Differential long non-coding RNA expression profiles in the peripheral blood and CD4+ 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+ 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+ 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+ 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+ 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+ T cells, which are aberrantly activated adaptive immune cells in multiple autoimmune diseases (13-15). Dysregulated differentiation of CD4+ T cells alter the balance of interactions between the CD4+ T-cell subgroups and may be an important factor leading...
to the development of RA disease (16). As such, it may be possible to identify potential therapeutic targets by analyzing the differential expression of IncRNAs. In the present study, the IncRNA expression profiles in the peripheral blood and CD4⁺ T cells of healthy donors were compared with those of patients with active RA using high-throughput sequencing. Further verification was performed using quantitative qPCR and the results supported the potential involvement of specific IncRNA in CD4⁺ 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 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 IncRNA 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⁺ T-cell isolation and total RNA extraction. CD4⁺ 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⁺ T cells were isolated using a BD IMag™ cell separation protocol following the following reagents: Anti-human CD4 particles-dm CD (cat. no. 557767) and IMag buffer (cat. no. 552362; both from BD Biosciences). Separated CD4⁺ 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 IncRNAs, 21 were selected (Table SI) according to the following criteria: i) ≥10-fold up- or downregulation in expression; ii) the sequence of the IncRNA was in the database (IncRNAdbase; http://cmbi.bjmu.edu.cn/IncnaRdbase) and was closely associated with RA. For RT reactions, SuperScript™ 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^ΔΔCq 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 for 20 min at 4°C. A total of 1x10^6 of cells were resuspended in 1% BSA‑PBS and the volume of fluorescent antibodies added to cells were as listed below: CD4: 1 µl; IL-17A: 2 µl; CD25: 2 µl and CD127: 2 µl. 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)‑β 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 (log2 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 ≥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 IncRNAs and clinical features. P<0.05 (bilateral) was considered to indicate a statistically significant difference.

Results

Differentially expressed IncRNAs and mRNAs in the peripheral blood of patients with RA. Aberrantly expressed mRNAs and IncRNAs associated with RA were identified based on the following criteria: ≥2-fold upregulation or <2-fold downregulation in expression and P<0.05. To visualize the differentially expressed IncRNAs 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 IncRNAs and 374 mRNAs were identified in the peripheral blood of patients with RA, of which 275 IncRNAs and 193 mRNAs were upregulated and the remaining ones were downregulated. The results of hierarchical clustering analysis showed distinguishable IncRNA and mRNA expression profiles between patients with active RA and normal people. (Fig. 1E and F).

Classification of IncRNAs. Among the 275 significantly upregulated IncRNAs, 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 IncRNAs, 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 IncRNA 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 IncRNAs and 7 of the 10 downregulated IncRNAs obtained by chip were confirmed as differentially expressed (Fig. 2).

Pathway and GO analyses. As IncRNAs 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 IncRNAs (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+ T cells, which serves an important role in the pathogenesis of RA.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>IL-17</th>
<th>TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA patients</td>
<td>12</td>
<td>392.09±71.69</td>
<td>218.47±49.81</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>307.27±53.87</td>
<td>274.28±75.43</td>
</tr>
<tr>
<td>P-values</td>
<td></td>
<td>0.011</td>
<td>0.061</td>
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*P<0.05 compared with the normal group. IL, interleukin; TGF, transforming growth factor; RA, rheumatoid arthritis.
qPCR validation of lncRNA in CD4+ T cells. To further investigate the alternative expression of lncRNAs in CD4+ T cells from patients with RA, peripheral blood was collected from 12 patients with RA and 8 healthy individuals. CD4+ 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+ 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+ T lymphocytes. Furthermore, two of the lncRNAs downregulated in plasma were also observed to be downregulated in CD4+ T lymphocytes (Fig. 4) differentiation of CD4+ T cells in patients with RA. Differentiation of CD4+
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.
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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+ 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, ENST00000420096, ENST00000563752, ENST00000572491, ENST00000569543, 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, 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
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 DAS28 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+ 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+ T-cell differentiation in peripheral blood of patients with active RA (8,31). In the present study, the expression of lncRNAs in CD4+ T cells from peripheral blood of patients with active RA was assessed to provide novel insight into the pathogenesis of RA.
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
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+ 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+ 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+ 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-β. 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

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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