Original Paper

Abnormal Expression of Long Noncoding RNAs in Primary Immune Thrombocytopenia: A Microarray Related Study

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Key Words

Long noncoding RNA • Primary immune thrombocytopenia • Microarray • Quantitative real-time polymerase chain reaction • Co-expression network construction.

Abstract

Background/Aims: Long noncoding RNAs (lncRNAs) are important regulators of biological processes and they contribute to the pathological developments of various diseases, including autoimmune diseases. To gain the further understanding, we estimate the expression of lncRNAs in primary immune thrombocytopenia (ITP). **Methods:** In this study, microarray studies were performed to characterize expression profiles of various lncRNAs and mRNAs in blood samples collected from ITP patients. Quantitative real-time PCR (qRT-PCR) was performed to confirm the results, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and gene ontology analysis were used to provide functional annotations, co-expression network construction (CNC) analysis was made to reveal the relations between lncRNAs and their targeted genes. **Results:** A total of 1177 and 632 lncRNAs were significantly up-regulated or down-regulated, respectively, in "newly diagnosed ITP" patients versus healthy individuals. In addition, 1182 genes and 737 genes were up-regulated or down-regulated, respectively, in "chronic recurrent ITP" patients versus healthy individuals. In a KEGG analysis, "TNF signaling pathway-Homo sapiens (human)" was a key result. In a gene ontology analysis, "Granulocyte macrophage colony-stimulating factor production (GO:0032604, ontology: Biological process, P = 1.69577E-05)" and "coreceptor activity (GO: 0015026, ontology: molecular function, P = 4.67594E-06)" were the two most critical results. Data from qRT-PCR and receiver operating characteristic curves further demonstrated that ENST00000440492, ENST00000528366,

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NR_038920, and ENST00000552576 can efficiently distinguish different stages of ITP, especially NR_038920 and ENST00000528366. In a CNC analysis, four lncRNAs were emphasized, and NR_038920 and ENST00000528366 were both associated with proteins with important roles in autoimmune diseases. **Conclusions:** These results suggest that lncRNAs act through targeted genes to mediate their functions and to mediate their functions and affect the pathogenesis of ITP.

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Introduction

Primary immune thrombocytopenia (ITP), also known as idiopathic thrombocytopenic purpura, is an autoimmune disorder characterized by a decrease in platelets due to autoantibody-mediated destruction of platelets or compromised platelet production [1, 2]. It has been reported that pathological autoantibodies produced by B cells, aberrant T lymphocytes, and especially the turbulent developed megakaryocytes, significantly contribute to ITP [3-6]. Previous studies have demonstrated that most immune disorders are caused by dysregulation of gene expression [7, 8], and changes in phenotypes are often manifestations of changes in gene regulation [9-11]. In addition to coding genes, noncoding genes such as microRNAs and lncRNAs have increasingly gained attention [12-14]. lncRNAs have recently been discovered to be noncoding RNAs with lengths greater than 200 nucleotides [15]. Based on proximal protein-coding mRNAs, lncRNAs have been classified as sense lncRNAs, antisense lncRNAs, intronic lncRNAs, bidirectional lncRNAs, and long intergenic ncRNAs (lincRNAs). lncRNAs have been shown to affect every stage of a gene's life cycle, including chromosome reconstruction, transcription, post-transcription, and intracellular metabolism [15-18]. In a recent study, the role of lncRNAs in the development of autoimmune diseases was highlighted [19]. For example, the lncRNAs, Gas5, Hotair and H19, and Tmevg1, have roles in systemic lupus erythematosus (SLE) [20, 21], rheumatoid arthritis (RA) [22, 23], and Sjögren syndrome (SS) [24], respectively. However, few studies have investigated lncRNAs in ITP. Among these studies, the lncRNA, TMEVPG1, has been shown to be related to Th1-type transcription factors, T-bet, STAT1, and STAT4, thereby indicating the importance of lncRNAs in the pathology of ITP [25]. In the present study, a microarray analysis of lncRNAs and mRNAs in blood samples collected from newly diagnosed ITP patients (n = 3), chronic recurrent ITP patients (n = 3), and healthy individuals (n = 3) was conducted (Fig. 1). The goal was to identify differentially expressed lncRNAs which may participate in the development of ITP. In addition, quantitative real-time PCR (qRT-PCR) was performed to detect selected lncRNAs in a larger number of patient samples, and bioinformatics methods were applied to reveal potential functions.

