Differential co-expression analysis of rheumatoid arthritis with microarray data

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Abstract. The aim of the present study was to investigate the underlying molecular mechanisms of rheumatoid arthritis (RA) using microarray expression profiles from osteoarthritis and RA patients, to improve diagnosis and treatment strategies for the condition. The gene expression profile of GSE27390 was downloaded from Gene Expression Omnibus, including 19 samples from patients with RA (n=9) or osteoarthritis (n=10). Firstly, the differentially expressed genes (DEGs) were obtained with the thresholds of |logFC|>1.0 and P<0.05, using the t-test method in LIMMA package. Then, differentially co-expressed genes (DCGs) and differentially co-expressed links (DCLs) were screened with q<0.25 by the differential coexpression analysis and differential regulation analysis of gene expression microarray data package. Secondly, pathway enrichment analysis for DCGs was performed by the Database for Annotation, Visualization and Integrated Discovery and the DCLs associated with RA were selected by comparing the obtained DCLs with known transcription factor (TF)-targets in the TRANSFAC database. Finally, the obtained TFs were mapped to the known TF-targets to construct the network using cytoscape software. A total of 1755 DEGs, 457 DCGs and 101988 DCLs were achieved and there were 20 TFs in the obtained six TF-target relations (STAT3-TNF, PBX1-PLAU, SOCS3-STAT3, GATA1-ETS2, ETS1-ICAM4 and CEBPE-GATA1) and 457 DCGs. A number of TF-target relations in the constructed network were not within DCLs when the TF and target gene were DCGs. The identified TFs may have an important role in the pathogenesis of RA and have the potential to be used as biomarkers for the development of novel diagnostic and therapeutic strategies for RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic destructive disease (1) affecting ~1% of the population worldwide, most commonly middle-aged females (2). RA is characterized by chronic inflammation of the synovium, particularly of the small joints, which commonly leads to destruction of articular cartilage and juxta-articular bone. It is often accompanied with systemic manifestations, including anemia, fatigue and osteoporosis. RA has a severe impact on quality of life and so the improvement in early diagnosis and effective therapies is urgently required.

With the development of genomics, the understanding of susceptibility and severity of RA has rapidly advanced, and numerous key RA-associated genes and metabolic pathways, which may have important roles in the pathogenesis and progression of RA, have been described. For example, the toll-like receptor (TLR) signaling pathway has been identified to contribute to the pathogenesis of RA (3). TLRs are a family of type I integral membrane glycoprotein pattern recognition receptors, which are well known for their role in the recognition of microbial ligands and in the development of adaptive immunity (4). TLRs are also important in the persistence of RA and the destruction of the joint by the chronic expression of pro-inflammatory cytokines and chemokines, including tumor necrosis factor-α, interleukin (IL)-1β, IL-6 and IL-8. In RA, TLRs are important for the generation of adaptive immunity, including the activation of T and B cells. However, instead of the normal dampening of the immune response (5) and the downregulation of innate immunity in patients with RA, there is the persistent pathogenic expression of inflammatory cytokines, including TNF-α, IL-1β and IL-6, each of which have been targeted successfully in patients with RA. Increasing evidence has demonstrated the role of TLRs in the persistent, progressive activation of macrophages that appears to drive this destructive inflammatory process (6). In addition, the p53 signaling pathway, another RA-associated metabolic pathway, has also been attracting significant attention. It is reported that overexpression and functional mutations of the tumor suppressor p53 protein have been demonstrated in RA synovial tissue, most extensively in patients with advanced destructive type disease (7). Recent studies suggest that p53 induction is a general phenomenon in inflammation, directed at modulating normal inflammatory responses (8). A higher

p53 expression has been demonstrated in synovial tissue from patients with destructive RA disease and the association between p53 expression and joint damage have also been examined (9). Numerous studies of p53 mutations and protein expression in RA and other inflammatory disorders have consistently demonstrated an association between p53 expression and joint damage (10-12). Other signaling pathways involved in RA, including the NOD-like receptor signaling pathway (10), the T cell receptor signaling pathway (11) and the Wnt signaling pathway (12) have also been investigated. A number of other factors, such as inflammatory cytokines (for example IL-6 and IL-1) (13), the human leucocyte antigen (14) and DNA methylation (15) have been identified to affect the development of RA and may be used as diagnostic markers for RA. Although the understanding of the mechanisms of RA has improved, further studies are required to further elucidate the pathogenesis of RA comprehensively, to facilitate the development of novel diagnostic markers and effective treatments.

