RNA-sequence analysis of samples from patients with idiopathic adhesive capsulitis

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Received March 16, 2017; Accepted August 4, 2017

DOI: 10.3892/mmr.2017.7579

Abstract. The present study aimed to investigate idiopathic adhesive capsulitis (frozen shoulder), to gain insights on its pathogenesis, diagnosis and therapeutic targets. Using RNA-sequencing (seq), the present study investigated differentially expressed genes (DEGs) in five samples from five idiopathic adhesive capsulitis patients and two samples from two acromioclavicular dislocation patients, without idiopathic adhesive capsulitis. The DEGs were analyzed using the following tools: Gene Ontology enrichment analysis, Kyoto Encyclopedia of Genes and Genomes pathways analysis and protein-protein interaction analysis. A total of 188 DEGs were identified and it was observed that 150 of these were upregulated and 38 were downregulated. It was hypothesized that various nutrient associated proteins may be associated with idiopathic adhesive capsulitis. The Matrix metalloproteinase family of proteins (MMPs), may exhibit a key role in the formation of abnormal collagen cross-links. Overall, the comprehensive and detailed information collected in the present study, regarding idiopathic adhesive capsulitis, may provide a foundation on which in-depth follow-up experiments may be based, aimed at identifying novel strategies for treatment of this disease.

Introduction

Adhesive Capsulitis, also known as Frozen Shoulder (1), pericapsulitis and periarthritis (2) is a common disease of unclear cause and significant morbidity (3). It is characterized by pain and a progressive loss of both active and passive range of motion. These symptoms can last up to 2 years or longer (4,5). Patients who underwent the arthroscopic capsular release procedure experienced significant reductions in pain, improvements in range of motion (6).

It has long been recognized that glucose and lipid metabolism disorders have a close association with idiopathic adhesive capsulitis. Although the incidence in the general population is <2% (7,8), the incidence is about 10% in Type I diabetics and up to 29% in Type II diabetics (9-11). Sung *et al* (12) demonstrated that hypercholesterolemia and inflammatory lipoproteinemias, especially hyper-low-density lipoproteinemia and hyper-non-high-density lipoprotein cholesterolemia, may contribute to the development of primary idiopathic adhesive capsulitis. Won *et al* (13) demonstrates that the anterior-inferior capsular portion is the main pathologic site of idiopathic adhesive capsulitis and reveals significant correlations with metabolic parameters on 18F-FDG PET/CT.

The diagnostic criteria, which still holds true today, was initially described by Codman (14) in 1934 based upon the recognition of selective restriction of passive external rotation with pain. The macroscopic and histological features of idiopathic adhesive capsulitis indicate that it is mediated by an inflammatory process (2,15), fibrotic process (16-21), or inflammatory process with subsequent reactive capsular fibrosis (22). However, the underlying pathological processes and molecular pathogenesis remain poorly understood (23).

Due to the lack of understanding of the causes of this disease, current treatment involves mainly relieving symptoms. We conducted a transcriptional analysis of samples from patients with idiopathic adhesive capsulitis and compared them with control healthy samples, in order to gain insight into the molecular mechanisms that contribute to the pathogenesis of this disease. Thus, to test this hypothesis and further understand this disease, we conducted a transcriptional analysis.

Materials and methods

To acquire broader and deeper insights into the mechanisms of idiopathic adhesive capsulitis development, we performed RNA-seq on five idiopathic adhesive capsulitis samples (part

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Abbreviations: DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; BP, biological process

Key words: idiopathic adhesive capsulitis, RNA-Seq, differentially expressed genes, matrix metalloproteinase

of shoulder capsule, subacromial bursa and synovial) and two matched adjacent normal tissues (some part of the shoulder capsule, subacromial bursa and synovial from the acromioclavicular dislocation patients).

RNA-seq and quality analysis of raw data. Each subject signed the informed consent form before participating in our study. This study was approved by the Ethics Committee of The First Affiliated Hospital of Shenzhen University and was conducted in conformity with the guidelines outlined in the Declaration of Helsinki statement. After obtaining the written informed consent, tissue samples for genetic analysis were obtained from the idiopathic adhesive capsulitis patients and control subjects.

