Int J Hum Genet, 18(2): 87-94 (2018)
DOI: 10.31901/24566330.2018/18.2.691

Integrated Analyses of Gene Profile Digs out Suitable Biomarkers for Ankylosing Spondylitis using Three Different Methods

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KEYWORDS Combining p-values. Convergent Evidence. Hub Genes. Mutual Information Network. Rank Product

ABSTRACT In the current study, the researchers aimed to extract suitable genes which contributed to ankylosing spondylitis (AS) using three popular meta-analysis approaches (combining p-values, convergent evidence, and rank product). Candidate genes across multiple methods were respectively identified. The common genes between any two methods were extracted as the optimal genes, following by functional enrichment analysis for the optimal genes. Context likelihood of relatedness algorithm was utilized to establish mutual information network (MIN) using the optimal genes. Simultaneously, classification ability analysis for hub genes was implemented. A MIN (93 genes and 2212 interactions) was constructed, and 3 hub genes (TALDO1, LCP1, and RPS27A) were identified. The classification analysis showed that hub genes separated AS from controls with the high accuracy, sensitivity, and specificity of 0.962, 0.937, and 0.953, respectively. Hub genes LCP1 and RPS27A might be used as genetic biomarkers for AS diagnosis and treatment.

INTRODUCTION

Ankylosing spondylitis (AS), a long term inflammation arthritis attacking sacroiliac joints as well as spine (Taurog et al. 2016). Back pain is a characteristic symptom of AS (Arthritis and Diseases 2013). Worriedly, AS can induce structural and functional impairments and reduce patients' quality of life (Braun and Sieper 2007). Though the cause of AS is unclear, it is believed that there is a close link between genetic factors and AS. For example, 30 years ago, the strong association between HLA-B27 and susceptibility to AS has been demonstrated, however, HLA-B27 accounts for about 5 percent of the genetic risk of AS (Reveille 2006). Non-HLA-B27 genetic factors might also exert important functions in the progression of AS. Recently, genome-wide association studies extracted several non-HLA genes, for example, IL23R, ARTS1 and ERAP1 in AS (Brionez and Reveille 2008; Brown 2009). Nevertheless, the current understanding of the pathogenesis of this disease remains still poor. Thus, there is an urgent need to explore the molecular mechanisms of AS.

DNA microarray is a powerful technology for monitoring the expression level of thousands of genes simultaneously, which provides the foundation for widespread applications such as disease classification, pathway modeling, as well as functional genomics. One of the common challenges of high-throughput technologies across-omics fields is that they generate a larger pool of candidates, yet only very few of these genes are of high relevance to the disease. Thus, gene prioritization is necessary aiming to explore the most promising genes among long lists of genes through integrative computational analysis of public genomic data (Moreau and Tranchevent 2012). Fortunately, several computational methods have been created to prioritize highly relevant candidate genes (Moreau and Tranchevent 2012). Furthermore, integrating gene-level data from multiple evidence layers has been demonstrated to be effective in extracting and prioritizing candidate genes of complex genetic traits (M et al. 2012). However, most of the existing meta-analysis methods have been exclusively developed to

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integrate microarray expression data, but do not serve the purpose of integrating gene-level data from multiple study-types. Thus, in the current study, the researchers implemented three methods including combining p-values (CP), convergent evidence (CE), and rank product (RP) method, to integrate gene-level data generated from multiple lines of evidences to identify the suitable genes for AS. In the section of Material and Methods, details and citations of these three methods are provided.

Objectives

In the current study, the researchers attempted to screen out the suitable genes which contributed to AS based on three popular meta-analysis approaches (CE, CP, RP). AS-related microarray expression profile was firstly derived from Gene Expression Omnibus (GEO) database. Then, candidate genes across multiple methods were respectively identified based on significance statistic or genes rank. Next, the common genes between any two methods were extracted and named as the optimal genes, following by functional enrichment analysis for the optimal genes. Afterwards, context likelihood of relatedness (CLR) algorithm was utilized to establish the mutual information network (MIN) based on the optimal genes. Simultaneously, hub genes were extracted from the MIN. Support vector machines (SVM) was utilized to measure the classification ability of hub genes. Results from the present study will provide the groundwork for the understanding of AS pathogenesis.