Materials and Methods

Patient selection

All of the subjects enrolled in this study received treatment at No. 100 Hospital of PLA (Suzhou, Jiangsu, China) or Changhai Hospital (Shanghai, China) between October 2014 and January 2015. There were

Fig. 1. Hierarchical clustering for lncRNAs(a) and mRNAs(b) detected in healthy individuals(N1-3), newly diagnosed ITP cases(P1a-c), and chronic recurrent ITP patients(P2a-c).

no statistically significant differences among the patients in regard to gender or age. Patients referred to as having “newly diagnosed ITP” (termed P1 below) included diagnosed patients without reliable predictive clinical or laboratory parameters of disease duration. The patients referred to as having “chronic recurrent ITP” (termed P2 below) were previously diagnosed with ITP that persisted for more than 12 months, as previously detailed [26]. Healthy individuals composed the N group and they were examined for the same period of time as the P1 and P2 patients. Table 1 summarizes the characteristics of the P1 (n = 30) and P2 (n = 30) patients. Pregnant patients and those with diabetic complications, cardiovascular disease, an active infection, hypertension, or other autoimmune diseases were excluded. The Ethics Committee of the People’s Liberation Army 100th Hospital and Changhai Hospital approved this research. The procedures performed were carefully explained to the patients and informed consents were signed by all of the participating patients. All experiments in the methods section were performed in accordance with approved international guidelines and ethical standards.

Patient specimens

Peripheral blood (6 mL) was collected from each subject. To isolate peripheral blood mononuclear cells (PBMCs), the collected blood samples were diluted with PBS (pH 7.2–7.4, Jiru Biology Company, Shanghai, China) and then slowly added to Ficoll-Hypaque (Thermo Fisher Scientific, USA). After centrifugation at 2500 rpm for 20 min, the obtained PBMCs were washed twice with PBS, were added into 1 ml Trizol reagent (Invitrogen, USA) and then were stored at -80 °C

RNA isolation

Prepared blood samples were thawed at room temperature (RT) before chloroform was added to each sample. After a brief vortexing step, the samples were centrifuged (12000 × g, 5 min, 4 °C) and the supernatants were transferred to RNase-free eppendorf tubes and mixed with isopropyl alcohol. After an incubation at RT for 30 min and another centrifugation step (12000 × g, 15 min, 4 °C), the supernatants were precipitated with cold 75% alcohol and centrifuged (12000 × g, 5 min, 4 °C). The pellets were dried at RT for 2–5 min and then 20 µl of RNase-free water was added to each. From each sample, a 1 µl aliquot was analyzed for RNA concentration and purity with a Nanodrop 2000 instrument (Thermo Scientific, USA).

Microarray to obtain expression profiles for lncRNAs and mRNAs

An Arraystar Human LncRNA Microarray V3.0 (Arraystar Inc., USA) was used based on its capacity to evaluate 30,586 lncRNAs and 26,109 coding transcripts. To prepare samples for analysis, total RNA was isolated as described above. Both RNA integrity and genomic DNA contamination were assayed by denaturing agarose gel electrophoresis performed with an Agilent Bioanalyzer 2100 instrument (Agilent Technologies, USA) by KangChen (Shanghai, China). Total RNA was purified with RNeasy Mini Kits (Qiagen, Germany) and Baseline-ZERO DNase (EPICENTRE, USA) according to the manufacturers’ protocols. A One-Color Quick Amp Labeling Kit (Agilent, USA) was used to label samples. The labeled samples were subsequently purified, along with labeled cRNA as a quality control, with RNeasy Mini Kits (Qiagen, Germany) according to the manufacturer’s protocol. Specific activity (pmol dye per g cRNA) was calculated as follows: specific activity = (pmol per µl dye)/(µg per µl cRNA). Array hybridization was performed according to the manufacturer’s protocol for the Agilent Gene Expression Hybridization Kit (Agilent, USA). The hybridized

Table 1. Characteristics of the patients with ITP. Abbreviations: ITP: immune thrombocytopenia; PLT: platelet; IVIG: intravenous immunoglobulin; GP: glycoprotein; GC: glucocorticoid; P1: newly diagnosed ITP; P2: chronic ITP

Variables	P1 No. patients(%)	P2 No. patients(%)
Age of the ITP patients (y)		
≤20	4(13.3%)	2(6.7%)
20–40	10(33.3%)	14(46.7%)
40–60	8(26.7%)	12(40.0%)
≥60	8(26.7%)	2(6.7%)
Male	4(13.3%)	16(53.3%)
Course(mo)		
≤1(P1) 3–100(P2)	26(86.7%)	22(73.3%)
1–3(P1) 100(P2)	4(13.3%)	8(26.7%)
PLT count ≤10×10 ⁹ /L	12(40.0%)	18(60.0%)
Anti-platelet antibodies		
GPIIb+	12(40.0%)	16(53.3%)
GPIIIa+	18(60.0%)	14(46.7%)
GPIb/IX+	14(46.7%)	8(26.7%)
GPIb+	14(46.7%)	16(53.3%)
Therapy		
GC	4(13.3%)	30(100.0%)
IVIG	2(6.7%)	16(53.3%)

array was washed with Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 (Agilent, USA) before being scanned by an Agilent Microarray Scanner. Data were subsequently extracted and analyzed with Agilent Feature Extraction Software (V11.0.1.1).

