Comprehensive analysis of long non-coding RNA and mRNA expression profiles in rheumatoid arthritis

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Received November 3, 2016; Accepted August 10, 2017

DOI: 10.3892/etm.2017.5284

Abstract. Abnormal expression of long non-coding RNA (lncRNA) has been demonstrated to be involved in a variety of human diseases. However, the role of lncRNA remains largely unknown in rheumatoid arthritis (RA). The present study aimed to investigate whether lncRNA are differentially expressed in RA. Differentially expressed lncRNA and mRNA in peripheral blood mononuclear cells from individuals with RA and healthy controls were detected using a human lncRNA microarray containing 30,586 lncRNA and 26,109 coding transcripts. Several candidate lncRNA and mRNA in 24 paired samples were verified by reverse transcription-quantitative polymerase chain reaction analysis. Bioinformatics analyses (Gene Ontology and Kyoto Encyclopedia of Genes and Genomes) were used to evaluate signaling pathways and biological functions influenced by the differentially expressed mRNA. A total of 5,045 IncRNA (upregulated, 2,410; downregulated, 2,635) and 3,289 mRNA (upregulated, 1,403; downregulated, 1,886) were differentially expressed in patients with RA (fold-change >2; P<0.05). The majority of abnormal IncRNA were from intergenic spacer regions (42%), natural antisense (19%) and intronic antisense (15%) to protein-coding loci. IncRNA target prediction indicated the presence of 135 potential lncRNA-mRNA target pairs for the 85 aberrant lncRNA and 109 aberrant mRNA. Significantly enriched (P<0.05) signaling pathways based on deregulated mRNA were mostly implicated in bile secretion, T cell receptor signaling pathway and systemic lupus erythematosus.

In summary, to the best of our knowledge, the present study executed global expression profiling of IncRNA and mRNA involved in RA for the first time. These results may provide important insights regarding lncRNA in RA pathogenesis and provide potential therapeutic targets.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that predominantly affects multiple peripheral joints and results in irreversible joint damage (1). Accumulating evidence suggests that the participation of inflammation-associated cells, as well as their production of proinflammatory mediators, including tumor necrosis factor (TNF)-α and interleukin-1β, serve a key role in the pathogenesis of RA (2). Although major strides in understanding the disease have been made, the specific mechanistic details underlying the pathogenesis of RA have not been fully clarified (2).

In the human genome, >70% is transcribed; however, only 1-2% encodes proteins (3,4). The majority of the remainder of the genome generates non-coding RNA (ncRNA) (5,6). According to their length, ncRNA may be divided into long ncRNA (lncRNA), which are arbitrarily defined as transcripts longer than 200 nt, or short ncRNA, including microRNA (miRNA) and short interfering RNA (7). It is well documented that miRNA-155, -146 and -21 are aberrantly expressed in RA (8-10), suggesting that miRNA serve critical roles in the regulation of RA pathogenesis. An increasing number of studies have suggested that lncRNA have a critical role in fundamental biological processes, including genomic imprinting, chromosome modification, immune response, inflammatory conditions, tumorigenesis, cellular development and metabolism, through comprehensive mechanisms (11,12). Emerging evidence has also indicated that the expression of lncRNA is dysregulated in a range of disorders, including cancer, intervertebral disc degeneration and Alzheimer's disease (13-15). Additionally, the deregulation of various lncRNA has been implicated in RA, including H19, Hotair, large intergenic non-coding (linc)RNA-p21, CST1lncRNA, LOC100652951 and LOC100506036 (16-20). Despite these notable developments, the alteration in lncRNA and mRNA expression levels induced by RA and the roles of these transcripts in modulating RA pathogenesis remain unclear.

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Key words: rheumatoid arthritis, long non-coding RNA, mRNA, peripheral blood mononuclear cells
The present study determined the expression patterns of lncRNA and mRNA in RA samples and compared them with healthy samples. Additionally, three lncRNA and three mRNA were confirmed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). lncRNA classification, subgroup analysis and genomic location analysis were performed to further analyze these differentially expressed lncRNA. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) analysis were used to predict the function and signaling pathways affected by the differentially expressed mRNA, which were target genes of aberrantly expressed lncRNA. The present results suggested that lncRNA expression patterns may provide pivotal insights into the pathogenesis of RA.

