers to stage the severity of OA (9,11). In addition, lubricin, as well as collagen type I, collagen type II are found to be associated with the formation of hyaline cartilage (10). Giunta *et al* (12) revealed that the expression of pituitary adenylate cyclase-activating polypeptide is reduced in OA and contributes to the inhibition of chondrocyte apoptosis induced by interleukin 1β. However, much less is known about the transcriptional alterations

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occurring in the acetabular labrum. Two recent studies compared the expression profiles of OA acetabular labrum cells and healthy cells (13,14). One of the studies collected and deposited the expression data in the Gene Expression Omnibus database under accession number GSE60762 (14). The study focused on small leucine rich repeat proteins (SLRPs) and indicated that genes coding for osteomodulin, osteoglycin, and asporin (*ASPN*) may be functionally important for OA (14). Another study using the same dataset revealed several distinct OA related genes, including cadherin 2 (*CDH2*), Wnt family member 5A, kinase insert domain receptor (*KDR*, also known as *VEGFR2*), Fms related tyrosine kinase 1 (*FLT1*) and *CDH5* (13). However, further studies are needed to identify additional essential genes related to OA acetabular labrum.

To get new insights into the molecular mechanisms underlying the pathological changes in the OA acetabular labrum, the GSE60762 dataset was reanalyzed using an optimized bioinformatics strategy as diverse bioinformatics approaches may lead to novel insights (15). Following the screening of important differentially expressed genes (DEGs), key genes associated with OA were identified using a combination of co-expression analysis and protein-protein interaction (PPI) analysis. Moreover, the functions of these key genes as well as potential drugs targeting the proteins encoded by these genes were investigated. Therefore, the present study may advance the understanding of the underlying molecular mechanisms of OA and may contribute to the diagnosis and treatment of OA.

Materials and methods

Data source and preprocessing. Expression profile data under the accession number GSE60762 was downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (16) database and used for the present study. In total, 5 OA samples and 3 normal samples from acetabular labrum cells were included in the present study. The Series Matrix File(s) was downloaded and the probe names were converted to gene symbols according to the annotation information provided on the GPL6244 platform. The expression values of genes were acquired by averaging the values of their corresponding probes using the aggregate function in base R (version 3.3.3; https://cran.r-project.org/). The expression value of probes with missing value was adjusted using the K-nearest neighbor method (17) (nearest neighbor average with the k value set as 10) from the impute package (18) in R. The expression profile data was normalized by quantile normalization using the preprocessCore package (19) in R.

Screening of significant DEGs. The significant DEGs obtained were screened using the limma package (20) in R. The differences of mean expression values between OA and normal samples were compared using a t test from the package. P-value was adjusted by the Benjamini-Hochberg method. Genes with |log$_2$FC (fold change)|>1 and P<0.05 were considered to be significant DEGs.

Weighted gene co-expression network analysis (WGCNA) of the significant DEGs. All the significant DEGs were analyzed using WGCNA (https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/) (21) package in R to reveal their co-expression relationships. The DEGs were divided into different WGCNA modules. The correlations between different WGCNA modules and OA were assessed using one-way analysis of variance. P<0.05 was set as the cut-off criteria.

Functional analysis of gene modules. Gene Ontology (GO) (22) enrichment analysis were performed for different WGCNA modules using clusterprofiler package (23) in R, with P<0.05 set as the cut-off criteria.

Construction of PPI network. The interactions among proteins encoded by all the DEGs were predicted using the STRING database (24). The PPI network was constructed and visualized with Cytoscape (version 3.2.0; http://www.cytoscape.org) software (25). Furthermore, WGCNA-PPI common subnetworks were constructed. The resulting common subnetworks contained genes with both the weighted correlations as well as PPIs.

Literature mining analysis of key genes. Gene Cluster with Literature Profiles (GenCLiP; version 2.0) is a software for clustering genes under functional keywords based on literature mining (26). The functions of DEGs were analyzed using the GenCLiP (version 2.0, http://ci.smu.edu.cn/GenCLiP2/analysis.php) (26) module 'Gene Cluster With Literature Profiles'. Previously studied functions of genes could therefore be obtained. Functional keywords with P<1x10^{-4} and those at least for 2 genes were selected.