The RNA extraction method was followed by the article (24), then the mRNA is enriched using oligo (dT) magnetic beads after incubating total issue samples with DNase I. Then the mRNA was fragmented into short fragments which were then used to synthesize the cDNA by using random hexamer-primer, Buffer, dNTPs, RNase H, and DNA polymerase I. Following cDNA purification, end repair, 3'-end single nucleotide A (adenine) addition, and sequencing adaptors ligation, we performed PCR amplification. RNA sequencing was performed via Illumina HiSeq™ 2000 after the QC step by using Agilent 2100 Bioanalyzer and ABI Step One Plus Real-Time PCR System. Primary sequencing data produced by Illumina HiSeq™ 2000 were subjected to quality control (QC) methods. Before data analysis, we removed the dirty raw reads, which contain adapter sequences, high content of unknown bases, and low quality reads.

Calculation of expression values and identification of differentially expressed genes (DEGs). First, we used Burrows-Wheeler Aligner (BWA) (25) and Bowtie software (v2.3.0) (26) to map clean reads to genome reference. Secondly, we used RSEM (27) to quantify gene expression level followed by FPKM (28) method to calculate expression level. We calculated FPKM value for normalization. The Hg19 version of the human genome reference was used in the present study.

Thirdly, we used Noiseq package method (29) to find differentially expressed genes using the following criteria: Fold change ≥2 and diverge probability ≥0.8. Noiseq is available at http://bioinfo.cipf.es/noiseq or Bioconductor.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Gene Ontology analysis was performed with QuickGO, which is a web-based tool for Gene Ontology annotations. Moreover, we performed the Biological Process (BP), Cell Component (CC) and Molecular Function (MF) enrichment analysis.

QuickGO (30) was used to conduct GO functional annotation and enrichment analysis of DEGs. The enrichment analysis by the hypergeometric test was done to test whether a GO term is statistically enriched for the given set of genes. KEGG (31), the major public pathway-related database, was used to perform pathway enrichment analysis of DEGs.

Protein-protein interaction (PPI) analysis. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) (32) was used to predict protein-protein interactions.

Table I. Summary of the RNA-seq results.

Sample name	Clean reads	Genome map rate	Gene map rate	Expressed gene
Control	12,833,093	0.882	0.8093	17,567
FS2_JAS	12,748,683	0.8865	0.7744	17,605
FS2D_JAS	13,116,962	0.8842	0.737	17,831
F2D	12,073,217	0.8864	0.808	17,826
F3C	12,153,733	0.8691	0.8389	17,651
F4	12,122,251	0.8921	0.7875	17,468
N2Y	12,148,799	0.8712	0.8348	17,679

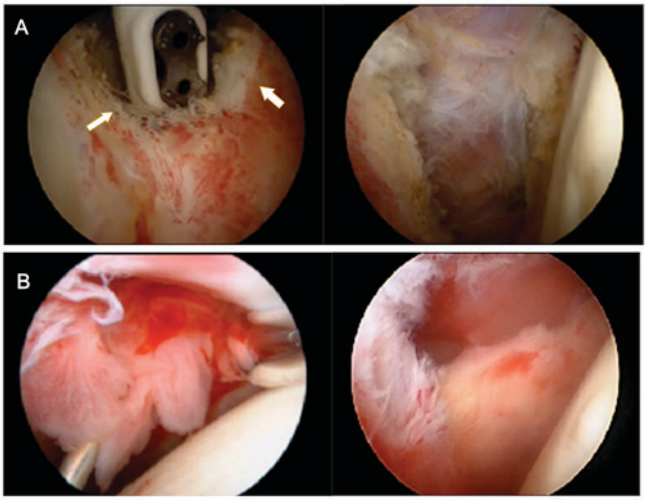

Figure 1. (A) Left: Before arthroscopic release and debridement; right: After arthroscopic release and debridement. (B) Left: synovium is rich in blood vessels; Right: the synovium after arthroscopic debridement.

Cytoscape analysis. Cytoscape is a software for visualizing complex networks and integrating these with any type of attribute data. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) provides protein-protein interactions data and cytoscape visualization.

Results

Identification of DEGs. Our previous clinical work found that patients with idiopathic adhesive capsulitis have a huge number of thick fibrous adhesive bands generated by the proliferation of fibrous connective tissue formed in the shoulder joint capsule (Fig. 1A left, the yellow arrows show the adhesive band being ablated by radio frequency). After arthroscopic release and debridement of these adhesive bands (Fig. 1A right), symptoms of limited mobility and shoulder pain has significantly improved. Our previous clinical work found that patients with idiopathic adhesive capsulitis show increased areas of bright red synovium. The synovium, which is rich in blood vessels, is more fragile and bleeds easier when touched than normal synovium (Fig. 1B left). After arthroscopic debridement of the synovium with blood vessels (Fig. 1B right), the symptoms of shoulder pain are significantly relieved.

