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Identification of differentially expressed long non-coding RNAs in CD4⁺ T cells response to latent tuberculosis infection

Zhengjun Yi ^{a,1}, Jianhua Li ^a, Kunshan Gao ^a, Yurong Fu ^{b,*,1}

^a Department of Laboratory Medicine of Affiliated Hospital of Weifang Medical University, Key Laboratory of Clinical Laboratory Diagnostics in Universities of Shandong, Weifang Medical University, Weifang 261031, China

^b Department of Medical Microbiology, Weifang Medical University, Weifang 261053, China

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KEYWORDS

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Summary Objective: To identify differentially expressed long non-coding RNAs (lncRNAs) in CD4⁺ T cells triggered upon latent tuberculosis (TB) infection.

Methods: Expression profiles of lncRNAs and mRNAs in CD4⁺ T cells from individuals with latent TB infection (LTBI), active TB and healthy controls were analyzed by microarray assay and four lncRNAs were selected for validation using real time-quantitative polymerase chain reaction (RT-qPCR). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway based approaches were used to investigate biological functions and signaling pathways affected by the differentially expressed mRNAs.

Results: LncRNAs and mRNAs in CD4⁺ T cells were involved in LTBI and active TB disease. Compared with healthy controls, 449 lncRNAs and 461 mRNAs were deregulated in LTBI group, 1,113 lncRNAs and 1,490 mRNAs were deregulated in active TB group, as well as 163 lncRNAs and 187 mRNAs were differentially expressed in both LTBI and active TB group. It was worth noting that 41 lncRNAs and 60 mRNAs were deregulated between three groups. Most deregulated lncRNAs were from intergenic regions (~50%), natural antisense to protein-coding loci (~20%), or intronic antisense to protein-coding loci (~10%). Significantly enriched signaling pathways based on deregulated mRNAs were mainly involved in mitogen-activated protein kinase (MAPK) signaling pathway, cytokine–cytokine receptor interaction, Toll-like receptor signaling pathway, etc.

* Corresponding author. Department of Medical Microbiology, Shandong Provincial Key Laboratory of Clinical Laboratory Diagnostics, Weifang Medical University, No. 7166, Baotong Xi (Western) Street, Weifang 261053, China. Tel.: +86 0536 8068955; fax: +86 0536 8462230. E-mail address: yifuyurong@163.com (Y. Fu).

¹ These two authors contributed equally to this work: conceived and designed the study, performed microarray experiment, bioinformatics analysis as well as wrote the paper.

Conclusions: The study was the first report of differentially expressed lncRNAs in CD4⁺ T cells response to TB infection and indicated that some lncRNAs may be involved in regulating host immune response to TB infection. Future studies are needed to further elucidate potential roles of these deregulated lncRNAs in LTBI and its reactivation.

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Introduction

Tuberculosis (TB), causing more than 1.3 million deaths in 2013, is still one of the biggest killers among the infectious diseases.¹ It is estimated that nearly one-third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb) and about 90% of them have asymptomatic, latent TB infection (LTBI). Overall, without treatment, about 10% of individuals with LTBI will develop active TB disease at some time in their lives.^{2,3} However, the underlying mechanisms of LTBI and its reactivation remain largely unknown. Host immune response against Mtb is complex and multifaceted. Identification of immune mediators which contribute to LTBI and reactivation is useful for early diagnosis, prevention and treatment of TB disease.

CD4⁺ T cells play an important role in host defense against intracellular pathogens, including Mtb.^{4–6} Genome-wide transcriptional analysis is a widely used method for the study of complex diseases. Noncoding RNAs play an important role in modulating gene transcription.⁷ Based on their length, noncoding RNAs are divided into 2 major classes: short (<200 nucleotides) and long (>200 nucleotides).⁸ MicroRNAs are a large group of short noncoding RNA and their roles have been relatively well studied in human biology and disease.⁹ In contrast to microRNAs, long noncoding RNAs (lncRNAs) are much less known concerning their functions. Recent evidence indicates that lncRNAs play important roles in many physiological and pathological processes, such as cell differentiation, apoptosis, proliferation and cancer.^{10–12} However, little is known about the role of lncRNAs in TB disease.

CD4⁺ T cell-mediated immunity plays a pivotal role in modulation of immune homeostasis and control of Mtb growth. In the study, to investigate whether lncRNAs were involved in TB infection, expression profiles of both lncRNAs and mRNAs in CD4⁺ T cells from subjects with LTBI or active TB were detected and the relationship between deregulated lncRNAs and their adjacent protein-coding genes as

well as deregulated mRNAs related to signaling pathways were analyzed.

Materials and methods

Human subjects

Patients with active pulmonary TB were enrolled from Weifang Chest Hospital in Shandong Province, China. Both LTBI subjects and healthy control individuals were recruited from the staff in Affiliated Hospital of Weifang Medical University and Weifang Chest Hospital. The clinical characteristics of 30 patients with active TB (ATB group), 25 subjects with LTBI (LTBI group) and 30 healthy controls (HCo group) were presented in Table 1. Diagnosis of active pulmonary TB was based on typical clinical symptoms, such as cough, fever, fibrocavitary lung infiltrate on chest radiography, and at least one sputum culture positive or both sputum smear and culture positive. Diagnosis of LTBI was based on the results of tuberculin skin test (TST) and interferon-gamma release assay (IGRA) (T-SPOT.TB, Oxford Immunotec, Oxfordshire, U.K.). All LTBI subjects had no history of recent contact with active TB patients. Before starting the study, pre-analytical evaluations were performed for detection of tumors and infectious diseases such as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections. Participants were excluded if they had a history of TB, diabetes, tumor or other infectious disease.

The study was approved by the Ethics Committee of Weifang Medical University and conducted in accordance with the Declaration of Helsinki. All participants provided informed consent before commencement of the study.

