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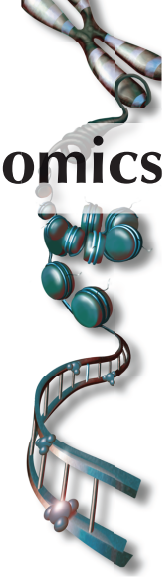
Epigenomics



Specific PIWI-interacting small noncoding RNA expression patterns in pulmonary tuberculosis patients

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Aim: PIWI-interacting RNAs (piRNAs) play crucial roles in germline development and carcinogenesis. The expression patterns of piRNAs in pulmonary tuberculosis (PTB) are still unclear. **Materials & methods:** Small RNA sequencing was applied to investigate peripheral blood piRNA expression patterns in PTB patients and healthy individuals. **Results:** A total of 428 upregulated and 349 downregulated piRNAs were identified from PTB patients. Target genes of dysregulated piRNAs were mainly involved in transcription and protein binding. Dysregulated piRNAs were enriched in many pathways related with immunity. Many target genes were regulated by the same piRNAs. Nucleotide bias of these piRNAs showed that piRNAs in peripheral blood may be formed from the primary biogenesis pathway. **Conclusion:** Findings demonstrated that the PIWI-piRNA pathway is active in human peripheral blood, where it may represent a new player in the PTB pathogenesis.

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Keywords: expression pattern • healthy individuals • peripheral blood • piRNA clusters • piRNA • PTB patients • pulmonary tuberculosis • regulatory interaction • small RNA sequencing • target genes

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. This mycobacterium not only affects the lungs but also affects other sites [1]. Only 5–15% of people infected with *M. tuberculosis* will develop TB during their lifetime [2]. This disease has remained one of the major threats to health [3]. TB pathogenesis and the immune response to *M. tuberculosis* infection have not been completely elucidated. Therefore, understanding the components of the host response will help us to a better understanding of the pathogenesis of TB in humans, and to find novel approaches to prevention and therapy of this infectivity disease [4–6].

Noncoding small RNAs, regulatory RNAs, regulate the expression of target genes at the transcriptional and translational levels [7–9]. miRNAs have emerged as important regulators of the immune response [10–14]. miRNA expression level is differentially regulated in macrophages following mycobacterial infection. Expression of miRNAs in macrophages can be induced by extracted mycobacterial cell–wall components [15]. A total of 15 miRNAs were expressed uniquely in latent TB infection with miRNA microarray [16]. miRNA (miR)-133a inhibits collagen degradation by downregulating matrix metalloproteinase-9 expression to attenuate the destructive effects of spinal TB on intervertebral discs [17]. The miR-26a/KLF4 and CREB-C/EBPβ signaling pathways play important roles in regulating the survival of *M. tuberculosis* in macrophages [18]. The presences of circulating extracellular RNA (ExRNAs) from the tissues injury have the potential to be as biomarker for a great variety of medical conditions [19]. Our previous studies have shown that the expression levels of six serum miRNAs in the peripheral blood of patients

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with pulmonary tuberculosis (PTB) are significantly different from those in healthy individuals. Logistic regression analysis of six serum miRNAs can be used for diagnosis of PTB [20]. Comprehensively understand the relationship between noncoding small RNAs and PTB will help us to better understand the PTB pathogenesis.

Compared with miRNAs, piRNAs are a novel type of noncoding small RNAs that interact with a subset of Argonaute proteins, and are a less-investigated type of small RNA [21,22]. This type of noncoding small RNA was first found in mammalian systems in 2006 [23–25]. They are 26–31 nucleotides (nt) long and are clearly distinct from miRNAs that are 21–24 nt [26]. PiRNAs have been studied extensively in *Drosophila* [27,28], mice [29], *Caenorhabditis elegans* [30,31], zebrafish [32], *Aedes aegypti* [33], *Tribolium castaneum* [34] and *Cubitus interruptus* [35]. Many roles of piRNAs were reported, such as maintaining the integrity of germline DNA, heterochromatin formation, sex determination and germline development [36,37]. However, most of piRNAs identified involved in inside of germline, and the roles played by piRNAs outside of germline are still poorly investigated [38]. However, there is an increasing number of reports about piRNA identification and function outside the germline, such as breast cancer [39,40], hepatocellular carcinoma [41], Alzheimer's disease [42,43], renal cell carcinoma [44], pancreatic cancer [45], bladder cancer [46] and gastric cancer [47]. In addition, piRNAs are stably expressed in human serum and plasma samples, which can serve as valuable blood-based biomarkers for disease detection and monitoring [48]. Recent findings on piRNAs suggest that these novel noncoding RNAs are crucial regulators that contribute to the progression of human diseases.