Table II. Top 20 DEGs (exclude microRNA and small nucleolar RNA).

Gene ID	Means of control group[a]	Means of case group[b]	log2Ratio (case/control group)	Up-/down-regulation	Probability	Symbol
58	67.798	1.045	-6.01966787	Down	0.95680825	ACTA1
1158	40.484	0.23	-7.45957417	Down	0.95341977	CKM
4632	26.866	0.225	-6.89971273	Down	0.93373733	MYL1
3039	1,486.478	157.21	-3.24113322	Down	0.91165282	HBA1
4151	30.018	1.085	-4.79006091	Down	0.91126009	MB
7138	18.974	0.2	-6.56788004	Down	0.91012755	TNNT1
3040	375.082	41.745	-3.16753072	Down	0.90738297	HBA2
3043	885.424	107.95	-3.03607206	Down	0.90423652	HBB
5346	52.46	4.19	-3.64619566	Down	0.90008996	PLIN1
7125	27.208	1.275	-4.41546176	Down	0.89780662	TNNC2
6029	45.314	352.66	2.960228742	Up	0.89624024	RN7SL1
6288	13.144	0.01	-10.3601887	Down	0.89516251	SAA1
125	36.534	2.465	-3.88958017	Down	0.89502094	ADH1B
4604	14.354	0.125	-6.84338092	Down	0.88850427	MYBPC1
4322	44.41	4.12	-3.43016833	Down	0.8870064	MMP13
8557	23.91	1.22	-4.29266108	Down	0.88602456	TCAP
2354	212.662	30.965	-2.77985191	Down	0.88517059	FOSB
4620	11.978	0.02	-9.22617132	Down	0.88275938	MYH2
729359	38.198	4.09	-3.22332435	Down	0.87474484	PLIN4
4633	17.344	0.695	-4.64127987	Down	0.87408267	MYL2

Gene ID: Identity of gene; [a]expression (FPKM) of control group; [b]mean expression (FPKM) of case; log2Ratio (case/control): Log2 (folds of mean expression in the two groups); Probability: probability of difference; Symbol: Gene symbol.

We generated an average of 12,899,579 clean reads, and 88.17% of the reads mapped to the human genome (Table I). After a series of analyses (see Materials and methods), 188 genes were identified as differentially expressed gene (DEG) compared to control sample according to our criteria (P>0.7, abs (log2 (case/control))>=1) (Table II).

Gene ontology analysis. We analyzed gene ontology using up to 10 significantly enriched terms in BP, CC, and MF categories, respectively. The cut-off of P-value was set to 0.05, and terms under the same category were ordered by P-values. The terms on the left side are more significant. Information on the percentage and number of involved genes/proteins in a term are shown on the left and right y-axis (Fig. 2). There are 1,802 biological processes (BPs) that are statistically significant among the whole enriched dataset of 3,309 BPs (Fig. 3). There are 318 cell components (CCs) enriched for this dataset and 120 of those are statistically significant (Fig. 4). There are 443 molecular functions (MFs) were enriched for this dataset and among that, 178 are statistically significant (Fig. 5).

Pathway enrichment analysis of DEGs. The 188 differentially expressed genes were found to be involved in 143 KEGG terms, such as PPAR signaling pathway, rheumatoid arthritis, osteoclast differentiation, regulation of lipolysis in adipocytes, p53 signaling pathway, and so on (Table III).

Protein-protein interaction (PPI) analysis. Protein-protein interaction analysis by STRING database and cytoscape web application provided 4 levels of functional analysis: Fold change of gene/protein, protein-protein interaction, KEGG pathway enrichment, and biological process enrichment (Fig. 6).

Discussion

Idiopathic adhesive capsulitis is a common disease of unclear cause and significant morbidity, which can last up to 2 years and longer. The diagnosis is still based upon the recognition of the characteristic features initially described by Codman (14) in 1934. The macroscopic and histological features of the idiopathic adhesive capsulitis have been described, but the underlying pathological processes and molecular pathogenesis remain poorly understood. Therefore, the identification and functional analysis of specific expression genes involved in idiopathic adhesive capsulitis are necessary to elucidate the disease molecular pathogenesis and the strategies of precision medicine in idiopathic adhesive capsulitis.