Isolation of CD4⁺ T cells

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood by use of Ficoll (TBD, Tianjin, China)

Table 1 Characteristics of the participants.

Characteristics	Hco group (n = 30)	LTBI group (n = 25)	ATB group (n = 30)
Male/female	18/12	16/9	19/11
Age, mean (range) years	38.8 ± 16.7 (21–55)	34.6 ± 13.2 (25–50)	43.9 ± 17.2 (18–58)
TST test	Negative	Positive	Not applicable
IGRA	Negative	Positive	Not applicable

IGRA, Interferon-Gamma release assays. All patients with active pulmonary TB had clinical signs and symptoms, such as cough, fever, and fibrocavitary lung infiltrate on chest radiograph. Healthy controls and LTBI involved in the study were free of clinical symptoms of any infectious disease.

HCo: healthy control; LTBI: latent TB infection; ATB: active TB. There were no significant differences in age ($P > 0.05$, one-way ANOVA test) or gender ($P > 0.05$, chi-square test) between three groups.

density gradient centrifugation before anti-TB treatment. CD4⁺ T cells were isolated from PBMCs by negative selection using CD4⁺ T Cell Isolation Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, PBMCs were resuspended in appropriate volume of cold MagCelect buffer and labeled with negative selection biotinylated antibodies. Labeled cells were incubated with MagCelect Streptavidin Ferrofluid and MagCelect Magnet. CD4⁺ T cells were then enriched by depletion of unwanted cells. To achieve high purity of isolation, CD4⁺ T cells were negatively selected again from the above enriched cells. Flow cytometric analysis showed that the purity of isolated CD4⁺ T cells was more than 90% (Fig. S1). All samples were stored in liquid nitrogen until further use. Four samples of each group were used for microarray expression analysis and all samples were used for real time-quantitative polymerase chain reaction (RT-qPCR).

RNA extraction and quality control

Total RNA was extracted from purified CD4⁺ T cells using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA, USA) and further purified with RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quantity and purity of isolated RNA for each sample were assessed on a NanoDrop Spectrophotometer ND-1000. RNA integrity for each sample was determined using denaturing gel

electrophoresis. For spectrophotometer analysis, samples with OD260/OD280 ratio between 1.8 and 2.1 and OD260/OD230 ratio >1.8 were only acceptable. For electrophoresis analysis, samples should be free of genomic DNA contamination and ratio of 28S/18S band intensities should be greater than 2.0.

Microarray analysis of lncRNA and mRNA expression

Sample labeling and array hybridization were performed using Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, 4 qualified RNA samples of each group were amplified and transcribed into fluorescent cRNA. Labeled cRNA samples were then purified using RNeasy Mini Kit (Qiagen, Hilden, Germany). Concentration and specific activity of labeled cRNA samples were subsequently measured using a NanoDrop ND-1000. After passing quality test, 1 µg labeled cRNA for each sample was used for hybridization on a Human LncRNA Array v 2.0 (8 × 60 K, Arraystar) with 33,045 lncRNA and 30,215 mRNA probes (each probe being replicated 4 times on each array). After hybridization, microarrays were washed, fixed, and then scanned using an Agilent DNA Microarray Scanner (G2505B) (Agilent, Santa Clara, CA, USA). Positive control probes of 20 housekeeping genes (NM_002455, NM_021009, NM_006013, NM_001536, NM_003746, NM_002107,

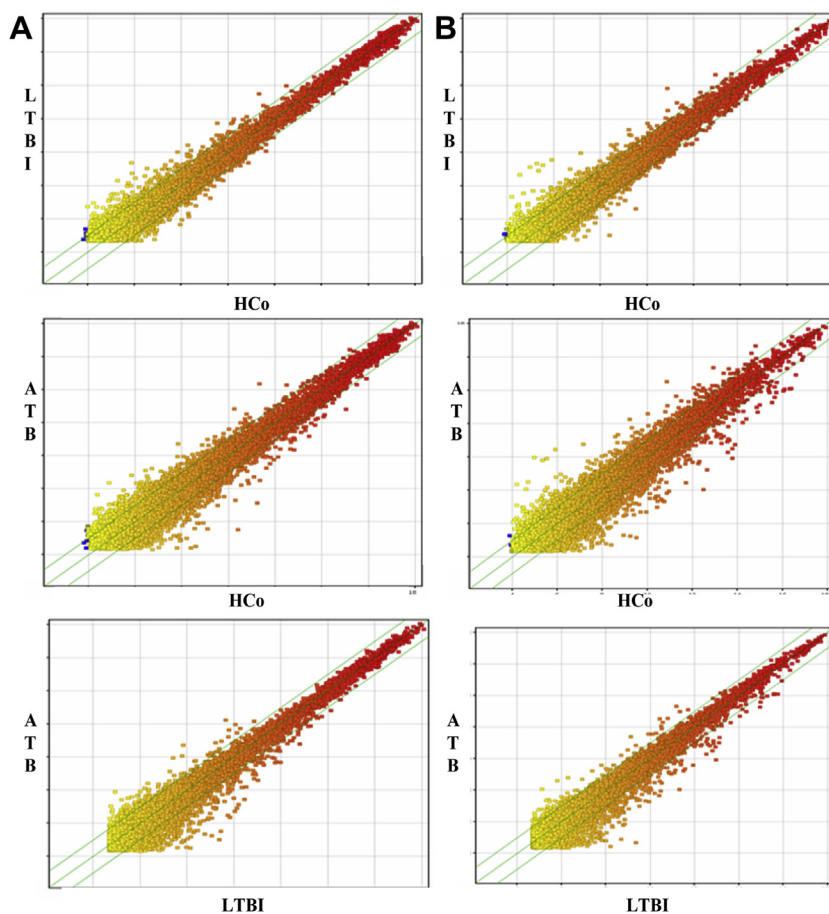


Figure 1 The scatter plots showed lncRNA variation (A) and mRNA variation (B) between chips. HCo: healthy control; LTBI: latent TB infection; ATB: active TB.

