

# Differential long non-coding RNA expression profiles in the peripheral blood and CD4<sup>+</sup> T cells of patients with active rheumatoid arthritis

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**Abstract.** The human transcriptome is primarily composed of long non-coding RNAs (lncRNAs), which are key regulatory molecules of multiple biological processes. In the present study, the expression profiles of lncRNAs in the peripheral blood and CD4<sup>+</sup> T cells of patients with active rheumatoid arthritis (RA) were determined. Based on the expression profiles, 493 lncRNAs and 374 mRNAs were identified to be differentially expressed in the peripheral blood of active RA patients and healthy donors. Further verification of lncRNAs was performed using reverse transcription-quantitative (RT-q) PCR analysis of peripheral blood from 5 healthy donors and 5 patients with active RA and 14 additional differentially expressed genes were identified. CD4<sup>+</sup> T cells in peripheral blood from 12 patients with active RA and 8 healthy donors were isolated using magnetic beads and qPCR was used to assess differentially expressed lncRNAs. The results suggested that 7 lncRNAs were upregulated and 2 were downregulated. The results indicated that these 9 lncRNAs may be involved in the pathogenesis of RA. An increased ratio of Th17: T-regulatory (Treg)

cells was also observed. It may be hypothesized that lncRNAs serve important roles in the differentiation of CD4<sup>+</sup> T cells. Receiver operating characteristic curve analysis suggested that these 9 lncRNAs are of potential clinical diagnostic value for RA. Pearson correlation analysis indicated that the correlation coefficient between Ensembl transcript (ENST)00000569543 and complement C4 was 0.623 (P<0.05), and that between ENST00000420096 and anti-cyclic citrullinated peptide antibody or disease activity evaluation score, the correlation coefficient was 0.662 and 0.605, respectively (P<0.05 for each). In conclusion, the results of the present study suggest a possible role of lncRNAs in the differentiation of CD4<sup>+</sup> T cells and the pathogenesis of RA, as well as the potential value as diagnostic biomarkers for active RA.

## Introduction

Rheumatoid arthritis (RA) is a multi-systemic inflammatory autoimmune disease that causes joint swelling, pain, loss of function and stiffness in affected appendages, and significantly impacts the health and quality of life of patients. The worldwide prevalence of RA is ~0.24% and it is 2-3 times higher in females compared with that in males (1-3). Although the pathogenesis remains to be fully elucidated, it is well known that inflammatory response-mediated immune dysfunction is a key factor involved in the pathogenesis (4,5).

High-throughput sequencing is a comprehensive analysis platform for studying differentially expressed genes in multiple diseases (6,7). High-throughput sequencing studies have indicated that various lncRNAs may be associated with a variety of autoimmune diseases, including RA, systemic lupus erythematosus, Sjogren's syndrome and multiple sclerosis (8-12). lncRNAs are also hypothesized to be involved in the differentiation and activation of CD4<sup>+</sup> T cells, which are aberrantly activated adaptive immune cells in multiple autoimmune diseases (13-15). Dysregulated differentiation of CD4<sup>+</sup> T cells alter the balance of interactions between the CD4<sup>+</sup> T-cell subgroups and may be an important factor leading

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to the development of RA disease (16). As such, it may be possible to identify potential therapeutic targets by analyzing the differential expression of lncRNAs. In the present study, the lncRNA expression profiles in the peripheral blood and CD4<sup>+</sup> T cells of healthy donors were compared with those of patients with active RA using high-throughput sequencing. Further verification was performed using quantitative (q)PCR and the results supported the potential involvement of specific lncRNA in CD4<sup>+</sup> T-cell differentiation during the development of RA. These results provide novel insight into the immunological mechanisms underlying the development of RA.

## Materials and methods

**Patients and specimens.** A total of 19 patients with RA (16 females and 3 males; mean age, 53 years) who visited the Department of Rheumatology and Immunology of Jiangsu Province Hospital of Chinese Medicine (Nanjing, China) between March 2016 and June 2016 were recruited. Patients were recruited if they met the criteria for the diagnosis of RA set out by the American College of Rheumatology in 1987 (17). The patients recruited were outpatients or hospitalized patients. Peripheral blood was collected and first used to detect RA-related indicators prior to administration of glucocorticoids and immunosuppressive agents. Erythrocyte sedimentation rate (ESR) was measured using the Westergren method. An ESR >20 mm/h was considered positive (18). Rheumatoid factor (RF) and C-reactive protein (CRP) were measured by immunonephelometry, RF >15 U/ml and CRP >8 mg/l were considered positive (19,20). Anti-cyclic citrullinated peptide (anti-CCP) antibody levels were measured by chemiluminescence microparticle immunoassay, anti-CCP titers <5.0 RU/ml were judged as negative, and ≥5.0 RU/ml as positive (21). The Disease Activity Score 28 (DAS28) were then used to assess RA disease activity. For each patient in the validation cohort, the disease severity was assessed according to the DAS28 (22). A total of 10 healthy donors (8 females and 2 males; mean age, 50 years) with no history of autoimmune diseases or administration of immunosuppressive therapy were used as the control group. The present study was approved by the Institutional Review Board (IRB) of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China; affiliated to Nanjing University of Chinese Medicine; approval no. 2016NL-KS14).

**RNA extraction.** Total RNA from peripheral blood was extracted using TRIzol<sup>®</sup> (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Subsequently, total RNA was evaluated by 1% agarose gel electrophoresis and observation under ultraviolet transmitted light, quantified using a NanoDrop ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

**High-throughput sequencing analysis.** High-throughput sequencing was performed using an Arraystar v.3.0 (Arraystar, Inc.). Genome-wide analysis of lncRNA and mRNA expression profiles in peripheral blood samples from two patients with RA and two healthy donors was performed using Arraystar Human lncRNA Microarray version 3.0. In brief, RNA extraction and evaluation were performed as described above. Sample labeling (Labeling Kit Agilent p n 5190-0442; Agilent Technologies, Inc.)