Drug screening. Connectivity Map (CMAP; https://portals.broadinstitute.org/cmap) is a database used to connect gene expression signatures with small molecules (27). Potential small drug molecules for arthritis treatment were screened using CMAP. The significant DEGs were converted to probes under HG-U133A platform by format conversion. Subsequently, the OA-related small drug molecules were identified by comparing the expression profiles of the DEGs with the gene expression profiles generated by small molecules from the CMAP database. P<0.05 was set as the cut-off criteria.

Results

DEGs between OA and normal labrum cells. expression data for a total of 19,952 genes were obtained after data preprocessing. Among these genes, 141 (50 upregulated and 93 downregulated genes) were identified to be differentially expressed in OA samples compared with normal samples (Fig. 1A and B). Hierarchical cluster analysis based on the expression value of DEGs could clearly classify OA and normal samples into two different clusters (Fig. 1C).

Co-expression modules of DEGs. In order to identify OA-related co-expression patterns, DEGs were analyzed using WGCNA, an R package for weighted gene co-expression network analysis (21). In total, 5 significant WGCNA modules were acquired and designated as brown, yellow, blue, turquoise, and grey. Most of the modules contained
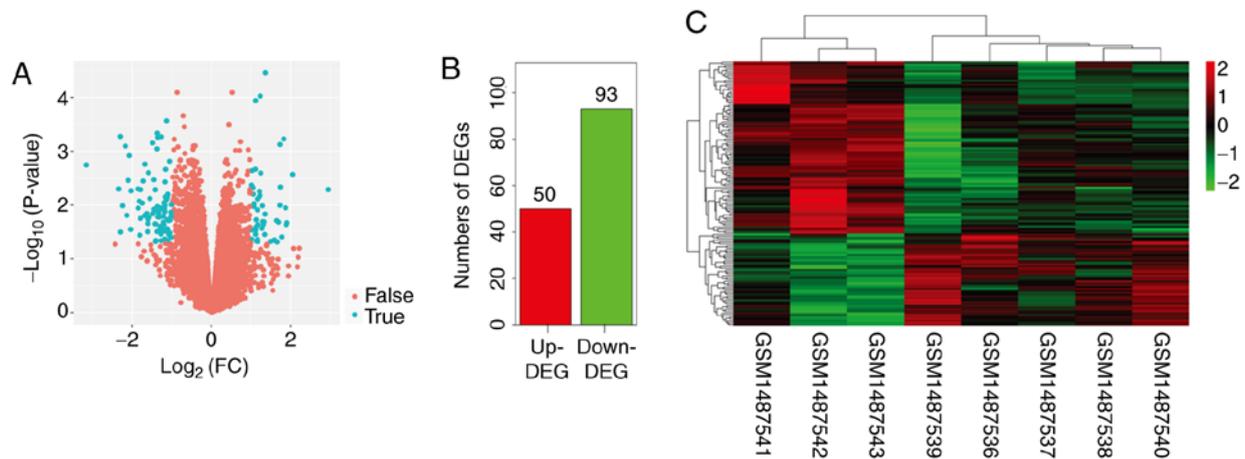
Figure 1. Screening of DEGs between OA and healthy labrum cells. (A) Volcano plot of DEGs. Cyan dots indicate significant DEGs (P<0.05, |logFC|>1.0) in OA in comparison to healthy labrum cells. (B) The numbers of DEGs are shown in the histogram. Upregulated and downregulated DEGs in OA labrum cells are shown as red and green, respectively. (C) Heatmap of DEGs based on hierarchical clustering analysis. The horizontal axis represents the sample codes and vertical axis, the DEGs. Upregulated and downregulated DEGs in OA labrum cells are shown in red and green, respectively. DEG, differentially expressed genes; OA, osteoarthritis.

