

N6-methyladenosine methyltransferase METTL14 is associated with macrophage polarization in rheumatoid arthritis

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Abstract. Rheumatoid arthritis (RA) is largely caused by the inflammatory response triggered by macrophage polarization. Through epigenetic reprogramming, the inflammatory state of macrophages can be modified. Macrophage polarization is associated with the RNA epigenetic alteration N6-methyladenosine (m6A) RNA methylation. However, the specific function and underlying mechanisms of m6A methylation in the role of macrophage polarization in RA remain to be elucidated. The mRNA expression levels of m6A methylase genes and signaling pathway components associated with RA macrophages were determined in the present study using reverse-transcription quantitative PCR. Methyltransferase 14 (METTL14) protein expression levels were determined using western blot analysis, and the levels of specific cellular secretion factors were determined using ELISA and flow cytometry. The results of the present study demonstrated that elevated METTL14 expression was associated with joint tenderness, and METTL14 expression was positively correlated with both C-reactive protein and rheumatoid factor expression levels. Moreover, METTL14 exhibited potential in the prediction of visual analogue scale. Pro-inflammatory cytokines (TNF- α) and M1 macrophage markers (CD68⁺CD86⁺) were also positively associated with METTL14 expression. The results of the Kyoto Encyclopedia of Genes and Genomes analysis revealed that METTL14 was strongly associated with the MAPK signaling pathway. Notably, JNK and ERK2 exhibited a positive correlation with the M1 macrophage marker, CD68⁺CD86⁺, which was positively associated with the pro-inflammatory factor, TNF- α . JNK and ERK2 expression levels were markedly increased in the METTL14 high-expression group, compared with in the low-expression group; however, p38

and ERK1 expression levels were not significantly different between these groups. Collectively, the results of the present study demonstrated that METTL14 expression was significantly increased in the peripheral blood and synovial tissue of patients with RA, highlighting the potential association with both immunoinflammatory markers and clinical symptoms. In addition, it was suggested that METTL14 may exacerbate the downstream inflammatory response, through mediating macrophage polarization via the MAPK pathway.

Introduction

An extended and severe inflammatory response may cause damage to bone and cartilage in the inflammatory autoimmune disease rheumatoid arthritis (RA) (1). The pathophysiology of RA is complex, involving immune cells, and environmental and genetic factors (2,3). Among them, macrophages are crucial to the pathophysiology of RA. Notably, macrophages induce the translocation of a high number of immune cells to the joints, where they secrete inflammatory cytokines to maintain an inflammatory environment (4,5). In different settings, macrophages develop into two distinct phenotypes; namely, M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages. In some cases, M1 and M2 macrophages may alternate between types, in order to control the inflammatory response of the body (6,7). Notably, the prognosis of patients with RA has been reported to be associated with the biological properties of macrophages (8). Targeted alterations in macrophage activity and inflammatory state may exhibit potential in the treatment of patients with RA.

Alterations in gene expression that are heritable and reversible are referred to as epigenetics. Epigenetic modifications may occur in factors, such as metal, organic pollutants and air particles, associated with signaling between the environment and gene. Notably, epigenetics may affect the expression of inflammatory and matrix degradation pathways, which are strongly associated with the onset of RA (9-11). The results of previous studies have revealed that epigenetic reprogramming and epigenetic enzymes modify the inflammatory state of macrophages (12,13). mRNA molecules most commonly undergo N6-methyladenosine (m6A) RNA methylation, which is a type of epitranscriptomic alteration (14,15). Methyltransferases (writers), demethylases (erasers) and m6A-associated binding proteins (readers) are responsible for

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processing m6A alterations (16,17). The results of previous studies have identified the significance of m6A methylation alterations in immune regulation and inflammatory response mechanisms (18-20). However, the effects of m6A methylation alterations on the clinical diagnosis of RA and their specific roles in macrophage polarization remain to be fully elucidated. Thus, the present study aimed to determine the potential association between symptoms of RA, clinical markers and m6A methylation modifier enzyme genes. In addition, the present study aimed to investigate whether genes encoding m6A methylation modifier enzymes may control the inflammatory state and promote distinct pathways of macrophage development. Collectively, the results of the present study may uncover novel treatment targets, and could provide a theoretical basis for the role of m6A methylation modifier enzyme genes in macrophage polarization in RA.

Materials and methods

Database analysis of m6A methylation and RA macrophages. Differentially expressed genes in RA macrophages (compared with healthy control macrophages) were obtained from the GSE97779 dataset using the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) (21). The GSE97779 dataset consisted of nine samples of synovial fluid-derived macrophages obtained from patients with RA, and five samples of macrophages obtained from healthy control (HC) human monocytes. The Affymetrix microarray platform was utilized for data analysis. The GEO2R backend uses the established Bioconductor R software package (Bioconductor 3.18; Fred Hutchinson Cancer Research Center) to transform and analyze GEO data and the results are presented as a genetic table sorted by significance that can be visualized via GEO Profile graphs (<https://www.ncbi.nlm.nih.gov/geo/info/geo2r.html>). The m6A2Target database (<http://rm2target.canceromics.org/>) provided the m6A methylation modifier enzyme genes. Among the 21 m6A methylation modifier enzyme genes, eight writers (METTL3, ZC3H13, METTL14, RBM15B, CBL1, WTAP, RBM15 and KIAA1429), two erasers (FTO and ALKBH5) and 11 readers (YTHDC1, YTHDC2, ELAVL1, YTHDF1, LRPPRC, YTHDF2, FMR1, YTHDF3, HNRNPC, HNRNPA2B1 and IGF2BP1) were selected.

Patients. RA and HC peripheral blood samples were collected to determine the mRNA expression levels of m6A. In addition, synovial tissue was collected from RA and HC knee joints to verify the protein expression levels of METTL14. The patients with RA had not received any treatment prior to blood or synovial tissue collection. The patients with RA from whom synovium was collected during surgery included one man and three women, with a mean \pm SD age of 45.05 \pm 10.82 years. The HC volunteers from whom synovium was collected during arthroscopy also included one man and three women, with a mean \pm SD age 40.89 \pm 11.62 years. The synovial tissue samples were all obtained from the Department of Orthopedics, The First Affiliated Hospital of Anhui University of Chinese Medicine (Hefei, China). The participants for synovial tissue collection were recruited between December 2022 and March 2024.