METHODOLOGY

Affymetrix Chip Data

AS-related microarray data (accession NO. GSE25101) were obtained from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) at National Center of Biotechnology Information (NCBI) (Barrett et al. 2007). The platform was GPL6947 (Illumina Human HT-12 whole-genome expression BeadChips). In GSE25101, 32 chips were available for subsequent analysis, including 16 active AS patients and 16 gender-and age-matched controls. The raw data and probe annotation files were downloaded for following analysis.

Preliminary Preprocessing

Before analysis, the raw expression data were carried on preprocessing. In this study, with an attempt to eliminate the effect of nonspecific hybridization, Affy package was used (Gautier et al. 2004). Next, background correction was done using robust multi-array average (RMA) method (Irizarry et al. 2003), and then normalization was implemented to obtain unbiased data (Bolstad et al. 2003). Subsequently, perfect match and mismatch correction was conducted based on Micro Array Suite 5.0 (MAS 5.0) algorithm (Hubbell et al. 2002). Probe sets matching either none were filtered, and if there were several probes matching to one gene, the mean value was utilized to represent the expression level of this gene. After probes were aligned to gene symbols, overall 11,587 genes were remained after preprocessing.

Analytical Techniques

In recent years, integrated analysis was utilized to combine information of multiple different analysis methods to extract significant candidate genes associated with diseases. In this study, three popular analysis methods, including CP, CE, and RP, were utilized in the evaluation and comparison. Below, these 3 methods that were selected for comparison were briefly described.

CP Method

CP is one of the traditional methods of meta-analysis. To combine p-values of a gene from multiple evidence layers, the p-values should have been estimated from the same null hypothesis. Popular approaches to combine p-values cover Fisher's and Stouffer's methods. In this study, the Fisher's exact test was used to extract the significant genes between AS and control groups. The raw P-values were corrected based on false discovery rate (FDR) using Benjamini and Hochberg method (Benjamini et al. 2001). Then, genes were ranked in ascending order according to the FDR values, and a FDR-value < 0.05 was chosen as the cutoff criteria.

CE Method

CE method is a modified form of PageRank algorithm (Page 1998). Previously, this variant of PageRank algorithm has been used to sort

genes in microarray-based gene expression experiments (Morrison et al. 2005). A conceptually similar gene-level integration has been successfully adopted to prioritize candidate genes in sychiatric diseases (M et al. 2012). Herein, the researchers used this modified PageRank algorithm to calculate the CE scores to further identify the optimal genes using the following formula:

$$CE(G)=CE(L_1)/n\ (L_1)+....CE(L_n)/n(L_n)$$

Where, $CE\ (L_i)$ denoted the expression values of evidence layer-*i* and $n(L_i)$ stood for the number of genes in evidence layer-*i*.

CE approach does not incorporate significance statistic but ranking genes. Based on the CE scores, genes were ranked in descending order, and the top 100 genes with higher CE scores were identified.

RP Method

RP is a non-parametric analysis method developed by Breitling et al. (Breitling et al. 2004), which is used to provide reliable significance thresholds to identify significant genes. In detail, for each gene *g* in *k* replicates *i*, each examining n_i genes, researchers can calculate the corresponding combined probability as a RP. In the current study, the genes were compared and sorted using RP method. Next, the ranks were aggregated to an overall value for all replications, and then a ranked gene list was obtained. Of note, genes with the smallest RP values were the most significant candidate genes and the biologist can then select some of them for further study. Thus, in this study, genes were sorted in ascending order based on RP scores, and the top 100 genes were identified.

The RP value was calculated based on the following equation:

$$RP\ (g) = \left(\Sigma_{i=1}^{k}\ r_{g,i}\right)$$

In this formula, $r_{g,i}$ the position of gene *g* in the list of genes in the i^{th} replicate sorted by fold change.

Prioritization of Candidate Genes

With the goal of better understanding the differences of genes in any two methods, gene compositions identified by each method were analyzed and compared using R/Bioconductor package GenRank. Specifically, to avoid the potential bias due to duplicated genes, duplicated genes were calculated only once (as a single vote) in each evidence layer in all the three methods. When reserving duplicated genes, those genes with significant test statistic (for example, smaller p-values or higher effect-size) were retained. Then, the common genes between any two methods were extracted and named as the optimal genes.