Data analysis

Raw data were imported into GeneSpring GX v12.1 software (Agilent, USA) for quantile standardization. Differentially expressed lncRNAs and mRNAs with statistically significant differences between each set of comparisons were identified with P-value/ false discovery rate filtering. For the differently expressed lncRNAs or mRNAs, we selected them according to the following standards: 1) the fold change was ≥ 2.0 and P-value ≤ 0.05 ; 2) the raw intensity was ≥ 200 to make the background intensity as small as possible; 3) the Xhyb column was blank to make sure that there was not non-specific detection. Hierarchical clustering and combined analysis were performed with homemade scripts. To better understand the functions of the identified lncRNAs, correlations between enhancer lncRNAs, antisense lncRNAs, lincRNAs, and nearby mRNAs were analyzed. A gene ontology (GO) analysis was performed and the categories included: Molecular Function (MF), Biological Process (BP), and Cellular Component (CC). P-values were obtained according to Fisher's exact test and were used to measure GO term enrichment for differentially expressed lncRNAs and mRNAs. To calculate fold enrichment of a GO term, the number of differentially expressed genes and those with an assigned GO term ("Count"), the number of genes annotated in the database ("Pop.Nits"), the number of different genes with GO annotations ("List.Total"), and the total number of genes in the database (background) with a GO annotation ("Pop.Total"), were used as follows: $(\text{Count} / \text{Pop.Nits}) / (\text{List.Total} / \text{Pop.Total})$, Enrichment Score = $-\log(\text{P-value})$. A Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis showed every gene/protein or small molecule in each Pathway Map it generated. Histograms presented the enrichment scores of the pathway terms. A Volcano plot was used to select differentially expressed mRNAs and lncRNAs (for all online suppl. material, see www.karger.com/doi/10.1159/000491890, Fig. S3a-c). A scatter plot was generated to present repeatability among the microarray data and groups (see online suppl. material, Fig. S3d-f). Probe distribution in the microarray is presented in a box plot, with changes before and after standardization shown (see online suppl. material, Fig. S3g-h). To display similar biological properties in multiple samples, hierarchical clustering was performed with Aligent GeneSpring GX software (version 11.5.1) (Fig. 1).

Fig. 2. CNC analysis results for 12 lncRNAs (a) and a subset of 4 immune-related lncRNAs [ENST00000528366, and ENST00000440492, NR_038920, and ENST00000552576] and their related genes (b). (a) The blue-green nodes represent coding genes, the red nodes represent lncRNAs. Solid lines indicate positive relationships between the lncRNAs and coding genes, and the dotted lines indicate negative relationships. (b) In this second CNC network, there were 47 nodes representing mRNAs, as well as related genes. The related genes were presented in colors that indicate their level of significance in immunity or autoimmunity (significance: blue-green > green > yellow-green > yellow).

Synthesis of cDNA and qRT-PCR

For the synthesis of cDNA, an annealing mixture composed of 1.5 µg RNA, 1 µl oligo(dT)18 (0.5 µg/µl), 1.6 µl dNTPs mix (2.5 mM) (HyTest Ltd., China), and 10.4 µl RNA-free H₂O was used. After the samples were incubated in the annealing mixture in a water bath at 65 °C for 5 min, and then in an ice bath for 2 min, the samples were subjected to a short centrifugation. The following reagents were then added in this order: 4 µl 5X First-Strand Buffer (Invitrogen, USA), 1 µl 0.1M DTT, 0.3 µl RNase Inhibitor (Epicentre, USA), and 0.2 µl SuperScript III RT (Invitrogen, USA). The samples were incubated at 37 °C for 1 min, at 50 °C for 60 min, and then at 70 °C 15 min in a thermostatic water tank (DK-8D, Senxin Instruments Inc., Shanghai, China). For immediate use, the samples were placed on ice, while samples were stored at -20 °C for later use.

PCR reactions were performed with 2X PCR Master Mix (Arraystar, USA) according to the manufacturer's protocol. See online suppl. material, Table S2 provides a list of the primers used. β-Actin was detected as a reference gene and relative quantification was performed to measure gene expression levels. PCR amplification was subsequently performed to verify selected genes from the lncRNA microarray analysis in 30 patients with "newly diagnosed ITP" (P1) and 30 patients with "chronic recurrent ITP" (P2) (Table 1). ROC curves and areas under the ROC curves (AUCs) for the qRT-PCR results of the selected genes were generated with GraphPad Prism 6.0 software (GraphPad, USA).