Details of the participants from whom blood samples were collected are included in Table SI. The participants for blood collection were recruited between January 2022 and August 2023. Patients with RA (30 cases) were admitted to the Rheumatology and Immunology Department, The First Affiliated Hospital of Anhui University of Chinese Medicine, and HC individuals (30 cases) were selected from the Physical Examination Center of The First Affiliated Hospital of Anhui University of Chinese Medicine.

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui University of Chinese Medicine (ethics approval no. 2019AH-12), and all patients provided written informed consent. Notably, there were no statistically significant differences in age or sex between the RA and HC groups. RA disease activity was distinguished according to the Disease Activity Score-28 (DAS28) of the patients (22). 2.6 < DAS28 < 5.10 indicates low disease activity, whereas DAS28 \geq 5.10 indicates high disease activity. The pain perception of patients with RA was evaluated by visual analogue scale (VAS) (23). The correlation between METTL14 and clinical immunoinflammatory indicators was analyzed to determine the potential association between METTL14 and immune inflammation. The patients with RA were divided into eight subgroups according to the presence or absence of clinical symptoms. These subgroups include the joint tenderness group (20 cases), the non-joint tenderness group (10 cases), the joint swelling group (13 cases), the non-joint swelling group (17 cases), the joint morning stiffness group (17 cases), the non-joint morning stiffness group (13 cases), the limitation of joint motion group (16 cases) and the normal joint activity group (14 cases). White blood cells, red blood cells, hemoglobin, hematocrit, platelets, mean platelet volume, platelet distribution width, procalcitonin, rheumatoid factor (RF), anti-cyclic peptide containing citrulline, C-reactive protein (CRP), immunoglobulin A, immunoglobulin G (IgG), immunoglobulin M and serum amyloid A were measured using a fully automated biochemical analyzer (LTS 008AS; Hitachi, Inc.). Erythrocyte sedimentation rate was measured using an automated erythrocyte sedimentation rate analyzer (Roller 20; Alifax, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Peripheral venous blood samples (3-5 ml) were collected from the subjects, lymphocyte separation fluid (cat. no. 1308106078; Shanghai Kelaman Reagent Co., Ltd.) was added, and samples were centrifuged at 2,500 \times g for 20 min 18°C. After centrifugation, there was obvious stratification, and the white membrane between the plasma and separation solution was considered the mononuclear cell layer. These cells were added to a clean 15-ml centrifuge tube, washed with 10 ml PBS and centrifuged at room temperature and 250 \times g for 10 min. The supernatant was discarded, the cells were resuspended in 5 ml PBS and centrifuged again at room temperature and 250 \times g for 10 min. Following removal of the supernatant, the collected cells were considered mononuclear cells. TRIzol[®] reagent (cat. no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract RNA from the cells. RMBase v2.0 (<https://rna.sysu.edu.cn/rmbase/>) and PrimerBank databases (<https://pga.mgh.harvard.edu/primerbank/index.html>) were used to create primers, and all sequences are displayed in Table I. An RT kit (cat. no. RR047A; Takara Bio, Inc.) was used to reverse

Table I. Quantitative PCR primers used in the present study.

Gene	Length, bp	Forward primer, 5'-3'	Reverse primer, 5'-3'
METTL14	169	CGGGGACTTCATTCATGCTA	CCAGCCTGGTCGAATTGTA
CBLL1	76	GATCCTTGGGTGGTCTTGAT	GGTTTCGCTTTGTTTGCTTG
RBM15	122	CTCCGACGACCCGCAACAAT	CCACCAGAGCCCCCTAACTT
KIAA1429	203	GAGTAAAGAGCCCATAGCAGT	TAGCACCAGACCATCAGTATTCAC
ERK1	145	TTTTCCCAAGTCAGACTCC	GACTGGCCCACCTCATC
ERK2	86	AACTTGTGTAGGGCTGTGA	AAGGTCTGAAGAACCACCTG
p38	169	CTCATTAACAGGATGCCAAGC	CTTGGGCCGCTGTAATTCTC
JNK	146	TGTGTGGAATCAAGCACCTTC	AGGCGTCATCATAAACTCGTTC
β-actin	96	CCCTGGAGAAGAGCTACGAG	GGAAGGAAGGCTGGAAGAGT

METTL14, methyltransferase 14.

transcribe mRNA into cDNA according to the manufacturer's instructions, and Novostart SYBR qPCR SuperMix Plus (cat. no. E096-01B; Novoprotein Scientific, Inc.) was used on the prepared cDNA. The thermocycling conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 sec, and annealing and extension at 60°C for 1 min. The relative expression levels of METTL14 were calculated using the $2^{-\Delta\Delta C_q}$ method (24).

Western blot analysis. Synovial tissue samples were collected and lysed using 600 μ l RIPA cell lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Total protein was extracted from tissue via centrifugation for 15 min at 4°C and 12,000 x g. The protein concentration was determined by an ultra micro spectrophotometer (OD1000+; Nanjing Wuyi Technology Co., Ltd.). Proteins (50 μ g/lane) were subsequently separated on 10% polyacrylamide gels using SDS-PAGE and transferred onto PVDF membranes (cat. no. IPVH00010; MilliporeSigma). Membranes were blocked with 5% skimmed milk powder at room temperature for 2-4 h, and then incubated with anti-METTL14 (1:1,000; cat. no. ab220030; Abcam) and anti-GAPDH (1:2,000; cat. no. TA-08; ZSGB-Bio, Inc.) at 4°C overnight. Subsequently, membranes were incubated with a HRP-conjugated goat anti-mouse IgG secondary antibody (1:20,000; cat. no. ZB-2305; OriGene Technologies, Inc.) at room temperature for 2 h. Super-sensitive ECL chemiluminescent substrate (cat. no. BL520A; Biosharp, Inc.) was used to develop the membrane and a Gel Imaging System (Shanghai Peiqing Technology Co., Ltd.) was used to capture, and protein expression was semi-quantified using using ImageJ software (version 1.80; National Institutes of Health).

Detection of total RNA m6A modification levels. TRIzol reagent was used to isolate total RNA from peripheral blood samples, and a NanoDrop 3000 (NanoDrop; Thermo Fisher Scientific, Inc.) was used to assess the quality of the extracted RNA. The EpiQuik m6A RNA Methylation Quantification kit (cat. no. P-9005-48; EpiGentek) was used to quantify the m6A content according to the manufacturer's protocols.