and array hybridization was performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Inc.). Specific experimental process: mRNA was purified from total RNA using an mRNA isolation kit after the removal of ribosomal RNA (mRNA-ONLY<sup>™</sup> Eukaryotic mRNA Isolation kit; Epicentre; Illumina, Inc.). Subsequently, each sample was amplified and transcribed into fluorescent cRNA (complementary RNA) using a mixture of oligo (dT) and random primers (Arraystar Flash RNA Labeling kit; Arraystar, Inc.). The labeled cRNAs were purified using an RNeasy Mini kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol cyanine 3/μg cRNA) were measured using a NanoDrop-1000 (Thermo Fisher Scientific, Inc.). A total of 1 μg of each labeled cRNA was fragmented by adding 5 μl 10X Blocking Agent (Agilent Technologies, Inc.) and 1 μl of 25X Fragmentation Buffer. The mixture was heated at 60°C for 30 min, after which 25 μl 2x GE Hybridization buffer (Agilent Technologies, Inc.) was added to dilute the labeled cRNA. A total of 50 μl of hybridization solution was dispensed into a gasket slide and assembled to obtain the lncRNA microarray slide. The slides were incubated at 65°C in an Agilent Hybridization oven for 17 h. The hybridized arrays were washed, fixed and scanned with the Agilent DNA Microarray Scanner (cat. no. G2505C; Agilent Technologies, Inc.).

**CD4<sup>+</sup> T-cell isolation and total RNA extraction.** CD4<sup>+</sup> T cells in the peripheral blood of each participant were separated by gradient centrifugation at 400 x g for 30 min at 4°C and enriched using magnetic beads. In brief, an equivalent volume of PBS was added to the peripheral blood and the sample was agitated until it was evenly distributed. Subsequently, the mixture was added to the lymphocyte separation solution, followed by centrifugation at 400 x g for 30 min at 4°C to obtain peripheral blood mononuclear cells. CD4<sup>+</sup> T cells were isolated using a BD IMag<sup>™</sup> cell separation protocol using the following reagents: Anti-human CD4 particles-dm CD (cat. no. 557767) and IMag buffer (cat. no. 552362; both from BD Biosciences). Separated CD4<sup>+</sup> T cells were frozen in pre-chilled RNase-free vials for 5 min in liquid nitrogen and stored at -78°C prior to RNA extraction.

**Reverse transcription (RT)-qPCR.** Of the differentially expressed lncRNAs, 21 were selected (Table SI) according to the following criteria: i) ≥10-fold up- or downregulation in expression; ii) the sequence of the lncRNA was in the database (LncRNADisease; <http://cmbi.bjmu.edu.cn/lncrnadisease>) and was closely associated with RA. For RT reactions, Super Script<sup>™</sup> III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) was used. qPCR was performed using specific primers, the sequences of which are provided in Table SII. The thermocycling conditions were as follows: PCR assay was set at an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 1 min. The ViiA 7 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for PCR amplification. PCR amplification was performed in triplicate. The results were quantified using the 2<sup>-ΔΔC<sub>q</sub></sup> method (23).

**Flow cytometry and ELISA.** Samples were washed with PBS and fixed with immobilization reagent (BD Biosciences). Type

17 T-helper (Th17) cells were stained with allophycocyanine mouse anti-human CD4 (cat. no. 561840; BD Biosciences) for 20 min at 37°C, phycoerythrin (PE) mouse anti-human interleukin (IL)-17A (cat. no. 580487; BD Biosciences) for 30 min at 4°C, PerCP-Cy™ 5.5 mouse anti-human CD25 (cat. no. 580503; BD Biosciences) for 20 min at 4°C, PE mouse anti-human CD127 (cat. no. 561028; BD Biosciences) for 20 min at 4°C. A total of  $1 \times 10^6$  of cells were resuspended in 1% BSA-PBS and the volume of fluorescent antibodies added in to cells were as listed below: CD4: 1  $\mu$ l; IL-17A: 2  $\mu$ l; CD25: 2  $\mu$ l and CD127: 2  $\mu$ l. After incubation with the antibodies, cells were analyzed using flow cytometry on a BD FACSCalibur (BD Biosciences).

ELISA kits (cat. nos. m1037279 and m1028599; Shanghai Enzyme Link) were used to detect the levels of IL-17 and transforming growth factor (TGF)- $\beta$  in human serum (1:5 dilution), according to the manufacturer's instructions.

**Volcano plots and hierarchical cluster analyses.** Microarray data were log-transformed and subjected to quantile normalization. After elimination of unreliable transcripts, the remaining data of lncRNA and mRNA expression were further analyzed. The transcripts were distributed according to statistical significance (y-axis) and magnitude of change (log<sub>2</sub> ratio of RA group/normal control group; x-axis). Volcano plots were used for visualizing genes that were differentially expressed between the two groups. Hierarchical cluster analysis was performed to display RNA expression profiles between different samples.

**Bioinformatics analysis.** Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.ad.jp/kegg) database and Gene Ontology (GO; www.geneontology.org) analysis were used to explore the potential role of differentially expressed mRNA in biological pathways, including biological process, cellular component and molecular function.

**Co-expression network.** Co-expression analysis was performed based on the Pearson correlation coefficient between the coding and non-coding gene transcripts. Pearson correlation coefficient  $\geq 0.90$ ,  $P < 0.01$  and false discovery rate  $< 0.01$  were used as thresholds and a coding-non-coding network was plotted using Cytoscape version 2.8.2 (<http://cytoscape.org>).

**Statistical analysis.** Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). A non-parametric Mann-Whitney U-test was used to compare gene expression between two groups. Spearman rank correlation analyses were performed and coefficients were determined to assess the correlation between expression levels of lncRNAs and clinical features.  $P < 0.05$  (bilateral) was considered to indicate a statistically significant difference.

## Results

**Differentially expressed lncRNAs and mRNAs in the peripheral blood of patients with RA.** Aberrantly expressed mRNAs and lncRNAs associated with RA were identified based on the following criteria:  $\geq 2$ -fold upregulation or  $< 2$ -fold downregulation in expression and  $P < 0.05$ . To visualize

Table I. Plasma levels of IL-17 and TGF- $\beta$  in the RA group and the normal control group (pg/ml).

Group	n	IL-17	TGF- $\beta$
RA patients	12	392.09 $\pm$ 71.69 <sup>a</sup>	218.47 $\pm$ 49.81
Normal	8	307.27 $\pm$ 53.87	274.28 $\pm$ 75.43
P-values		0.011	0.061

<sup>a</sup> $P < 0.05$  compared with the normal group. IL, interleukin; TGF, transforming growth factor; RA, rheumatoid arthritis.

the differentially expressed lncRNAs and mRNAs, scatter plots (Fig. 1A and B) and volcano plots (Fig. 1C and D) were analyzed to further explore the differences in peripheral blood between patients with active RA and normal people. A total of 493 differentially expressed lncRNAs and 374 mRNAs were identified in the peripheral blood of patients with RA, of which 275 lncRNAs and 193 mRNAs were upregulated and the remaining ones were downregulated. The results of hierarchical clustering analysis showed distinguishable lncRNA and mRNA expression profiles between patients with active RA and normal people. (Fig. 1E and F).