The use of RNA-seq to assess the level of gene expression of idiopathic adhesive capsulitis is novel in the field of idiopathic adhesive capsulitis research. Cohen *et al* (33) observed that the synovium/capsule samples from the patients with adhesive capsulitis had significantly higher TNC and FN1 expression than those from the controls. They targeted the following proteins; TGFβ1, TGFβR1, LOX, PLOD1, PLOD2,

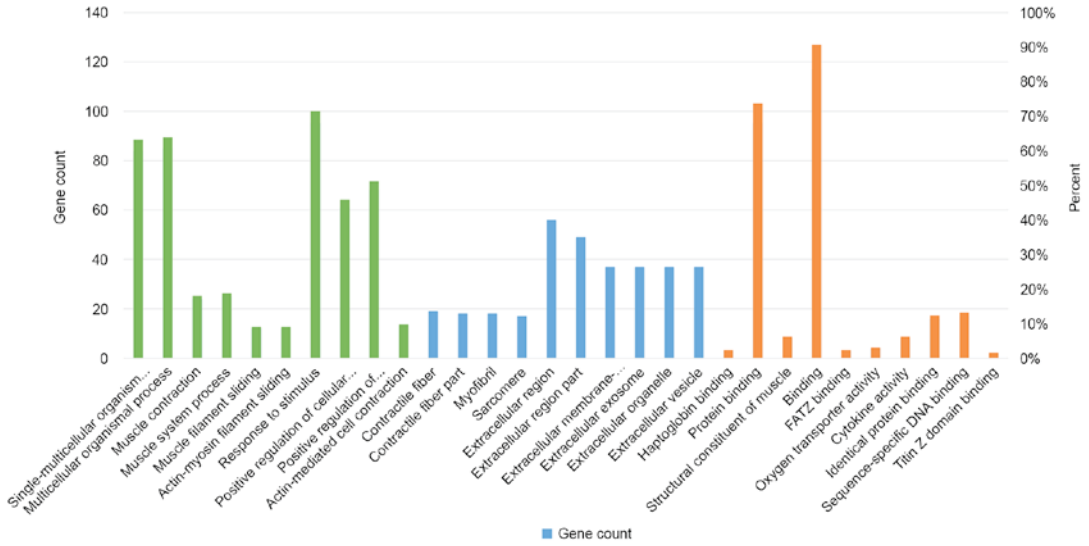

Figure 2. An overview of the gene ontology analysis with up to 10 significantly enriched terms in BP, CC, MF categories, respectively. The cut-off of P-value is set to 0.05, Terms of same category are ordered by P-values. Left terms are more significant. Information of percentage and number of involved genes/proteins in a term are shown on the left and right y-axis.

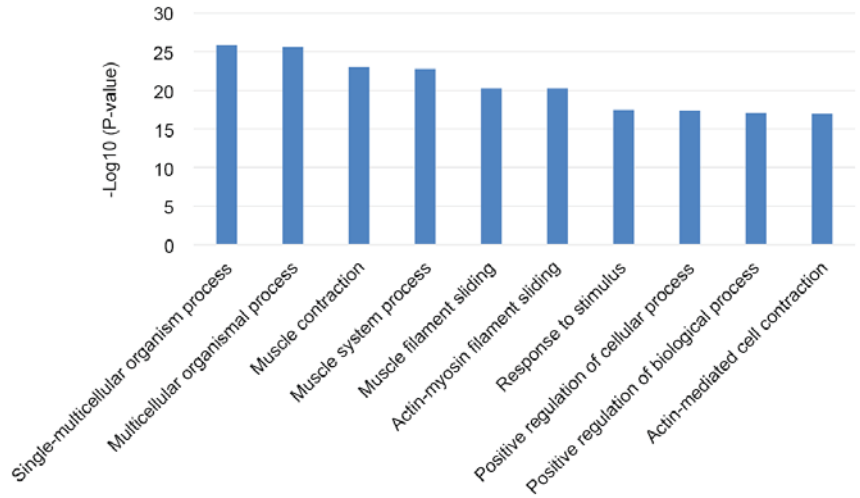

Figure 3. Significantly enriched biological processes.