>20 genes except the grey module, which contained only 8 genes and therefore was not included in the subsequent analysis. The expression of genes in the blue, yellow and turquoise modules correlated negatively with OA, whereas that of genes in the other two modules correlated positively with OA (Table I). Consistent with this, the expression of genes in the blue, yellow and turquoise modules was lower and the expression of genes in the brown module was increased in OA samples compared with the normal samples (Fig. 2A).

Furthermore, GO enrichment analysis was performed for genes in each WGCNA module. The blue module was significantly enriched in multicellular organismal development and system development (P<0.05; Fig. 2B). The brown module was significantly enriched in terms such as regulation of biological quality, single-organism localization and single-organism transport (P<0.01; Fig. 2C). The turquoise module was significantly enriched in terms related to extracellular region, extracellular region part, development process, single-organism developmental process, multicellular organismal process and single-multicellular organism process (P<0.001; Fig. 2D). The yellow module was significantly enriched in terms such as membrane, membrane part, intrinsic component of membrane and integral components of membrane (P<0.001; Fig. 2E).

PPI network of DEGs. The PPIs between proteins encoded by DEGs were analyzed using the STRING database. In total, 102 PPIs were obtained for 66 DEGs (Fig. 3A). *KDR* (degree=12, downregulated), *CD34* (degree=10, downregulated), *CDH5* (degree=10, downregulated), *FLT1* (degree=10, downregulated) and *ASPN* (degree=7, downregulated) were considered to be hub nodes in the network, as they had the highest connectivity degree.

The PPIs between proteins encoded by genes in each WGCNA module could also be identified based on the PPI network. Therefore, 4 WGCNA-PPI common subnetworks were constructed to delineate the PPIs within each WGCNA

module (Fig. 3B). Both the blue and brown subnetwork consisted of only 4 nodes and 2 PPIs, whereas the yellow and turquoise subnetwork consisted of several nodes and PPIs. In total, 9 nodes and 14 PPIs were included in the yellow common subnetwork, and 12 nodes and 16 PPIs were included in the turquoise common subnetwork. All the downregulated DEGs in the yellow and turquoise subnetwork are shown in OA (Fig. 3B; Table II). Moreover, all the 5 hub nodes were present in either the yellow or turquoise subnetworks and the blue and brown subnetworks did not contain any hub nodes. Specifically, *KDR*, *CD34* and *CDH5* were present in the yellow subnetwork, and *FLT1* and *ASPN* were present in the turquoise subnetwork. Consequently, the 23 downregulated genes in yellow and turquoise subnetworks were considered to be key genes associated with OA.

Literature mining analysis of key genes. In order to study the roles of the 23 key genes in the development and progression of OA, literature mining analysis was performed. Using GenCLiP, abundant functional study results were found for all the 23 genes in yellow and turquoise subnetwork (Fig. 4A). *FLT1*, *KDR*, *CD34* and *CDH5* were the key genes associated with the most abundant biological functions. The published literature and related function of the *FLT1*, *KDR*, *CD34*, and *CDH5* in OA are shown in Table SI. Functional clusters with the highest scores included transforming growth factor (TGF), alkaline phosphatase, bone morphogenetic protein (BMP) and extracellular matrix (ECM).

Small drug molecules targeting proteins encoded by key genes. Potential small drug molecules targeting proteins encoded by the key genes were also explored using CMAP. As a result, 25 drug molecules were obtained associated with OA, including 15 positively associated and 10 negatively associated drug molecules (Fig. 4B; Table III). Among the top 10 associated drug molecules, 3 were positively associated and 7 were negatively associated with OA (Fig. 4B). The top 3 drugs were arachidonic acid, yohimbic acid and mimosine.

Table I. WGCNA modules.