NM_000754, NM_003753, NM_001003, NM_001101, NM_006098, NM_002539, NM_022551, NM_004309, NM_002046, NM_001861, NM_000291, NM_005022, NM_001614 and NM_000841) and negative control probes were used as hybridization quality control. Quantile normalization and subsequent data processing were performed using a GeneSpring GX v12.0 software package (Agilent, Santa Clara, CA, USA). Differentially expressed lncRNAs and mRNAs were identified. It is considered statistically significant only if a *P* value adjusted with false discovery rate (FDR) is less than 0.05. Results were presented as fold change. Hierarchical clustering was performed using Agilent GeneSpring GX software (version 12.0).

RT-qPCR analysis

To validate the microarray results, 4 dysregulated lncRNAs were randomly selected and used for confirmation 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, Dalian, China) according to the manufacturer's protocol. Following first strand cDNA synthesis, PCR reaction was carried out in a 10 µL reaction volume containing 1 × SYBR Green PCR Master Mix, 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 s and 60 °C for 1 min with an ABI PRISM 7900 system (Applied Biosystems, USA). The primers used in the study (their sequences were available if necessary) were designed by optimization of parameters including melting temperature, GC content, complementarity, and secondary structure as well as primer length. No template control (containing all the reagents except template) was run to reduce the possible cross contamination from reagents as well as surfaces and no amplification control (including all the reagents other than reverse transcriptase) was used to eliminate genomic DNA contamination. All reactions were performed in triplicate. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Relative expression levels of lncRNAs were calculated using $2^{-\Delta\Delta C_t}$ method and a *P*-value of less than 0.05 was considered statistically significant.

Gene ontology and Kyoto encyclopedia of genes and genomes pathway analysis based on differentially expressed mRNAs

Gene ontology (GO) (www.geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.ad.jp/kegg/>) were used to investigate biological functions and signaling pathways affected by differentially expressed mRNAs.^{13,14} It was considered statistically significant only if a *P*-value was less than 0.05.

Analysis of relationship between lncRNAs and adjacent protein-coding genes

lncRNA originates from complex transcriptional loci and regulates gene expression through epigenetic regulation

of chromatin modification, transcriptional and post-transcriptional processing.¹⁵ To reveal the potential roles of lncRNA in TB infection and shed light on the underlying mechanisms of LTBI and its reactivation, the study mainly focused on analysis of the deregulated lncRNAs and relationship between them and adjacent protein-coding genes.

Statistical analysis

Data were presented as mean ± standard deviation (SD). ANOVA test or student's *t* test was used for statistical analysis. *P* < 0.05 was considered statistically significant.

Results

Differential expression of lncRNAs and mRNAs in CD4⁺ T cells between groups

Microarray analysis showed lncRNA and mRNA expression variations between chips (Fig. 1). In order to identify the most significant candidates, lncRNAs or mRNAs with at least two-fold expression changes were selected. After data normalization, we determined hundreds of differentially expressed lncRNAs between groups in Ensembl, RNAdb, NRED, lincRNA, misc_RNA, UCSC_knowngene, H-invDB, HOX cluster, RefSeq_NR and UCR (FDR < 0.05).

Compared with HCo group, 449 lncRNAs (305 of them up-regulated) and 1, 113 lncRNAs (550 of them up-regulated) were deregulated in LTBI group and ATB group, respectively, and 163 lncRNAs (94 of them up-regulated) were differentially expressed in both LTBI group and ATB group (FDR < 0.05). Moreover, compared with ATB group, 685 lncRNAs (521 of them up-regulated) were differentially

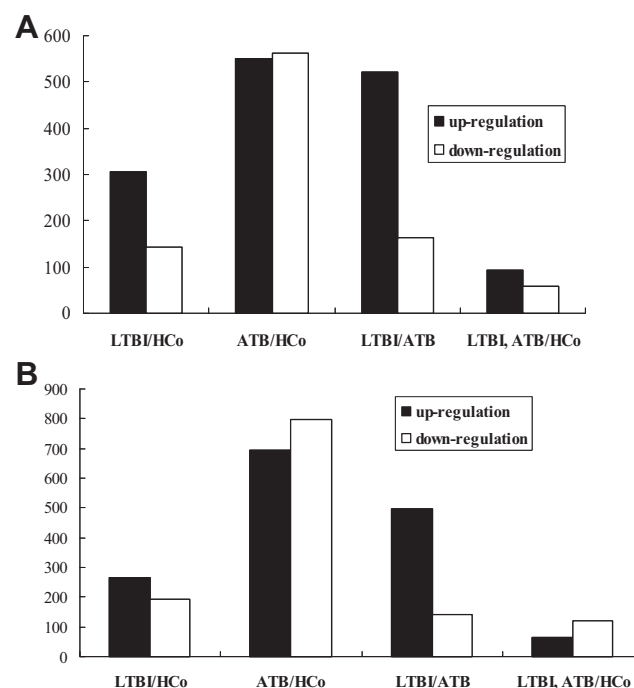


Figure 2 Number of up-regulated and down-regulated lncRNAs (A) and mRNAs (B).