Flow cytometry. Peripheral blood was extracted from patients and combined with 10 μ l anti-FITC-CD68 (cat.

no. MA1-82715; Thermo Fisher Scientific, Inc.), anti-PE-CD86 (cat. no. MHCD8604; Thermo Fisher Scientific, Inc.) or anti-PE-CD206 (cat. no. MA5-23594; Thermo Fisher Scientific, Inc.). Samples were incubated for 30 min at 4°C in the dark. Subsequently, samples were centrifuged at 1,500 x g for 5 min at room temperature, and the supernatant was discarded. Samples were washed twice with saline, and resuspended in 500 μ l saline for detection. Flow cytometry was conducted using a CytoFLEX flow cytometer (Beckman Coulter, Inc.), and FlowJo analysis software (version 10.8.1; Beckman Coulter, Inc.) was used to assess the levels of CD68/CD86/CD206 in cells.

ELISA. Peripheral blood samples were separated via centrifugation at 600 x g for 10 min at room temperature, and were subsequently stored at -80°C. The levels of inflammatory components, TNF- α (cat. no. E-EL-H0109c; Elabscience Biotechnology, Inc.) and IL-10 (cat. no. E-EL-H6154; Elabscience Biotechnology, Inc.) were determined using ELISA kits, following the manufacturer's protocols.

Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology (GO) enrichment analyses. The linear models for microarray data package (<https://bioconductor.org/packages/release/bioc/html/limma.html>) in the R programming language was used to analyze the GEO dataset. Genes associated with METTL14 ($P < 0.01$ and $|R| > 0.6$) were subjected to functional term enrichment analysis using the GO database and signaling pathway enrichment analysis using the KEGG database. The DAVID database (<https://david-d.ncifcrf.gov/>) was used to perform these enrichment analyses. GO signaling pathway enrichment analysis included three main components; namely, biological process (BP), cellular component (CC) and molecular function (MF).

Statistical analysis. Quantitative data conforming to a normal distribution are presented as the mean \pm SD. The Mann-Whitney U test was used for data that did not follow a normal distribution. Comparisons between multiple groups were made using one-way ANOVA followed by the least significant difference post hoc test. Differences between two groups were analyzed using unpaired Student's t-test. Spearman's rank correlation

coefficient tests were used to assess correlations. The χ^2 test was used for statistical analysis of sex comparisons among groups. Receiver operating characteristic (ROC) curves were used to evaluate the accuracy of METTL14 in predicting pain indicators (VAS). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of m6A methylation modifier enzyme genes are associated with differential expression in RA macrophages. RA disease activity was distinguished according to the Disease Activity Score-28 (DAS28) of the patients. Through the determination of m6A levels in the peripheral blood, the results of the present study demonstrated that m6A expression levels in the low-activity RA group were significantly increased compared with those in the HC group (Fig. 1A). In addition, m6A levels in the high-activity RA group were significantly increased compared with those in the low-activity RA group and the HC group (Fig. 1A). Using the GEO2R function, 5,476 differentially expressed genes were identified in RA macrophages in the GSE97779 dataset. Subsequently, m6A methylation modifier enzyme genes were intersected with the differentially expressed genes in RA macrophages. The results of the present study demonstrated that four genes (METTL14, CBL1, RBM15 and KIAA1429) were methyltransferases (Fig. 1B and C). Therefore, it was hypothesized that the polarization process of RA macrophages may involve these four genes. Subsequently, the results of the present study demonstrated that the mRNA expression levels of METTL14 were significantly higher in the RA group than those in the HC group (Fig. 1D and E). There was no significant difference between RA and HC groups regarding the other three methyltransferases. In addition, the results of western blot analysis demonstrated that the protein expression levels of METTL14 were significantly increased in the synovial tissue of patients with RA, compared with those in the HC group (Fig. 1F).

METTL14 is associated with markers of immunological inflammation and clinical manifestation in patients with RA. The results of the present study demonstrated that METTL14 was differentially expressed in patients with RA, compared with in HC individuals. Thus, the potential association of METTL14 with immunological inflammation was determined in patients with RA, and the results demonstrated that METTL14 was positively associated with CRP and RF (Fig. 2A). Subsequently, the mRNA expression levels of METTL14 were compared between the subgroups of patients with RA, who were classified according to the presence of joint tenderness, morning stiffness, joint swelling, joint mobility problems and other clinical symptoms. The results demonstrated that patients in the RA group experienced joint tenderness, and these patients exhibited significantly higher METTL14 expression levels compared with those in the group without joint pain (Fig. 2B). In addition, METTL14 has a high accuracy in predicting pain indicators of VAS, according to the results of the ROC curve analysis (Fig. 2C).

METTL14 is associated with cytokines and markers of macrophage polarization in patients with RA. The levels of the M1 macrophage marker CD68⁺CD86⁺ and the M2 macrophage

marker CD68⁺CD206⁺ were determined. The results of the present study demonstrated that the levels of the M1 macrophage marker CD68⁺CD86⁺ and the pro-inflammatory factor TNF- α were significantly increased in the RA group, compared with those in the HC group (Fig. 3A). By contrast, the levels of the M2 macrophage marker CD68⁺CD206⁺ and the anti-inflammatory factor IL-10 were significantly lower in the RA group, compared with those in the HC group (Fig. 3A). The results also demonstrated that CD68⁺CD86⁺ was positively correlated with TNF- α , and CD68⁺CD206⁺ was positively correlated with IL-10 (Fig. 3B). Collectively, these results highlighted that RA may be associated with macrophage polarization and an unbalanced inflammatory factor profile. In addition, the results revealed a positive correlation between METTL14, TNF- α and CD68⁺CD86⁺ (Fig. 3C). Based on the median METTL14 expression level, patients with RA were divided into two groups; namely, METTL14 low-expression and METTL14 high-expression groups. While there were no significant differences in the levels of CD68⁺CD206⁺ and IL-10 between these groups, the METTL14 high-expression group exhibited significantly higher levels of TNF- α and CD68⁺CD86⁺ than those in the low-expression group (Fig. 3D). These results suggested that high METTL14 expression may be closely associated with M1 macrophage markers and pro-inflammatory factors. The flow cytometry plots of macrophage polarization markers are shown in Fig. S1.