Variable	Brown	Yellow	Blue	Turquoise	Grey
Correlation[a]	0.74	-0.8	-0.87	-0.79	0.82
P-value	0.035237748	0.016851893	0.005226361	0.019352318	0.013654178
Gene number[b]	24	21	37	53	8

[a]Correlation between each WGCNA module and osteoarthritis. [b]The number of genes within each module. WGCNA, weighted gene co-expression network analysis.
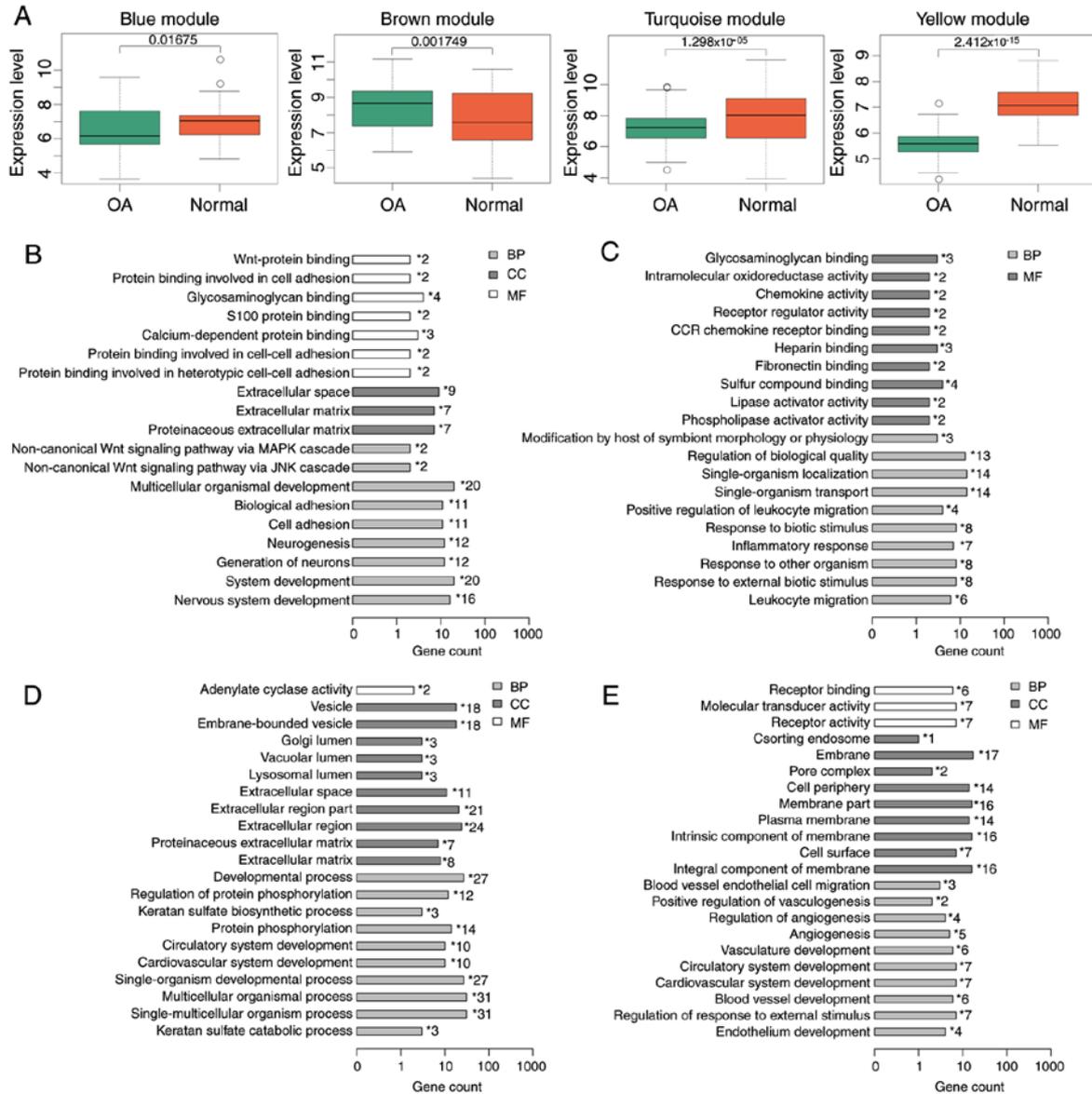

Figure 2. Expression and functional annotation of DEGs in WGCNA modules. (A) Expression of DEGs in OA (green) and normal (red) labrum cells is shown by box-plot. Gene Ontology annotation of DEGs in (B) blue, (C) brown, (D) turquoise, and (E) yellow modules. BP, biological process; CC, cellular component; MF, molecular function; WGCNA, weighted gene co-expression network analysis; DEG, differentially expressed genes.

