Identification of *RRM2* as a Potential Biomarker for the Diagnosis and Prognosis of Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a common autoimmune disease that can occur at any age. If treatment is delayed, RA can seriously affect the patients' quality of life. However, there is no diagnostic criteria for RA and the positive predictive value of the current biomarkers is moderate.

Objective: to identify RA-associated susceptibility genes and explore their potential as a novel biomarker for diagnosis and evaluation of the prognosis of RA.

Methods: Peripheral blood mononuclear cells (PBMCs) were collected from healthy human donors and RA patients. RNA-seq analyses were performed to identify the differentially expressed genes (DEGs) between RA and control samples. The PBMCs-mRNA in DEGs were further subjected to enrichment analysis. Furthermore, the hub genes and key modules associated with RA were screened by bioinformatics analyses. Then, the expression of hub genes in RA were assessed in mRNA expression profiles. Next, real time-quantitative PCR (RT-qPCR) analyses were performed to further confirm the expression of the hub genes from the PBMCs that collected from 47 patients with RA and 40 healthy controls. Finally, we evaluated the clinical characters for the candidate mRNAs.

Results: RNA-seq analyses revealed the expression of 178 mRNAs from PBMCs were disregulated between the healthy controls and the RA patients. Bioinformatics analyses revealed 10 hub mRNAs. The top 3 significant functional modules screened from PPI network functionally were involved in DNA replication origin binding, chemokine activity, etc. After validating the 10 hub mRNAs in GSE93272 dataset and clinical samples, we identified 3 candidate mRNAs, including ASPM, DTL and RRM2. Among which, RRM2 showed great capacity in discriminating between remissive RA and active RA. Significant correlations were observed between DTL and IL-8, TNF-α, between RRM2 and CDAI, DAS-28, tender joints and swollen joints, respectively. The AUC values of ASPM, DTL and RRM2 were 0.654, 0.995 and 0.990, respectively.

Conclusion: We successfully identified multiple candidate mRNAs associated with RA. RRM2 showed high diagnosis efficiency with the AUC of 0.990 (sensitivity=100%, specificity=97.5%). And RRM2 severed as an additional biomarker for evaluating disease activity. The findings provided a novel candidate biomarker for diagnosis and evaluation of the prognosis of RA.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic, inflammatory, autoimmune disease which can occur at any age[1]. It is characterized by a progressive synovitis that is initiated and maintained by a complex interplay among different immune cells as well as tissue cells[2, 3]. The average prevalence of RA ranges from 0.5–1.0% globally[4]. Once treatment is inadequate or delayed, RA will lead to accumulating cartilage destruction, bone erosion, irreversible physical challenges and systemic complications[1, 5].

The disease is complex and is known to be prominently associated with some risk factors, including sex, genetic component and environmental factors[1]. The females are generally two to three folds more likely to be affected with RA than males[6]. The females with higher prevalence of RA is partly attributed to the dual (stimulatory and inhibitory) effects of estrogen on the immune system[7]. With the development of Genome-Wide Associated Study, plenty of genetic association studies show that RA have a strong genetic element which can account for 60% of the risk of developing RA[1, 8] and more than 100 RA risk genetics loci have been identified, such as HLA-DRB1, CD28, STAT4, PADI4, PTPN22 as well as epigenetic modification and so on[4, 9, 10]. Other risk factors are consist of environmental factors, including inhaled pollutants, nutritional habits, chronic infection, microbiota and geographic or ethnic differences[11, 12]. However, studies for some of these risk factors are not robust enough. And plenty of RA-associated susceptibility genes still need to be further explored.

In the treatment strategies of RA, early and precise diagnosis is pivotal to prevent damage from occurring, since timely treatment can protect as many as 90% patients with early RA from disease progression and subsequent joint damage[2, 3]. However, among most RA patients, when the clinical manifestations such as swelling, morning stiffness and tenderness of joints are appeared evidently, the pathogenesis has begun for many years[1, 13]. Moreover, there is still no ideal diagnostic criteria for RA[13]. Although the current ACR/EULAR 2010 classification criteria has a higher sensitivity for early recognition of RA than the older ACR 1987 classification system[13–15], it has only a specificity of 61%[2]. Currently there are some ways for clinicians to evaluate a patient with possible early RA, including inquiry symptoms, medical history, physical examination, imaging test and laboratory blood tests[16]. Yet the imaging tests, i.e., magnetic resonance imaging (MRI), ultrasonography or X-ray are very sensitive for detecting synovitis and pannus before bone erosion and cartilage degradation, but they are some limitations in specificity or routine application[3, 13, 17, 18]. Additionally, the positive predictive value of the biomarkers (anti-CCP, RF) is moderate. The sensitivity is 67% and 70% for anti-CCP and RF, respectively[19], which remain challenges facing rheumatologists. Therefore, the development of novel diagnostic biomarkers for RA remains an unmet need.

In the present study, to identify the novel RA-associated susceptibility genes and test their potential as new biomarkers for the diagnosis and prognosis of RA, we collected PBMCs from 40 healthy human donors and 47 RA patients. Partial samples were used for RNA-seq analyses to identify the differentially expressed genes (DEGs) between RA patients and healthy donors. The PBMCs-mRNA in DEGs were further subjected to Gene Ontology (GO) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Furthermore, the hub genes and key modules associated with RA were screened by bioinformatics analyses and made a functional exploration by GO and KEGG. Then, the expression of hub genes in RA were assessed and screened in GSE93272 dataset which included 23 healthy control subjects and 136 patients with RA. Next, real time-quantitative PCR analyses were performed to further confirm the expression of the hub genes from the PBMCs that collected from 47 RA patients and 40 healthy volunteers. To assess whether the identified candidate mRNAs can be biomarkers for the diagnosis and prognosis of RA, we further investigated correlation between candidate mRNAs with clinical characteristics of RA patients (Fig. 1A).