KEGG and GO enrichment analyses. The linear models for microarray data package in the R programming language was used to analyze the GSE97779 dataset and produce the differentially expressed gene expression matrix. Correlation analysis was used to determine genes correlated with METTL14, using $P < 0.01$ and $|r| > 0.6$. For enrichment analysis, genes that were highly correlated with METTL14 were submitted to the DAVID database. According to the GO analysis, the enriched BP terms were associated with an 'inflammatory response', an 'intimate immune response', 'IL-6 positive regulation' and 'cytokine-mediated signaling' (Fig. 4B). In addition, the enriched CC and MF terms were associated with 'peroxisome and peroxisomal membrane processes' (Fig. 4C and D). The results of the KEGG analysis demonstrated that METTL14 was highly associated with the 'MAPK signaling pathway' (Fig. 4A). The MAPK pathway was selected for subsequent analysis, based on the P-value and count value of the result as the most enriched pathway relevant to the topic of this study.

METTL14 is associated with the MAPK signaling pathway. The results of the present study demonstrated that the expression levels of JNK, ERK2, p38 and ERK1 were markedly increased in the RA group, compared with those in the HC group (Fig. 5A). Notably, the results of the correlation analysis revealed that there was no statistically significant association between METTL14 and p38; however, there was a statistically significant correlation between METTL14 and JNK, ERK1 and ERK2 (Fig. 5B). In addition, there was a significant correlation between JNK, ERK2, TNF- α and CD68⁺CD86⁺ (Fig. 5C). Based on the median METTL14 expression level, patients with RA were divided into two groups; namely, METTL14 low-expression and METTL14 high-expression groups. It was revealed that the METTL14 high-expression group exhibited

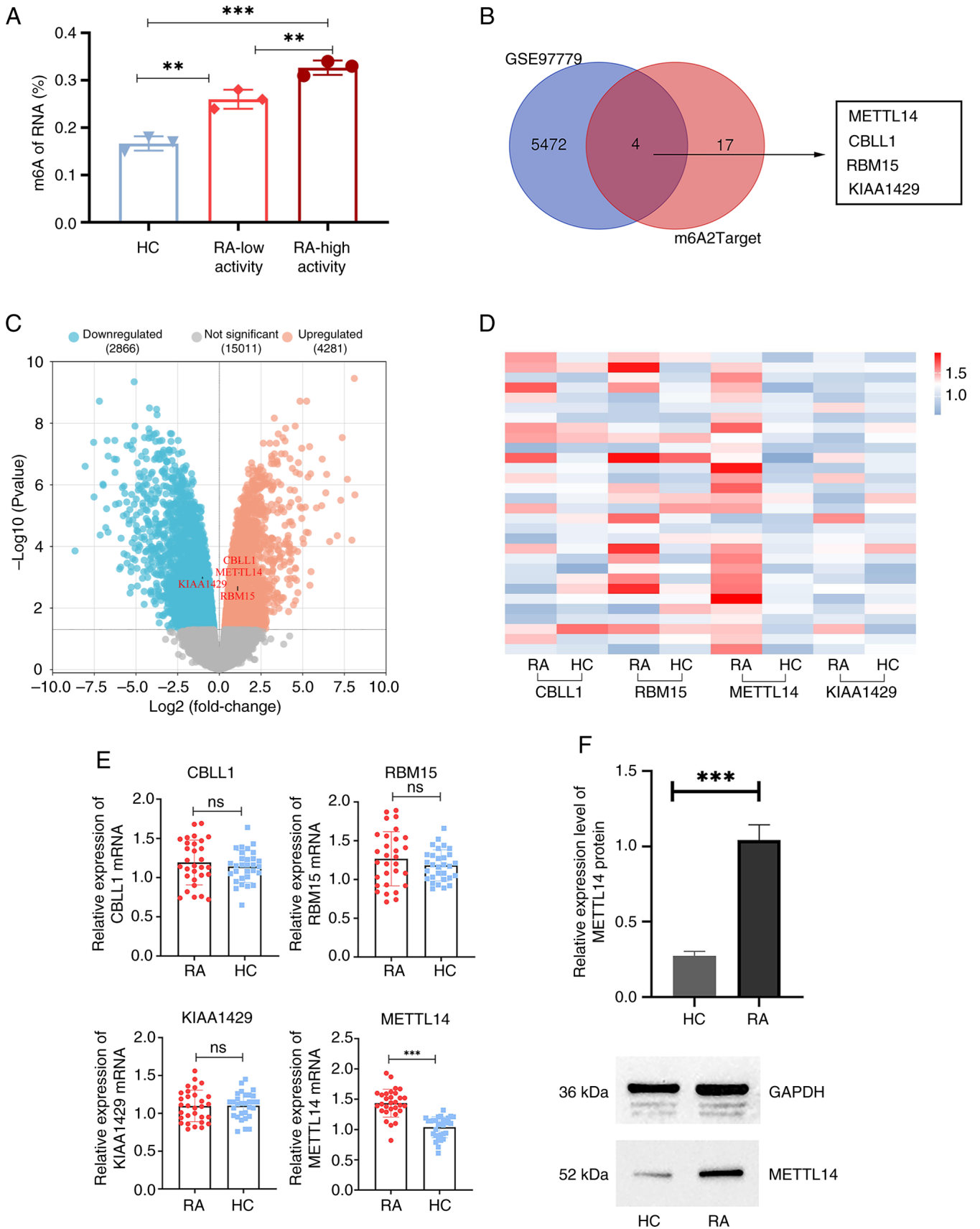


Figure 1. RA macrophages were screened for differentially expressed genes associated with m6A methylation modifier enzyme genes. (A) Determination of serum m6A expression levels (n=3). **P<0.01, ***P<0.001. Data were analyzed using one-way ANOVA. (B) Venn diagram demonstrating the intersection of m6A methylation modifier enzyme genes and differentially expressed RA macrophage genes. (C) Volcano plot of differentially expressed genes in RA macrophages. (D) Heatmap of RA macrophages associated with differentially expressed m6A methylation modifier enzyme genes. (E) Evaluation of mRNA expression levels of m6A methylation modifier enzyme genes (n=30). ***P<0.001. (F) Comparison of METTL14 protein expression between the two groups (n=3). ***P<0.001. Data are presented as the mean \pm SD in A, E and F, and were statistically analyzed using the unpaired two-independent sample t-test in E and F. RA, rheumatoid arthritis; HC, healthy control; m6A, N6-methyladenosine; METTL14, methyltransferase 14; ns, not significant.