Discussion

In the present study, 141 significant DEGs were screened between OA and healthy labrum cells. A total of 23 DEGs were identified to be key genes associated with OA. All the key genes were downregulated in OA labrum cells and could be grouped into two different WGCNA co-expression modules. Moreover, among the key genes, *FLT1*, *KDR*, *CD34* and *CDH5* were associated with the most abundant biological functions and may be substantially involved in the development and progression of OA.
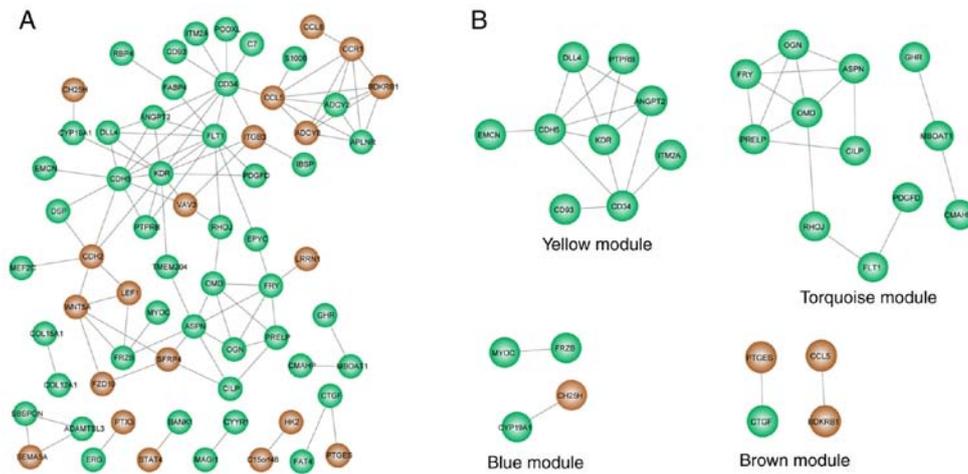
Figure 3. PPI analysis of DEGs. (A) PPI network of proteins encoded by DEGs. (B) WGCNA-PPI common subnetworks. Upregulated and downregulated genes are shown as red and green spheres, respectively. Interactions between the proteins are indicated by black lines. DEG, differentially expressed genes; WGCNA, weighted gene co-expression network analysis; PPI, protein-protein interaction.
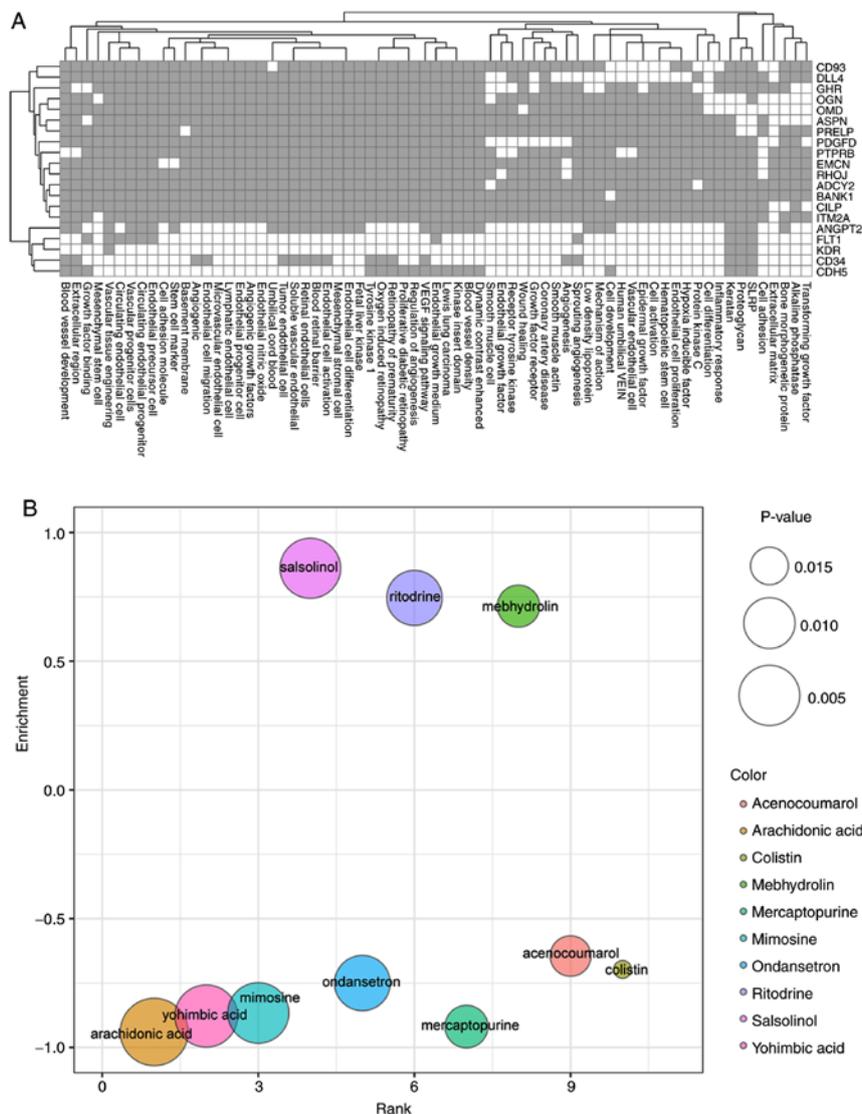