In this study, small RNA sequencing showed that piRNAs were aberrantly expressed in the peripheral blood of PTB patients and healthy individuals. Many dysregulated piRNAs were found and these aberrantly expressed piRNAs may be associated with the pathogensis of PTB. This study provided the first evidence of piRNAs in the peripheral blood of PTB patients, which will provide the foundation to determine the regulatory role of piRNAs in PTB.

Materials & methods

Patients

All the PTB patients and healthy individuals were recruited from the First People's Hospital of Zhangjiagang, Jiangsu Province, China. PTB patients were diagnosed based on clinical manifestations. Healthy individuals had no family history of hereditary diseases or low immune function. The characters for PTB patients and healthy individuals were described in our published paper [49,50]. Three PTB patients and three healthy individuals were selected for detecting small RNAs with small RNA sequencing. Other 20 normal healthy people as control group and 20 PTB patients as test group were recruited for further validation.

Small RNA sequencing

Peripheral blood was collected from PTB patients and healthy individuals and the total RNAs was extracted from these samples according to our published paper [49,50]. All RNA samples were submitted to Shanghai Oebiotech (China) and sequencing was performed on an Illumina Hiseq4000.

Bioinformatics analysis

Obtained raw data/reads were filtered remove the low-quality reads, reads with 5′-primer contaminants and poly (A). Reads without 3′-adapter and insert tags, and reads shorter than 15 nt and longer than 41 nt from raw data were filtered to obtain clean reads. Samll RNAs were aligned and subjected to BLAST (v2.2.28+) search against Rfam (version 10.0) (http://www.sanger.ac.uk/software/Rfam) with E-value $\leq$0.01, miRBase database (http://www.mirbase.org/) and piRBase (http://www.regulatoryrna.org/database/piRNA/). Through the above several methods to filter, obtained clean reads were aligned with the known piRNA from the piRBase with Bowtie software without mismatches. The novel piRNAs were predicted with Piano (http://ento.njau.edu.cn/Piano.html) according to the structure and sequence characteristics of the interaction between transposon and piRNAs [51]. Transcript per million (TPM) was used to calculate the expression levels of these piRNAs [52]. piRNAs were considered differentially expressed (DE) when showing absolute fold change $\geq$2 with false discovery rate (FDR) $\leq$0.05, as determined by DESeq method (http://bioconductor.org/packages/release/bioc/html/DESeq.html). The biological processes associated with mRNAs targeted by the DE piRNAs were predicted with Miranda algorithm (single-residue-pair match scores$\geq$150, specific Gibbs free energy (ΔG) $\leq$-30 kcal/mol and Demand strict 5′ seed pairing). Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of DE piRNA target genes were based on hypergeometric distribution ($p \leq 0.05$).

Western blotting

To estimate the expression levels of PIWIL2 and PIWIL4 proteins in the peripheral blood, three PTB patients and three healthy individuals were included. Total protein was extracted from the peripheral blood with a whole blood protein extraction kit. 30 μg total proteins were run on a 12% polyacrylamide gel. The antibodies included rabbit polyclonal anti-PIWIL2 and anti-PIWIL4 (1: 1000; Abcam, MA, USA). Next, goat anti-rabbit horseradish-peroxidase-conjugated secondary antibody (1: 2000; Abcam) was used and then visualized using enhanced chemiluminescence (Millipore, MA, USA). GAPDH (Abcam, MA, USA) was selected as the protein internal reference control.

piRNA–mRNA network construction

To explore the relationship between piRNAs and target mRNAs in PTB progression, the coexpression networks were constructed based on the correlation analysis between the DE piRNAs and target mRNAs. One network was constructed with upregulated piRNAs and downregulated mRNAs, and another with downregulated piRNAs and upregulated mRNAs. Many interaction pairs were identified, and only the top 100 interacting pairs were shown according to the lowest p-value.

piRNA clusters analysis

Many piRNAs derived from particular genomic sites are termed as piRNA clusters. the presence of piRNA clusters in the peripheral blood of PTBs were searched with proTRAC software with a scanning window of length 5 kb and a window shift of 1 kb. Significant ($p \leq 0.01$) hit density was calculated based on observed hit distribution.