**Classification of lncRNAs.** Among the 275 significantly upregulated lncRNAs, 116 were associated with intergenic interaction, 53 were natural antisense, 39 were intronic antisense, 36 were exon sense overlapping, 12 were bidirectional and 12 were other types of interactions. Of the 208 significantly downregulated lncRNAs, 108 of these were associated with intergenic interactions, 26 were natural antisense, 21 were intronic antisense, 45 were exon sense overlapping, 4 were bidirectional and 14 were other types of interactions (Fig. 1G and H).

**qPCR validation of lncRNA in peripheral blood.** Further identification was performed with qPCR on peripheral blood plasma of 5 patients and 5 healthy individuals. Based on these results, 7 of the 11 upregulated lncRNAs and 7 of the 10 downregulated lncRNAs obtained by chip were confirmed as differentially expressed (Fig. 2).

**Pathway and GO analyses.** As lncRNAs are non-coding RNAs that are not translated and the majority of them are structurally similar to mRNA, their function may be reflected in the associated mRNAs. Pathway and GO enrichment analysis of differentially expressed mRNAs further demonstrated the regulatory role of lncRNAs (Fig. 3). Pathway analysis indicated that differentially expressed mRNAs in peripheral blood from patients with RA were associated with multiple signaling pathways, including mitogen-activated protein kinase (MAPK) and P53 (Fig. 3G and H). These mRNAs were further characterized as being involved in the regulation of processes associated with cell growth and immune regulation (Fig. 3A-F). Of note, the MAPK and P53 signaling pathways are involved in the differentiation of CD4<sup>+</sup> T cells, which serves an important role in the pathogenesis of RA.

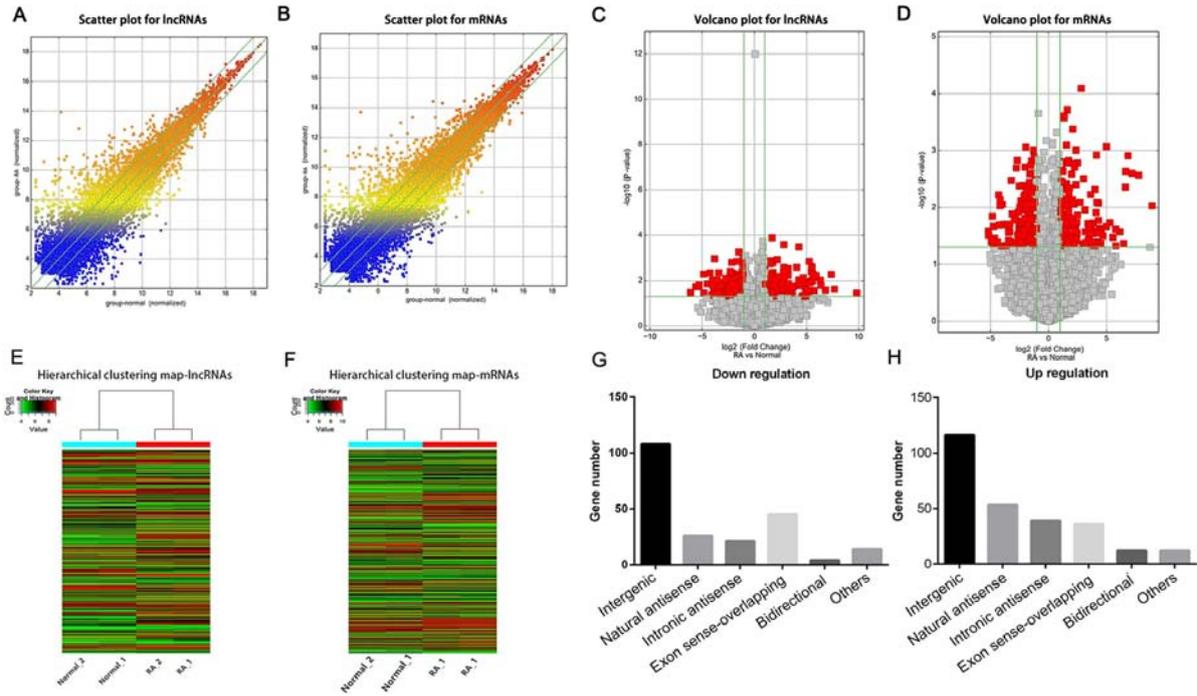


Figure 1. Analysis of lncRNAs and mRNAs in patients with RA and healthy individuals. Scatter-plots of differences in the (A) lncRNA and (B) mRNA expression. The x and y axes display the mean normalized signal values (log<sub>2</sub> scaled). The green lines represent a 2-fold change. Volcano plots of differentially expressed (C) lncRNAs and (D) mRNAs. The horizontal green lines represent a P-value of 0.05 and vertical green lines represent a 2.0-fold change. The x-axis displays the fold change values (log<sub>2</sub> scaled) and the y-axis displays the P-value (log<sub>10</sub> scaled). Hierarchical clustering analysis of (E) lncRNAs and (F) mRNAs with expression changes >2-fold and P<0.05. Relatively high expression is indicated by red shading and relatively low expression is indicated by green shading. Classification analyses of (G) upregulated and (H) downregulated lncRNAs in peripheral blood of patients with RA as detected by microarray analysis. Groups: RA1 and RA2, RA patient group; Normal 1 and Normal 2, healthy control group. lncRNA, long non-coding RNA; RA, rheumatoid arthritis.