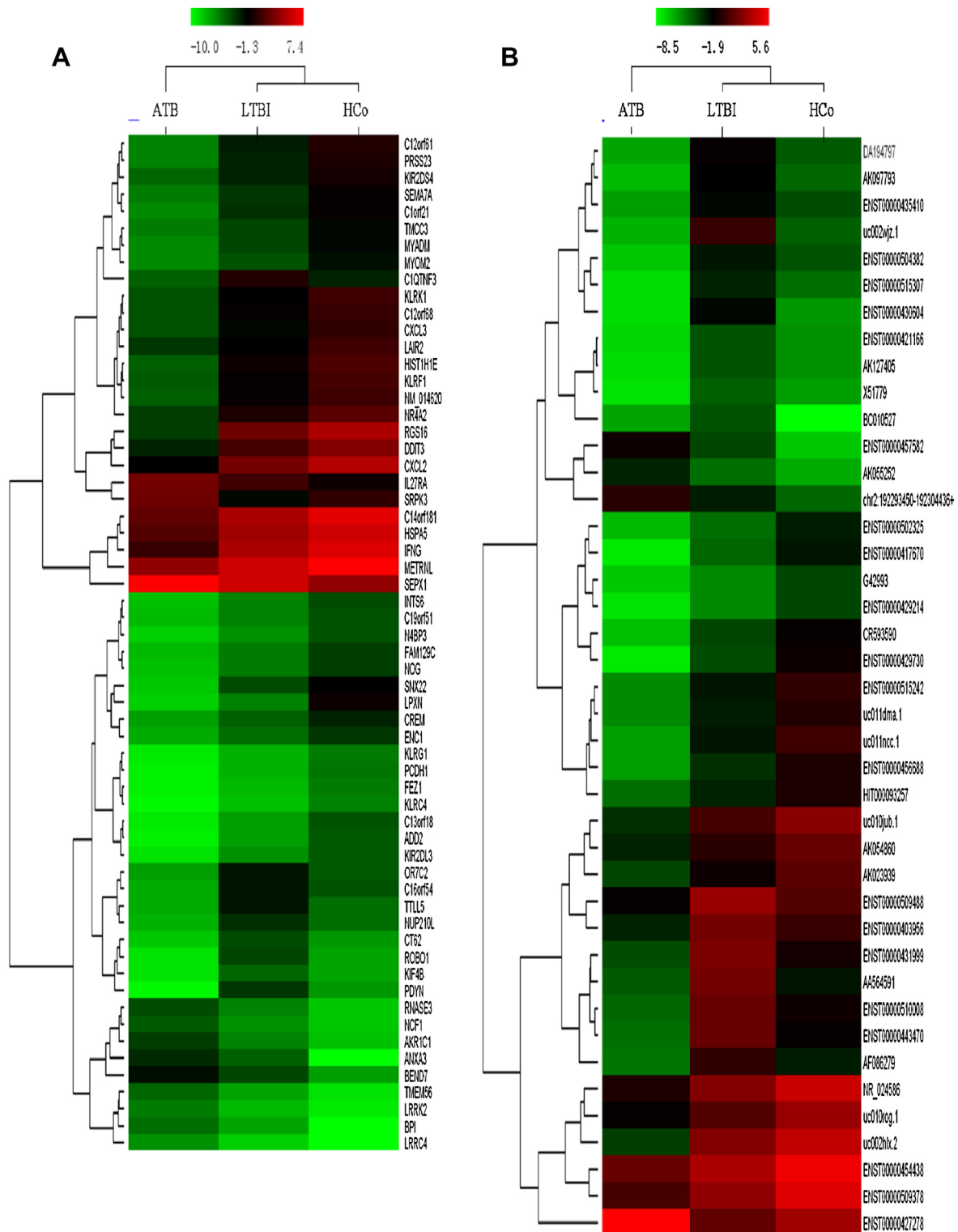


Figure 3 Differentially expressed mRNAs (A) and lncRNAs (B) between three groups. HCo: healthy control ($n = 4$); ATB: active TB ($n = 4$); LTBI: latent TB infection ($n = 4$). Red indicated high relative expression and green indicated low relative expression. One ANOVA test was used for statistical analysis. LncRNA or mRNA with expression fold change >2 and with FDR adjusted P value <0.05 was considered statistically significant.

Table 2 Differentially expressed lncRNAs between three groups.

Seqname	LTBI vs HCo	ATB vs HCo	ATB vs LTBI
DA194797	3.10	-2.26	-7.05
AK097793	3.12	-2.50	-7.82
ENST00000435410	2.24	-2.54	-5.7
uc002wjz.1	5.65	-2.41	-13.65
ENST00000504382	2.09	-3.61	-7.57
ENST00000515307	2.32	-3.51	-8.17
ENST00000430604	4.89	-2.15	-10.55
ENST00000421166	2.00	-2.05	-4.12
AK127405	2.00	-2.27	-4.54
X51779	2.02	-2.17	-4.40
BC010527	6.73	2.72	-2.47
ENST00000457582	4.58	11.3	2.47
AK055252	2.0	4.45	2.26
chr2:192293450-192304436+	2.34	4.85	2.07
ENST00000502325	-2.40	-5.65	-2.34
ENST00000417670	-2.44	-10.4	-2.87
G42993	-2.13	-4.46	-2.10
ENST00000429214	-2.15	-5.90	-2.73
CR593590	-2.37	-9.23	-3.89
ENST00000429730	-2.76	-15.5	-5.62
ENST00000515242	-2.17	-7.80	-3.58
uc011dma.1	-2.03	-6.61	-3.25
uc011ncc.1	-2.54	-11.5	-4.52
ENST00000456688	-2.33	-7.57	-3.24
HIT000093257	-2.09	-4.65	-2.23
uc010jub.1	-2.16	-7.89	-3.64
AK054860	-2.04	-4.82	-2.36
AK023939	-2.24	-5.70	-2.54
ENST00000509488	2.05	-2.41	-4.95
ENST00000403956	2.02	-2.70	-5.47
ENST00000431999	3.26	-2.80	-9.14
AA564591	4.54	-2.14	-9.74
ENST00000510008	2.52	-3.68	-9.29
ENST00000443470	2.97	-3.66	-10.88
AF086279	2.24	-2.74	-6.17
NR_024586	-2.11	-7.08	-3.35
uc010rog.1 (NEAT1)	-2.12	-4.95	-2.33
uc002hlx.2	-2.05	-16.8	-2.73
ENST00000454438	-2.08	-4.54	-2.18
ENST00000509378	-2.19	-5.06	-2.10
ENST00000427278	-2.08	2.93	6.12