Real-time PCR

To examine the dysregulation of piRNAs indentified from the PTB patients from RNA sequencing, three upregulated piRNAs (piRNA-1007467, piR-hsa-1344 and piR-hsa-1944) and three downregulated piRNAs (piR-hsa-32157, piRNA-276256 and piRNA-1088346) were selected and validated with real-time PCR. According to the Bulge-loop™ miRNA validation method, the forward primers of six DE piRNAs were designed based on the mature sequence of these piRNAs, and the reverse primers of them were designed according to the published method [53]. U6 gene was used to normalize the expression level of these piRNAs, respectively. All of the primers were shown in the Supplementary Table 1. Total RNAs extraction and real-time PCR validation were conducted according to the methods described in our previous reports [49]. Relative expression levels of piRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was performed using T-test.

Results

PIWIL proteins profiles in the peripheral blood of PTB patients & healthy individuals

The PIWI protein subfamily is essential for piRNAs biogenesis and the expression of PIWILs is related with the presence of active piRNAs pathways. In our previous study, *PIWIL2* and *PIWIL4* genes were expressed in the peripheral blood of PTB patients and healthy individuals by transcriptome sequencing (Figure 1A). To investigate the active piRNA pathways in the peripheral blood of PTB patients, the expression of two human PIWIL proteins (PIWIL2 and PIWIL4) were surveyed in PTB and healthy samples by Western blotting. Two human PIWI orthologs, PIWIL2 and PIWIL4, were present in PTB patients and healthy individuals (Figure 1B). Except these, the related genes (*EXD*1, *FKBP*6, *HENMT*1, *MAEL*, *MOV10L*1, *PLD*6, *TDRD*1, *TDRD*12, *TDRD*9 and *TDRKH*) with piRNA metabolic processes were checked from the transcriptome sequencing, and these genes were expressed in peripheral blood (Figure 1C). Hence, we suggest that active piRNA pathways appear in the peripheral blood of PTB patients and may play some key regulatory roles in PTB pathogenesis.

Small RNA sequencing reveals piRNA in peripheral blood of PTB patients & healthy individuals

To understand the piRNA expression patterns in the peripheral blood of PTB patients and healthy individuals, small RNA sequencing was applied to investigate the small RNA expression pattern. The peripheral blood of PTB patients and healthy individuals were used for total RNAs extraction, and the length of 15–41 nt small RNA were sequenced. Small RNA profiling of mapped reads revealed two distinct peaks, one at the 21–23 nt position, corresponding to miRNA in size, and another at the 31–33 nt position, corresponding to piRNAs in size (Figure 2A). From each sample, 22,672,995–26,645,820 reads aligned to the human genome sequence dataset (GRCh38.p7) were obtained, which included miRNA (0.61%), rRNA (0.03%), tRNA (0.01%), snRNA (0.03%), cis-reg (0.03%),