At present, numerous conventional methods exist for the analysis of gene expression data, including contrasting of differentially expressed genes and clustering. However, an increasing number of studies suggest that to reflect the interaction between genes, only searching for genes with significantly differential expression levels at different conditions lacks sufficient accuracy. Rather, the identification of a set of genes with the same expression profiles that functionally interact in different states, may better reflect the interaction between genes under different conditions, and this set of genes are defined as differentially co-expressed genes (16,17). The aim of the present study was to analyze the differentially co-expressed genes (DCGs) and differentially co-expressed links (DCLs) of RA and osteoarthritis based on microarray expression data. The results may improve the understanding of the underlying molecular mechanisms of RA and facilitate the development of novel approaches for improving the diagnosis or treatment of RA.

Materials and Methods

Gene expression data of RA. Firstly, GSE27390 (18) chip expression data were selected from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), and samples in the chip GPL570 (HG-U133_Plus_2) Affymetrix Human Genome U133 Plus 2.0 Array platform (Affymetrix, Inc., Santa Clara, CA, USA) were selected for analysis. There were a total of 19 samples under this platform, among which 10 samples were from patients with RA, while the other nine samples were from patients with osteoarthritis. Following this, the sample data and platform annotation files were downloaded.

Analysis of differential co-expression. The chip data obtained were standardized by the robust multi-array average method (19) using the Affy package of R software (www.r-project.org). Then, the samples were divided into two groups (RA vs. osteoarthritis) and the t-test method in the LIMMA package (a set of tools for background correction and scaling) of R software (20) was used to calculate the differentially expressed genes (DEGs), with threshold of $|logFC|>1.0$ and a P-value <0.05. Finally, the DCGs and DCLs were calculated with the cutoff criterion of $q<0.25$ using the functions of DCe, DCp and DCsum in the differential coexpression analysis and differential regulation analysis of gene expression microarray data (DCGL) package (21,22). The DCGL package included four modules: Gene screening, relations screening, analysis of differential co-expression and differential regulation analysis.

Functional enrichment analysis of DCGs. As the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (23) is a relatively common and comprehensive database that contains a variety of biochemical pathways, it was also selected to continue the enrichment analysis of the DCGs screened during the analysis of RA microarray data. This database was utilized to identify which biochemical pathways, with their associated biological functions, the co-expressed genes are more likely to affect during the pathological development of RA. The online tool, the Database for Annotation, Visualization and Integrated Discovery (DAVID) (24) was used to conduct pathway enrichment analysis for DCGs.

Transcriptional regulation correlation. TRANSFAC (25) is a database associated with transcription factors and their genomic binding sites on DNA-binding profiles. It is composed of SITE, GENE, FACTOR, CLASS, MATRIX, CELLS, METHOD and REFERENCE, etc. All associations between human transcription factors and target genes in TRANSFAC were downloaded and compiled, which contained a total of 298 transcription factors (TF) and 6458 associations.

Construction of RA-related transcription regulation network. The obtained differential co-expression correlations were mapped to the associations between human TFs and their target genes, and the TFs were corresponded to the already known target genes. Then the transcription regulation associations of DCGs were obtained. Finally, cytoscape was used for network description (26).

Results

Differential co-expression analysis. Affy and LIMMA packages in R software were used to calculate the chip data and 1,858 DEGs were obtained with the cut-off criteria of $|logFC|>1.0$ and a P-value <0.05. The DCGL method in R software was used to perform differential co-expression analysis of the expression data of DEGs in RA to obtain a P-value and q value corresponding to each gene, and the DCLs between genes. Following selection of $q<0.25$ as the threshold for screening DCGs and DCLs, 457 DCGs and 101,988 DCLs with significant correlations were obtained.

Biological pathways closely associated with RA. Following identification of the DCGs, the online tool, DAVID, was used to perform KEGG pathway significant enrichment analysis of 457 DCGs associated with RA and the ten metabolic pathways with the smallest P-values were selected, as revealed in Fig. 1. From this, it was identified that the major metabolic pathways of the DCGs were associated with the following mechanisms: TLR signaling (27,28), graft-versus-host disease (29), the NOD-like receptor signaling (30), cytokine-

Table I. The known transcriptional regulation associations contained in DCLs.