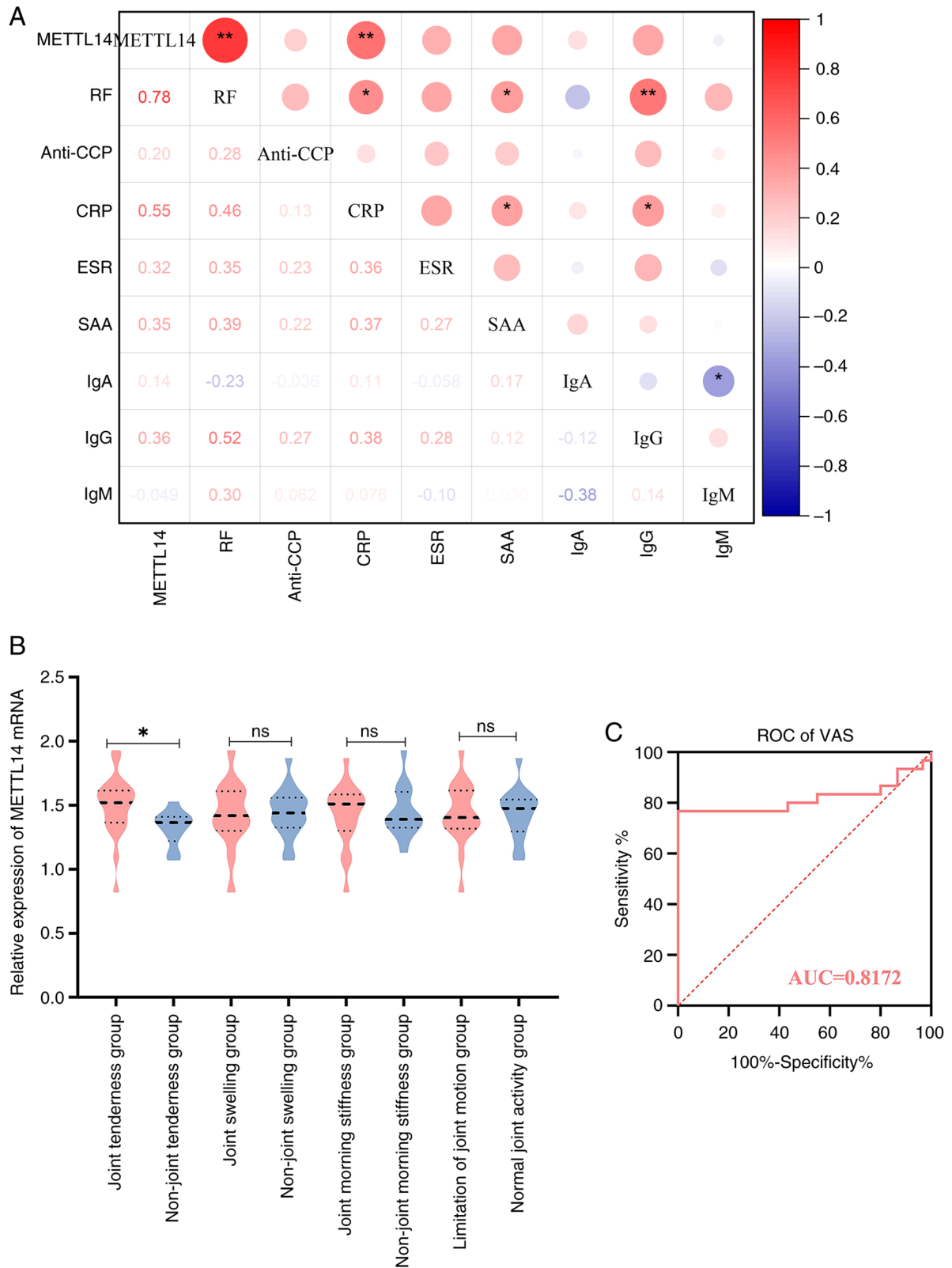


Figure 2. Association of immune-inflammatory markers, clinical symptoms and METTL14 in patients with RA. (A) Correlation analysis of METTL14 and immune-inflammatory markers in patients with RA. Spearman correlation analysis was used to analyze the data. * $P < 0.05$, ** $P < 0.01$. (B) Analysis of METTL14 and clinical symptoms in patients with RA. Data are presented as median \pm IQR, and data were statistically analyzed using the Mann-Whitney U test. * $P < 0.05$. (C) ROC curve. RA, rheumatoid arthritis; METTL14, methyltransferase 14; RF, rheumatoid factor; CRP, C-reactive protein; ROC, receiver operative characteristic; VAS, Visual Analogue Scale; AUC, area under the curve; ns, not significant.

increased JNK and ERK2 expression levels compared with those in the METTL14 low-expression group; however, no statistically significant differences were observed in the expression levels of

p38 and ERK1 between the groups (Fig. 5D). These findings indicated that METTL14 may increase downstream inflammatory responses via the MAPK signaling pathway.