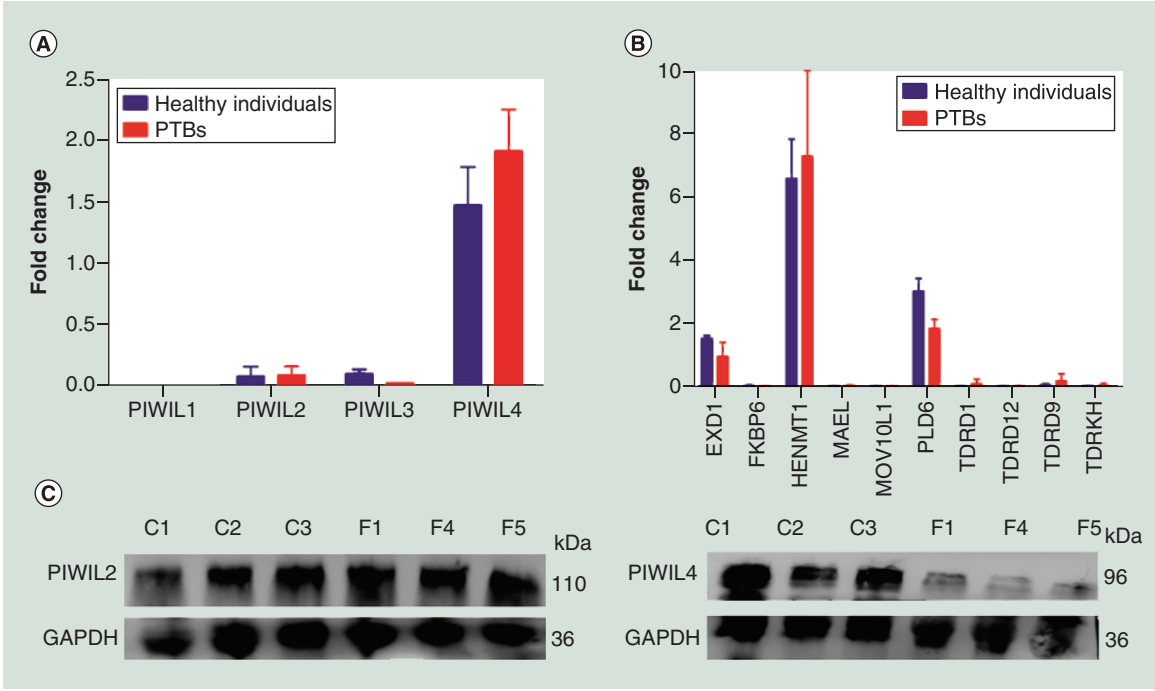

Figure 1. Expression of PIWIL mRNAs and proteins in the peripheral blood of pulmonary tuberculosis patients and healthy individuals. (A) Expression of *PIWIL1*, *PIWIL2*, *PIWIL3* and *PIWIL4* genes in the peripheral blood of pulmonary tuberculosis (PTB) patients and healthy individuals with transcriptome sequencing. *PIWIL1*, *PIWIL2*, *PIWIL3* and *PIWIL4* represent PIWI-like RNA-mediated gene silencing 1, 2, 3 and 4. **(B)** PIWIL2 and PIWIL4 proteins were detected by Western blotting in the peripheral blood of three PTB patients and three healthy individuals. GAPDH was used as the reference control. C1, C2 and C3 represent healthy individuals, and F1, F4 and F5 represent PTB patients. **(C)** Expression of genes related to piRNA metabolism with transcriptome sequencing.
PTB: Pulmonary tuberculosis.

others (0.07%) and unannotated (98.69%) (Figure 2B). In addition to the small RNAs aligned to known sncRNA genes, the remaining reads were aligned to piRBase (http://www.regulatoryrna.org/database/piRNA/). The piRBase currently contains 7.7 billion piRNAs sequences originally discovered from nine species, such as humans, mice, fruitfly, zebrafish and other species (Figure 2C), which can be aligned to the GRCh38.p7 genome without mismatches. Through the repeated sequences filtered, obtained clean reads were aligned with the known piRNA from the piRBase with Bowtie software without mismatches. Nearly 500 known piRNAs identified from each sample. Piano (http://ento.njau.edu.cn/Piano.html) was used to predict piRNAs. A total of 454,320 piRNAs were predicted and 39 481 piRNAs were expressed in peripheral blood cells (Supplementary Table 2). These finding demonstrated that piRNAs were present in the peripheral blood of PTB patients and healthy individuals, which also indicated that piRNAs are highly conserved across species and present in the somatic tissues.

piRNAs are DE in the peripheral blood of PTB patients & healthy individuals

To investigate DE piRNAs in the peripheral blood of PTB patients and healthy individuals, the identified piRNAs (known and novel piRNAs) were subjected to differential expression analysis with the DESeq method. Expression of piRNAs in PTB patients and healthy individuals was measured based on TPM (transcript per million), which indicated no abnormal expression in the six samples (Figure 3A). The number and distribution of piRNAs from the different samples were displayed in the volcano plot (Figure 3B and 3C). Hierarchical clustering revealed a clear segregation of the samples from PTB patients from healthy individuals (Figure 3D). Total 777 DE piRNAs were identified from the PTB patients, including 428 upregulated and 349 downregulated piRNAs (fold change >2, and FDR < 0.05). A total of 142 and 192 DE piRNAs were only specifically expressed in healthy individuals and PTB patients, respectively (Figure 4A). To understand the authenticity of these dysregulated piRNAs from the sequencing data, a set of piRNAs (piRNA-1007467, piR-hsa-1344, piR-hsa-1944, piR-hsa-32157, piRNA-276256