TF	Target	cor. 1	cor. 2	Type	cor. diff
STAT3	TNF	0.014514	0.723764	same signed	0.709251
PBX1	PLAU	-0.74973	-0.13143	same signed	0.618294
SOCS3	STAT3	0.033164	0.628008	same signed	0.594844
GATA1	ETS2	-0.114	-0.66228	same signed	0.548279
ETS1	ICAM4	-0.53926	-0.04391	same signed	0.495352
CEBPE	GATA1	-0.57329	-0.09175	same signed	0.481536

cor. 1 and cor. 2 represent the maximum absolute correlation coefficient of TF and the gene, respectively. Type refers to the type of association between the TF and target gene. cor.diff means the absolute difference between cor.1 and cor.2. TF, transcription factor; target, target gene; DCLS, differentially co-expressed links.

Table II. Text mining results of TFs.

	TF											
	IL-6	STAT3	FOS	STAT1	RUNX1	EGR1	ERG	ETS1	JUNB	PRDM1	EGR2	ETS2
Count	211	132	131	73	19	18	16	8	7	7	3	2

TF name refers to the name of the transcription factor in differentially co-expressed genes and the count refers to the number of literatures on TF and RA. TF, transcription factor; RA, rheumatoid arthiritis.

cytokine receptor interaction, T cell receptor signaling, p53 signaling, RIG-I-like receptor signaling, Jak-STAT signaling and chemokine signaling.

Analysis of transcriptional regulation. The 101,988 pairs of DCLs were compared with the known links between TFs and target genes. Six links between TFs and target genes that are known to be involved in RA were obtained, including STAT3-TNF, PBX1-PLAU, SOCS3-STAT3, GATA1-ETS2, ETS1-ICAM4 and CEBPE-GATA1, as summarized in Table I. These six associations and 457 DCGs contained 20 TFs, including CEBPE, EGR1, EGR2, EGR3, ERG, ETS1, ETS2, FOS, HOXA5, IKZF1, IL6, JUN, JUNB, KLF9, PLAU, PRDM1, RUNX1, STAT1, PBX1 and STAT3. Using text mining methods, the literature on these 20 TFs associated with RA were investigated and the results are summarized in Table II. Then, the 20 TFs were mapped to the known TF-target regulatory associations and 233 regulatory associations were achieved. Cytoscape software was utilized to describe the associations observed, including those between the 20 transcription factors and 572 target genes (Fig. 2). In addition, a number of TF-target associations in the constructed network were not within DCLs when TFs and target genes were DCGs, which indicated that the TFs and target genes may have acted synergistically with other factors (Table III).

Table III. TFs and target genes in DCGs.

TF	Target gene
EGR1	FGFR3, FGFR3, IFNG, TNG, PLAU
ETS1	OAS2, SPRY2, CCL3, SOCS1, MDM2, IFNG, TNG, PLAU, OAS2
ETS2	GBA, CD163, MDM2, ERG, JUNB, PLAU
FOS	PLAU, CCL4, IFNG
IL6	PLAU
JUN	TNF, PLAU
JUNB	PLAU
STAT1	PLAU, GBP1, PSMB9, ISG15, IFI6, IRF7, TAP1, SOCS3, IFNG

TF, transcription factor; DCGs, differentially co-expressed genes.

Discussion

RA is a disease characterized by chronic inflammatory processes that targets the synovial lining of diarthrodial joints. Investigating the mechanisms underlying RA development has been the focus of numerous studies, in an attempt to improve the disabling impact RA has on quality of life. In the present study, a DCGL method was utilized to perform a differential co-expression analysis of microarray data of RA and a total of 457 DCGs and 101,988 DCLs were obtained. From significant enrichment analysis of the KEGG pathway for DCGs, it was identified that the major metabolic pathways associated with these DCGs were as follows: The TLR signaling pathway, graft-versus-host disease, the NOD-like receptor signaling pathway, the cytokine-cytokine receptor interaction, the T cell receptor signaling pathway, the p53