Data as fold change. Positive value indicated up-regulation and negative value indicated down-regulation. HCo: healthy control; LTBI: latent TB infection; ATB: active TB. One ANOVA test was used for statistical analysis. LncRNA with expression fold change >2 and with FDR adjusted *P* value <0.05 was considered statistically significant.

expressed in LTBI group (FDR <0.05) (Fig. 2). It was worth noting that, 41 lncRNAs were deregulated between three groups, 23 of which showed similar expression tendency in both LTBI group and ATB group compared with HCo group (FDR <0.05) (Fig. 3, Table 2).

Compared with HCo group, 461 mRNAs (267 of them up-regulated) were differentially expressed in LTBI group, 1,490 mRNAs (694 of them up-regulated) were differentially

Table 3 Differentially expressed mRNAs between the three groups.

GeneSymbol	LTBI vs HCo	ATB vs HCo	ATB vs LTBI
C12orf61	-2.48	-9.39	-3.78
PRSS23	-2.17	-7.98	-3.66
KIR2DS4	-2.21	-5.46	-2.46
SEMA7A	-2.20	-5.67	-2.57
C1orf21	-2.13	-7.20	-3.36
TMCC3	-2.30	-4.80	-2.09
MYADM	-2.12	-5.51	-2.58
MYOM2	-2.49	-5.47	-2.19
C1QTNF3	2.72	-2.30	-6.27
KLRK1	-2.35	-6.64	-2.82
C12orf68	-2.03	-6.89	-3.39
CXCL3	-2.03	-5.93	-2.91
LAIR2	-2.29	-4.88	-2.12
HIST1H1E	-2.22	-9.79	-4.40
KLRF1	-2.28	-8.60	-3.77
NM_014620	-2.10	-8.47	-4.03
NR4A2	-2.25	-7.87	-3.48
RGS16	-2.25	-21.7	-9.62
DDIT3	-2.27	-9.35	-4.10
CXCL2	-2.22	-10.6	-4.78
IL27RA	2.01	4.05	2.02
SRPK3	-2.00	2.46	4.94
C14orf181	-2.09	-5.62	-2.68
HSPA5	-2.02	-5.45	-2.68
IFNG	-2.03	-9.48	-4.65
METRNL	-2.04	-4.41	-2.16
SEPX1	2.17	4.38	2.01
INTS6	-2.03	-5.04	-2.47
C19orf51	-2.00	-4.19	-2.08
N4BP3	-2.23	-5.38	-2.41
FAM129C	-2.21	-5.05	-2.28
NOG	-2.42	-5.96	-2.46
SNX22	-2.77	-15.7	-5.66
LPXN	-7.40	-20.2	-2.73
CREM	-2.40	-5.68	-2.36
ENC1	-2.10	-5.26	-2.50
KLRG1	-2.09	-4.23	-2.02
PCDH1	-2.31	-5.33	-2.30
FEZ1	-2.27	-4.65	-2.04
KLRC4	-2.42	-4.94	-2.03
C13orf18	-2.69	-7.21	-2.68
ADD2	-2.70	-7.75	-2.86
KIR2DL3	-2.17	-6.67	-3.06
OR7C2	2.60	-2.47	-6.46
C16orf54	2.16	-3.61	-7.82
TLL5	3.38	-2.30	-7.78
NUP210L	2.35	-2.60	-6.09
CT62	2.66	-2.05	-5.49
ROBO1	3.43	-2.29	-7.89
KIF4B	2.25	-2.31	-5.20
PDYN	3.82	-3.52	-13.5
RNASE3	2.44	5.08	2.07
NCF1	2.18	4.71	2.15
AKR1C1	2.21	5.79	2.62
ANXA3	8.92	18.05	2.02
BEND7	3.26	7.10	2.17

(continued on next page)

Table 3 (continued)

GeneSymbol	LTBI vs HCo	ATB vs HCo	ATB vs LTBI
TMEM56	2.17	4.91	2.26
LRRK2	2.06	4.43	2.15
BPI	3.63	7.62	2.10
LRRC4	2.03	4.45	2.19

Data as fold change. Positive value indicated up-regulation and negative value indicated down-regulation. HCo: healthy control; LTBI: latent TB infection; ATB: active TB. One ANOVA test was used for statistical analysis. mRNA with expression fold change >2 and with FDR adjusted *P* value <0.05 was considered statistically significant.

expressed in ATB group, and 187 mRNAs (66 of them up-regulated) were differentially expressed in both LTBI group and ATB group (FDR <0.05). Compared with ATB group, 639 mRNAs (497 of them up-regulated) were differentially expressed in LTBI group (FDR <0.05) (Fig. 2). It was worth noting that 60 mRNAs were deregulated between three groups, 50 of which showed similar expression tendency in both LTBI group and ATB group compared with HCo group (FDR <0.05) (Fig. 3, Table 3).

Confirmation of microarray results by RT-qPCR

Four lncRNAs were randomly selected for confirmation of microarray results using RT-qPCR. Data showed that, compared with HCo group, 4 lncRNAs showed similar expression tendency in both LTBI group and ATB group: ENST00000429730 and uc011ncc.1 were down-regulated, while ENST00000457582 and chr2:192293450-192304436+ were up-regulated in both LTBI group and ATB group; compared with ATB group, ENST00000429730 and uc011ncc.1 were increased, while ENST00000457582 and chr2:192293450-192304436+ were decreased in LTBI group (Fig. 4). The results were consistent with those obtained by microarray analysis.