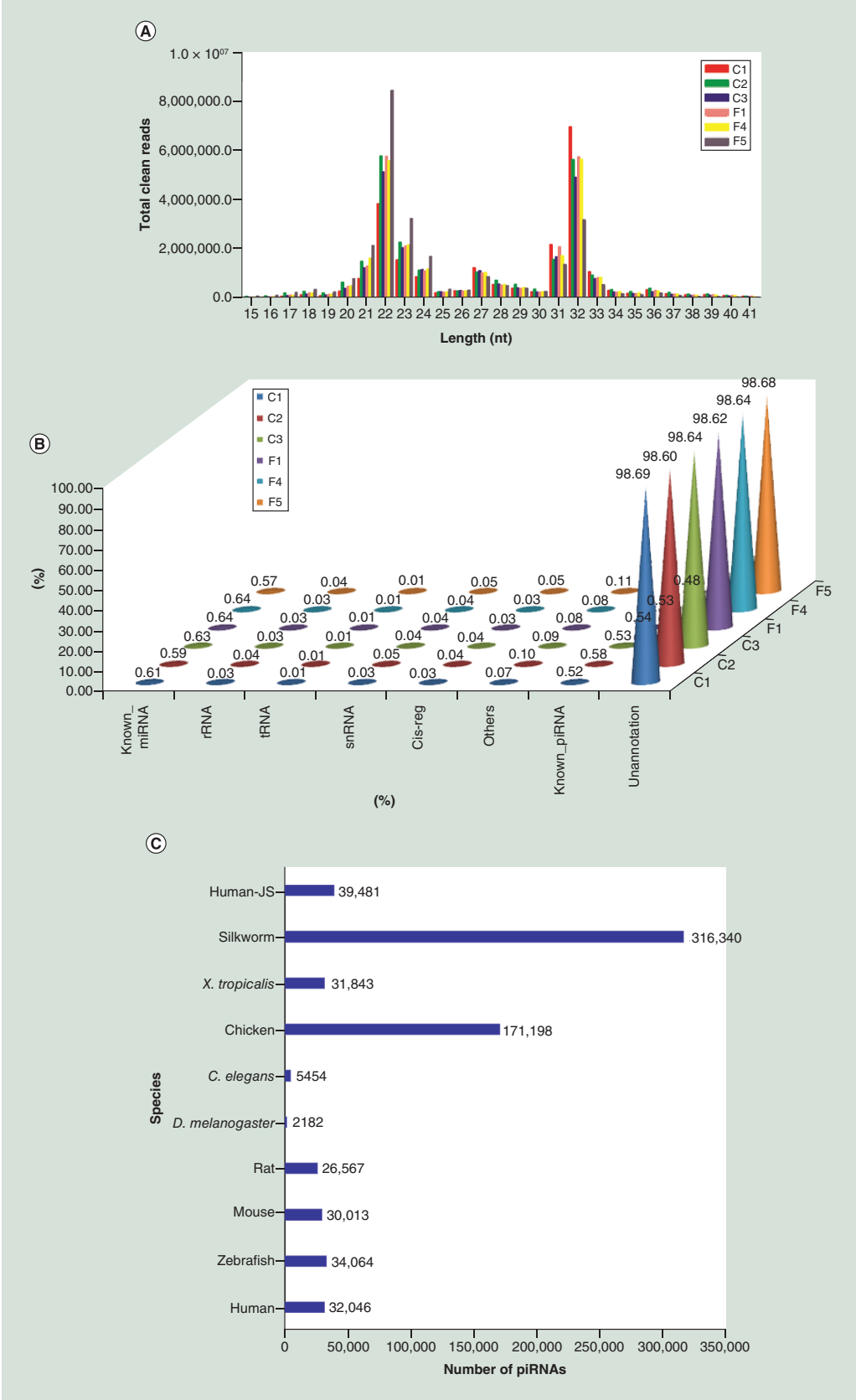

Figure 2. Characterization of PIWI-interacting RNAs present in the peripheral blood of pulmonary tuberculosis patients and healthy individuals. (A) Length distribution of the small RNAs identified with small RNA sqeuencing. **(B)** Category of small RNA with uniq-clean reads from the small RNA sequencing. **(C)** Number of piRNAs from different species (humans, silkworm, *X. tropicalis*, chicken, *C. elegans*, *D. melanogaster*, rat, mouse, zebrafish) and the data were collected from the piRBase (http://www.regulatoryrna.org/database/piRNA/).
piRNA: PIWI-interacting RNA.