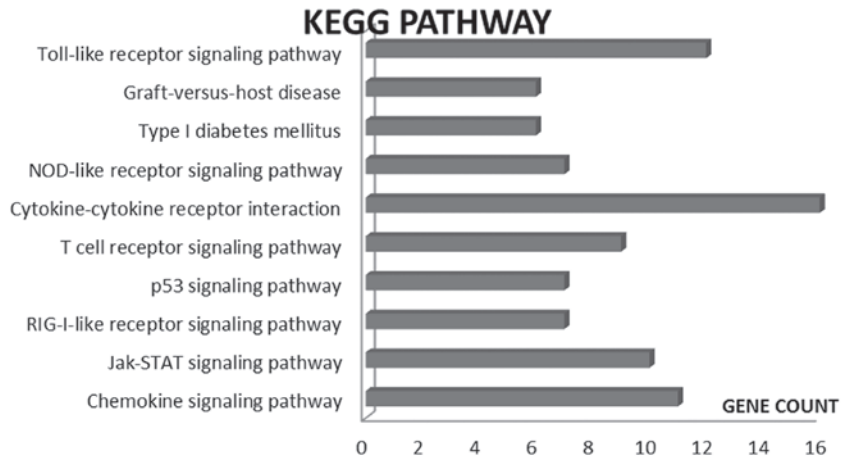

Figure 1. Significant metabolic pathways of differentially co-expressed genes. Abscissa represents the number of genes enriched, the vertical axis represents the name of the pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes.