COMP, FN1, TNC, TNXB, B2 M and HPRT1 (34). We did not find these genes elevated or changed in our RNA-seq study.

In the present study, we identified a total of 188 genes to be differentially expressed. These code for proteins of the matrix metalloproteinase (MMP) family (MMP-9, MMP19, ADAMTS), serum amyloid A1 (SAA1), glutathione S-transferase θ 1 (GSTT1), myosin heavy chain family (MYH1, MYH2), amphiregulin (AREG), major histocompatibility complex (HLA-DRB4), interleukin 6 (IL6), and CD248 molecules.

Matrix turnover is a dynamic equilibrium between synthesis and degradation and controlled by Matrix metalloproteinases and other related proteins. Disruption of this equilibrium may lead to fibrosis (35). The using of MMP inhibitors in clinical trials, reported to be associated with idiopathic adhesive capsulitis, rapidly resolved after cessation of therapy (36). Johnston *et al* (37) found that the level of MMP19 in the Dupuytren's nodule is increased compared to cord, while the level of ADAMTS is decreased. The dynamic equilibrium turnover of extracellular matrix can be catalyzed by matrix metalloproteinases and other related enzymes at neutral pH (38). A previous study showed that the levels of MMP-8 and -9 in the systemic circulation are representative of the levels of these enzymes in the inflamed joint, and suggested that MMP-9 and MMP-1 may be involved in degradation of the joint collagen (39).

The *IL6* gene encodes a cytokine that acts as a mediator in inflammatory and immune responses. The SAA1 gene encodes a member of the serum amyloid family of apolipoproteins. These differentially expressed genes are reported to be associated with chronic inflammatory diseases such as atherosclerosis and rheumatoid arthritis, which suggest that idiopathic adhesive capsulitis is an inflammatory condition. These findings are similar to those of Kabbabe *et al* (40) and Asleh *et al* (41).

The GO enrichment analysis revealed that DEGs are enriched for a total of 3309 BP terms, 318 CC terms, and 443

Table III. Top10 significantly pathway enrichment.

Pathway ID	Pathway name	P-value	Genes count
hsa03320	PPAR signaling pathway	5.27E-08	9
hsa05219	Bladder cancer	6.59E-05	5
hsa05144	Malaria	0.000157	5
hsa05323	Rheumatoid arthritis	0.000384	6
hsa05143	African trypanosomiasis	0.00048	4
hsa04380	Osteoclast differentiation	0.00268	6
hsa04923	Regulation of lipolysis in adipocytes	0.00285	4
hsa04710	Circadian rhythm	0.00419	3
hsa04145	Phagosome	0.00589	6
hsa04115	p53 signaling pathway	0.00604	4

Regulation of lipolysis in adipocytes pathway (hsa04923).

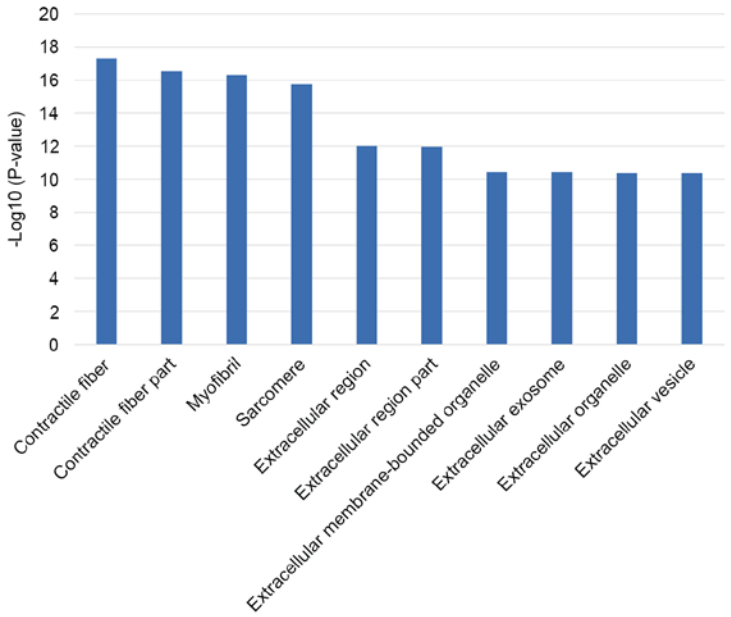

Figure 4. Significantly enriched cell components.