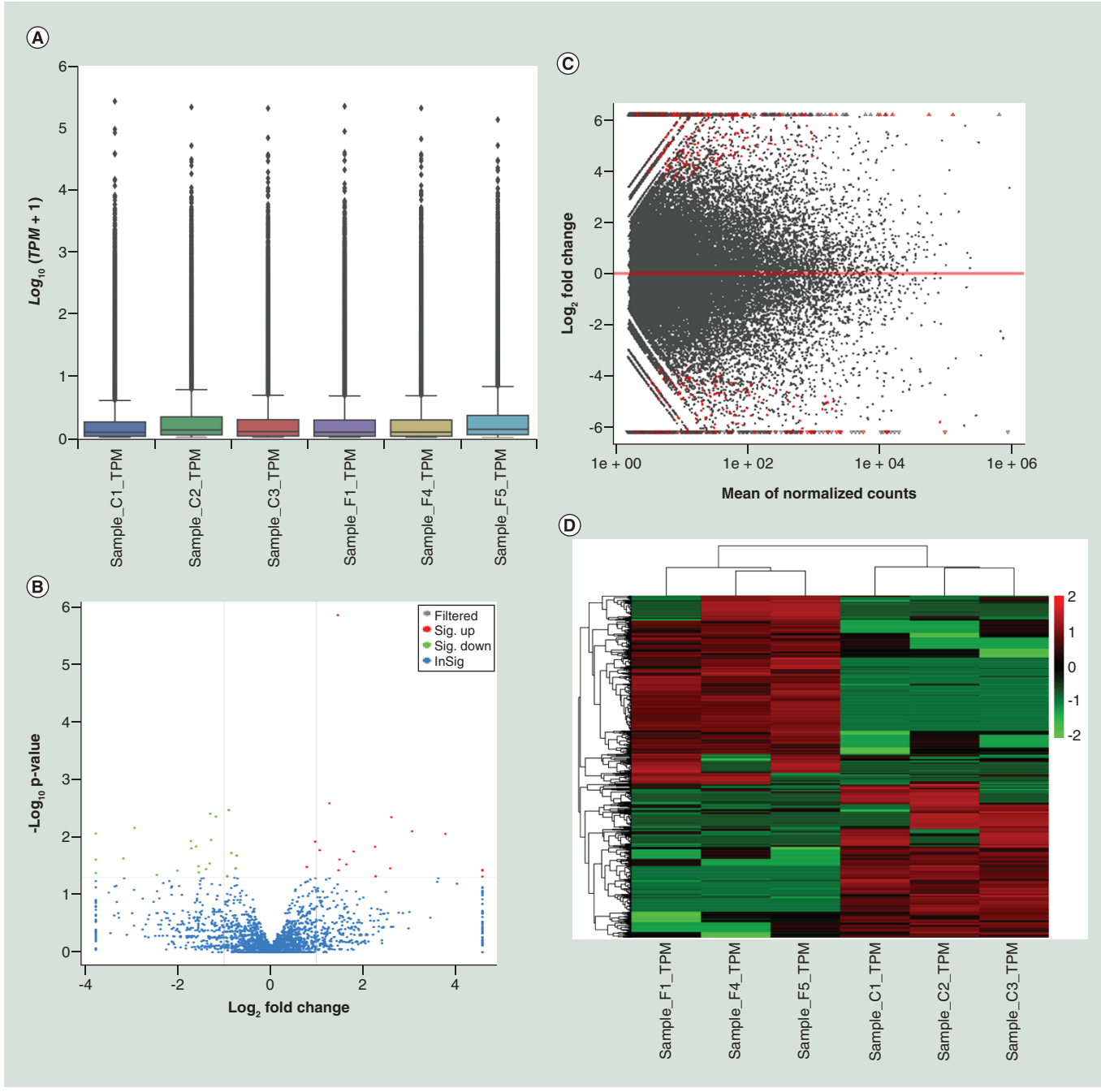

Figure 3. Expression of PIWI-interacting RNAs in the peripheral blood of pulmonary tuberculosis patients and healthy individuals. (A) Box plot showing quality assessment of PIWI-interacting RNA (piRNA) data after filtering. **(B)** MA plot of differentially expressed piRNAs in peripheral blood of pulmonary tuberculosis (PTB) patients and healthy individuals. **(C)** Volcano plots showing differentially expressed piRNAs in peripheral blood of PTB patients and healthy individuals. The vertical lines correspond to a 2.0-fold increase and decrease, respectively, and the horizontal line represents a p-value of 0.05. The red point in the plot represents the significantly differentially expressed piRNAs. **(D)** Heatmaps representing mean centered and normalized data relative to differentially expressed piRNAs in the peripheral blood of three PTBs and three healthy individuals with average fold change > 2; $p < 0.05$ and FDR < 0.05.

and piRNA-1088346) was investigated using real-time PCR (Figure 4B). The results from the real-time PCR were consistent with the sequencing data, which indicated that the sequencing data were accurate and reproducible.