Figure 4. Literature mining and small drug molecule screening of key genes. (A) Functional clustering of key genes based on literature mining. The vertical axis denotes the key genes and horizontal axis shows the functional terms. The white block represents the relationship between a corresponding gene and its functional term, and gray block represents no relationship between a corresponding gene and its functional term. (B) Top 10 small drug molecules targeting the key genes. The horizontal axis shows the ranks of the drug molecules and vertical axis shows the enrichment scores. The bubbles represent drug molecules. The size of a bubble is negatively correlated with its P-value.

Table II. Genes in the turquoise and yellow WGCNA-PPI common subnetworks.

Module	Gene symbol	log$_2$FC	P-value
Turquoise	ADCY2	-1.231891879	0.02107479
	ASPN	-3.174936812	0.001813696
	BANK1	-1.039158068	0.040643831
	CILP	-1.337322006	0.017005166
	CMAHP	-1.69454791	0.015905385
	FLT1	-2.083203641	0.001212928
	FRY	-1.126732775	0.047399489
	GHR	-1.127449211	0.007123746
	MBOAT1	-1.193125199	0.030776301
	OGN	-2.05075982	0.028726148
	OMD	-2.042591674	0.005478374
	PDGFD	-1.082416359	0.035278215
	PRELP	-1.07748275	0.018850184
	RHOJ	-1.586566225	0.01714161
Yellow	ANGPT2	-1.800427427	0.005234895
	CD34	-1.734034415	0.003612027
	CD93	-1.183506958	0.010186758
	CDH5	-1.428997713	0.005249021
	DLL4	-1.032847908	0.016503115
	EMCN	-2.351818405	0.005082473
	ITM2A	-1.377567348	0.010768885
	KDR	-2.312597602	0.000534604
	PTPRB	-1.028136925	0.009858035

FC, fold change; WGCNA, weighted gene co-expression network analysis; PPI, protein-protein interaction.