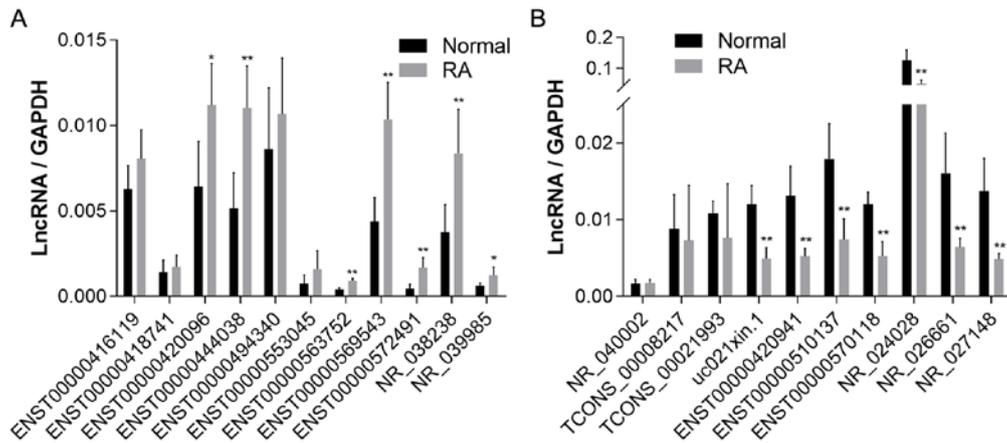


Figure 2. Relative expression of lncRNAs in peripheral blood determined using quantitative PCR. (A) Expression of the 11 upregulated lncRNAs (ENST00000416119, ENST00000563752, ENST00000572491, ENST00000553045, ENST00000494340, ENST00000418741, ENST00000569543, ENST00000420096, ENST00000444038, NR\_038238 and NR\_039985) in the peripheral blood of patients with RA. (B) Expression of the 10 downregulated lncRNAs (ENST00000510137, ENST00000570118, NR\_024028, TCONS\_00021993, uc021xin.1, TCONS\_00008217, ENST00000420941, NR\_026661, NR\_040002 and NR\_027148) in the peripheral blood of patients with RA. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001 vs. normal. ENST, Ensembl transcript; lncRNA, long non-coding RNA; RA, rheumatoid arthritis.

*qPCR validation of lncRNA in CD4<sup>+</sup> T cells.* To further investigate the alternative expression of lncRNAs in CD4<sup>+</sup> T cells from patients with RA, peripheral blood was collected from 12 patients with RA and 8 healthy individuals. CD4<sup>+</sup> T cells were isolated with magnetic beads. Based on the qPCR results, one of the less differentially expressed lncRNAs were excluded for analysis in CD4<sup>+</sup> T cells and finally, a total of

13 lncRNAs (those previously identified in plasma) were validated. The results suggested that the differential expression of 7 of the lncRNAs upregulated in plasma was consistent with that observed in CD4<sup>+</sup> T lymphocytes. Furthermore, two of the lncRNAs downregulated in plasma were also observed to be downregulated in CD4<sup>+</sup> T lymphocytes (Fig. 4) *differentiation of CD4<sup>+</sup> T cells in patients with RA.* Differentiation of CD4<sup>+</sup>