Functional Enrichment Analysis

Cytoscape, as an open-source software, is used to display molecular interaction networks and combining those interactions with microarray profiles or genomics data (Shannon et al. 2003). Biological networks gene ontology (BiNGO) is a plugin of Cytoscape for assessing the overrepresentation of gene ontology (GO) terms in a graph of a biological network, or any other set of genes (Maere et al. 2005). In the current study, to further investigate the functional enrichment of the optimal genes with high classification accuracy, GO biological process (BP) terms enrichment analysis was performed using BiNGO. A FDR-value < 0.01 was set as threshold.

Identification of Hub Genes via CLR Algorithm

As reported, MIN is a subclass of network inference approach, whose theoretical foundation is to infer a connection among a few of nodes when it possesses a high value on the basis of mutual information (Meyer et al. 2007). CLR algorithm (Faith et al. 2007), as an extension of the relevance network approach, is utilized to count the mutual information for each pair of nodes and derive a score related to the empirical distribution of mutual information values. In the present work, the optimal genes were used as vertices, and microarray profiles were taken as the mutual information between vertices. Next, CLR was used to calculate the edge score for each pair of genes using an inference approach which took the square weighted value as input. Then, the adjacency matrix was established, following by the MIN construction by means of Igraph package.

Subsequently, to further uncover the significance of nodes in the MIN, degree analysis for the MIN was conducted. Degree is the total number of interactions of a given node connected with its neighboring nodes (Haythornthwaite

1996). Commonly, the nodes with more interactions were determined as hub genes. In this work, the genes with degree > 60 were extracted, and regarded as hub genes.

Analysis of Classification Capability Using SVM

To evaluate the classification performance of the hub genes between AS and control samples, SVM was employed in this study (Chang and Lin 2007). In detail, to measure the classification ability of hub genes, several terms of accuracy, sensitivity, as well as specificity were employed (Mohammadi et al. 2011). During the classification, all samples were divided into two parts (training and testing groups) using 5-fold cross-validation (5-CV) method. Specifically, samples were separated according to the ratio of 6 to 4, and the 19 samples were used as a training set, the remaining 13 samples as a testing group to verify the classification models.

RESULTS

Identification of the Optimal Genes

To prioritize the candidate genes of complicated genetic traits, three different methods (CP, CE, and RP) were used. The numbers of identified candidate genes from different methods under the thresholds of FDR values or effect-size was generated. Using the CP method, a total of 326 candidate genes were identified based on the FDR < 0.05. In the CE and RP methods, only the top 100 genes were selected for further analysis. The top 20 candidate genes detected by each method were listed in Table 1. Significantly, there was one common gene (MYL6) in CP and RP method, and there was also one common gene (AAMDC) in CP and CE methods. However, no overlap was observed in CE and RP methods. Then, R/Bioconductor package GenRank provided in turn was used to read the data sets and then the common genes across three different methods were extracted, and duplicated genes were filtered out. The corresponding number of genes identified by each method was shown in the Venn diagram of Figure 1. Among the 429 genes, 7 genes were common between CP and CE methods, 90 genes were the intersection of CE and RP, and 4 genes were common of CP and RP. Significantly, there were 4 genes (S100A4, MYL6, ATP5EP2, and S100A8) identified by CE, CP and RP simultaneously. Finally, 93 optimal genes were reserved for further analysis.

Table 1: The top 20 candidate genes identified by each method

CP	*CE*	*RP*
PTPN1	AAAS	OAZ1
LAMTOR2	AACS	HBA2
IL27RA	AAED1	RN7SL1
XPC	AAK1	HBB
FAM222B	AAMDC	ITIH5
WBP5	AAMP	LCP1
AAMDC	AAR2	ORC6
MRI1	AARS	HBG2
HOXB1	AARS2	IFITM2
DGKQ	AASDH	HBG1
TXN	AASDHPPT	ARHGDIB
CDK13	AATBC	TYROBP
S100A4	AATF	SLC25A39
LSM10	AATK	FTL
TNNC2	ABAT	HLA-B
CASP2	ABCA1	MKRN1
MYCBP2	ABCA13	UBB
GMFG	ABCA2	MYL6
BBS1	ABCA7	S100A9
MYL6	ABCB1	ACTB