2. Materials And Methods

Study population and data resources

For RNA-seq analysis as well as hub genes expression validation and clinical evaluation, a total of 84 participants (47 RA patients and 40 healthy individuals) were recruited in 2019. All the RA patients met the classification criteria of ACR/EULAR 2010 and were recruited in The Fifth Affiliated Hospital of Sun-Yat-Sen University. And all the healthy controls (HCs) without renal failure, heart failure, or autoimmune disease, and free from other inflammatory conditions, were recruited from the same hospital. All the participants had examinations and evaluations of swollen and tender joints, clinical disease activity index (CDAI) and disease activity score 28 (DAS28). Laboratory tests included anti-CCP, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and blood routine parameters. The characteristics of healthy controls and RA patients were showed in Table 1. All study protocols were approved by the ethics committee of The Fifth Affiliated Hospital of Sun-Yat-Sen University. All participants in this study were informed and signed written consent. For testing the expression of hub genes between RA and HCs, we are in searched of the Gene Expression Omnibus(GEO)database[20]https://www.ncbi.nlm.nih.gov/geo/ for microarray datasets using the keyword “rheumatoid arthritis”. Datasets were included if they met all of the following criteria: (1) were from humans; (2) included expression data from blood mRNA of both RA and HCs; (3) the number of rows in each platform was > 50,000; (4) the number of RA samples was ≥ 20, and the number of HCs samples was ≥ 20. Finally, one dataset GSE93272 was selected[21].

Table 1
Characteristics of healthy controls and patients with rheumatoid arthritis.

Characteristics	RA	HC
Number	47	40
Age(years)[a]	57.13 ± 12.27	61.15 ± 11.03
Sex (M/F)	16/31	11/29
Disease duration (Month)[b]	1008.00(158.40,1872.00)	NA
Anti-CCP(U/ml)[b]	179.40(57.10,200.00)	NA
DAS28-CRP[a]	4.06 ± 1.14	NA
DAS28-ESR[b]	4.90(4.10,5.70)	NA
CRP (mg/l)[b]	25.14(5.10,73.40)	13.35(1.18,53.06)
ESR (mm/h)[a]	61.17 ± 34.13	36.26 ± 28.36
Swollen joints[b]	6.00(2.00,10.00)	NA
Tender joints[b]	6.00(3.00,10.00)	NA
ILD positive (%)	17.02%	NA
[a]Expressed as mean ± standard deviation.		
[b]Expressed as the median (25th to 75th percentile).		

Preparation of Peripheral Blood Samples and Isolation of plasma and RNA

For whole-blood transcriptome analysis, peripheral blood samples (8ml) were collected from each RA patient and healthy control into EDTA-2K-containing tubes. Peripheral blood mononuclear cells (PBMCs) were extracted by using the Histopaque-1077 (Sigma-Aldrich, UK) from blood samples. And the plasma was isolated and stored at -80°C for Elisa assay. Follow-up total RNA was extracted from PBMCs using the Total RNA Kit I (Omega Bio-tek, USA) according to manufacturer's instructions. The concentrations of the RNA were quantified by NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, USA) and assessed via absorbance ratios of A260/A280 nm > 1.8 and RNA Integrity Number > 7.

RNA-seq analysis

Before RNA-sequencing, the quality of RNA was tested by Agilent 2100 Bioanalyzer (Agilent Technology). 2 μg of RNA sample was taken for RNA-sequencing. RNase R digested and rRNA depleted RNAs were taken to generated the sequencing libraries by using Total RNA-seq (H/M/R) Library Prep Kit for Illumina

(Vazyme Biotech) according to manufacturer's instructions. The library preparations were sequenced on Hiseq X Ten (Illumina).

Identification of differentially expressed genes

For identification of the differentially expressed mRNA in all DEGs between RA patients and healthy control samples, the limma package (version 3.46.0) was performed with the predefined criterion (|log2FoldChange| >1 and $P < 0.05$)[22].

Functional exploration for DEGs and key modules

To explore and visualize the potential functions of the identified mRNAs in DEGs and key modules, clusterProfiler package[23] in R was utilized for Gene Ontology (GO) enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment.

Protein–protein interaction (PPI) network analysis

To establish the interactions of up-or down-regulated RA-related mRNAs in DEGs, we constructed PPI network by STRING V11.0 database (https://string-db.org/)[24], and subsequently visualized it by Cytoscape software v3.8.2[25]. Typically, the most widely applied gene centrality measures are degree, closeness and betweenness. Degree depicts the overall connectivity of a node with other nodes in the network. Closeness represents the average distance from a node to all other nodes in the network. The betweenness counts the number of times a node acts as a bridge along the shortest path between two other nodes in the network.

Identification of hub genes and key modules

For screening hub genes, CytoHubba v0.1, a Cytoscape plug-in which can predict and explore important nodes/hubs by topological algorithms, which was used based on the genes in PPI networks[26]. Meanwhile, the Cytoscape plug-in Molecular Complex Detection v2.0.0 (MCODE) was employed to analyze the RA-associated key modules with MCODE score ≥ 5 and nodes ≥ 5[27].

Real time-quantitative PCR (RT-qPCR) analysis

Firstly, RNA integrity was assessed prior to cDNA synthesis by agarose gel electrophoresis followed by ethidium bromide staining. Then we synthesized first strand cDNA from total RNA templates using RevertAid Master Mix (Thermo Scientific, Lithuania). Briefly, the reverse transcription reactions were incubated for 30min at 42℃, 5min at 95℃ and hold at 4℃. Last, real time-quantitative PCR (RT- qPCR) was performed to measure the relative genes expression on a CFX-96 Touch™ (BIO-RAD, USA) using Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium, USA) according to the product' protocol. The primer sequences were listed in Supplementary Table S1. The expression was determined by the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as an internal control.

ELISA

To determine the concentration of inflammatory cytokines in all plasma samples, such as IL-1α, IL-1β, IL-6, IL-8, TNF-α and IFN-γ, enzyme-linked immunosorbent assay (ELISA) was performed by corresponding ELISA Kit (Saicheng Bio-tek, China) according to the manufacturer's instruction.