Relationship between lncRNAs and adjacent protein-coding genes

More and more evidence suggests that lncRNAs play important roles in regulation of gene expression.^{16,17} To reveal potential roles of lncRNAs in TB infection, we analyzed the relationship between differentially expressed lncRNAs and their adjacent protein-coding genes. The majority of differentially expressed lncRNAs are from intergenic regions (~50%), natural antisense to protein-coding loci (~20%), or intronic antisense to protein-coding loci (~10%), with the others representing overlapping transcripts from exons or introns in both sense and antisense directions, exon sense-overlapping, or bidirectional regions. Among the 685 differentially expressed lncRNAs between LTBI group and ATB group, 463 of them are oriented in or around a known protein-coding region (i.e., not intergenic). Among the 41 differentially expressed lncRNAs between three groups, 16 of them are oriented in or around a known protein-coding region (Table 4). However, expression levels of adjacent protein-coding genes of the 16 deregulated lncRNAs were largely unaltered. Only a few lncRNAs and

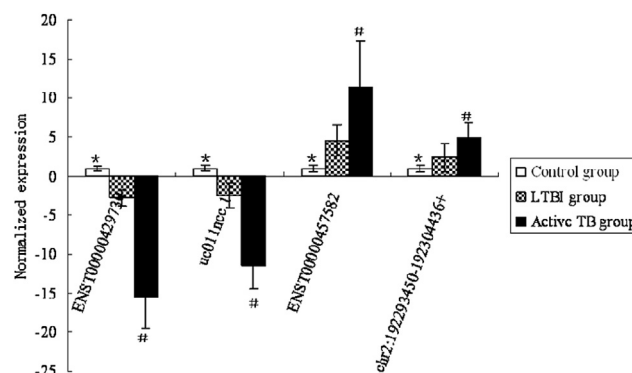


Figure 4 Confirmation of lncRNAs expression by RT-qPCR. After normalization to GAPDH expression, data were presented as mean \pm SD and obtained average expression value for each lncRNA was used for statistics. One ANOVA test for three groups or student's *t* test for two groups was used for statistical analysis. Four lncRNAs were differentially expressed between three groups. * Significant difference between healthy controls ($n = 30$) and LTBI group ($n = 25$), as well as between healthy controls and active TB group ($n = 30$). # Significant difference between LTBI group and active TB group. *P*-value <0.05 was considered statistically significant. Error bars in graphs referred to standard deviation. Each reaction was run three separate times, with technical triplicates in each reaction.

their associated protein-coding genes were differentially expressed simultaneously between two groups: both uc002hlx.2 and its adjacent protein coding gene CCL4L1 mRNA were down-regulated in ATB group compared with LTBI group as well as HCo group. Levels of NR_024586 lncRNA and ENST00000431999 lncRNA as well as their associated protein coding genes MLL5 mRNA and CYP1B1 mRNA were down-regulated in ATB group compared with HCo group, respectively.

GO and KEGG pathway analysis of deregulated mRNAs

GO analysis showed that the differentially expressed mRNAs between LTBI group and ATB group were significantly enriched in cell communication, immune response, MAPKKK cascade, toll signaling pathway, etc. Pathway analysis indicated that the deregulated mRNAs between LTBI group and ATB group were mainly involved in MAPK signaling pathway, cytokine–cytokine receptor interaction, chemokine signaling pathway, natural killer cell mediated cytotoxicity, toll-like receptor signaling pathway, Fc gamma R-mediated phagocytosis, antigen processing and presentation, p53 signaling pathway, NOD-like receptor signaling pathway and metabolism of xenobiotics by cytochrome P450 (*P*-value <0.05 after multiple testing correction) (Table 5).

Discussion

Expression profiles of genes involved in the pathogenesis of LTBI and active TB have been widely studied, however, the molecular regulatory mechanisms underlying TB disease

Table 4 Analysis of the differentially expressed lncRNAs and adjacent protein-coding genes.