Note: CP, combining p-values; CE, convergent evidence; RP, rank product

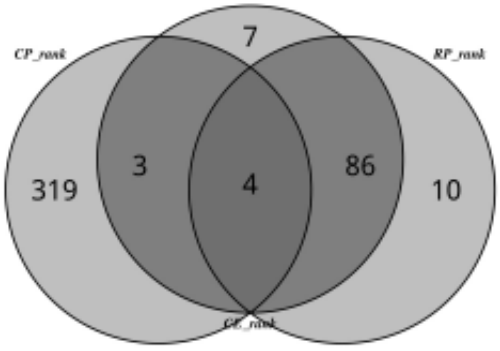

Fig. 1. Venn diagrams showing the overlap between lists of genes generated by any two analysis of CE, CP, and RP. Only intersecting genes with similar expression pattern were considered. Abbreviations: CP, combining p-values; CE, convergent Evidence; RP, rank product
Source: Author

Functional Enrichment Analysis for the Optimal Genes

Then, the functions overrepresented by the 93 optimal genes were extracted using BiNGO, a

Cytoscape plugin. A GO tree representing a hierarchical structure of GO-BPs was shown in Figure 2, where yellow nodes in the GO tree demonstrated significantly overrepresented GO-BPs (*FDR* < 0.01). The overrepresented BPs included 13 terms. Among these terms, the top 5 functions were translational elongation (FDR = 5.71E-10), translation (FDR=2.83E-06), immune system process (FDR =1.11E-03), gas transport (FDR = 1.27E-03), and actin filament-based process (FDR = 2.05E-03).

Identification of Hub Genes

Before hub genes identification, a MIN was firstly constructed for the optimal genes using CLA algorithm. The property of the MIN covering 93 genes and 2212 interactions was de-