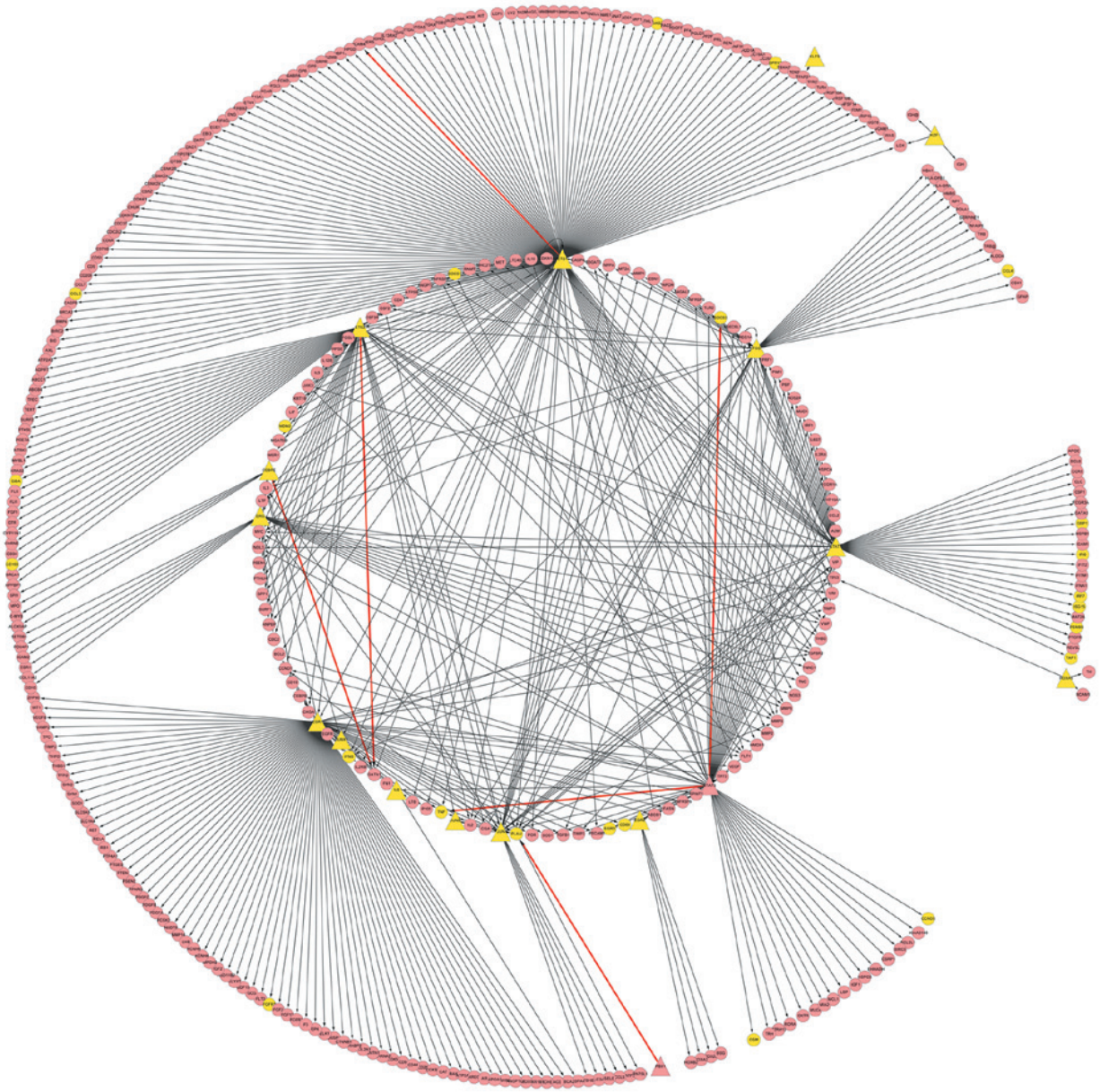

Figure 2. Transcriptional regulatory network diagram. The diamonds represent TFs, circles represent target genes, yellow dots represent the differentially co-expressed genes, pink dots represent the non-differentially co-expressed genes, red lines represent the six associations contained in differentially co-expressed links and the black edges represent the known transcription regulation associations. TF, transcription factor; DEGs, differentially co-expressed genes.

signaling pathway, the RIG-I-like receptor signaling pathway, the Jak-STAT signaling pathway and the chemokine signaling pathway. The six TF-target associations included STAT3-TNF, PBX1-PLAU, SOCS3-STAT3, GATA1-ETS2, ETS1-ICAM4 and CEBPE-GATA1. Among which, the focus of the present study was SOCS.

SOCS family proteins consists of SOCS1-SOCS7 and CIS1, with SOCS1, SOCS2, SOCS3 and CIS1 being the best-characterized members of the family. Once expressed, they inhibit the JAK-STAT pathway by several mechanisms, including the suppression of JAK catalytic activity and prevention of STAT recruitment to activated cytokine receptors. Evidence has revealed that SOCS proteins are important regulators of immune and inflammatory responses *in vivo* (31,32). SOCS proteins also appear to regulate the development and progression of arthritis. Egan *et al* (33) demonstrated increased arthritis severity in mice lacking SOCS1 and interferon-γ, and STAT1 gene-knockout mice demonstrated exacerbated zymosan-induced arthritis, possibly due to a reduction in SOCS1 (34). Furthermore, overexpression of SOCS has inhibitory effects on arthritis, as demonstrated by the ability of adenoviral-mediated induction of SOCS3 to markedly reduce the severity of collagen-induced arthritis (35). Isomäki *et al* (35) demonstrated increased SOCS3 mRNA expression in synovial tissues from patients with RA when compared with those with osteoarthritis, which indicates SOCS3 is generally upregulated in RA. Furthermore, in patients with long-standing RA, increased levels of SOCS1 and SOCS3 may suppress cytokine signaling and potentially prevent the deleterious actions of pro-inflammatory cytokines. In general, upregulation of SOCS expression may significantly affect cellular responsiveness to cytokines in chronic RA and may be involved in disease progression by inducing unresponsiveness to anti-inflammatory cytokines.

It was observed that the target gene of SOC3 was STAT3, however STAT3 was also a TF in another TF-target association, and its target gene was TNF. STAT3 has been demonstrated to have an important role in cellular proliferation and anti-apoptotic mechanisms (36). Evidence reveals that hyperactivation of STAT3 may be involved in proliferation and/or prevent apoptosis of RA synoviocytes. In addition, STAT3 activation is identified exclusively in synovial tissue from RA, but not osteoarthritis patients. Therefore, STAT3 may have an important role in the mechanisms associated with RA. Furthermore, although the cause of RA remains unclear, it has been suggested that cytokines, particularly pro-inflammatory cytokines, including TNF-α, IL-1 and IL-6 derived from activated synovial cells, are important in the pathology of the disease (37). Among these cytokines, TNF-α has been the most extensively investigated as a target in the treatment of RA. TNF is the target gene of STAT3, which further confirms the role of STAT3-TNF in RA. Several studies have demonstrated that anti-TNF-α mAbs markedly ameliorate joint involvement in the majority of patients with RA (38,39). It is also of note that IL-6, a major target gene of TNF-α, has been proposed to contribute to the development of arthritis, and may induce proliferation of synovial fibroblastic cells (40) and the formation of osteoclasts in the presence of soluble IL-6 receptors (41).

In conclusion, the six TF-target associations in DCLs and the obtained TFs may have important roles in the pathogenesis of RA. However, the number of samples in the present study were limited. Therefore, whether the obtained TFs may be used as biomarkers and whether they are a cause or consequence of the disease remains to be elucidated in a larger prospective study.

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