Seqname	GeneSymbol	Source	RNA length	Chrom	Relationship	Associated_gene_name
DA194797	lincRNA-ZMIZ1-6	lincRNA	549	chr10	Intergenic	
AK097793		misc_RNA	1698	chr18	Intronic antisense	C18orf1
ENST00000435410	LA16c-59E1.2	Ensembl	822	Chr22	Intergenic	
uc002wjz.1	BC012193	UCSC_ knowngene	1317	chr20	Bidirectional	PANK2
ENST00000504382	CTD-2227C6.3	Ensembl	526	chr5	Intergenic	
ENST00000515307	AL590666.2	Ensembl	2293	chr1	Natural antisense	NES
ENST00000430604	RP11-3N2.4	Ensembl	821	chr7	Intergenic	
ENST00000421166	RP4-681L3.2	Ensembl	230	chr1	Natural antisense	SLC26A9
AK127405		misc_RNA	1750	Chr3	Intergenic	
X51779		misc_RNA	3444	Chr5	Intergenic	
BC010527		RNAdb	1447	chr8	Intergenic	
ENST00000457582	DEFA8P	Ensembl	285	chr8	Intergenic	
AK055252		misc_RNA	2452	chr15	Intron sense-overlapping	C15orf38-AP352
chr2:192293450-192304436+	lincRNA-OBFC2A-4	lincRNA	10987	chr2	Intergenic	
ENST00000502325	AC005593.2	Ensembl	515	chr5	Bidirectional	FNIP1
ENST00000417670	CD8BP	Ensembl	633	chr2	Intergenic	
G42993		misc_RNA	150	chr6	Intergenic	
ENST00000429214	RP11-162G10.5	Ensembl	826	chr10	Bidirectional	ZNF248
CR593590		NRED	2329	chr18	Natural antisense	MPPE1
ENST00000429730	AC079767.4	Ensembl	492	chr2	Intergenic	
ENST00000515242	AC215219.2	Ensembl	1643	chr12	Intergenic	
uc011dma.1	BC068238	UCSC_ knowngene	2289	chr6	Intergenic	
uc011ncc.1	DDX11L family member	UCSC_ knowngene	1643	chrY	Intergenic	
ENST00000456688	RP11-327P2.5	Ensembl	569	chr13	Bidirectional	DHRS12
HIT000093257		H-invDB	499	chr4	Bidirectional	NFKB1
uc010jub.1	AK293020	UCSC_ knowngene	375	chr6	Intergenic	
AK054860		NRED	2659	chrX	Intergenic	
AK023939		misc_RNA	3065	Chr17	Intergenic	
ENST00000509488	RP11-241F15.6	Ensembl	521	Chr4	Intergenic	
ENST00000403956	RP3-406A7.1	Ensembl	242	Chr6	Intergenic	
ENST00000431999	C2orf58	Ensembl	527	chr2	Natural antisense	CYP1B1
AA564591	lincRNA-PPYR1-2	lincRNA	385	chr10	Intron sense-overlapping intronic antisense	GPRIN2 SYT15 PPYR1 AGAP9 ANXA8 FAM25C FAM25B ANXA8L1 FAM25G
ENST00000510008	AC068137.8	Ensembl	774	chr2	Intergenic	
ENST00000443470	RP4-696P19.2	Ensembl	744	chr6	Intergenic	
AF086279		misc_RNA	603	chr14	Intergenic	
NR_024586	LOC100216545	RefSeq_NR	3615	chr7	Bidirectional	MLL5
uc010rog.1	NEAT1	UCSC_ knowngene	17357	chr11	Intergenic	
uc002hlx.2	CCL4L	UCSC_ knowngene	541	chr17	Exon sense-overlapping	CCL4L1

(continued on next page)

Table 4 (continued)

Seqname	GeneSymbol	Source	RNA length	Chrom	Relationship	Associated_gene_name
ENST00000454438	AC108039.3	Ensembl	364	chr2	Intergenic	
ENST00000509378	RP11-39C10.1	Ensembl	2057	chr4	Intronic antisense	1-Mar
ENST00000427278	ZEB2AS	Ensembl	1187	chr2	Natural antisense	ZEB2
ENST00000446001	RP11-501119.3	Ensembl	332	chr6	Natural antisense	GFOD1

UCSC_knowngene: UCSC known genes annotated as “noncoding”, near coding” or “antisense” (<http://genome.ucsc.edu/cgi-bin/hgTables/>); Ensembl: Ensembl (<http://www.ensembl.org/index.html>); H-invDB: H-invDB (<http://www.h-invitational.jp/>); RNAdb: RNAdb2.0 (<http://research.imb.uq.edu.au/rnadb/>); NRED: NRED (<http://jrm-research.imb.uq.edu.au/nred/cgi-bin/ncrnadb.pl>); lincRNA: lincRNA identified by John Rinn’s group (Guttman et al., 2009; Khalil et al., 2009); misc_lincRNA: other sources.

Relationship: the relationship of lincRNA and its nearby coding gene. “sense_overlapping”: the lincRNA’s exon is overlapping a coding transcript exon on the same genomic strand; “intronic”: the lincRNA is overlapping the intron of a coding transcript on the same genomic strand; “natural antisense”: the lincRNA is transcribed from the antisense strand and overlapping with a coding transcript; “bidirectional”: the lincRNA is oriented head to head to a coding transcript within 1000 bp; “intergenic”: there are no overlapping or bidirectional coding transcripts nearby the lincRNA.

Source: The source of lincRNA is collected from. RefSeq_NR: RefSeq validated noncoding RNA.

remains unclear. LncRNAs have been shown to be involved in major mechanisms of gene expression regulation, such as initiating chromatin remodeling, targeting transcription factors, directing transcriptional and post-transcriptional processing.¹⁸ Increasing studies have reported that many lncRNAs are deregulated in human diseases.^{19,20} The mechanisms of action of lncRNAs, however, are still unclear. Therefore, we performed the study to better reveal the potential role of lncRNAs in contributing to TB infection.

It was interesting to note that more lncRNAs and mRNAs were deregulated in ATB group than LTBI group. The data indicated that more genes in CD4⁺ T cells were involved in active TB than LTBI. Compared with ATB group, 685 lncRNAs (521 of them up-regulated) and 639 mRNAs (497 of them up-regulated) were differentially expressed in LTBI group, respectively. The result suggested that transcriptional activity was more decreased in active TB than that in LTBI.

Moreover, 41 lncRNAs were differentially expressed between three groups and 23 of them showed similar expression tendency in both LTBI group and ATB group compared with HCo group. The results suggested that both active TB and LTBI might possess, at least partly, similar regulatory mechanisms. Increasing evidence indicates that many lncRNAs are associated with some infectious diseases.

More than 4,800 lncRNAs were involved in the host response to EV71 infection.²¹ Approximately 500 lncRNAs were differentially expressed during coronavirus infection.²² A genetic variant in HULC lncRNA contributed to risk of HBV-related hepatocellular carcinoma in a Chinese population.²³ LncRNAs can be used as biomarkers for many infectious diseases.²⁴ The data suggested that lncRNAs might be widely involved in various infectious diseases. Recent data showed that NEAT1 (uc010rog.1) played an important role in the innate immune response to influenza virus and herpes simplex virus by facilitating the expression of antiviral genes including cytokines such as interleukin-8 (IL-8).²⁵ Another data reported that NEAT1 was involved in the replication of HIV-1 and knockdown of NEAT1 enhanced virus production.²⁶ Our results showed that NEAT1 lncRNA was down-regulated in both ATB group and LTBI group compared with HCo group. The data suggested that NEAT1 may be involved in host response to pathogen infection such as TB disease. Despite much progress, function of NEAT1 is still not well-defined. Only a few lncRNAs were found to be associated with various diseases. However, their potential functions of most deregulated lncRNAs, such as ENST00000393334, AK125689, ENST00000457582 and NR_002191, are still largely unknown.