Statistical Analysis

Data was tabulated using Microsoft Excel 2019 and analyzed using SPSS software v24 (IBM, USA) and Graphpad Prism v8.0.1 (GraphPad Software, Inc, San Diego, CA). Student's t-test was employed to assess the gene expression between RA cases and healthy controls in GSE93272 dataset which was normally distributed parameters. Mann- Whitney's U test was applied for skewed distribution including genes expression in RA course and states. Pearson's analysis or Spearman method was performed for testing the correlation between mRNA expression levels and the clinical variables including CDAI, DAS28, counts of swollen/tender joints and inflammatory factors, as appropriate. Receiver Operating Characteristic (ROC) Curves were conducted to evaluate sensitivity and specificity of candidate mRNAs. $P < 0.05$ was considered to be statistically significant threshold.

3. Results

Identification of DEGs

To identify the different expression genes between control and RA, we collected whole blood samples from 4 healthy individuals and 4 RA patients,and then performed RNA-seq analyses. Analysis of the dataset revealed 178 mRNAs that were differentially expressed between the healthy controls and the rheumatoid arthritis patients ($|\log_2 \text{FoldChange}| > 1$ and $P < 0.05$). Among the 178 mRNAs identified, 124 mRNAs were upregulated and 54 mRNAs were downregulated. Their distribution was presented using a volcano plot (Fig. 1B).

Functional exploration

To further understand the functions of the mRNAs that were differentially expressed and connections among them, GO and KEGG pathway enrichment analyses were performed on the up or down-regulated mRNAs by the DAVID functional annotation clustering tool, respectively. The analysis results showed that under the "biological processes" category,up-regulated mRNAs were significantly enriched in nucleosome assembly, mitotic nuclear division, cell division, cellular protein metabolic process and gene silencing by RNA. On the other hand, down-regulated mRNAs were enriched in inflammatory response, immune response, cell adhesion, proteolysis, and bicarbonate transport (Supplementary Table S2). The top 5 pathways of KEGG enrichment for the mRNAs upregulated in rheumatoid arthritis patients were systemic lupus erythematosus, alcoholism, viral carcinogenesis, cell cycle and transcriptional misregulation in cancer. The top 5 KEGG pathways for the mRNAs downregulated in rheumatoid arthritis patients were

complement and coagulation cascades, staphylococcus aureus infection, chagas disease, african trypanosomiasis and rheumatoid arthritis (Supplementary Table S2).

Protein-protein interaction (PPI) network construction and key module analysis

To further narrow and target the key regulatory mRNAs, a PPI network analysis was conducted by using STRING (Fig. 2A). The top 10 hub mRNAs with the highest degrees of interaction were identified with the cytoHubba in Cytoscape. These hub genes were CCNB1, CDC6, TOP2A, BIRC5, KIF2C, RRM2, CDC20, ASPM, KIF23 and DTL. Their degree, closeness and betweenness were showed in Table 2.

Table 2
Degree, Closeness and Beetweenness scores for the top 10 upregulated genes.

Gene ID	Gene name	Degree	Closeness	Betweenness
CCNB1	cyclin B1	48	82.08333	597.6878
CDC6	cell division cycle 6	47	81.83333	582.0712
TOP2A	topoisomerase (DNA) II alpha	40	76.95	380.6416
BIRC5	baculoviral IAP repeat containing 5	36	78.58333	824.264
KIF2C	kinesin family member 2C	35	73.11667	51.22497
RRM2	ribonucleotide reductase M2	35	74.91667	152.8886
CDC20	cell division cycle 20	35	73.11667	51.22497
ASPM	abnormal spindle microtubule assembly	35	73.11667	128.5843
KIF23	kinesin family member 23	34	72.61667	12.64816
DTL	denticleless E3 ubiquitin protein ligase homolog	34	72.61667	12.64816

Subsequently, the top 3 significant functional modules were screened from the PPI network by the Molecular Complex Detection (MCODE) plug-in with MCODE score ≥ 5 and nodes ≥ 5(Supplementary Table S3. All genes in the three modules were utilized to perform GO term and KEGG pathway enrichment analyses, and these terms based on their adjusted P-values are illustrated in Fig. 3. The top five biological process terms for GO analysis were nuclear division, organelle fission, mitotic nuclear division, sister chromatid segregation, and chromosome segregation. Interestingly, in the cellular component terms, the top five GO terms were all enriched in condensed chromosome. In addition, the top five molecular function terms for GO analysis were DNA replication origin binding, chemokine activity, cyclin-dependent protein serine/threonine kinase regulator activity, microtubule binding, and chemokine receptor binding. For KEGG pathway analysis, cell cycle, complement and coagulation cascades, oocyte meiosis, staphylococcus aureus infection, and progesterone-mediated oocyte maturation were mostly enriched.

Validation of the Expression of Hub Genes

All of these 10 hub genes underwent expression validation in GSE93272 dataset which included 23 healthy control patients and 136 RA patients. The results, as shown in Fig. 4A, indicated that CCNB1, CDC6, RRM2, ASPM and DTL were significantly up-regulated in RA samples compared to healthy control samples ($P < 0.05$, 0.01, 0.001, or 0.0001). However, the five genes have been little studied on the associations with RA. To further confirm their roles in rheumatoid arthritis, we collected peripheral blood from 47 patients with RA and 40 healthy donors. Then RT-PCR analysis was performed to detect the expression of the five mRNAs (Fig. 4B-F). Eventually, we found that ASPM, DLT and RMM2 were significantly up-regulated in RA group compared to healthy controls.

Clinical evaluation for the 3 candidate mRNAs

Next, we assessed whether the expression of examined mRNAs could distinguish early RA patients from established RA patients and distinguish RA patients in remissive stages from that in active stages. Consistent with the validation data above, the expression of all the three mRNAs showed great difference between RA and HCs. But the expression of ASPM, DTL and RRM2 have no significant difference between early RA and established RA (Fig. 5A). And only RRM2 showed great capacity in discriminating between remissive RA and active RA (Fig. 5B).