Materials and methods

Patients and control individuals. A total of 34 patients with RA (29 females and 5 males; mean age, 53.7 years) admitted to the Department of Rheumatology and Immunology, The First Affiliated Hospital of Nanchang University (Nanchang, China) between October 2014 and April 2016, were enrolled in the present study. The diagnosis of RA was established by the American College of Rheumatology criteria (21). The baseline characteristics of 10 of these patients that were used in microarray analysis are summarized in Table I. For RA patients, Disease activity was calculated using the disease activity score 28 system (22), anti-cyclic citrullinated peptide (CCP) antibody was detected by Anti-CCP Enzyme Immunoassay Test kit (cat. no. EC170502; Shanghai Kexin Biotech Co., Ltd., Shanghai, China) and values >25 RU/ml were considered positive. Erythrocyte sedimentation rate was examined by the Westergren method (23), and values ≥15 mm/h for males and ≥20 mm/h for females were considered normal. Serum C-reactive protein (CRP) and rheumatoid factor (RF) were measured by nephelometry. Values >8 mg/l for CRP and >20 IU/ml were considered positive for RF. A total of 34 healthy controls (29 females and 5 males; mean age, 50.2 years) were selected based on no history of autoimmune disease. Among the patients and controls, 10 patients with RA (Table I) and 10 age-matched and sex-matched normal controls were recruited for isolating peripheral blood mononuclear cells (PBMCs) for microarray analysis. The remaining 24 healthy controls and 24 patients with RA were used to validate the reliability of microarray results by RT-qPCR analysis.

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (approval no. 019) and was conducted in compliance with the Declaration of Helsinki. Informed consent was obtained from all participants, including patients and normal controls, prior to initiation of the study.

PBMC collection and RNA extraction. Blood samples were collected in vacutainer tubes containing EDTA, and PBMCs were isolated by centrifugation against a density. Gradient (1.000 x g, 20 min, 22˚C). Total RNA was isolated from freshly obtained PBMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The integrity of the RNA was assessed by electrophoresis on a 2% denaturing agarose gel and visualized using ethidium bromide. The concentration and quality of the RNA were assessed by absorbance spectrometry measuring absorbance ratios of A260/A280 and A260/A230 using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Microarray and computational analysis. Arraystar Human LncRNA Microarray V3.0 (8x60 K; Arraystar, Inc., Rockville, MD, USA) is designed for the global profiling of human LncRNA and mRNA, and is updated from the previous Microarray V2.0. The sample preparation and microarray hybridization were performed according to the manufacturer's protocol. Briefly, ribosomal RNA was removed from total RNA and subsequently mRNA was obtained using an mRNA-ONLY™ Eukaryotic mRNA Isolation kit (Epigencentre; Illumina, Inc., San Diego, CA, USA). The random priming method (24) was utilized to amplify each sample according to the manufacturer's protocol (Quick Amp Labeling kit; 5190-0442 Agilent Technologies, Inc., Santa Clara, CA, USA; amplification assay was set at 40˚C for 2 h, followed by 65˚C for 15 min and 0˚C for 5 min), and mRNA was transcribed into fluorescent cRNA without bias. Labeled cRNA were hybridized to the Human LncRNA Microarray, designed for 30,586 lncRNA and 26,109 mRNA. After washing with Gene Expression Wash Buffer 1 (Agilent Technologies, Inc.), and Gene Expression Wash Buffer 2 (Agilent Technologies, Inc.), the Agilent Scanner G2505C and Agilent feature extraction software (version 11.0.1.1; Agilent Technologies, Inc.) were used to scan and analyze the arrays. Quantile normalization and subsequent data processing were executed using the GeneSpring GX v12.0 software package (Agilent Technologies, Inc.). The microarray work was performed by Shanghai Kangchen Bio-tech, Inc., (Shanghai, China).