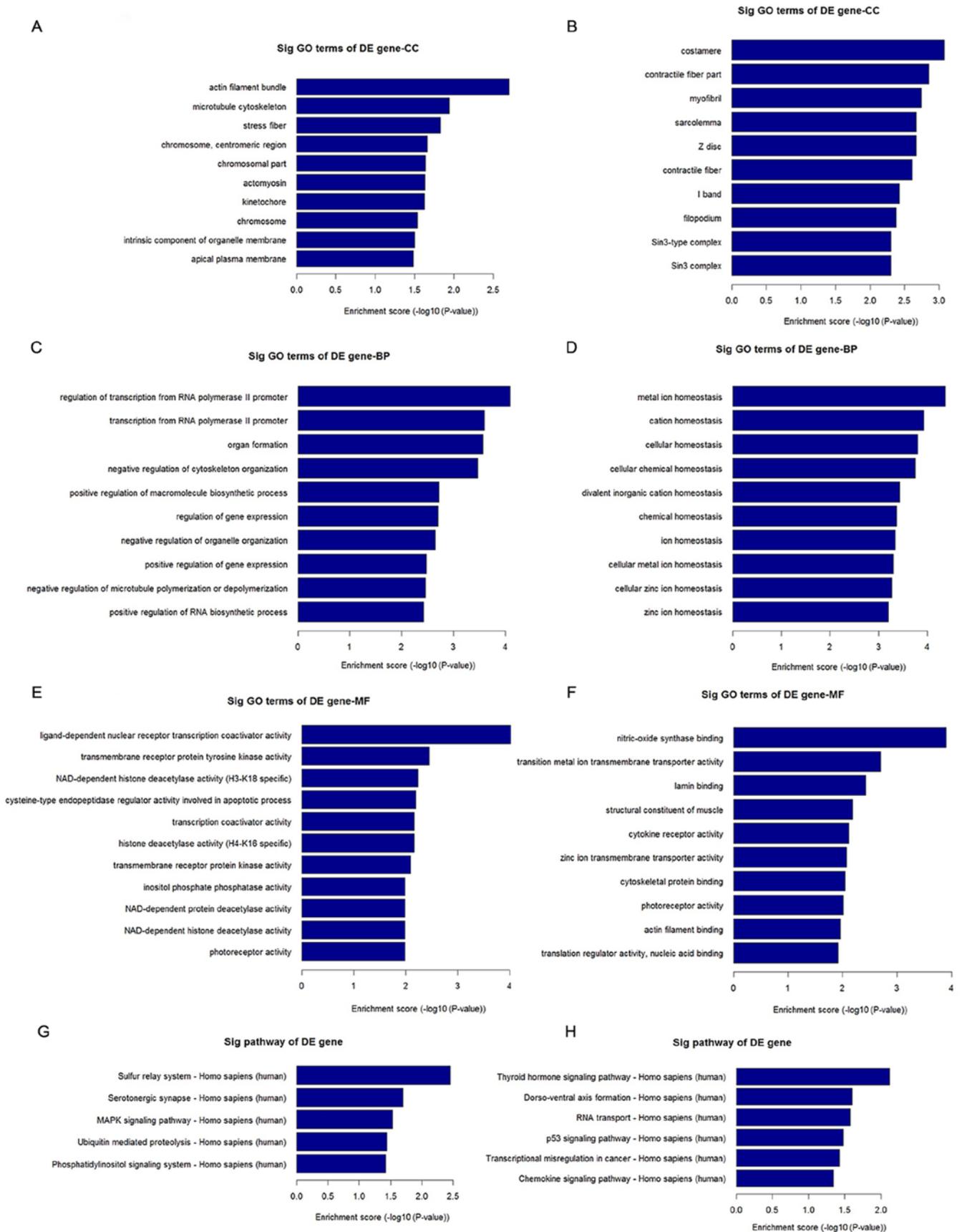


Figure 3. Enrichment analysis of GO terms and pathways of the differentially expressed mRNAs. (A) Top 10 GO terms in the categories CC for upregulated mRNA; (B) Top 10 GO terms in the categories CC for downregulated mRNA; (C) Top 10 GO terms in the categories BP for upregulated mRNA; (D) Top 10 GO terms in the categories BP for downregulated mRNA; (E) Top 10 GO terms in the categories MF for upregulated mRNA; (F) Top 10 GO terms in the categories MF for downregulated mRNA. (G and H) Top 10 pathways that exhibited significant differences between the patients with RA and healthy controls. From left to right, the upregulated and downregulated coding genes are displayed. Sig, significant; DE, differentially expressed; GO, gene ontology; CC, cellular component; MF, molecular function; BP, biological process; RA, rheumatoid arthritis; MAPK, mitogen-activated protein kinase.

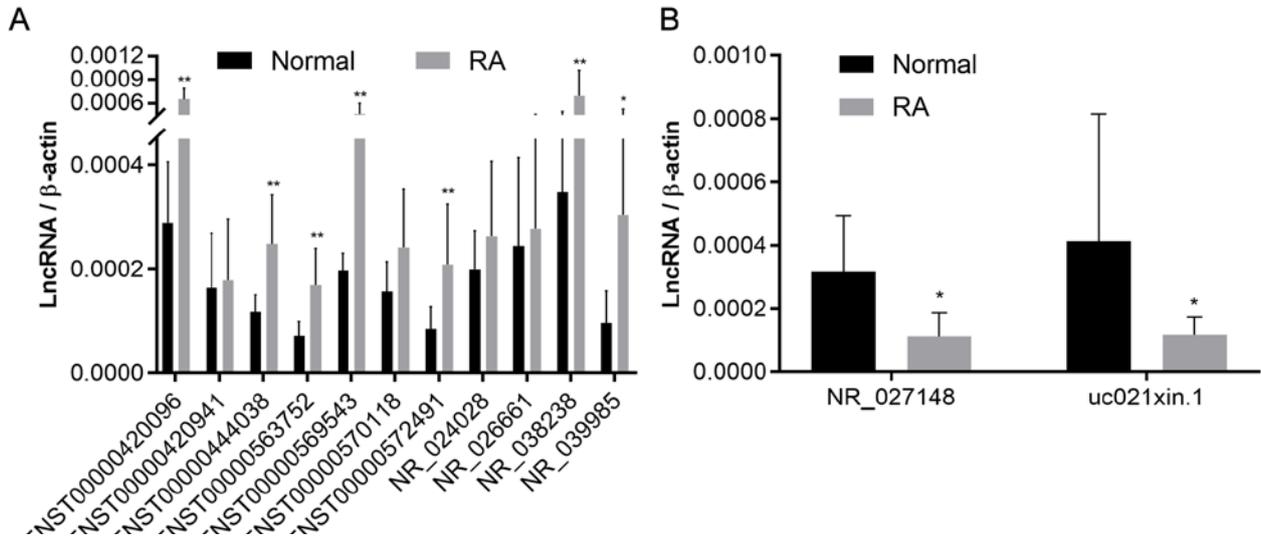


Figure 4. Relative expression of lncRNAs in CD4<sup>+</sup> T cells determined using quantitative PCR. (A) Expression of ENST00000420096, ENST00000563752, ENST00000572491, ENST00000444038, NR\_039985, NR\_038238, NR\_026661, NR\_024028, ENST000004200941, ENST00000570118 in CD4<sup>+</sup> T cells. (B) Expression of uc021xin.1 and NR\_027148 in CD4<sup>+</sup> T cells. \*P < 0.05, \*\*P < 0.01 vs. normal. ENST, Ensembl transcript; RA, rheumatoid arthritis; lncRNA, long non-coding RNA.

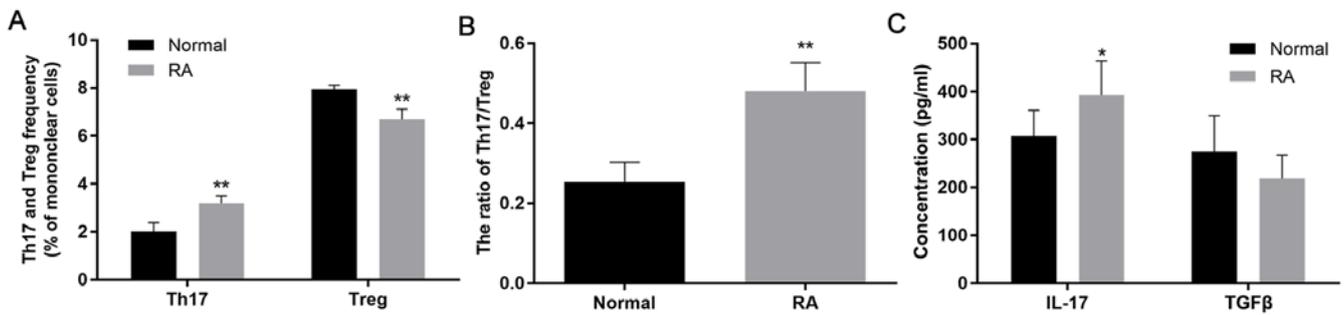


Figure 5. Differentiation of CD4<sup>+</sup> T cells and expression of IL-17 and TGF-β. (A) The percentage of Treg and Th17 cells was determined using flow cytometry and compared to that in the healthy controls. The portion of Th17 was increased in the patients with RA and the portion of Treg cells was significantly reduced compared with that in the healthy controls. (B) The ratio of Th17/Treg was significantly increased in patients with RA. (C) ELISA was used to detect the levels of Th17 and TGF-β. \*P < 0.05, \*\*P < 0.01 vs. normal. RA, rheumatoid arthritis; IL, interleukin; TGF, transforming growth factor; Treg, T-regulatory cells.

T cells from patients with RA was observed. The ratio of Th17/T-regulatory (Treg) cells was upregulated in patients with active RA by flow cytometry (Fig. 5A and B). IL-17 and TGF-β are important cytokines that are considered as regulatory sensors in RA (24,25). The results also suggested an increase in the levels of IL-17 and a decrease in the release of TGF-β in patients with RA, although there was no significant difference in TGF-β compared to the normal control group (Fig. 5C; Table I), demonstrating a difference in the differentiation of CD4<sup>+</sup> T cells.