Co-expression network construction

Normalized intensities of coding genes were calculated to perform a co-expression network construction (CNC) analysis. When there were varying intensity values for different transcripts of the same gene, the median value of these intensities was used as the expressed value of the gene. The Pearson correlation coefficient (pcc) of the normalized intensities of the selected lncRNAs and all of their related genes was also calculated. When [abs(pcc)] was > 0.998 and false discovery rate (FDR) was < 0.05, the record was acquired. All of the records were plotted with Cytoscape (version 3.4.0) to construct a co-expression network that is shown in Fig. 2. Considering the immune functions demonstrated for the related genes in previous reports [27-42], 12 lncRNAs were selected for CNC analysis (Fig. 2a). Subsequently, the four most important lncRNAs (ENST00000440492, ENST00000528366, ENST00000552576, and NR_038920) and related genes according to their immunological function were selected for a CNC analysis (Fig. 2b).

Results

Differentially expressed lncRNAs

Compared with healthy controls (the N group), a total of 7878 lncRNAs were found to be up-regulated in newly diagnosed ITP patients (the P1 group), among which only 451 lncRNAs exhibited significant differences according to the standards as described above. The lncRNA that exhibited the greatest fold-change was NR_037166 (fold-change: 18.2). Meanwhile, a total of 7692 lncRNAs (312 of which exhibited significant differences) were down-regulated between the P1 and N groups. The most remarkable decrease in expression was exhibited by TCONS_00003870 (fold change: 93.6).

The total number of up-regulated lncRNAs between the chronic recurrent group (P2) and the N group was 9313 (with 376 lncRNAs exhibiting significant differences). The total number of down-regulated lncRNAs was 6257 (with 393 of these exhibiting significant differences) between the P2 and N groups. The most notably up-regulated lncRNA was TCONS_00000918 (fold-change: 13.9), while TCONS_00003870 (fold-change: 186.4) was the most down-regulated lncRNA.

Between the P2 and P1 groups, a total of 9392 up-regulated lncRNAs (with 100 exhibiting significant differences) and 6178 down-regulated lncRNAs (with 191 exhibiting significant differences) and their related genes were analyzed. The antisense lncRNAs that exhibited the greatest change between the P1 and N groups, between the P2 and N groups, and between the P2 and P1 groups were: uc010kun.2, NST00000559553, and ENST00000559553, respectively. Detailed information regarding these analysis is presented (see online suppl. material,) in Table S1 (a, b, and e). A hierarchical clustering analysis of lncRNAs detected in three healthy controls, three newly diagnosed ITP patients, and three chronic recurrent ITP

patients is shown in Fig. 1a. We further analyzed the chromosomal derivation of the top10 lncRNAs in each group and found that in up-regulated groups (including P1 vs N, P2 vs N, P2 vs P1), those lncRNAs were focused on chr8 and chr11, while in down-regulated groups, they were mainly on chr2, chr12.

Differentially expressed mRNAs

An mRNA analysis of the P1 versus N samples, the P2 versus N samples, and the P2 versus P1 samples identified 9519 up-regulated mRNAs (with 363 of these mRNAs exhibiting significant differences), 9795 up-regulated mRNAs (with 234 of these mRNAs exhibiting significant differences), and 9997 up-regulated mRNAs (with 40 of these mRNAs exhibiting significant differences), respectively. The most up-regulated mRNAs in each analysis were: NM_001008 (fold-change: 68.2), NM_001008 (fold-change: 84.0), and ENST00000361255 (fold-change: 5.4), respectively. Conversely, there were 10779 mRNAs that were down-regulated in the P1 group versus the N group (with 353 of these mRNAs exhibiting significant differences), 10503 mRNAs down-regulated in the P2 versus N group (with 516 of these mRNAs exhibiting significant differences), and 10301 mRNAs down-regulated in the P2 versus P1 groups (with 183 of these mRNAs exhibiting significant differences). The most-downregulated mRNAs in each group were: NM_002090 (fold-change: 48.4), NM_002090 (fold-change: 84.4), and NM_003246 (fold-change: 8.4), respectively. Details of these analyses are summarized (see online suppl. material) in Table S1 (c,d).

GO analysis

A GO analysis of the P1 group versus the N group data showed that the greatest fold enrichment of GO terms for the up-regulated transcripts included: long-chain fatty acid binding (GO:0036041, ontology: MF, P = 0.000686924), response to bacterium (GO:0009617, ontology: BP, P = 9.31494E-09), and retromer complex (GO:0030904, ontology: CC, P = 0.000642907). The highest fold enrichment of GO terms for the down-regulated transcripts were: granulocyte macrophage colony-stimulating factor production (GO:0032604, ontology: BP, P = 1.69577E-05), coreceptor activity (GO:0015026, ontology: MF, P = 4.67594E-06), and T cell receptor complex (GO:0042101, ontology: CC, P = 0.012832272). Detailed information regarding these analyses are provided (see online suppl. material) in Fig. S1.