Further, the correlations analysis between the 3 candidate mRNAs and inflammatory factors (IL-1α, IL-1β, TNF-α, IFN-γ, IL-6, IL-8), blood cells (white blood cell, lymphocyte, monocyte), acute phase reactants (CRP, ESR), RA disease activity scores (DAS28, SDAI, CDAI), disease duration, and clinical symptoms (swollen joints, tender joints) (Supplementary Table S4). As showed in Fig. 6C-H, significant correlations were observed between DTL and IL-8, TNF-α (r = -0.3226, $P = 0.0270$; r = -0.3010, P = 0.0398), between RRM2 and CDAI, DAS-28, tender joints, swollen joints (r = 0.3360, 0.3504, 0.3618 and 0.2927, $P = 0.0210$, 0.0158, 0.0125 and 0.0459), respectively.

Additionally, to verify whether the 3 mRNAs could discriminate RA patients from healthy subjects, we performed Receiver-Operating Characteristic (ROC) Curve analysis and measured the area under the curve (AUC) to assess their diagnostic potential, respectively. The AUC of ASPM, DTL and RRM2 were 0.654 (95% CI: 0.5265–0.7812; $P < 0.0172$; sensitivity = 0.571; specificity = 0.821), 0.995 (95% CI: 0.9851–1.000; $P < 0.0001$; sensitivity = 1.000; specificity = 0.975) and 0.990 (95% CI: 0.9713–1.000; $P < 0.0001$; sensitivity = 1.000; specificity = 0.975), respectively (Fig. 5I-K) (Supplementary Table S5). The above results taken together showed great power of RRM2 in discriminating RA patients from healthy subjects. Moreover, RRM2 could not only distinguish remissive RA from active RA, but also have positive correlations with inflammatory factors and clinical manifestation.

4. Discussion

Here, we performed RNA-seq analyses of cohorts of patients with RA and HCs. By using bioinformatics analysis, this study comprehensively identified PBMCs differentially expressed mRNAs associated with RA and provided a novel diagnostic mRNA biomarker for rheumatoid arthritis. RRM2, as supported by both GEO dataset and clinical samples validations, importantly, had high discriminative power with the AUC of

0.990 (sensitivity = 1.000; specificity = 0.975). Moreover, we observed that PBMCs-mRNA-RRM2 levels in RA patients was raised following exacerbation of clinical symptoms and disease activity, including CDAI, DAS28-ESR, tender joints, swollen joints and a transformation of disease state from remission to activity. These results suggested that the potential role of RRM2 as a novel biomarker for diagnosing disease and monitoring therapy outcome. As we know, the predictive value of the existing biomarkers (anti-CCP and RF) is moderate[19]. The PBMCs-RRM2 level could be used as an supplement biomarker for RA patients who have possibility of missing diagnosis due to the limitations of laboratory test results.

Early diagnosis of RA is an important step to realize a more effective prevention of the progression and subsequent damage[28]. The RF and anti-CCP are the well-known serological biomarkers for RA diagnosis. Raised serum titer of RF is associated with disease activity, longer disease duration and extraarticular manifestations[29–31]. And circulating anti-CCP can be detected even 10 years before the first symptoms onset[32, 33]. Therefore, the presence of RF and anti-CCP are commonly used as diagnostic biomarkers as well as a prognostic biomarkers[34]. However, the sensitivity of anti-CCP and RF is just 67% and 70%, respectively[19], which means that negative results do not exclude RA[13], which remains challenges facing rheumatologists. With the development of sequencing technology and bioinformatics analysis, many scientists try to find out a novel highly sensitive and specific biomarker for the diagnosis of disease. Mounting data have suggested that non-coding RNAs (ncRNAs) are playing pivotal role in regulating inflammation and autoimmunity, which can be regarded as diagnostic biomarkers for RA[35]. For example, miR-146a, miR-150 and miR-223 have be found to be high expressed in peripheral blood and joint tissues, and can be served as promising biomarkers for RA[36]. Another candidate Long Non-Coding RNA biomarker is GAPLINC, which is participated in proliferation, invasion and migration of fibroblast-like synoviocytes, and whose expression was increased in the peripheral blood, T cell, and synovial tissues of RA patients[37]. Intriguingly, the ncRNAs were participated in the pathogenesis of RA through the lncRNA/circRNA-miRNA-mRNA network[35]. And only a few of them have been employed in clinical diagnosis. Yet it's worth noting that many of these biomarkers only can detected from tissue, such as synovium tissues, obtained by biopsies. By contrast, those can be detectable in peripheral blood through non- invasion are more useful[38].

Ribonucleotide reductase M2 (RRM2) acts as a subunit of Ribonucleotide reductase (RR) which is important for DNA replication and damage repair via providing deoxy- ribonucleoside triphosphates (dNTPs)[39, 40]. Many studied has showed that RRM2 plays a key role in tumor cell proliferation, apoptosis, DNA damage and epithelial mesenchymal [41–43]. Therefore, RRM2 is also considered as an important gene in tumor metastasis, progression and a promising biomarker for many diseases[44, 45]. Meanwhile, previous studies indicated that both mRNA and protein of RRM2 were responsible for chemotherapy sensitivity and resistance[46], such as imatinib-based therapy resistance in chronic myeloid leukemia[47], adriamycin resistance in breast cancer[48], gemcitabine resistance in advanced lung squamous cell carcinoma[49] and so on. Interestingly, only one report about RRM2 was associated with RA, using liposome-polycation-dNA (LPd) complex loaded with RRM2 small interfering RNA, which suggested that suppressed RRM2 gene may cause the downregulation of the levels of proinflammatory cytokines TNF-α and IL-6 in RA-fibroblast-like synoviocytes (RA-FLSs) via increasing the levels of

apoptosis and inhibiting the proliferation of RA-FLSs [50]. Evidence from this study elucidated that RRM2 could play a critical role in the pathogenesis of RA. However, we could not find positive correlations between RRM2 and inflammatory factors including TNF-α and IL-6 in plasma. These inconsistent results indicated that RRM2 has a cell and tissue-specific expression pattern in RA. In the light of the complex biological role of RRM2, more studies are needed to explore the mechanism and function of RRM2 in RA.