SYBR Green I RT-qPCR. The expression levels of differentially expressed LncRNA and mRNA were confirmed by RT-qPCR. Briefly, 5 µg of total RNA from each sample was used for the synthesis of first strand cDNA using a PrimeScript™ RT Reagent Kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RT reaction was carried out in a 10 µl reaction volume containing 5X PrimeScript™ Buffer, 1 µl RT specific primer, 0.5 µl PrimeScript™RT Enzyme Mix, 5 µg of total RNA. RT assay was set at an initial denaturation step at 37˚C for 15 min, followed by 85˚C for 5 sec. Following first strand cDNA synthesis, PCR reaction was performed in a 10 µl reaction volume containing 1X SYBR Green PCR Master mix (Takara Biotechnology Co., Ltd.), 0.4 µM of each specific forward and reverse primer, 0.5 µl of cDNA template. PCR assay was set at an initial denaturation step at 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min with an ABI 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were as shown in Table II. The gene expression levels were normalized to that of GAPDH in cDNA samples, and all samples were measured in triplicate. The relative expression levels of the genes were calculated using the ΔCt method where ΔCt=cT median gene-Ct median GAPDH (25,26).

Analysis of relationship between lncRNA and adjacent protein-coding genes. Target genes were predicted for
differentially expressed lncRNAs. LncRNAs originate in complex transcriptional loci and regulate gene expression through epigenetic regulation of chromatin modification, and transcriptional and post-transcriptional processing (27). In order to determine the underlying roles of lncRNA in RA and shed light on the potential mechanisms of RA, the present study mainly focused on unveiling the relationship between lncRNA and mRNA. Abnormal lncRNA (fold-change, ≥3.0) were subjected to bioinformatics analysis for target gene prediction. To increase the accuracy of target prediction, abnormal mRNA (fold-change, ≥3.0) were integrated with the predicted lncRNA targets.

**GO and KEGG pathway analysis based on differentially expressed mRNA.** GO (www.geneontology.org) and KEGG (www.genome.ad.jp/kegg) databases were utilized to analyze biological functions and signaling pathways affected by abnormally expressed mRNA (28,29). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**lncRNA and mRNA expression profiles in PBMCs of the different groups.** To profile differentially expressed lncRNA in RA, a microarray analysis of lncRNA and mRNA expression in the RA group and healthy group was performed (Figs. 1 and 2). The results revealed that 139 lncRNA (88 up and 51 down) and 92 mRNA (67 up and 25 down) were significantly differentially expressed between the RA group and healthy group (fold-change ≥10.0; P<0.05). Additionally, 5,045 lncRNA (2,410 up and 2,635 down) and 3,289 mRNA (1,403 up and 1,886 down) were differentially expressed with a fold change >2 (P<0.05). The most significantly deregulated lncRNA were ENST00000583574 (up fold-change, 257.7358) and ENST00000559539 (down fold-change, 4726.8003). The most significantly deregulated mRNA were ALAS2 (up fold-change, 52.16745) and KCNMB3 (down fold-change, 4999.96853) (data not shown).

**RT-qPCR validation.** To validate the reliability of the microarray results, three differentially expressed lncRNA and three differentially expressed mRNA were randomly selected and analyzed using RT-qPCR. For lncRNA, as demonstrated in Fig. 3, the results demonstrated that ENST00000445339 was significantly downregulated (P=0.042) and ENST00000506982 was significantly upregulated (P=0.019) in the 24 patients with RA compared to the levels in the healthy group (Fig. 3A and 3B). The primer sequences for RT-qPCR are listed in Table II.

**Table II. LncRNA and mRNA gene primers for polymerase chain reaction.**

| Gene          | Primer         |
|---------------|----------------|
| ENST00000445339 | S: GATGGAACGAGGAATGGTACGCA            |
|               | A: CAGTTTACGATCAGGAGTGTGGTC           |
| ENST00000569920 | S: TACCATCTGCATCCTCCCTACCCA            |
|               | A: CGGCCATTTACCTACCCCTCTTCA            |
| ENST00000506982 | S: CCTGTTAGCGGCTGATCGTGCA              |
|               | A: TCCGTCATGGTCACTGCGTTGTA            |
| PGLYRP1       | S: CATATGAAAGAACATGGCAAGTT               |
|               | A: CATGAACTGATCGCAATGGAC                |
| LRRN3         | S: GACACGCTTTTGCTCAAGACTGGA            |
|               | A: CAGGGTGCACCTTTGGTGT                 |
| CX3CR1        | S: AACTCCAGTGGTCTCCGTGGAACAAATCA      |
|               | A: CCACAACCTGGACACAGCA                 |
| GAPDH         | S: CCGGGAACACTGGCTGGTCATGGA           |
|               | A: AGCTGGGAGAGTGCTGGTGTCTGTT          |