A GO analysis of the P2 group versus the N group data showed that the greatest fold enrichment of GO terms for the up-regulated transcripts included: oxygen transporter activity (GO:0005344, ontology: MF, P = 1.60566E-06), hemoglobin complex (GO:0005833, ontology: CC, P = 3.78385E-07), and oxygen transport (GO:0015671, ontology: BP, P = 3.65271E-06). For the down-regulated transcripts, the greatest fold enrichment of GO terms included: mRNA cap binding complex (GO:0005845, ontology: CC, P = 0.000243535), fever generation (GO:0001660, ontology: BP, P = 0.000102935), and activin binding (GO:0048185, ontology: MF, P = 0.003340754).

Finally, a GO analysis of the P2 group versus the P1 group showed that the greatest fold enrichment of GO terms for the up-related transcripts included: hemoglobin complex (GO:0005833, ontology: CC, P = 9.60704E-10), oxygen transport (GO:0015671, ontology: BP, P = 6.65012E-07), and oxygen transporter activity (GO:0005344, ontology: MF, P = 4.09006E-07). For the most down-regulated transcripts, the most enriched GO terms were: extracellular region (GO:0005576, ontology: CC, P = 6.75631E-09), cGMP-mediated signaling (GO:0019934, ontology: BP, P = 7.52627E-05), and IgG binding (GO:0019864, ontology: MF, P = 0.003234109).

Pathway analysis

For up-regulated transcripts, the highest enrichment score pathways included: Legionellosis-Homo sapiens (human) (Fisher P-value = 2.51334E-05) in the P1 versus N analysis, Legionellosis-Homo sapiens (human) (Fisher P-value = 0.001259538) in the P2 versus N analysis, and Glycine, serine, and threonine metabolism - Homo sapiens (human) (Fisher P-value = 0.00173499) in the P2 versus P1 analysis (see online suppl. material, Fig.

S2d-f). For the down-regulated transcripts, the most enriched pathways were: TNF signaling pathway - Homo sapiens (human) (Fisher P-value = $1.22301\text{E-}08$) in the P1 versus N analysis, NF-kappa B signaling pathway - Homo sapiens (human) (Fisher P-value = $5.63797\text{E-}08$) in the P2 versus N analysis, and Rheumatoid arthritis - Homo sapiens (human) (Fisher P-value = $9.70212\text{E-}06$) in the P2 versus P1 analysis (see online suppl. material, Fig. S2a-c). The two most common pathways were the “NF APPA signaling pathway” and the “TNF signaling pathway” (see online suppl. material, Fig. S2, g and h).

CNC analysis

A total of 12 lncRNAs were selected from among the differentially expressed lncRNAs for CNC analysis. There were also nodes representing mRNAs related to the lncRNAs (a total of 912 different mRNAs) that were analyzed (Fig. 2a). Many of the mRNAs in the network were shared by two or more lncRNAs, yet the relationships were distinct. For example, CXCL11 was positively related to ENST00000528366 and uc010kun.2 (both connected by a solid line), yet was negatively related to ENST00000509463 (connected by a dotted line). Similarly, CD69 was positively related to ENST00000528366 and negatively related to ENST00000509463. More proximal lncRNAs shared a greater number of mRNAs. For example, ENST00000417932 shared 235 mRNAs with nearby NR_038920, yet had just two mRNAs that it shared with ENST00000440436, and it shared no mRNAs with ENST00000528366.

Next, mRNAs with previously described associations with immune functions [36-53] were considered (Fig. 2a). Accordingly, 4 important lncRNAs and 47 related mRNAs from the 12 lncRNAs network (described above) were selected. In this second CNC network, there were 47 nodes representing mRNAs, as well as related genes (Fig. 2b). Of note, PTPN1 was found to be shared by ENST00000440492 and ENST00000528366, thereby implying that both of these lncRNAs exert their functions by regulating expression of PTPN1. ENST00000440492 was also found to be associated with CCR2 and CD36, two proteins which have been shown to play important roles in immune-related diseases. Therefore, ENST00000440492 may mediate its effects on ITP pathogenesis via these two genes. Similarly, CCRL2 and CD274 may be important for the function of ENST00000528366 in relation to ITP. Meanwhile, ENST00000552576 and NR_038920 were found to be associated with CCR7, CXCR1, PILRA, CD86, CD79B, and CCR7. A KEGG pathway analysis further revealed that related mRNAs for the 4 lncRNAs were enriched in pathway hsa04060: cytokine-cytokine receptor interaction - Homo sapiens (human) and hsa04062: chemokine signaling pathway - Homo sapiens (human) (see online suppl. material, Fig. S4a-b). Moreover, in the GO analysis, these mRNAs were closely associated with GO:0050896 (response to stimulus) and GO: 0009987 (cellular process) (see online suppl. material, Fig. S4c), which both belong to the biological process group and indicate possible immune functions.