DTL (Denticleless E3 Ubiquitin Protein Ligase Homolog), also known as CDT2, is a substrate receptor for CRL4 ubiquitin ligases[51]. DTL is important in degrading some proteins that regulating DNA replication and repair and cell cycle through ubiquitin-dependent pathway [52, 53]. In this study, we found DTL have a high diagnosis efficiency for RA. DLT also have significant correlations with IL-8 and TNF-α. However, DTL have no correlation with clinical symptoms and disease activity. Whether DTL can serve as a diagnostic biomarker for RA, the further study needs to do.

Our study has several potential limitations. The first weakness was the population of cohorts used for high-throughput sequencing analyses was small. Secondly, the individual samples we collected and that in GSE93272 dataset used to validate the hub genes and evaluate their correlation with clinical characters were all Japanese and Chinese; more samples from other countries are needed to verify these results. Thirdly, the specific mechanism by which RRM2 cause the pathogenesis of RA remains to be *further* studied. Lastly, further research is also required to explore the value of RRM2 for *differential diagnosis with other* autoimmune diseases, such as osteoarthritis and ankylosing spondylitis.

5. Conclusion

In summary, this study identified 3 newly discovered mRNA from PBMCs and their expression levels significantly increased in RA. With the validations in vivo and vitro, RRM2 showed high diagnosis power with extremely high levels of sensitivity (100%) and specificity (97.5%). And RRM2 might be used as a new biomarker for evaluating disease activity. The findings provided a novel diagnostic and prognosis biomarker for RA.

Abbreviations

RA
Rheumatoid arthritis
PBMCs
Peripheral blood mononuclear cells
HCs
Healthy controls
DEGs
Differentially expressed genes
ACPAs
Anti-citrullinated protein antibodies

ACR/EULAR
American College of Rheumatology/ European League Against Rheumatism
MRI
Magnetic resonance imaging
RF
Rheumatoid factor
anti-CCP
Anti-cyclic citrullinated peptide antibody
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
GEO
Gene Expression Omnibus
PPI
Protein–protein interaction
MCODE
Molecular Complex Detection
PCR
Polymerase chain reaction
qRT-PCR
Quantitative real-time PCR
ELISA
Enzyme-linked immunosorbent assay
RRM2
Ribonucleotide reductase M2
DTL
Denticleless E3 Ubiquitin Protein Ligase Homolog
IL-1α
Interleukin-1α
IL-1β
Interleukin-1β
IL-8
Interleukin-8
TNF-α
Tumor necrosis factor alpha
IFN-γ
Interferon gamma
WBC
White blood cell

CRP
C-reactive protein
ESR
Erythrocyte sedimentation rate
DAS28
Disease Activity Score 28
SDAI
Simplified Disease Activity Index
CDAI
Clinical Disease Activity Index
ROC
Receiver operating characteristic curves

Declarations

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Contributions

Conception and design of the work, Biao Wu, Xianghe Xu and Huading Lu; acquisition and analysis of data, Biao Wu, Yufeng Chen and Shuping Zhong; interpretation of data, Xuegang Li, Junlong Zhong, Bin Wang, Jie Shang, Ning Jiang; writing and preparing the original draft, Biao wu, Junlong Zhong, Jie Shang; writing, reviewing and editing the paper, Biao Wu, Shuping Zhong and Yufeng Chen; supervision, Xianghe Xu and Bin Wang; project administration, Xianghe Xu and Huading Lu; and funding acquisition, Xianghe Xu and Huading Lu. All authors have read and agreed to the published version of the manuscript and to have agreed to both be personally accountable for the author's contributions and ensure to answer any questions related to the accuracy or integrity of any part of the work. all authors approved the final version to be published.

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Ethics declarations

The use of human blood samples was approved by medical ethic committee (□□□□[2020]□□□ (K62-1)) of the Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai, China.

Consent for publication

Not applicable

Competing interests

The authors declare that they do not have competing interests.

Availability of data and materials

The datasets analyzed during the current study are available from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/gds) accession number: GSE93272.