S, sense; A, antisense; lncRNA, long noncoding RNA.

**Table I. Demographic, clinical and laboratory information of patients with RA.**

| Subject | Sex | Age, years | DAS28 | Erythrocyte sedimentation rate, mm/h | C-reactive protein, mg/l | Rheumatoid factor, IU/ml | Anti-cyclic citrullinated peptide, RU/ml |
|---------|-----|------------|-------|-------------------------------------|--------------------------|--------------------------|----------------------------------------|
| RA1     | Female | 55        | 5.03  | 37                                  | 8.30                     | 142.00                   | 670.70                                 |
| RA2     | Female | 54        | 4.81  | 14                                  | 2.15                     | 124.00                   | 106.30                                 |
| RA3     | Female | 43        | 5.50  | 12                                  | 2.04                     | 46.50                    | 155.60                                 |
| RA4     | Male    | 68        | 6.01  | 19                                  | 21.60                    | <20.00                   | 5.66                                   |
| RA5     | Male    | 37        | 6.39  | 33                                  | 16.10                    | 59.60                    | 20.61                                   |
| RA6     | Female | 68        | 4.46  | 24                                  | 27.90                    | <20.00                   | 39.90                                   |
| RA7     | Female | 68        | 4.12  | 10                                  | 5.02                     | 115.00                   | 175.10                                 |
| RA8     | Female | 49        | 7.33  | 126                                 | 81.50                    | 1,190.00                 | 493.60                                 |
| RA9     | Female | 43        | 7.38  | 84                                  | 6.95                     | 32.30                    | 382.00                                 |
| RA10    | Female | 52        | 5.25  | 40                                  | 4.06                     | 80.90                    | 1,600.00                               |

RA, rheumatoid arthritis; DAS28, disease activity score 28.
and C, respectively). No significant difference was observed in the expression of ENST00000569920 between RA patients and healthy controls (P=0.57; Fig. 3B). For mRNA, the expression of PGLYRP1 was significantly downregulated (P<0.0001), LRRN3 was significantly upregulated (P=0.0003) and CX3CR1 was significantly upregulated (P=0.048) in patients with RA compared with the levels in healthy controls (Fig. 3D-F). This demonstrated that RT-qPCR results were consistent with those of the microarray analysis.

lncRNA classification and subgroup analysis. Evidence from previous reports has indicated that lncRNA may be classified into lncRNA with enhancer-like functions, antisense lncRNA and lincRNA (26,30). The results of the present study indicated that 610 intergenic lncRNA (304 upregulated and 306 downregulated), 251 enhancer-like lncRNA (138 upregulated and 113 downregulated) and 134 antisense lncRNA (57 upregulated and 77 downregulated) were differentially expressed (fold-change ≥2.0; P<0.05) between the RA group and healthy group. Some nearby coding genes that may be regulated by these differentially expressed lncRNA were also identified (data not shown).

Integration of differentially expressed mRNA into the predicted targets of differentially expressed lncRNA. The
Recent results revealed that the majority of differentially expressed lncRNA were from intergenic regions (42%), natural antisense to protein-coding loci (19%), or intronic antisense to protein-coding loci (15%), with the others representing intronic sense-overlapping, exon sense-overlapping, and bidirectional regions. In addition, 2,859 of 5,045 differentially expressed lncRNA were oriented in or around a known protein-coding region (i.e. not intergenic). Therefore, differentially expressed lncRNA (fold-change, ≥3.0) were integrated with predicted mRNA (fold-change, ≥3.0). The result demonstrated that 109 differentially expressed protein-coding genes were markedly associated with each of the 85 differentially expressed lncRNA to generate 135 lncRNA-mRNA target pairs. Among them, 81 pairs were differentially expressed unidirectionally (upregulated or downregulated), while 54 pairs were differentially expressed bidirectionally (data not shown).