qRT-PCR

To confirm the microarray results obtained, expression levels of 12 lncRNAs in CNC network and TCONS_00000918, TCONS_00003870 listed (see online suppl. material), in Table S1 were assayed by qRT-PCR in 30 P1 patients and 30 P2 patients. Characteristics of these patients are summarized in Table 1. According to the P values, NR_038920, ENST00000528366, ENST00000552576, ENST00000521756, ENST00000440492 showed better examination efficiency in distinguishing

Table 2. Results of qRT-PCR for some of the vital lncRNAs and their fold changes. Abbreviations: P1: newly diagnosed ITP; P2: chronic ITP; N, normal. * If the lncRNAs were presented in different groups with different fold changes, then the average values would be calculated

Sequence Name	P-Value			Average* Fold Change
	P1 vs N	P2 vs N	P2 vs P1	
ENST00000440492	4.915E-19	0.376	6.985E-13	3.187
ENST00000509463	0.581	0.626	0.466	13.468
uc010kun.2	0.00739	0.000717	0.186	12.296
ENST00000436506	0.0402	0.00387	0.582	2.634
ENST00000528366	1.623E-15	5.097E-08	0.000765	18.546
ENST00000440436	0.0466	0.0401	0.678	9.855
uc002ubt.1	0.447	0.828	0.416	2.493
ENST00000417932	0.0220	0.0170	0.597	2.457
NR_038920	2.781E-08	3.636E-11	2.655E-08	8.943
ENST00000412084	0.000187	0.0419	0.121	5.271
ENST00000552576	0.000111	0.000261	0.231	3.503
ENST00000521756	0.0337	0.0127	0.620	2.521
TCONS_00000918	0.00815	0.00133	0.302	30.577
TCONS_00003870	0.0106	0.00258	0.579	140.009

P1 from N, P2 from N, and P2 from P1 than other lncRNAs (Table 2). In detail, expression of NR_038920 significantly differed between the P1 and N groups, between the P2 and N groups, and between the P2 and P1 groups. ENST00000528366 also significantly differed between these three groups. In addition, ENST00000552576 distinguished the P1 samples from the N samples and the P2 samples from the N samples, while ENST00000440492 significantly differed between the P1 and N groups and between the P2 and P1 groups (Fig. 3a–d, P-values were shown in Table 2). AUCs of the ROC curves for the four lncRNAs ranged from 0.531 to 0.997, implying that these lncRNAs can distinguish different stages of ITP and the presence or absence of ITP, particularly NR_038920 (AUC: 0.857–0.958) and ENST00000528366 (AUC: 0.758–0.962) (Fig. 3e–h).

Furthermore, we also examined the gene expression of CCR7, CXCR1, CD274, CCRL2 by qRT-PCR as above, the results showed that the gene expressions of CXCR1 and CD274 were significantly different in P1 vs N, P2 vs N, P2 vs P1 groups. For CXCR1, the P-values were 0.000409, 1.11E-08, 0.000309, separately; for CD274, 0.00444, 3.696E-07, 0.0109, separately. CCR7 and CCRL2 were statistically different in P1 vs N, and P2 vs N groups. For CCR7, P-values were 0.00316, 2.529E-05, individually; for CCRL2, 0.00160, 3.86E-05, separately (see online suppl. material, Fig. S5).

Discussion

ITP is an autoimmune disorder that is characterized by pathologically produced lymphocytes, abnormally developed megakaryocytes, and/or biologically disordered platelets [5]. With the development of second generation sequence analysis (especially RNA-seq), an increasing number of lncRNAs are being identified, as well as their roles in various types of diseases, including cancers (e.g., hepatocellular carcinoma, prostate cancer, etc.) and autoimmune diseases (e.g., SLE, SS, etc.) [54–56]. Previous studies have shown that lncRNAs potentially influence the functions of dendritic cells (DCs) and T lymphocytes, and also affect the differentiation of Th1/Th2 cells which play an important role in the development of ITP [57–59]. For example, targeting of STAT3 by lnc-DC involves the differentiation of DCs, and this in turn can induce the activation of an immune response [57]. In addition, linc-MAF-4 influences the differentiation of CD4⁺ T lymphocytes by targeting the chromatin modifiers, EZH2 and LSD1 [60]. Considering the potential roles that lncRNAs may have in autoimmune disorders, they have received greater attention in recent years. As a result, specific roles for lncRNAs in autoimmune diseases such as SLE, RA, and SS have been elucidated [20–22].

In the present study, lncRNAs were profiled in three newly diagnosed ITP patients, in three chronic recurrent ITP patients, and in three healthy individuals by microarray (Fig. 1). The lncRNAs which exhibited the greatest fold-changes were NR_047631, ENST00000417071, and uc010idt.2 (see online suppl. material, Table S1a–b). We hypothesize that these lncRNAs may critically affect ITP pathology. To gain insight into the biological functions of these lncRNAs, bioinformatics were applied. As a result, ENST00000559553 was found to be related to “normal mucosa of esophagus-specific gene 1 protein”. Previously, normal mucosa

Fig. 3. Box plots (a–d) and ROC curves (e–h) for gene expression of ENST00000440492, ENST00000528366, NR_038920, and ENST00000552576. Blue, P1 vs N, Green, P2 vs N, Red, P2 vs P1, ns: not significant, *P<0.001, **P<0.0001.

of esophagus-specific gene 1 protein was overexpressed in an esophageal squamous cell carcinoma cell line and cell motility was found to be affected [61].