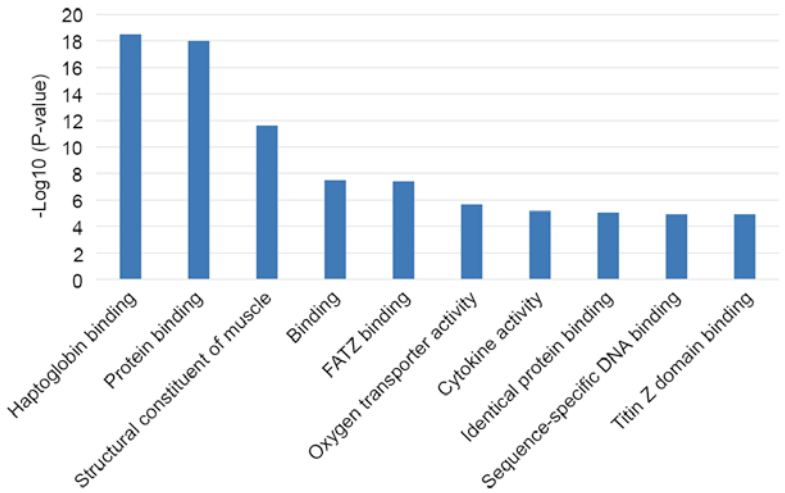

Figure 5. Significantly enriched molecular functions.

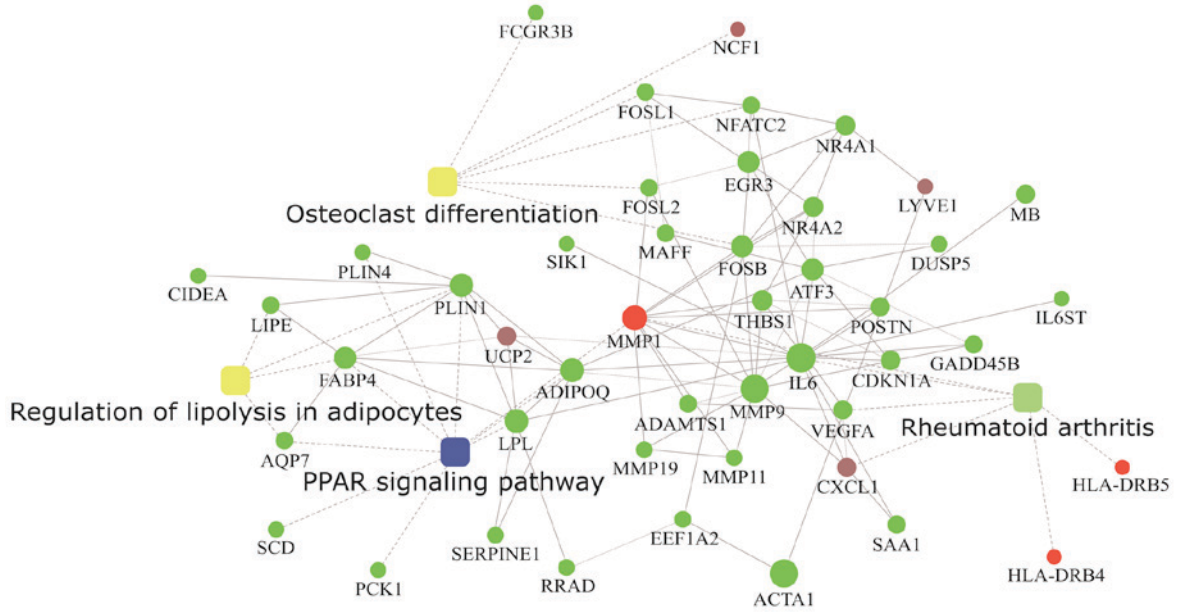

Figure 6. Genes/proteins are represented by circle nodes, and KEGG pathways or biological processes are represented by rectangles. Pathways are presented in gradient color from yellow to blue, with yellow for smaller P-values and blue for bigger P-values. Biological processes are presented in red. In case of fold change analysis, genes/proteins are presented in red (upregulation) or in green (down-regulation). A default confidence cutoff of 400 was used: interactions with bigger confident score are shown as solid lines between genes/proteins (otherwise in dashed lines).