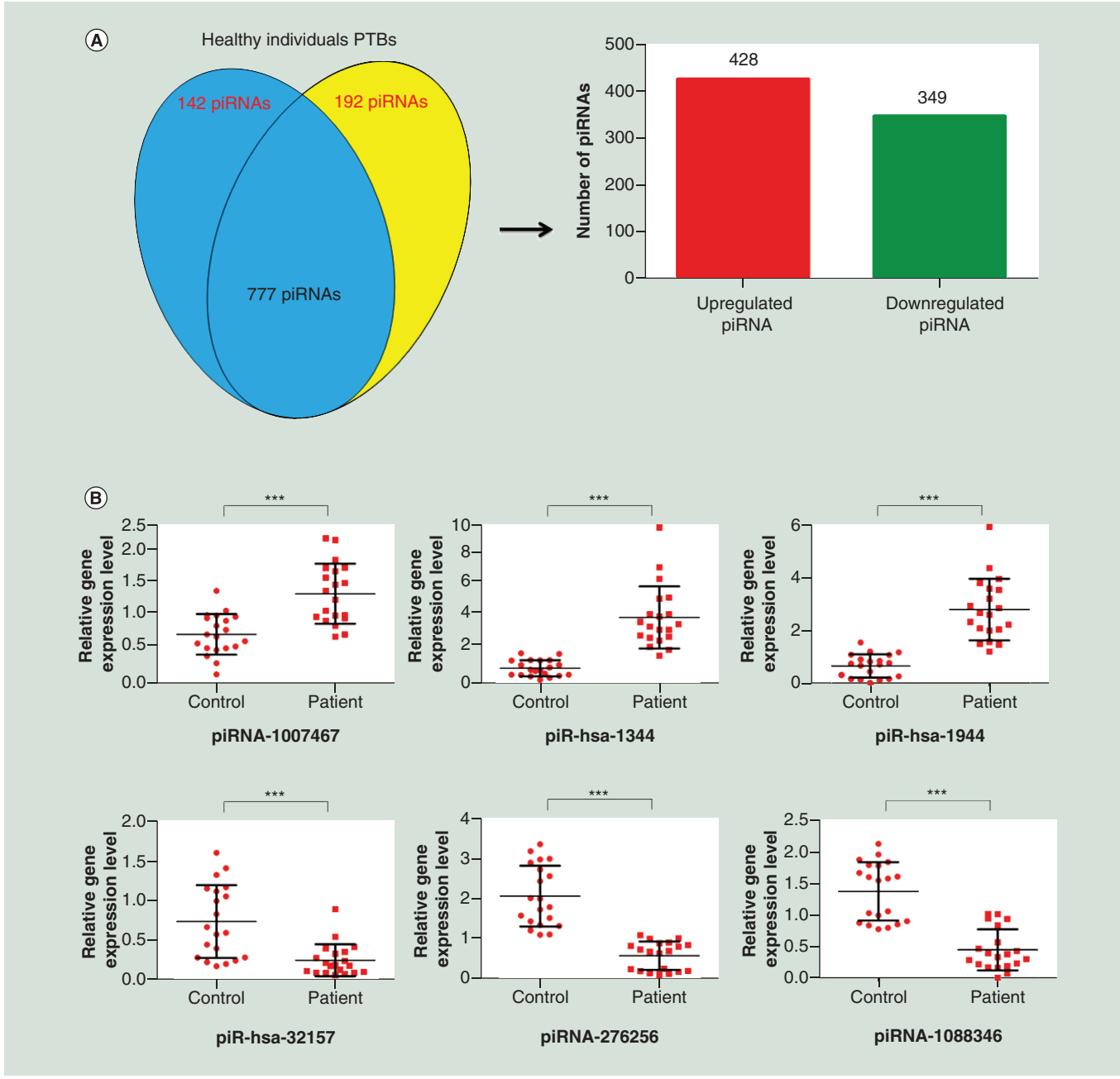

Figure 4. Identification of differentially expressed PIWI-interacting RNAs in peripheral blood of pulmonary tuberculosis patients and healthy individuals. (A) PiRNA Venn diagram for pulmonary tuberculosis patients versus healthy individuals and the differentially expressed piRNAs in peripheral blood of PTB patients and healthy individuals. **(B)** Validation of piRNAs expression levels with real-time PCR. Six differentially expressed piRNAs (piRNA-1007467, piR-hsa-1344, piR-hsa-1944, piR-hsa-32157, piRNA-276256 and piRNA-1088346) from the small RNA sequencing were further validated with real-time PCR. The expression levels of six piRNAs were examined in 20 pulmonary tuberculosis patients and 20 healthy individual. U6 was used as the reference gene. Relative expression levels of piRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method. Statistical analysis was performed using t-test. ***$p < 0.001$.
piRNA: PIWI-interacting RNAs; PTB: Pulmonary tuberculosis.

Target prediction of DE piRNAs

To understand the potential roles of these DE piRNAs, mRNAs targeted by the DE piRNAs were predicted with Miranda algorithm. All the target genes were derived from our whole transcriptome data. Many key genes involved in important signaling pathways were targeted by the dysregulated piRNAs, such as pathways in cancer,

regulation of actin cytoskeleton, proteoglycans in cancer, rap1 signaling pathway and cGMP–PKG signaling pathway (Supplementary Table 3).