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Figures

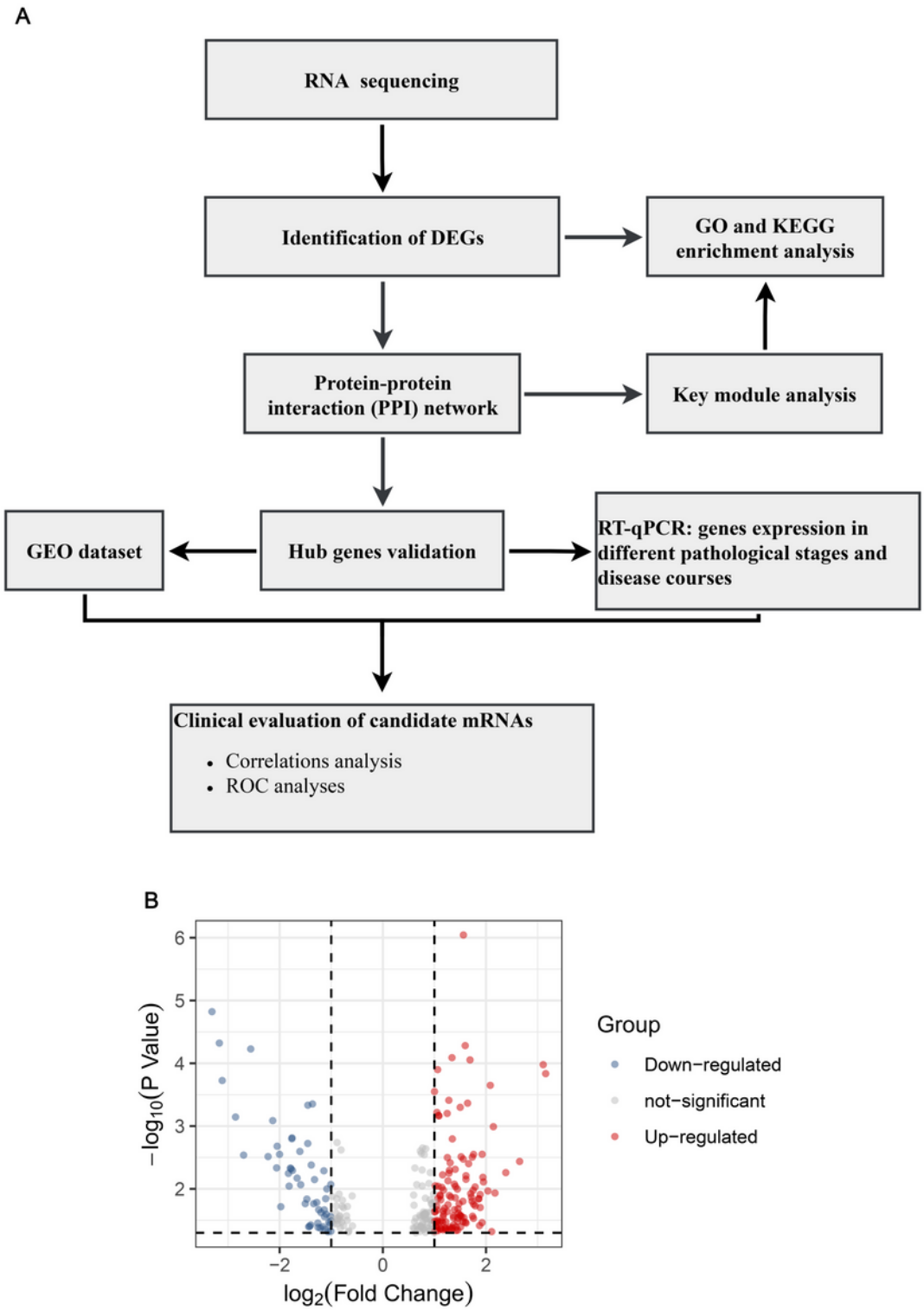

Figure 1

(A) Flow chart of the study. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein protein interaction; ROC, Receiver operating characteristic curves. CDAI, Crohn's Disease Activity Index; DAS28, Disease Activity Score 28; (B) Volcano plots showing the DEGs in the two comparison groups. The red dots represent up-regulated genes (log2FC > 1), and the blue dots represent down-regulated genes (log2FC < -1).

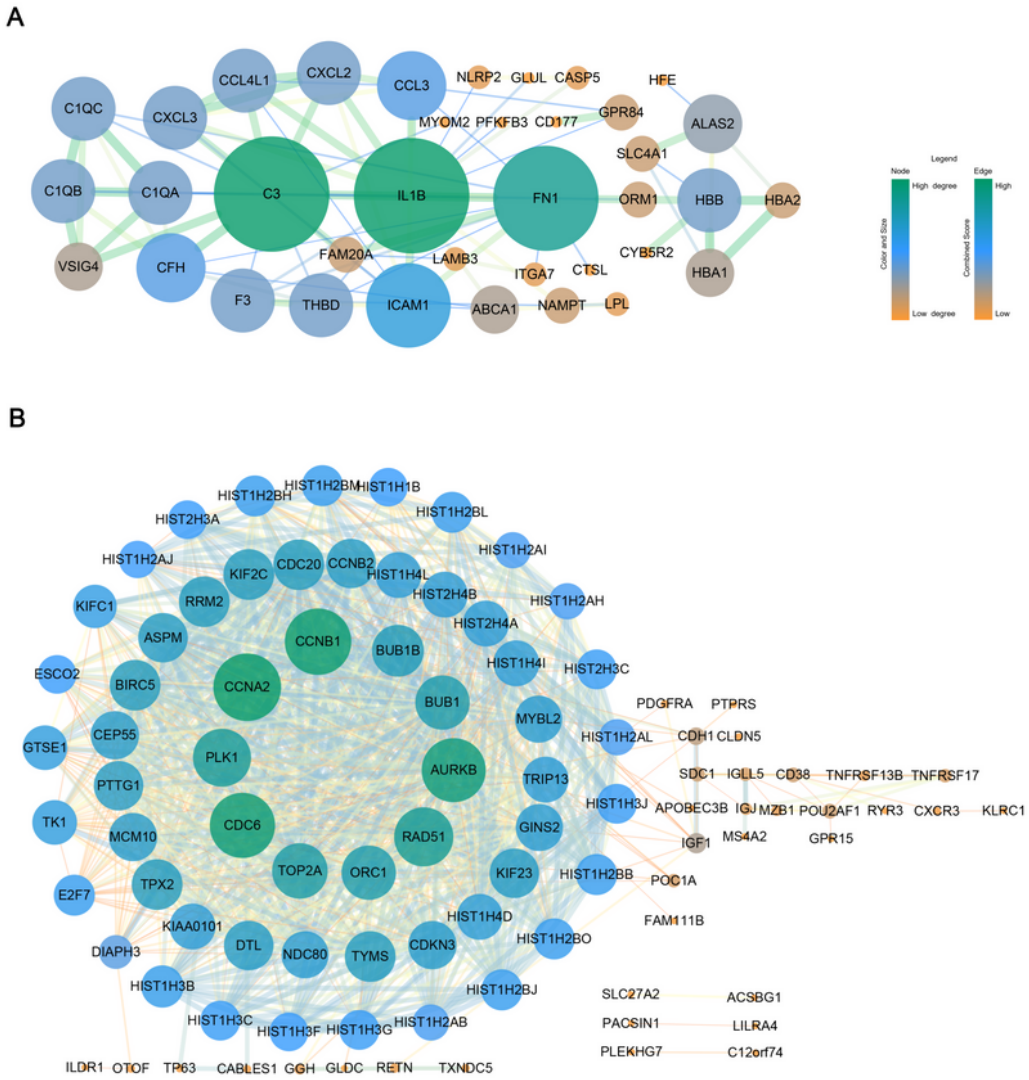

Figure 2

Protein-Protein Interaction network of RA-related mRNAs in DEGs. (A) PPI network of up-regulated RA-related mRNAs. (B) PPI network of down-regulated RA-related mRNAs. The size and color of the node depends on the degree. The width of the edge is determined by the combined score of STRING.