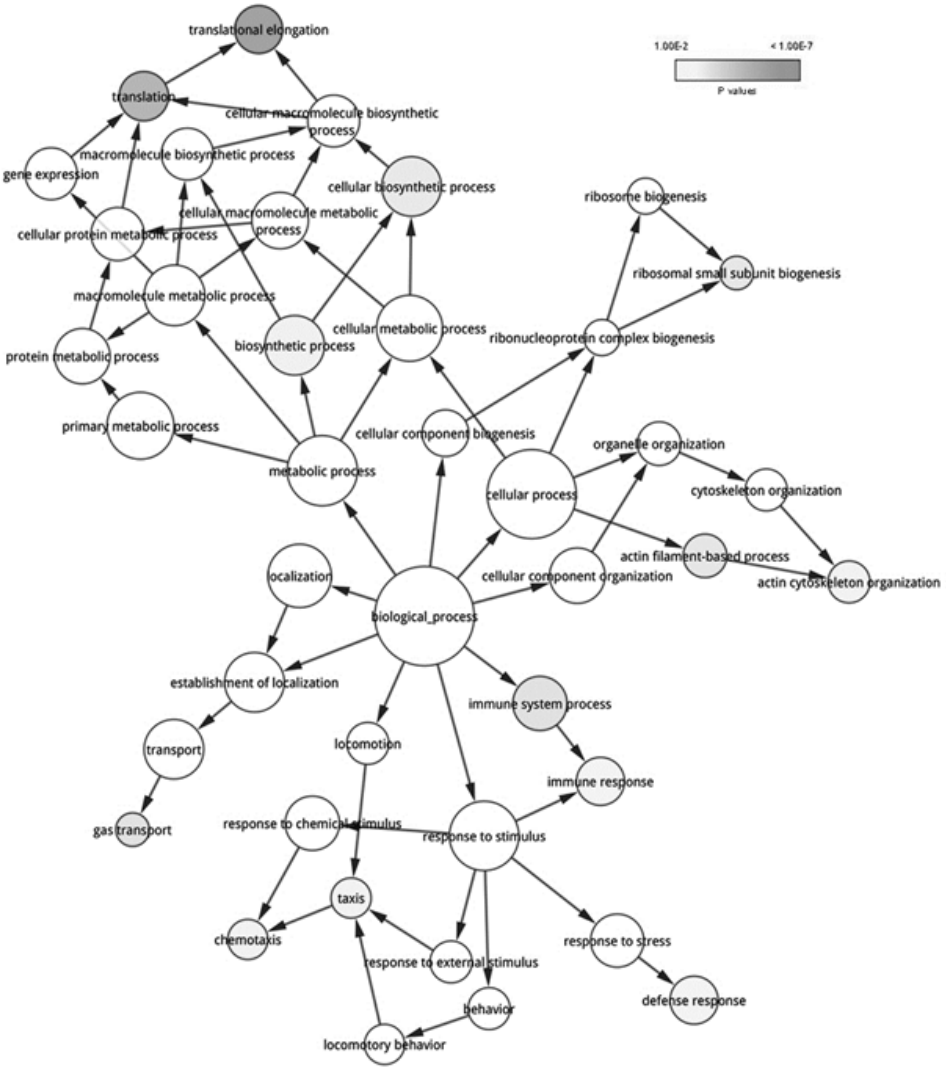

Fig. 2. Functional enrichment analysis for the optimal genes, as visualized using Cytoscape. Node colors (yellow) represented the statistical significance of functional enrichment of the corresponding gene oncology (GO) biological processes (BPs). White nodes (FDR > 0.01) were not significantly overrepresented, they were added to demonstrate the relationships among the significant BPs and non-significant BPs. The area of a node was proportional to the number of genes annotated to the corresponding GO term
Source: Author

scribed in Figure 3. The degree distribution of every gene in the MIN was different. Based on the degree > 60, a total of 3 hub genes were identified, including TALDO1 (degree = 66), LCP1 (degree = 64), and RPS27A (degree = 61).

Classification Ability of Hub Genes

SVM was used in this study to assess the classification performance of the 3 hub genes between AS and control patients. This method separated AS from control samples with the high accuracy of 0.962, specificity of 0.937, and sensitivity of 0.953.

Accordingly, these 3 genes can classify unknown samples from AS patients with high accuracy.

DISCUSSION

AS is a common inflammatory rheumatic disease that affects the predominantly axial skeleton and causes characteristic inflammatory back pain, which can decrease the quality of life (Du-