Table 5 The list of enriched KEGG pathways of the differentially expressed mRNAs in CD4⁺ T cells in active TB group versus LTBI group.

Pathway ID	Definition	Gene count	P value
hsa04010	MAPK signaling pathway	268	0.0012
hsa04060	Cytokine–cytokine receptor interaction	265	0.0001
hsa04062	Chemokine signaling pathway	189	0.0035
hsa04650	Natural killer cell mediated cytotoxicity	137	0.0002
hsa04620	Toll-like receptor signaling pathway	102	0.0164
hsa04666	Fc gamma R-mediated phagocytosis	95	0.0241
hsa04612	Antigen processing and presentation	78	0.0002
hsa04115	p53 signaling pathway	69	0.0087
hsa04621	NOD-like receptor signaling pathway	59	0.0180
hsa00980	Metabolism of xenobiotics by cytochrome P450	53	0.0161

Enriched KEGG pathways were used for analysis of the differentially expressed mRNAs between active TB group and LTBI group. P-values after multiple testing corrections <0.05.

A recent report showed that a 251-gene signature from PBMC could accurately distinguish patients with active TB from both individuals with LTBI and healthy controls.²⁷ One hundred and forty-four blood transcripts were able to distinguish TB from controls and other lung diseases, such as pneumonia and lung cancer.²⁸ A distinct whole blood 86-gene transcriptional signature of active TB is distinct from other diseases.²⁹ Specific molecular signatures in PBMC could distinguish active TB from LTBI after antigenic stimulation with purified protein derivative of tuberculin (PPD) *in vitro*.³⁰ The data suggested that PBMCs play important roles in the immune response to TB infection. CD4⁺ T cells are important components of PBMCs and here we investigated transcriptional signature of CD4⁺ T cells in TB infection. In the study, 60 mRNAs were differentially expressed between three groups and 50 of them showed similar expression tendency in both LTBI group and ATB group compared with HCo group, which suggested that both active TB and LTBI might have, at least partly, similar regulatory mechanisms. In the study, it was worth mentioning that most of the differentially expressed mRNAs in PBMCs^{27–30} were not deregulated in CD4⁺ T cells, which was consistent with our recent miRNA expression results.³¹ These data indicated that the major cell type in PBMCs that showed deregulated mRNAs may not be CD4⁺ T cells and response to TB infection of PBMCs may not be consistent with that of CD4⁺ T cells. Further studies are needed to explore the inconsistency between our results and published data.

Most of the differentially expressed lncRNAs between LTBI group and ATB group were oriented in or around a known protein-coding region (i.e., not intergenic), and approximately 40% of which were cis-antisense lncRNAs in the study. It was reported that knockdown or down-regulation of certain lncRNA could lead to decreased expression of its neighboring protein-coding gene, which suggested that lncRNAs and nearby coding genes could have shared upstream regulation or local transcriptional effects.^{32,33} We found that only a few lncRNAs and their associated coding genes were differentially expressed simultaneously, such as both NR_024586 lncRNA and MLL5 mRNA, both ENST00000431999 lncRNA and CYP1B1 mRNA, which were all down-regulated in ATB group compared with HCo group. This result suggested that these lncRNAs and mRNAs were all associated with TB, but might be independently regulated. Future studies using knockdown or over-expression techniques in a relevant model system would be a reasonable approach to uncover their potential regulatory roles in TB disease. However, most of the deregulated lncRNAs associated protein genes were not differentially expressed, which suggested that more complex mechanisms than simple cis-regulation existed for these lncRNAs.

MAPK signaling pathway was involved in extensive tissue inflammation in TB disease.³⁴ Up-regulation of GM-CSF by Mtb was mediated by MAPK-associated signaling pathway.³⁵ Increased cytokine response was involved in TB disease.³⁶ Mtb RpfB driven Th1-type T cell immunity via a TLR4-dependent activation of dendritic cells.³⁷ These results indicated that MAPK signaling pathway and Toll-like receptor signaling pathway played important roles in TB infection. Our data showed that the differentially expressed mRNAs were also mainly involved in MAPK signaling pathway, Toll-like receptor signaling pathway, etc. This result was in

part agreement with that of a recent study in CD4⁺ T cells.³⁸ Analysis of PBMC revealed that active TB was closely associated with IFN signaling pathway, and IFN-inducible transcripts were over-expressed in purified blood neutrophils, to a lesser extent monocytes, but not CD4⁺ and CD8⁺ T cells.^{28,29} The data also suggested that response to TB infection of PBMCs may not be consistent with that of CD4⁺ T cells. Therefore, it is necessary to study the functions of CD4⁺ T cells in the pathogenesis of TB disease.

In summary, we identified differentially expressed lncRNAs and mRNAs in CD4⁺ T cells from subjects with LTBI and active TB, which opened up a new and interesting avenue toward an improved understanding of the relationship between lncRNA homeostasis in CD4⁺ T cells and TB infection. Role of lncRNAs in TB infection was just beginning to be explored and it was difficult to synthesize our results to reach a definitive conclusion based on this single study. As only 4 samples from each group were extensively evaluated, future studies are necessary to replicate, expand these findings in more patient samples. Moreover, further investigation of lncRNA roles may help to elucidate the underlying mechanisms of LTBI and its reactivation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jinf.2014.06.016>

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