In the GO analysis of down-regulated lncRNAs identified from a comparison of P1 versus N group data, three terms were predominant: “regulation of granulocyte macrophage colony-stimulating factor (GM-CSF) production”, “coreceptor activity”, and “T cell receptor complex” (see online suppl. material, Fig. S1d-f). These results suggest that differentially expressed lncRNAs may contribute to the pathology of ITP by influencing production of GM-CSF, coreceptor activity, and/or T cell receptors, and these correlations are consistent with the results of previous studies which have characterized the pathogenesis of autoimmune diseases [62]. For example, in patients with multiple sclerosis (MS), an increase in GM-CSF-expressing B cells has been detected, and this increase could activate myeloid cells in a GM-CSF-dependent manner [63]. In relation to B cells, CD22 is a co-receptor of these cells and is reported to control the B cell response to antigens. Thus, dysfunction of CD22 could lead to the development of autoimmune diseases such as SLE. Correspondingly, epratuzumab, a monoclonal antibody recognizing CD22, is a potential treatment for SLE[64]. T cells and their receptors have also been characterized as critical immune factors in immune-related diseases, with T cell receptors (TCRs) being able to recognize restricted antigenic peptide fragments and mediate signaling via transduction pathways which are critical for T cell activation [65]. Regarding the latter, decreased tyrosine phosphorylation of the TCR zeta-chain has been linked to defective TCR signaling that may underlie the hyporesponsiveness of synovial T cells in RA[66].

Metabolism-associated GO terms were also identified in the present study. These included: “long-chain fatty acid binding”, “oxygen transporter activity”, “oxygen transport”, and inflammation-related terms such as “response to bacterium” and “fever generation”. It is well established that ITP represents a complex disease that is accompanied by an inflammatory reaction mediated by a variety of immune cytokines including IL-17, IFN- γ , and TNF- α , among others. It has also been observed that the metabolism of these cytokines can change over the course of this disease, including their oxidant and antioxidant states, and these changes can potentially influence the development of ITP[33, 67-69]. It is possible that differentially expressed lncRNAs exert their functions by influencing inflammatory or metabolism processes, and these effects could influence the development of ITP. In the pathway analysis conducted in the present study, the “TNF signaling pathway - Homo sapiens (human)” was markedly enriched in a comparison of the down-regulated lncRNAs between the P1 and N groups (see online suppl. material, Fig. S2a). Previous studies have shown that TNFR1 and TNFR2 are two important receptors that mediate signaling downstream of TNF activation and binding [70]. In general, signaling via TNF-TNFR1 involves the MAPK and NF- κ B pathways. In contrast, signaling via TNF-TNFR2 is limited to CD4⁺ T cells, CD8⁺ T cells, and endothelial cells, and signaling via NF- κ B can be stimulated as well [70-74]. Between the P2 and N group data, the most enriched pathway that was identified was the, “NF-kappa B signaling pathway - Homo sapiens (human)” (see online suppl. material, Fig. S2g); and between the P2 and P1 group data, the “Rheumatoid arthritis - Homo sapiens (human)” pathway was identified (see online suppl. material, Fig. S2b-c). A role for the NF- κ B signaling pathway in maintaining immune tolerance in the body has previously been demonstrated, including its roles in central and peripheral tolerance which include an inhibition of regulatory T cells [75, 76]. Thus, NF- κ B signaling is an important mediator in regulating chronic inflammatory diseases and autoimmune diseases [77]. In brain tissues affected by MS, NF- κ B has also been shown to localize to astrocytes and infiltrating macrophages in or near lesions in the central nervous system [77, 78]. Furthermore, genetic studies have shown that abnormal levels of NF- κ B-related genes may be related to MS relapses [79, 80]. Regarding the RA pathway that was identified in a comparison of the P2 and P1 groups, enrichment of this pathway may be due to similarities in the underlying mechanisms of these two diseases, which include the involvement of CD4⁺ T cells, the NF- κ B signaling pathway, and aberrant expression of cytokines such as IFN- γ . Moreover, clinically, patients with RA are often complicated by

immune thrombocytopenic purpura, thereby further supporting the relationship between these two diseases [81-84].