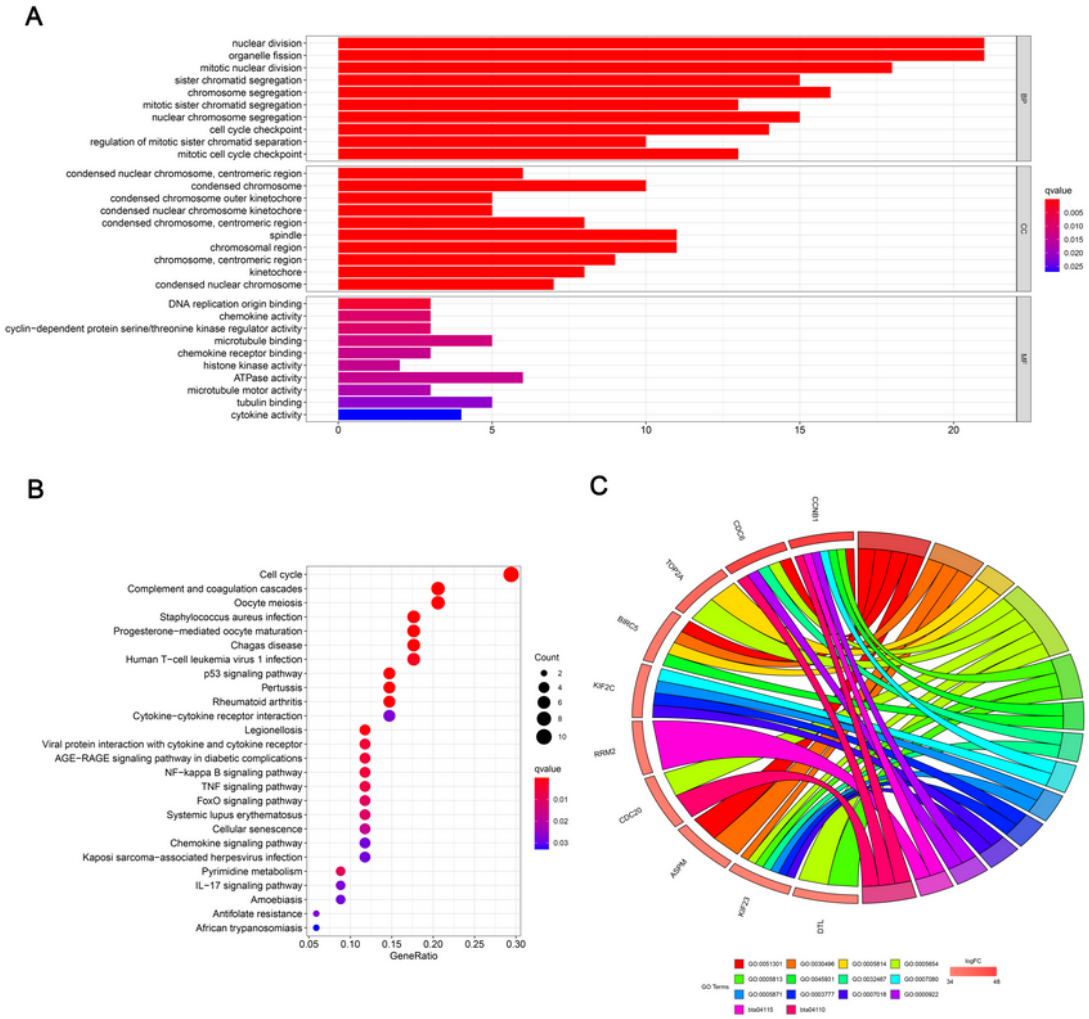

Figure 3

Functional and pathway enrichment analysis of the mRNAs in each module. (A) GO terms analysis of all mRNAs in 3 functional moduals. (B) KEGG pathway enrichment analyses of all mRNAs in 3 functional moduals. (C). GO terms/KEGG pathways associated with top 10 enriched hub genes. GO terms: GO:0051301, cell division; GO:0030496, midbody; GO:0005814, centriole; GO:0005654, nucleoplasm; GO:0005813, centrosome; GO:0045931, positive regulation of mitotic cell cycle; GO:0032467, positive

regulation of cytokinesis; GO:0007080, mitotic metaphase plate congression; GO:0005871, kinesin complex; GO:0003777, microtubule motor activity; GO:0007018, microtubule-based movement; GO:0000922, spindle pole; bta04115, p53 signaling pathway; KEGG pathway module: bta04110, Cell cycle.