Table III. Small drug molecules screened by CMAP analysis.

Rank	Drug molecule[a]	Mean score[b]	P-value
1	Arachidonic acid	-0.671	0.00032
2	Yohimbic acid	-0.393	0.00373
3	Mimosine	-0.392	0.00477
4	Salsolinol	0.505	0.00505
5	Ondansetron	-0.332	0.00778
6	Ritodrine	0.394	0.0079
7	Mercaptopurine	-0.513	0.0134
8	Mebhydrolin	0.623	0.01359
9	Acenocoumarol	-0.339	0.0141
10	Colistin	-0.421	0.01778
11	Alimemazine	0.327	0.01908
12	Imatinib	0.507	0.02058
13	Econazole	0.515	0.02109
14	Bretylium tosilate	-0.316	0.02532
15	Clebopride	0.449	0.0266
16	Etifenin	-0.421	0.02672
17	Oxybenzone	0.511	0.02711
18	Kanamycin	0.323	0.02851
19	Spaglumic acid	-0.439	0.02897
20	Semustine	0.469	0.03083
21	Buflomedil	0.311	0.03943
22	Quercetin	0.338	0.04007
23	11-deoxy-16,16-dimethylprostaglandin E2	0.338	0.0401
24	Calmidazolium	0.24	0.04036
25	Digitoxigenin	0.373	0.04752

[a]The names of small drug molecules used in CMAP database; [b]Mean scores of the connectivity between corresponding drug molecules and the key genes. CMAP, Connectivity Map.

In the PPI analysis in the present study, it was demonstrated that *FLT1*, *KDR*, *CD34* and *CDH5* together with *ASPN*, were hub nodes in the PPI network of DEGs. As mentioned above, a previous bioinformatic study using the same GSE60762 dataset also demonstrated that *FLT1*, *KDR* and *CDH5* were among the hub nodes (13). *FLT1* and *KDR* are vascular endothelial growth factor (VEGF) receptors activated upon VEGF binding (28). VEGF signaling has been reported to promote the formation of cartilage matrix, indicating that inhibition of VEGF signaling may cause cartilage damage in OA (29). It was demonstrated that both *FLT1* and *KDR* were downregulated in the OA labrum, suggesting that inhibition of VEGF signaling may also lead to labrum damage in OA. In addition, it has reported that *FLT1*/VEGF are associated with the recruitment and differentiation of osteoclast cells in bone-resorbing and bone-forming (30,31). Moreover, Hopwood *et al* (32) reveals that the expression of *FLT1* is decreased in OA bone and low expression of *FLT1* in OA can promote the activity of VEGF in angiogenesis and osteogenesis. Similarly, the phosphorylation of *KDR*/Flk-1 has been selected in the regulation of angiogenesis induced by VEGF in several studies (33,34). The angiogenesis and inflammation causes and effects each other in the synovium in OA; the inflamed synovium produces VEGF which accelerates angiogenesis, leading to a further inflammatory response (35). Also, Mifune *et al* (36)

has demonstrated that the upregulation of FLT1 is involved in promoting collagen synthesis in articular cartilage repair. These findings were all consistent with the results of literature mining analysis, suggesting downregulation of those genes might be associated with angiogenesis and osteogenesis in OA by interacting with VEGF. *CDH5* is a specific marker for endothelial cells, essential for vascular integrity and endothelial functions (37). It also functions through binding to *KDR* to suppress VEGF-driven sprouting (38), further confirming the involvement of labrum VEGF signaling in OA. *ASPN* is a member of the SLRP family, which was found to be downregulated in OA labrum cells by the analysis of the present study and another bioinformatics study (14). In contrast, *ASPN* was found to be upregulated in OA chondrocytes (39), indicating that distinct pathological processes could exist in OA cartilage and labrum. The other hub node, *CD34*, is ubiquitously expressed on the surface of hematopoietic cells and represents a marker of hematopoietic cells (40). It has been shown that *CD34* facilitates the recruitment of hematopoietic stem cells in the niches of bone marrow (40), which suggests that a loss of function of *CD34* may cause damage to bone and result in OA.