**Construction of the coding-non-coding gene co-expression networks.** Functional prediction of lncRNAs was performed based on the function of the co-expressed mRNAs. A co-expression network for patients with RA was constructed based on the correlation analysis between differentially expressed lncRNAs and mRNAs. A total of 9 central lncRNAs were selected for the co-expression networks (uc021xin.1, ENST00000444038, NR\_038238, NR\_039985, NR\_027148, ENST00000563752, ENST00000572491, ENST00000569543, ENST00000492209 and ENST00000570118), which were

verified using qPCR (Fig. 6). The results indicated that C-C motif chemokine ligand (CCL)19, CD74 and adaptor-related protein complex 3 subunit δ1 were correlated with lncRNAs detected in the network, which were also closely associated with T-cell differentiation. RT-qPCR further validated CCL19 as an upregulated signaling factor in patients with RA (Fig. 7).

**Diagnostic value of lncRNA for RA.** ROC curves were used to assess the potential diagnostic value of these 9 lncRNAs in RA. The areas under the ROC curves of ENST00000420096, ENST00000444038, ENST00000563752, ENST00000569543, ENST00000572491, NR\_027148, NR\_038238, NR\_039985 and uc021xin.1 were 0.9792, 0.9479, 0.9896, 1.0000, 0.9167, 0.8750, 0.8333, 0.8958 and 0.8438, respectively (Table II, Fig. 8).

**Correlation analysis between lncRNA expression and clinical indicators of RA.** ESR, CRP, RF and CCP antibodies are conventional indicators for clinical diagnosis of RA (26). DAS28 is an internationally recognized method for determining RA activity. Complement 3 (C3) and C4 are involved in RA autoimmune disorders and inflammatory pathological

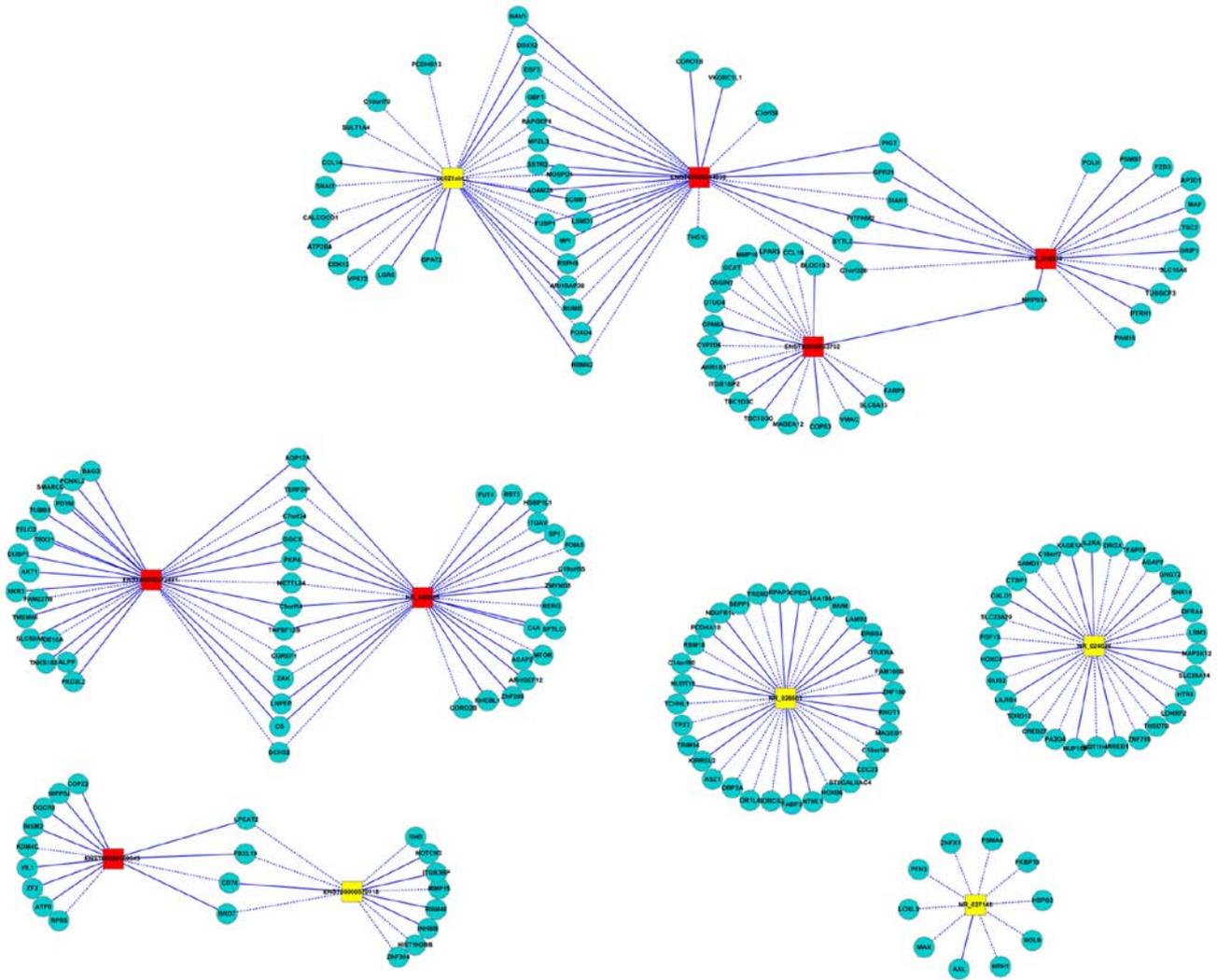


Figure 6. Co-expression network of differentially expressed lncRNAs and mRNAs. The blue circular nodes represent coding genes; the red square nodes represent upregulated non-coding lncRNAs; the yellow square nodes represent downregulated non-coding mRNAs; solid lines between two nodes represent a positive correlation and dashed lines represent a negative correlation. lncRNA, long non-coding RNA.