MF terms. Among these, 1802 BPs, 120 CCs, and 178 MFs are statistically significant. The top 10 BPs mainly referred to various processes including those related to to the muscular system and actin-mediated cell contraction. The top 10 CCs are mostly located in the extracellular region. The top 10 MFs mainly are related to protein binding, cytokine activity, and oxygen transporter activity.

The KEGG signaling pathway analysis showed that the DEGs are possibly involved in 179 pathways including pathways related PPAR signaling, regulation of lipolysis in adipocytes, circadian rhythm, Phagosomes, p53 signaling, malaria, bladder cancer, rheumatoid arthritis, African trypanosomiasis, and osteoclast differentiation.

PPAR signaling pathway plays an important role not only in the regulation of lipid and carbohydrate metabolism but also in many signaling pathways (immunity, inflammation, apoptosis and cell differentiation) (42,43). Many studies have found that PPAR signaling pathway is involved in many diseases related to prolonged nutrient excess such as type II diabetes, hyperlipoproteinemia, and hyperalphalipoproteinemia (44). Growing evidence suggests that idiopathic adhesive capsulitis is associated with glucose and lipid metabolic diseases, but not much is known beyond this correlation. Our study found that PPAR signaling pathway and fatty acid degradation might play a significant role in the pathogenesis of idiopathic adhesive capsulitis.

Osteoclasts are responsible for bone resorption. *NFATC2*, *FOSB*, *FOSL2*, *FOSL1*, and *FCGR3B* are expressed at higher levels in disease samples than in control samples. This suggests that bone resorption is enhanced in idiopathic adhesive capsulitis. This is consistent with some studies (45-47) looking at bone mineral density of the shoulder joint in idiopathic adhesive capsulitis. Waldburger *et al* (48) obtained satisfactory results after treatment with calcitonin to increase bone mass.

We also performed protein-protein interaction analysis with PPAR signaling pathway, cytokine-cytokine receptor interaction, rheumatoid arthritis and osteoclast differentiation (Fig. 5) and found that MMP-9 seems to be a node that directly or indirectly connects these pathways. The down regulation of MMP-9, which is a protein involved in the degradation of extracellular matrix collagen, leads to degeneration of collagen accumulation, which can facilitate development of idiopathic adhesive capsulitis. Further investigations are necessary to validate the molecular mechanism (s) underlying the development of idiopathic adhesive capsulitis and its link to glucose or lipid metabolism disorders.

Meanwhile, in the present study, the differentially expressed genes were found to be involved in the regulation of lipolysis in adipocytes. Currently, some articles reported that idiopathic adhesive capsulitis is significantly correlated with diabetes mellitus (49) and hyperlipidaemia (50). A nationwide population-based cohort study (49) found that hyperlipidemia is an independent risk factor for idiopathic adhesive capsulitis. In addition, hyperlipidemia can have cumulative detrimental effects to tendon properties. For example, some studies have revealed that the risk of rotator cuff disease is increased in patients with hypercholesterolemia (51) and can eventually lead to secondary idiopathic adhesive capsulitis (52). Moreover, patients taking hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have an increased risk of shoulder stiffness (53) that may predispose these patients to idiopathic adhesive capsulitis. Proteins such as adiponectin, leptin, resistin, and adipokines, which are normally involved in metabolism, have recently been implicated in the development of idiopathic adhesive capsulitis, as reviewed by Gómez *et al* (54) and Schäffler *et al* (55). Leptin has been shown to have proinflammatory and catabolic roles in OA (54,56,57). Thus, idiopathic adhesive capsulitis may correlate with the metabolism in adipose tissue. Adiponectin

in human synovial fibroblasts appear to act as a mediator of arthritis pathophysiology. Based on these observations, we presume that proliferation of synovium and fibrosis of shoulder capsule is because of the metabolic abnormalities in lipids.

In conclusion, this is a novel study investigating the transcriptome of idiopathic adhesive capsulitis. The data have provided important insights into the transcriptional regulation of gene expression. We found 24 genes to be downregulated and 147 genes to be up-regulated in disease tissues vs. controls, and this finding may be used to identify therapeutic targets. However, it is still necessary to validate the DEGs identified in this study in large patient populations and elucidate their specific functions in the pathogenesis of idiopathic adhesive capsulitis.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81672234); the Guangdong Provincial Science and Technology Department (grant no. 2015A020212001) and the Shenzhen Science Technology Innovation Council (grant nos. GCZX2015043017241191 and JCYJ20160226192924528).

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