However, the roles of these hub nodes in OA labrum have not been experimentally studied so far. Further studies are needed to clarify the specific roles of *FLT1*, *KDR*, *CD34*, *CDH5* and *ASPN* in the OA labrum.

Various biological functions are dysregulated in OA. TGFs are essential regulators of cell fate control, such as cell proliferation, differentiation and apoptosis (41). It has been demonstrated that the TGFβ signaling pathway is dysregulated in OA cartilage and bone (42). Reduced TGFβ signaling may cause a reduction in ECM synthesis of cartilage and therefore lead to increased susceptibility to OA (43). BMPs are growth factors promoting chondrocyte proliferation and ECM synthesis (44). Both *BMP-4* and *BMP-7* have been demonstrated to have a beneficial effect on OA by improving cartilage repair (44). Alkaline phosphatase is essential for controlled bone mineralization (45). Dysregulation of alkaline phosphatase activity leads to mineralized bones (45,46). In the present study, it was demonstrated that TGF signaling, ECM, BMP and alkaline phosphatase may also be dysregulated in OA labrum. According to the literature mining analysis of the present study, TGF signaling, ECM, BMP and alkaline phosphatase were the leading moieties associated with the key genes, including but not limited to *FLT1*, *KDR*, *CD34*, and *CDH5*. However, the roles of TGF signaling, ECM, BMP and alkaline phosphatase have not been directly studied in OA labrum to date; therefore, further studies are needed to elucidate their roles in OA.

Although OA is traditionally considered as a non-inflammatory disease, increasing evidence supports the involvement of inflammation in OA (47). Previously, synovitis has been recognized as a major feature of OA (47,48). In addition, inflammatory events in other joint tissues, such as cartilage, bone and ligament also contribute to OA (47,48). A variety of inflammatory mediators are released during OA progression. As mentioned above, inflammatory genes including *CD55*, *PTGES* and *TNFAIP6* are significantly upregulated in articular cartilage (7). In the present study, links were also found between inflammation and OA. Both arachidonic acid and mimosine, two top small molecular drugs that were identified by CMAP analysis to target the OA-associated key genes, are involved in the inflammatory process. L-mimosine is an anti-inflammatory compound that functions by inhibiting chemokines, such as monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 in OA (49). Consistent with this, a negative correlation between mimosine and OA was identified, indicating the role of inflammation in OA progression. Unlike mimosine, arachidonic acid is known for its pro-inflammatory effect (50). Enzymatic oxidation of arachidonic acid by COX2 produces prostaglandins (50), type of mediators of inflammation, which promote the production of matrix metallopeptidases (51). However, it was demonstrated that arachidonic acid was also negatively correlated with OA, which further complicated the roles of inflammation in OA. Nevertheless, it was hypothesized that both arachidonic acid and mimosine may serve as potential candidate drugs for OA treatment, though the effects of arachidonic acid and mimosine should be evaluated in future experimental research.

The obvious advantage of the present study is that an optimized bioinformatics strategy combining WGCNA analysis and PPI analysis was used to reveal a well-defined and more comprehensive set of key genes associated with OA. However,

the present study was also limited by the small sample size included in the original dataset. Due to the particularity of the osteoarthritic labrum cell samples used, appropriate data for data validation and clinical samples for experimental validation was not found. Therefore, studies with a larger number of samples will be used to verify the results of the present in the future. To conclude, 23 OA-associated key genes in labrum cells were identified, among which *FLT1*, *KDR*, *CD34* and *CDH5* may be involved in the development and progression of OA. It was hypothesized that the findings would greatly contribute to the investigation of OA progression; these genes may serve as novel prognostic and diagnostic biomarker candidates and potential therapeutic targets of OA, but further studies with large sample sizes are needed to verify the results of the present study.

Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

SW and CJ made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data and drafted the manuscript. KZ revised the manuscript for important intellectual content and conducted the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interest.

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