For the CNC analysis, we initially selected 12 lncRNAs. Then, four of these 12 lncRNAs were selected for further analysis (Fig. 2, a and b). As a result, NR_038920 appear to share related genes with ENST00000552576, such as CCR7 and CXCR1 (Fig. 2b). CCR7 has been shown to play an important role in adaptive immune activities as part of a CCR7-CCL19/CCL21 signaling axis. This axis has been shown to affect T lymphocyte function, DCs, and T cell priming or adaptive immunity in lymph nodes [85, 86]. Furthermore, CCR7 has also been shown to promote T cell development and lymphorganogenesis, two processes which are significant in the progression of autoimmune diseases [85, 86]. Thus, inappropriate regulation of the CCR7-CCL19/CCL21 axis may lead to immune disorders, including the development of autoimmune diseases [85]. Regarding CXCR1, it has always been found to function in combination with CXCR2. The CXCR1/2 complex has been detected on neutrophils, macrophages, and lymphocytes, and these cell types are critical in inflammatory processes and autoimmune diseases [50, 87, 88]. Inhibition of CXCR1/2 has been shown to prevent islet damage due to immune disorders, and this inhibition also promotes autoimmune throiditis [50]. Alternatively, the CXCR1/CXCL8 axis has been associated with the accumulation of NK cells in the liver of patients with primary biliary disease, thereby contributing to the pathological development of this disease [89]. ENST00000528366 is shown to be related with CD274 and CCRL2. CD274, also known as ligand for programmed cell death 1 (PD-L1), is expressed on the surface of lymphocytes, macrophages or the dendritic cells [48]. After interacting with PD-1, it can regulate the proliferation or the immune-tolerance of T cells, it is emphasized in the T cell-mediated autoimmune diseases such as SLE, MS, et al [90, 91]. In patients with active SLE, PD-L1 is found to be overexpressed in monocytes and negatively related with the level of TGF- β , while interestingly when patients are in the recovery stage, PD-L1 is showed to be positively related to TNF- α , implying that PD-L1 may involve in the pathogenesis of SLE by influencing the expression of TGF- β or TNF- α [90]. Chemokine (CC motif) receptor-like 2 (CCRL2) is a 7-transmembrane protein related to the family of the typical chemokine receptors that participate in the inflammation process, for example, in the murine models of experimental autoimmune encephalitis, those short of CCRL2 develop exacerbated nonresolving disease and recurrent inflammatory reactions, suggesting that it may take part in the control of multiple sclerosis [46]. Based on the roles CCRL2 and CD274 played in the process of autoimmune diseases, in ITP ENST00000528366 may also involve in its pathogenesis through the pathways as described above.

According to the literature regarding ITP, ENST00000440492, ENST00000528366, NR_038920, and ENST00000552576 may act through similar pathways. For example, when the pathways or GO terms associated with the mRNAs in the CNC network were searched to reveal their related lncRNAs' functions, hsa04060: cytokine-cytokine receptor interaction - Homo sapiens (human) (see online suppl. material, Fig. S4b) and hsa04062: chemokine signaling pathway - Homo sapiens (human) (see online suppl. material, Fig. S4a) pathways were identified. The GO results further suggest that these lncRNAs may mediate functions associated with stimulus response (GO:0050896) and cellular processes (GO: 0009987) (see online suppl. material, Fig. S4c).

To further verify the microarray results of the present study, qRT-PCR was performed to detect ENST00000440492, ENST00000528366, NR_038920, and ENST00000552576 in 30 patients with newly diagnosed ITP (P1) and 30 patients with chronic recurrent ITP (P2). These results (Fig. 3a-d), in combination with the ROC curves data (Fig. 3e-h), confirm that these four lncRNAs can efficiently distinguish different stages of ITP, especially NR_038920 and ENST00000528366 which can distinguish P1, P2 from N and P1 from P2 (ENST00000440492 cannot distinguish P2 from N, and ENST00000552576 cannot distinguish P2 from P1). Consequently, these lncRNAs may represent potential targets for monitoring, diagnosing, and/or treating ITP.

To our knowledge, this is the first microarray-based study to investigate lncRNAs in ITP. The identification of differentially expressed lncRNAs in this study provides support

for further analyses regarding their functions in this disease process. As described above, lncRNAs may exert their functions by influencing other molecules or via actions through certain pathways. Accordingly, the latter has been demonstrated for a subset of lncRNAs in relation to the development of certain autoimmune diseases. Thus, it appears that lncRNAs have the capacity to facilitate monitoring of ITP progression and to serve as targets for the treatment of this disease, and further studies of lncRNAs are warranted.

Acknowledgements

This research was supported by grants from the National 973 Foundation (2013CB531606), the National Science Foundation of China (81701608, 81671556, 81601406, 81501397, 31500721, 81501398, 81471605, 81401358, 81302579, 81273282 and 81202353), Shanghai Shengkang (SHDC22014014), Shanghai Educational Science (D14017), Army Scientific Research (BWS14J023, 15ZD009, 15XD007), China Postdoctoral Science Foundation (2017M623440) and MJD Founding (MJR20150019).

Disclosure Statement

The authors have no competing interests to declare.

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