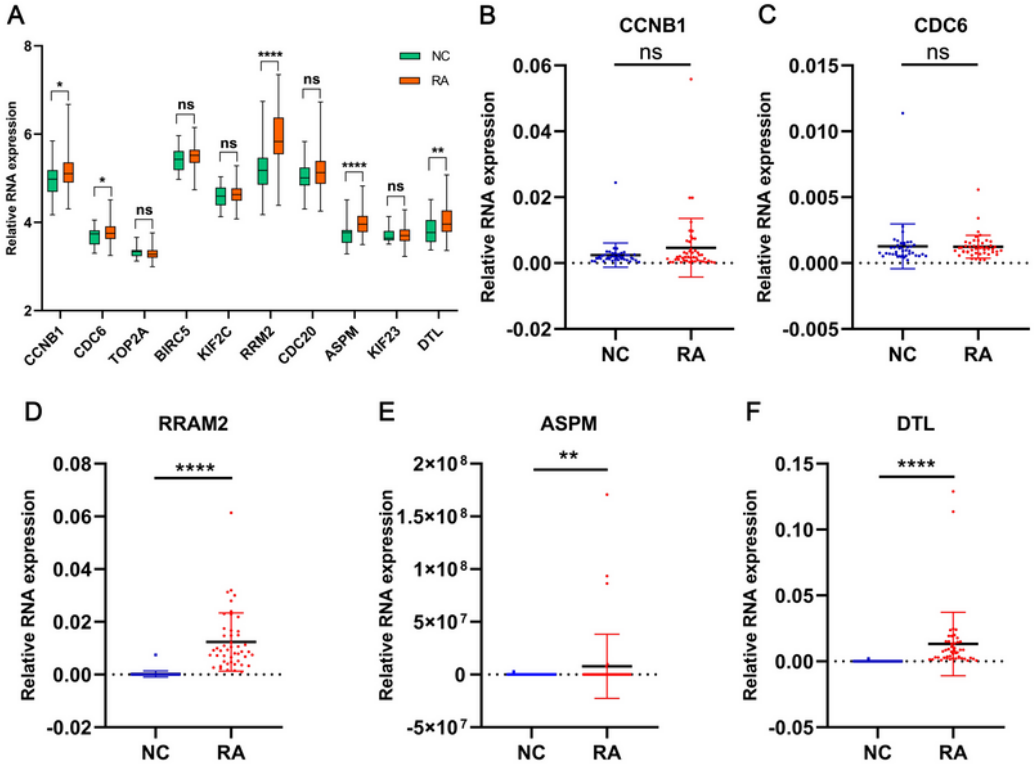

Figure 4

Verification of the 10 hub genes by GSE93272 dataset and patients' samples. (A). the expression of 10 hub genes in GSE93272 dataset. (B). expression of the hub genes in RA patients. Only hub genes that had

significantly differentially expression between RA and normal control groups were listed. The green box indicated normal control group, and the orange box indicated the RA group. Mann Whitney test was performed to compare the means of two groups. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. ns, no significance.

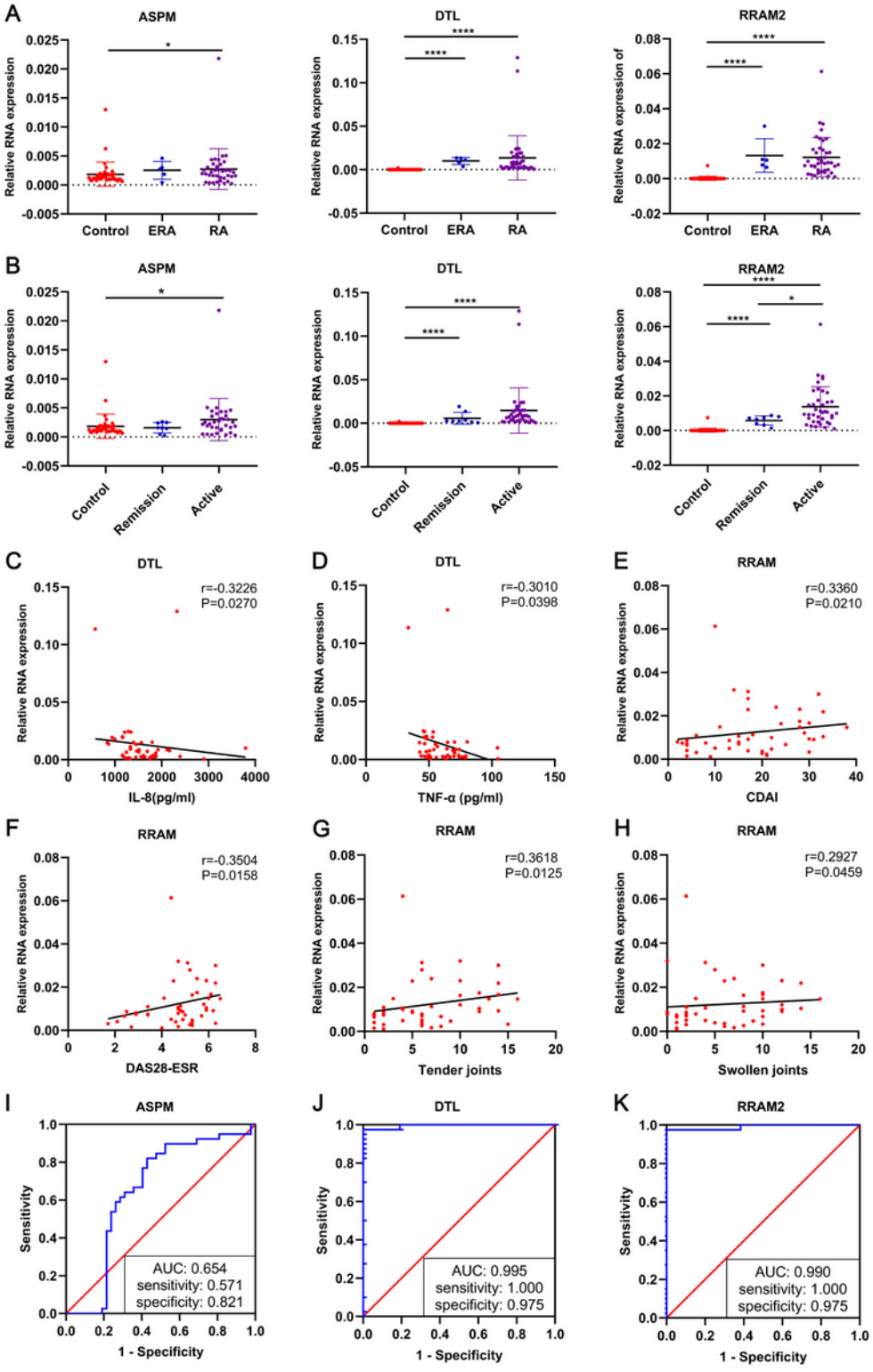

Figure 5

Clinical evaluation for the 3 candidate mRNAs. (A) levels of the 3 candidate mRNAs expression in PBMCs from healthy volunteers (control)(n=40), early RA patients (eRA)(n=5) and established RA patients (RA) (n=42). Bars show the mean with standard deviation. P values were determined by Mann-Whitney test. (B) levels of the 3 candidate mRNAs expression in PBMCs from RA patients in an active disease state(n=39) and those with RA in remission(n=8), and healthy volunteers(n=40). Bars show the mean with standard deviation. P values were determined by Mann-Whitney test. (C-H) Spearman correlation analyses for the relationships between the 3 candidate mRNAs and inflammatory factors(C-D), CDAI(E), DAS28(F), tender(G) and swollen joints(H). (I-K) ROC curve of the 3 candidate mRNAs in PBMCs from RA samples. AUC =area under the ROC curve.

Supplementary Files

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