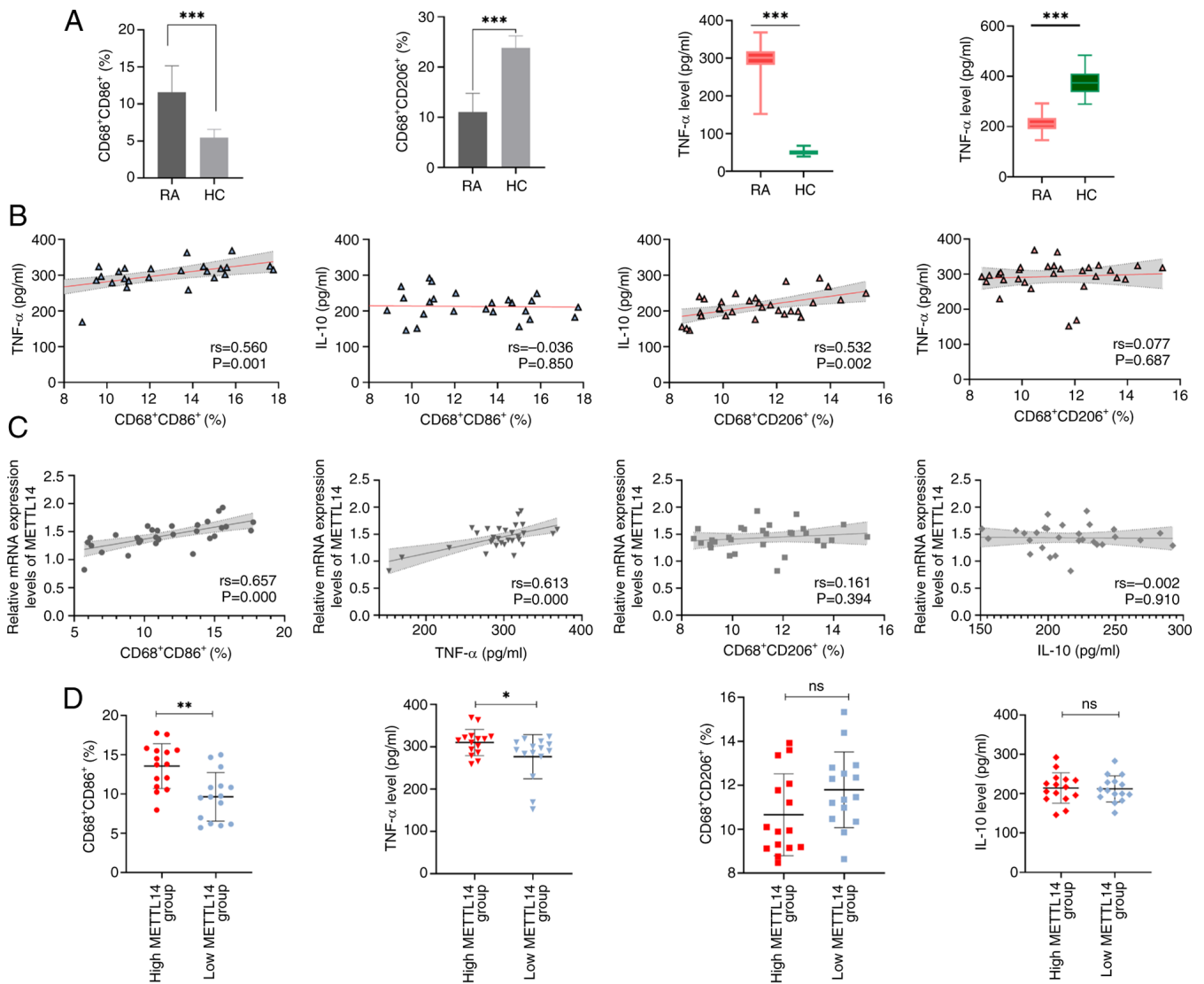


Figure 3. METTL14 expression is associated with cytokines and indicators of macrophage polarization. (A) Levels of inflammatory factors, TNF- α and IL-10, and macrophage markers, CD68⁺CD86⁺ and CD68⁺CD206⁺ (n=30). Data are presented as the mean \pm SD and were statistically analyzed using the unpaired two-independent sample t-test. ***P<0.001. (B) Correlation between TNF- α , IL-10, CD68⁺CD86⁺ and CD68⁺CD206⁺. Spearman correlation analysis was used to analyze the data. (C) Correlation between METTL14, TNF- α , IL-10, CD68⁺CD86⁺ and CD68⁺CD206⁺. Spearman correlation analysis was used to analyze the data. (D) CD68⁺CD86⁺ and CD68⁺CD206⁺, TNF- α and IL-10 expression levels in the METTL14 low-expression and METTL14 high-expression groups. Data are presented as the median \pm IQR, and data were statistically analyzed using the Mann-Whitney U test. *P<0.05, **P<0.01. RA, rheumatoid arthritis; HC, healthy control; METTL14, methyltransferase 14; ns, not significant.

Discussion

Antitumor immunity and healthy immunological responses are impacted by alterations in m6A methylation (14). The results of previous studies have indicated that m6A methylation alterations control the immune response mechanism in innate immune cells, including natural killer cells, dendritic cells and macrophages (25,26). Furthermore, it has been suggested that m6A methyltransferase-activated cells, such as synovial macrophages, may serve a role in the pathophysiology of RA and ultimately promote joint destruction (27,28). Through the determination of m6A levels in the peripheral blood of patients, the present study demonstrated that m6A levels in the low-activity RA group were significantly increased compared with those in the HC group, and the m6A levels in the high-activity RA group were significantly increased compared with those in the low-activity RA group.

Using bioinformatics analysis, differentially expressed m6A methylation modifier enzyme genes in macrophages were predicted. In total, it was suggested that four genes (METTL14, CBL1, RBM15 and KIAA1429) that alter methylation may be implicated in the process of differential expression in RA macrophages. The present study demonstrated that the peripheral blood and synovial tissue of patients with RA exhibited higher expression levels of METTL14 compared with those of HC individuals; however, there was no significant difference in the expression levels of KIAA1429, RBM15 or CBL1. The methyltransferase complex that stabilizes METTL3 and identifies target RNAs includes METTL14 as a crucial component (29,30). Li *et al* (31) previously demonstrated that the synovial tissues of rats with RA exhibited considerably higher levels of METTL14 expression, compared with HCs. Moreover, the potential association between METTL14 and