**GO and KEGG pathway analysis of deregulated mRNA.**

Through GO analysis, it was demonstrated that these abnormal transcripts of lncRNA were associated with biological process, cellular components and molecular function (Fig. 4). The analysis indicated that the differentially expressed mRNA between the RA group and healthy group were significantly enriched (P<0.05) in response to wounding, DNA packaging complex, chemokine activity, RNA metabolic process, nuclear lumen and DNA binding. Pathway analysis indicated that 23 upregulated pathways were identified, including systemic lupus erythematosus and alcoholism pathways. A total of 23 downregulated pathways were identified, including bile secretion and T cell receptor signaling pathway (Fig. 5).

**Discussion**

Over the past decades, expression profiles of genes involved in the pathogenesis of RA have been extensively explored (31,32). However, the molecular regulatory mechanisms underlying RA have not been fully elucidated. Increasing evidence indicates that lncRNA may serve crucial roles in various biological processes and may regulate gene expression in human disease (33,34). Few studies have reported that lncRNA have an important role in RA (16-20); however, fewer studies have investigated the expression profile of lncRNA in RA (16-20). Therefore, the present study aimed to better determine the potential role of lncRNA in contributing to RA.

PBMCs are crucial sentinels of host defense response, being used to identify novel disease mediators, disease variants
Figure 4. GO enrichment analysis for differentially regulated mRNA. GO analysis of (A) downregulated and (B) upregulated mRNA according to biological process. GO analysis of (C) downregulated.
Figure 4. Continued. (D) Upregulated mRNA according to cellular component. GO analysis of (E) downregulated and (F) upregulated mRNA according to molecular function. GO, Gene Ontology.
As we know, there are five kinds of lncRNA, including sense, antisense, bidirectional, intronic and intergenic. Various studies have revealed a variety of mechanisms by which lncRNA function; however, a common and key function of lncRNA is to alter the expression of nearby encoding genes by impacting the process of transcription (36,37). The present study identified notably different expression patterns of enhancer-like elements (36). In addition, the present study revealed that not all lncRNAs that were differentially expressed also exhibited differential expression in their associated protein-coding genes. This result suggested that these abnormal RA-associated lncRNA and mRNA may be independently regulated. Future studies are required to uncover their potential functional roles in RA disease by RNA interference or overexpression methods in an appropriate model system. However, the majority of the differentially expressed lncRNA associated protein-coding genes were not deregulated, suggesting that more complex mechanisms of regulation existed for these lncRNA.

To understand the underlying roles of lncRNA, GO category and KEGG pathway analyses were utilized to determine the target gene pool. These results predicted that downregulated and upregulated transcripts of lncRNA were associated with biological process, cellular component and molecular function, which were associated with 46 gene pathways that corresponded to transcripts, including ‘rheumatoid arthritis’ and ‘TNF signaling pathway’. These pathways may have essential roles in the occurrence and development of RA.

The present study only detected one RA group and one control group by microarray of gene chip. This is indeed a limitation of the study. In order to minimize the variation between groups, the chip assay was performed in the mode of a pooling test, according to previous reports (26,38) in the same research field. In future studies, we intend to investigate the microarray of gene chip in a larger sample size in triplicate.

In conclusion, the present study described the global expression profiling of lncRNA and mRNA in RA by microarray technology for the first time, which opened up a new and interesting pathway to provide novel insights into the relationship between lncRNA in PBMCs and RA. Additionally, the roles for the abnormal lncRNA in the regulation of the TNF signaling pathway and RA signaling pathways were researched.

Acknowledgements

The present study was financially supported by the National Natural Science Foundation of China (grant no. 81360459) and Jiangxi Provincial Natural Science Foundation of China (grant no. 20151BAB215031). We are grateful to Dr Rui Wu (Department of Rheumatology, The First Affiliated Hospital of Nanchang University, Nanchang, China) for providing help.
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