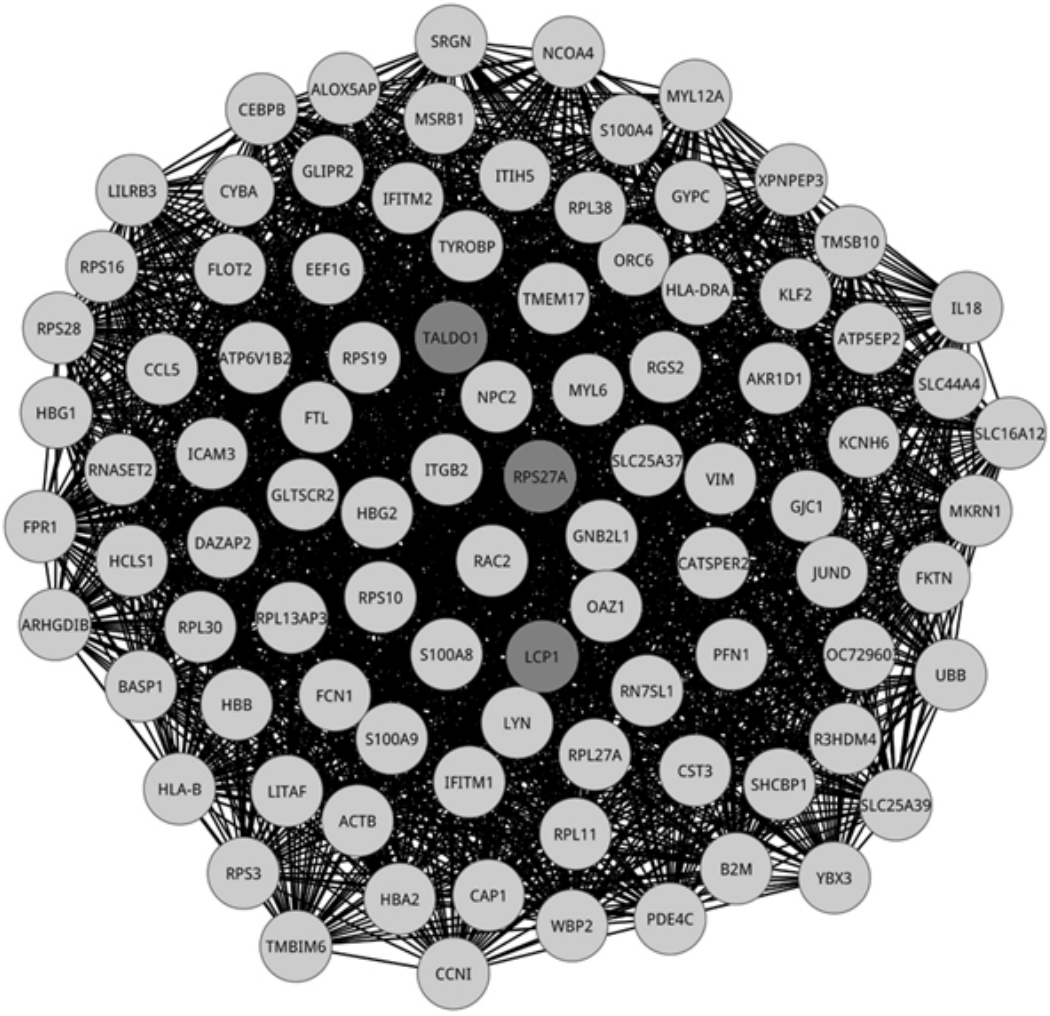

Fig. 3. Construction of mutual information network (MIN) using context likelihood of relatedness (CLR) algorithm. In the MIN, there were 93 genes and 2212 interactions. Yellow nodes denoted the hub genes, blue nodes stood for other optimal genes, and edges represented the interaction between any two nodes
Source: Author

ran et al. 2016; Osman and Maksymowych 2016). In the present research, the aim was to explore the potential molecular mechanism of AS by means of three meta-analyses approaches. This study demonstrates that several promising genes including LCP1 and RPS27A might provide important clues to the development process of RA.

Hub genes are believed to play major roles in a highly interacted network. In this study, several highly connected hub genes in the MIN were defined. In the current study, LCP1 was identified as a hub. As we all know, the alias of LCP1 is L-plastin and significantly, L-plastin is a member of the fimbrin family of actin-binding proteins. Moreover, the actin-cytoskeleton play important roles in the regulation of integrin function and leukocyte integrin avidity is crucial for inflammation and immunity (López-Posadas et al. 2017). Significantly, inflammatory stimuli can activate polymorphonuclear neutrophils (PMN) rapidly. Remarkably, improper release of oxygen free radicals from the activated PMNs, in synovial fluid of patients with rheumatoid arthritis, contribute to the damage of joint and other tissues (Zamudio-Cuevas et al. 2015). Moreover, AS, like rheumatoid arthritis, is one of the inflammatory arthritic diseases, has been addressed that free radicals might be the causative factors in the AS onset (Ho et al. 2000). Through the literatures, LCP1 involvement in AS is not well defined, and studies have rarely been reported previously. However, based on these results, the researchers infer that LCP might have crucial roles in the onset and progression of AS, partially through regulating the release of free radicals.

In the present study, RPS27 was another hub gene. RPS27A is one of ribosomal proteins (RPs) which are emerging as novel regulators of cell growth linking aberrant ribosome biogenesis to cell cycle arrest (Han et al. 2017). RPs have been suggested to regulate p53 activity (Cokariæ et al. 2015; Russo and Russo 2017). Growing evidence reveals that p53 is involved in many cellular activities including immune response (Li et al. 2017; Tsuda et al. 2017). Significantly, inflammatory is closely associated with AS which causes characteristic inflammatory back pain. Through consulting literature materials, there were no studies about RPS27A roles in AS development and progression previously. Demonstrated herein, the result indicates that the abnormal expression of RPS27A might cause disordered immune response, triggering the occurrence and development of AS.

Functional analysis demonstrated that translational elongation and translation were the most two significant GO terms. Translation, including three distinguished stages of translation (initiation, elongation as well as termination), is a part of the whole process of gene expression. In this process of translation, mRNA is decoded to generate a polypeptide on the basis of genetic code. Of note, translational elongation factor 2 has been revealed to control TNF-a translation (Gonzlez-Tern et al. 2013). A previous study has reported that TNF-α can cause systemic and local inflammation resulting in the clinical signs and symptoms of AS (Szalay et al. 2012). The results prove that translation might regulate AS through the inflammatory response.

CONCLUSION

Taken together, 3 hub genes and 13 significant functional terms were identified by integrating three different methods. LCP1 and RPS27A might be used as genetic biomarkers for AS diagnosis and treatment in the future. However, several limitations must be noted. Sample size was relatively small. Moreover, the identified genes were predicted using the bioinformatics approaches, yet detailed analyses of their expression were not implemented by experiments. These results warrant further study, and should generate hypotheses for patient or population-based studies.

RECOMMENDATIONS

Results from the current study will provide the groundwork for the understanding of AS pathogenesis.

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Paper received for publication on March 2018
Paper accepted for publication on May 2018