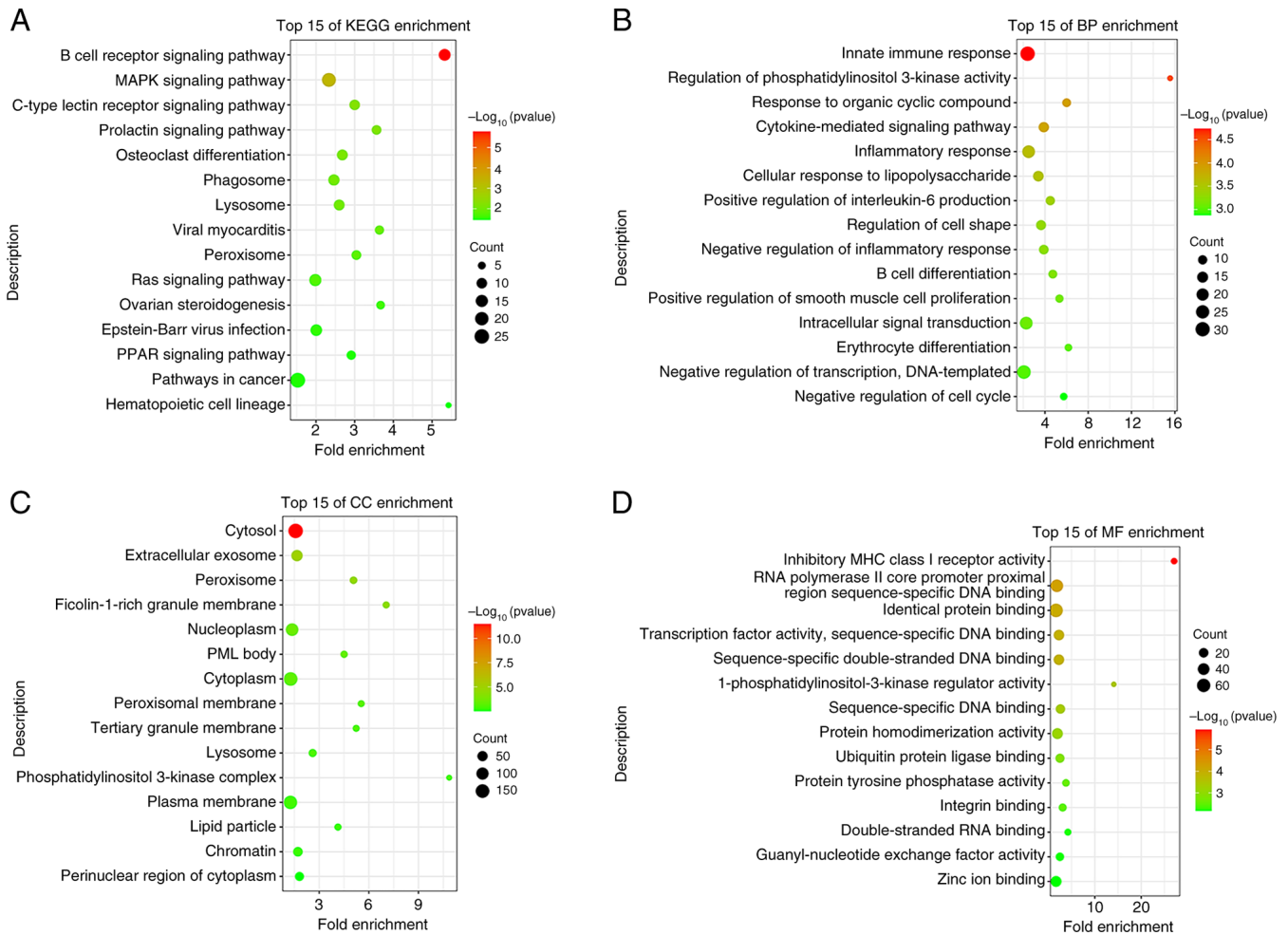


Figure 4. KEGG and GO enrichment analyses. Enrichment of METTL14-associated genes with (A) KEGG signaling pathways, (B) GO BP terms, (C) GO CC terms and (D) GO MF terms. The closer the color of the dot is to red, the smaller the P-value, and the larger the area of the dot, the more genes are enriched in this order. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology; BP, biological process; CC, cellular component; MF, molecular function; METTL14, methyltransferase 14.

clinical symptoms was determined in the present study. The results of the correlation analysis revealed a positive correlation between METTL14 and both RF and CRP. These two indicators are markers of RA disease activity. The present study also revealed that patients with RA experiencing joint pain exhibited higher levels of METTL14 expression than those without joint pain. In addition, METTL14 exhibited a high sensitivity in predicting VAS, highlighting that METTL14 may be strongly associated with clinical inflammatory markers in RA. Thus, METTL14 may exhibit potential as a molecular target for the treatment of RA.

Macrophage polarization includes the imbalance between pro-inflammatory M1 and anti-inflammatory M2 macrophage activity (32,33). Macrophage polarization causes the breakdown of articular bone and cartilage, and induces synovial inflammation, autoimmunity and joint injury (34,35). Flow cytometry was used to determine the levels of M1 and M2 macrophages in the peripheral blood samples of patients, to further explore the potential association between METTL14 expression, macrophage polarization and inflammatory response. The results of the present study revealed a positive correlation between the pro-inflammatory cytokine TNF- α , the M1 macrophage marker CD68⁺CD86⁺ and METTL14. The

METTL14 high-expression group exhibited markedly higher levels of TNF- α and CD68⁺CD86⁺ compared with those in the METTL14 low-expression group. The results of a previous study demonstrated that METTL14 may suppress TICAM2 and block the macrophage Toll-like receptor 4 (TLR4) pathway. In addition, macrophage polarization towards the M1 type was revealed to be promoted by a combination of TLR4 and METTL14 agonists (36). The results of another previous study also revealed that METTL14 knockdown can facilitate macrophage M2 polarization (37). In a rat model of RA, METTL14 knockdown was shown to significantly reduce fibroblast-like synovial cell activation and the generation of the inflammatory markers, IL-6, IL-18 and CXCL10 (31). Thus, METTL14 may facilitate macrophage conversion to the M1 type through upregulation of its expression. This, in turn, triggers a large-scale release of pro-inflammatory factors, which causes an imbalance in joint inflammation.

METTL14 expression is associated with numerous clinical characteristics and carries out a crucial additive methylation function during m6A alteration (38). Thus, METTL14 may serve a role in modulating signaling pathways and the expression of genes involved in RA. GO enrichment analysis revealed that METTL14 was enriched in immune-inflammatory