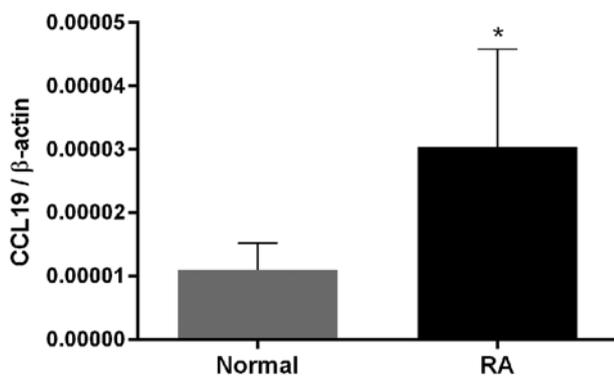


Figure 7. Relative expression of CCL19 determined using quantitative PCR. \*P<0.05 vs. normal. CCL19, C-C motif chemokine ligand 19.

processes (27). Pearson correlation analysis was used to analyze the correlation between target lncRNAs and ESR, CRP, RF, anti-CCP antibody and DAS28. The correlation coefficient between ENST00000569543 and C4 was 0.623 (P<0.05) and

the correlation coefficient between ENST00000420096 and anti-CCP antibody or the DAS 28 score was 0.662 and 0.605, respectively (P<0.05; Table III).

### Discussion

With the development of high-throughput gene sequencing technology, numerous lncRNAs have been identified and demonstrated to exert biological functions through interactions with other cellular macromolecules (28). lncRNAs are expressed in various types of immune cells and participate in the differentiation and activation of these cells (29). RA is an autoimmune disorder, with CD4<sup>+</sup> T cells differentiating into various Th-cell subsets that are thought to be deeply involved in the pathogenesis of RA (30). However, only few studies have assessed lncRNAs associated with CD4<sup>+</sup> T-cell differentiation in peripheral blood of patients with active RA (8,31). In the present study, the expression of lncRNAs in CD4<sup>+</sup> T cells from peripheral blood of patients with active RA was assessed to provide novel insight into the pathogenesis of RA.

Table II. Parameters of the receiver operating characteristic curve analysis.

lncRNA	AUC	Std. Error	95% confidence interval	P-value
ENST00000420096	0.9792	0.02681	0.9266-1.032	0.0003903
ENST00000444038	0.9479	0.05363	0.8428-1.053	0.0009147
ENST00000563752	0.9896	0.01678	0.9567-1.022	0.0002906
ENST00000569543	1.000	0.0000	1.000-1.000	0.0002151
ENST00000572491	0.9167	0.06784	0.7837-1.050	0.002039
NR_027148	0.8750	0.07743	0.7232-1.027	0.005499
NR_038238	0.8333	0.09317	0.6507-1.016	0.01359
NR_039985	0.8958	0.07068	0.7573-1.034	0.003386
uc021xin.1	0.8438	0.09240	0.6626-1.025	0.01093

AUC, area under curve; Std, standard.

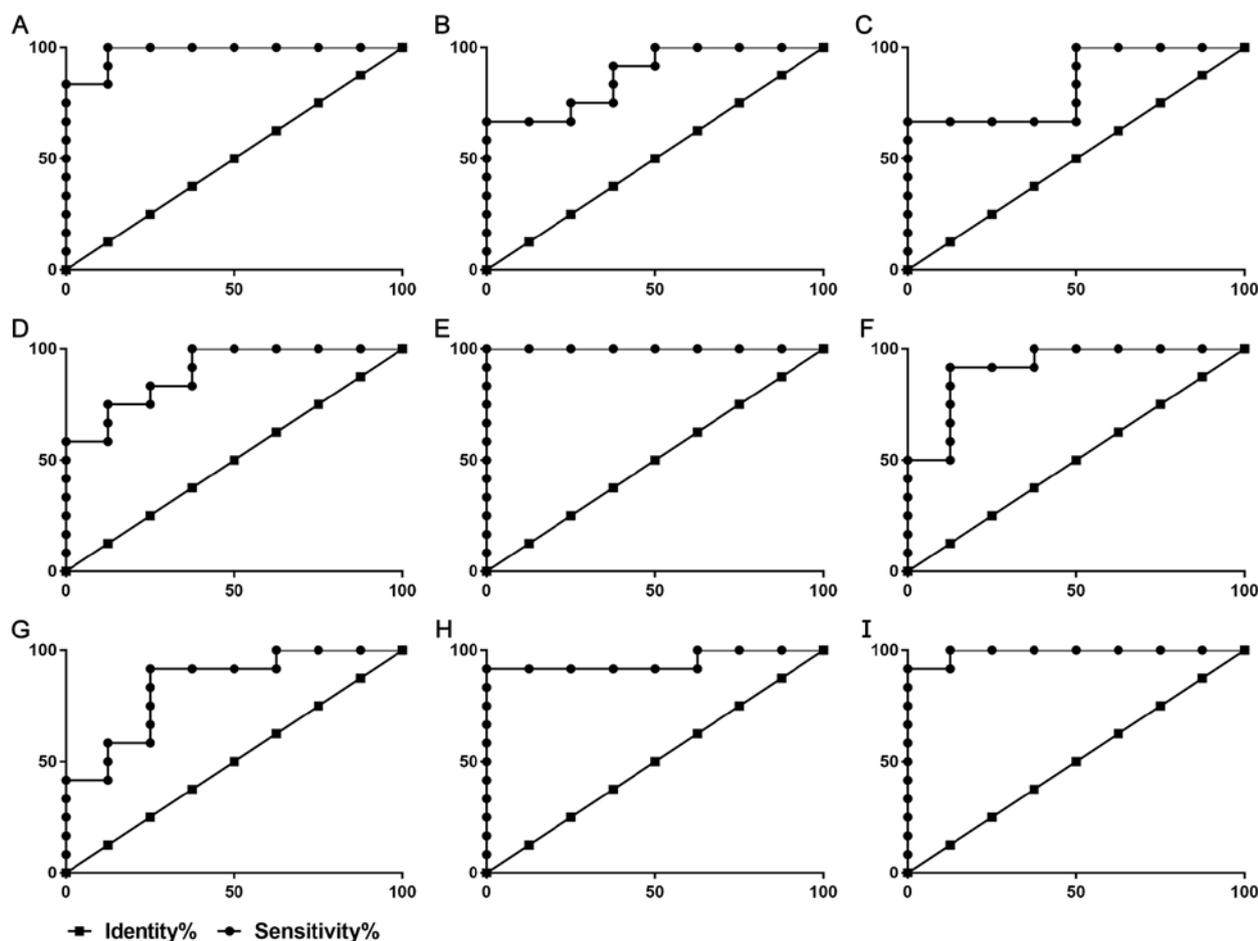


Figure 8. ROC curves for various long non-coding RNAs to differentiate between healthy individuals and patients with rheumatoid arthritis. The x-axis displays the Specificity % and the y-axis displays the Sensitivity %. ROC curves for patients RA based on the expression of (A) ENST00000420096, (B) NR\_027148, (C) NR\_038238, (D) NR\_039985, (E) ENST00000569543, (F) ENST00000572491, (G) uc021xin.1, (H) ENST00000444038, (I) ENST00000563752. RA, rheumatoid arthritis; ROC, receiver operating characteristic.