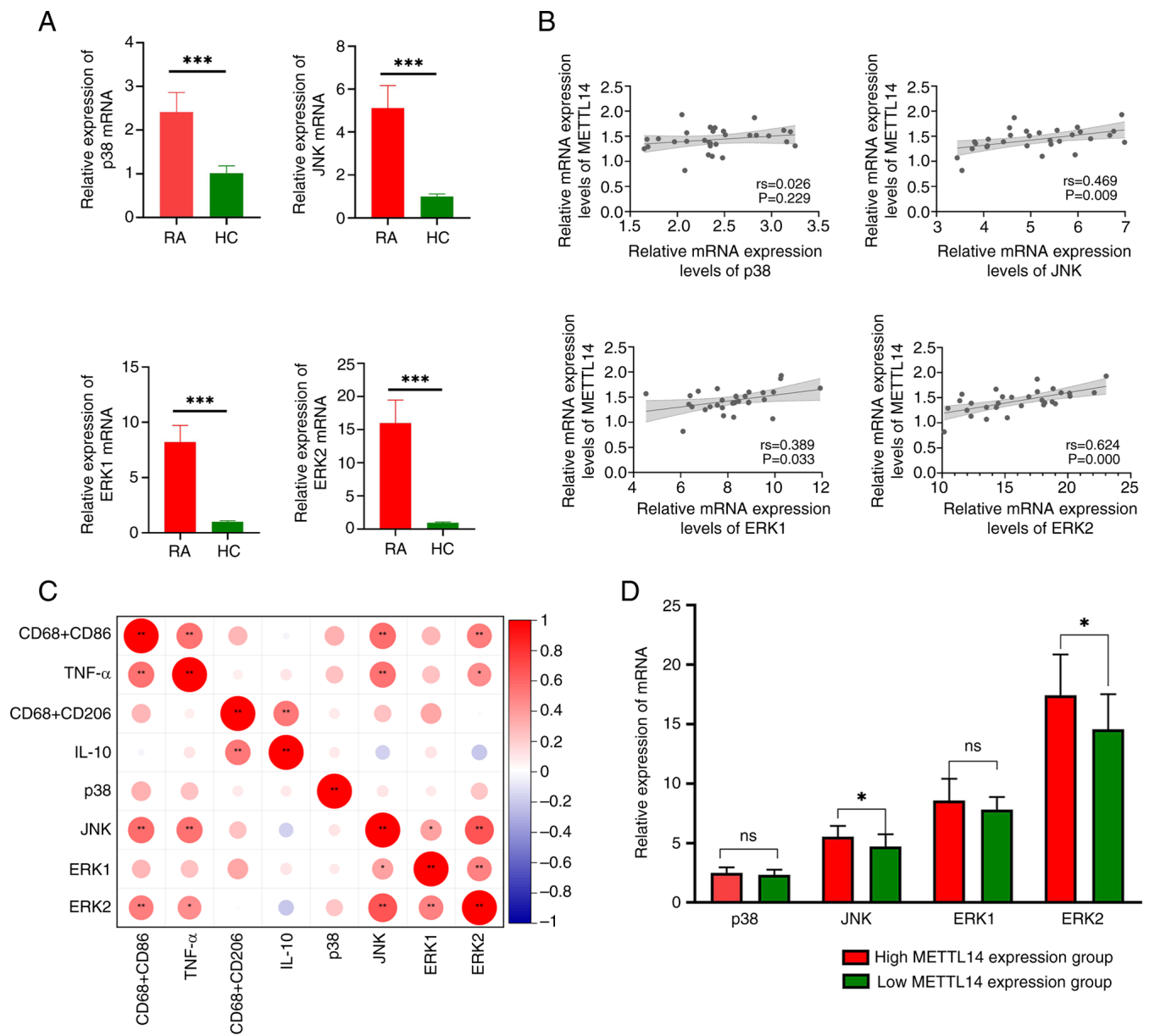


Figure 5. Analysis of the association between the MAPK signaling pathway and METTL14. (A) Expression levels of genes associated with the MAPK pathway (n=30). Data are presented as the mean ± SD and were statistically tested using the unpaired two-independent sample t-test. ***P<0.001. (B) Correlation between METTL14 and components of the MAPK pathway. Spearman correlation analysis was used to analyze the data. (C) Correlation between components associated with macrophage polarization and components of the MAPK pathway. Spearman correlation analysis was used to analyze the data. *P<0.05 and **P<0.01. (D) Expression levels of genes associated with the MAPK pathway in the METTL14 low-expression and METTL14 high-expression groups. Data are presented as the mean ± SD and were statistically tested using the unpaired two-independent sample t-test. *P<0.05. RA, rheumatoid arthritis; HC, healthy control; METTL14, methyltransferase 14; ns, not significant.

response biological activities, including the positive regulation of IL-6 production, innate immune response and inflammatory response. These findings indicated that METTL14 interacts with several targets and pathways. Moreover, METTL14 has been reported to regulate autophagy, glycolysis, ATPase activity and immunological response (39). Notably, among pathways associated with inflammation, KEGG enrichment analysis demonstrated that METTL14 was associated with the MAPK signaling pathway. ERK, p38 and JNK are members of the MAPK family. The results of the present study demonstrated that the expression levels of JNK, ERK2, p38 and ERK1 were higher in the RA group compared with those in the HC group. The results of previous studies have

highlighted that the MAPK signaling pathway is crucial for the transcriptional activation of cytokines associated with the pathophysiology of RA (2,40). The pro-inflammatory factor TNF-α and M1 macrophage marker CD68⁺CD86⁺ were significantly correlated with JNK and ERK2 in the present study. The results of a previous study have revealed that MAPK serves a crucial role in controlling macrophage polarization (41). Thus, RA macrophage polarization may involve activation of the MAPK signaling pathway. The results of the present study also revealed a significant positive correlation between METTL14 and JNK, ERK1 and ERK2. Furthermore, when compared with the METTL14 low-expression group, JNK and ERK2 expression levels were significantly higher

in the high-expression group. Therefore, METTL14 may mediate the transition of RA macrophages into the M1 type and result in the increased production of inflammatory markers, which may lead to activation of the JNK/ERK2 signaling axis through upregulated expression. The JNK signaling pathway modifies macrophage polarization through upregulating the expression of the downstream transcription factor, c-Myc. The JNK signaling pathway is key in regulating macrophage polarization and the production of inflammatory cytokines (42,43). The results of a previous study have demonstrated that macrophage inflammatory responses are markedly reduced following METTL14 knockdown (37). Collectively, these results highlight the critical roles of m6A methylation and METTL14 expression in macrophage polarization and the potential immunomodulation of RA. The present study indicated that, via the JNK/ERK2 signaling axis, METTL14 may mediate the polarization of RA macrophages.

Notably, the present study has some limitations. For example, bias may be present due to the inclusion of one institution, from which the samples were obtained. In addition, *in vitro* and *in vivo* experiments were not carried out due to time constraints. Thus, future investigations should be carried out with increased sample sizes, animals and cells, to verify the involvement of METTL14 in RA macrophage polarization.

In conclusion, the present study revealed that joint pain in RA was associated with the increased expression of METTL14. RF and CRP levels were also positively correlated with METTL14, and METTL14 exhibited a high sensitivity in predicting VAS. The results of the present study also demonstrated that inflammatory cytokines, such as TNF- α , were associated with proteins in the MAPK pathway, such as JNK and ERK2, and the M1 macrophage marker CD68⁺CD86⁺ was positively correlated with the expression levels of METTL14. In addition, JNK and ERK2 were positively correlated with TNF- α and CD68⁺CD86⁺. Compared with the METTL14 low-expression group, JNK and ERK2 expression levels were markedly increased in the METTL14 high-expression group. Notably, via the JNK/ERK2 signaling pathway, the m6A methyltransferase METTL14 may promote the conversion of macrophages to the M1 type in RA; therefore, intensifying the inflammatory response.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

LW was responsible for designing the study, and writing, reviewing and editing the manuscript. ZZ was responsible for data analysis, specimen and data collection, and writing the original draft. LW and ZZ confirm the authenticity of all the raw data. Both authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study complied with the Declaration of Helsinki and was approved by The Institutional Review Board Ethics Committee of The First Affiliated Hospital of Anhui University of Chinese Medicine (ethics approval no. 2019AH-12). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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