Based on the microarray data, 493 lncRNAs and 374 differentially expressed mRNAs were identified. lncRNA functional annotations are still not fully available; however, the majority of the mRNAs identified are well annotated. Pathway and GO enrichment analysis of significantly differentially

expressed mRNAs revealed that these mRNAs in the peripheral blood of patients with RA were primarily involved in the regulation of processes associated with cell growth and immune regulation. Further analysis demonstrated that these differentially expressed mRNAs were associated with the

Table III. Correlation analysis between lncRNA and major indicators of RA.

lncRNA	Correlation coefficient with major indicators of RA							
	ESR	RF	CRP	C3	C4	CRP	Anti-CCP	DAS 28
ENST00000420096	0.419	-0.013	0.490	-0.298	-0.095	0.490	0.662 <sup>a</sup>	0.605 <sup>a</sup>
ENST00000444038	0.551	0.314	-0.203	-0.371	0.437	-0.203	0.136	0.560
ENST00000563752	0.263	-0.085	-0.180	-0.095	0.206	-0.180	-0.126	0.147
ENST00000569543	0.190	0.376	-0.327	-0.237	0.623 <sup>a</sup>	-0.327	0.236	0.207
ENST00000572491	0.460	0.068	0.002	-0.207	0.181	0.002	-0.146	0.401
NR_027148	-0.002	-0.492	0.200	0.166	-0.026	0.200	-0.325	-0.123
NR_038238	0.182	0.331	-0.169	0.215	0.241	-0.169	-0.028	-0.002
NR_039985	0.049	0.400	-0.412	-0.245	0.529	-0.412	0.172	0.100
uc021xin.1	-0.111	-0.097	-0.198	-0.561	-0.123	-0.198	-0.126	0.044

<sup>a</sup>P<0.05. RA, rheumatoid arthritis; lncRNA, long non-coding RNA; RF, rheumatoid factor; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; C3, component 3; DAS, disease activity score; anti-CCP, anti-cyclic citrullinated peptide antibody. Correlation coefficient was calculated using the Pearson coefficient method.

activation of the MAPK and P53 signaling pathways. MAPK phosphorylation results in the activation of the downstream inflammatory transcription factor NF- $\kappa$ B, which may control the activation of the Th17-associated cytokine IL17 and the Treg-associated cytokine TGF- $\beta$  (32,33); this is consistent with the present results. P53 serves an important role in the differentiation of Treg cells and the homeostasis of CD4<sup>+</sup> T cells (33-35). The present study suggested that lncRNAs are involved in the pathogenesis of RA, which is possibly regulated by the differentiation of CD4<sup>+</sup> T lymphocytes.

A co-expression network based on 9 lncRNAs was constructed. Of these, CCL19 was an overexpressed chemokine in patients with RA. Various studies have indicated that CCL19 serves a chemotactic role in B cells, dendritic cells and naïve T cells via binding to the C-C motif chemokine receptor (CCR)7 (36-38). CCR7 is an important antigen molecule expressed on the surface of Treg cells and CCL19 may specifically bind to the receptor CCR7, which in turn exerts chemotactic activity on Treg cells (39-41). The results of the present study suggested that CCL19 is upregulated in patients with active RA, implying that lncRNA may affect CD4<sup>+</sup> T lymphocytes by regulating mRNA-CCL19, which in turn affects the differentiation of cells. Conversely, the CCL19/CCR7 signaling pathway is primarily activated through ERK and p38-associated proteins of the MAPK family (42), which suggests that the activity of MAPK is closely associated with CCL19/CCR7. These results confirmed that lncRNA is closely involved in the differentiation of CD4<sup>+</sup> T cells in patients with RA.

Numerous studies have examined the diagnostic value of various lncRNAs in different types of diseases (43-47). Studies have investigated the possibility of lncRNAs in fibroblast synoviocytes as biomarkers for RA (48). The present study explored the diagnostic value of peripheral blood lncRNAs in RA. ROC curves are generally used to assess the diagnostic value of markers for a certain disease (49,50). The association between lncRNAs and the degree of RA activity was also analyzed. Pearson correlation analysis suggested that there was a strong correlation between ENST00000569543 and C4 and

between ENST00000420096 with anti-CCP antibody or DAS. Anti-CCP is a marker that may be used to diagnose RA (51). DAS is generally considered the 'gold standard' for measuring RA disease activity (52,53). In the present study, these two lncRNAs identified in the screen and confirmed by PCR were most closely associated with RA, which suggested that they may be potential biomarkers for judging RA disease activity.

In conclusion, a total of 9 lncRNAs were identified to be involved in the pathogenesis of RA and serve an important role in the differentiation of CD4<sup>+</sup> T cells. ROC curve analysis indicated that these lncRNAs may also possess diagnostic value for RA, and ENST00000569543 and ENST00000420096 were most relevant. These results provide preliminary evidence for the further study of lncRNAs and their association with RA. Due to the difficulty in obtaining samples, the sample size was small, which is a limitation of the present study, also regarding the detection of IL-17 and TGF- $\beta$ . Furthermore, the value of lncRNAs in the diagnosis of RA was preliminarily explored, while horizontal comparative studies of other autoimmune diseases or osteoarthritis remain to be performed in the future.

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#### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on

reasonable request. All of the data from the microarray trials are stored in the Gene Expression Omnibus database (dataset no. GSE134087, <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE134087>).

### Authors' contributions

XZ and LZ conceived and designed the study. ML, KM and JW performed the experiments. ML and ZF analyzed and interpreted the experimental data. ML and KM wrote the manuscript. XZ, LZ and ZF reviewed and edited the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

All patients signed informed consent forms and the study was approved by the IRB of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (Nanjing, China; no. 2016NL-KS14).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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