Functional annotation of DE piRNAs

To understand the roles of DE piRNAs in PTB patients, GO annotation and KEGG pathway analysis were used. If the GO function set was significantly enriched in the DE piRNAs, a hypergeometric distribution test was used to calculate the p-value of them, which was then corrected by multiple Benjamini–Hochberg tests.

According to the routine GO classification algorithms, the top 10 GO classification of biological process (BP), cell components (CC) and molecular function (MF) were shown according to the enriched dysregulated piRNAs derived from the gene annotation ($p < 0.05$). The top three GO processes of upregulated/downregulated piRNAs included transcription, regulation of transcription and signal transduction in the BP subgroup. Cytoplasm, nucleus and plasma membrane were the top three processes in the CC subgroup. Protein binding, metal ion binding, and ATP binding were the top three processes in the MF subgroup (Figure 5A & B).

Three hundred and one KEGG pathways (multiple test of Benjamini & Hochberg) were identified among the dysregulated piRNAs. Enrichment of the signaling pathways of these dysregulated piRNAs showed that upregulated piRNAs were enriched in key pathways, such as pathway in cancer, regulation of actin cytoskeleton and Rap1 signaling pathway (Figure 6A). Downregulated piRNAs were enriched important pathways, such as pathway in cancer, regulation of actin cytoskeleton and Wnt signaling pathway (Figure 6B). These results support that the piRNAs represent a novel type of regulators in the pathogenesis of PTB.

piRNA–mRNA interaction networks construction

To know the regulatory mechanism of piRNAs in PTB, the targets of DE piRNAs were predicted with the Miranda algorithm. piRNAs have a similar function to miRNAs to degrade specific mRNAs. Therefore, the upregulated piRNAs (downregulated piRNAs)/downregulated (upregulated) mRNAs of piRNA targets were selected to construct the networks. Because of the reverse regulation of piRNAs to the targets, one network was constructed with upregulated piRNAs and downregulated mRNAs, and another with downregulated piRNAs and upregulated mRNAs. Many interaction pairs were identified, and only the top 100 interacting pairs were shown according to the p-value. From the correlation analysis of upregulated piRNAs and downregulated mRNAs network, piRNA-881565, piRNA-489848, piRNA-1869760, piRNA-784007 and piRNA-1503138 regulated 34, 32, 28, 21 and 18 mRNA targets, respectively (Figure 7A). piRNA-1216758, piRNA-1577991, piRNA-866055, piRNA-1403892 and piRNA-1154851 regulated 41, 32, 27, 20 and 13 mRNA targets, respectively (Figure 7B). One mRNA can be regulated by several piRNAs. The results showed that many targets interacted with piRNAs, which indicated dysregulated expression of piRNAs in the peripheral blood of PTB patients.

piRNA clusters in PTBs

To reveal the genomic piRNA clusters in PTB, proTRAC software was used. We found 24 highly dense piRNA clusters including 115 DE piRNAs from the genome of PTB patients. High cluster density was observed on chromosomes of 16 and 19, it harbored four and two piRNA clusters with 22 and 15 piRNAs in PTB. No clusters were detected on chromosomes 9, 13, 14, 21, 22, X and Y (Figure 8A). Nucleotides bias of these piRNAs identified in piRNA clusters was investigated, which showed that piRNAs encoded from the plus strand had the highest bias for 1 U as compared with the minus strand, while a higher bias for 10 A was observed in the minus strand (Figure 8B). Thus, we speculated that piRNAs in peripheral blood may be mainly generated from the primary biogenesis mechanism.

Discussion

piRNAs, a novel noncoding RNAs, are abundant and extensively expressed in germline and gonads, but little is known about their expression patterns and roles in the peripheral blood of patients with infectious diseases, especially PTB. Four PIWI proteins in piRNAs biogenesis, *PIWIL1*, *PIWIL2*, *PIWIL3* and *PIWIL4* were encoded by human genome [54,55]. piRNAs can bind to PIWI proteins to form complexes to induce target gene silencing effect [56,57]. A variety roles of piRNAs are reported, such as, regulation of transposable elements in the germline [58], regulation of histone modifications at the binding site [59] and post-transcriptional regulation of gene expression [60]. In the present study, piRNA expression patterns were first investigated in the peripheral blood of PTB patients and healthy individuals and 39481 expressed piRNAs were identified. We found that PIWIL2 and PIWIL4 proteins

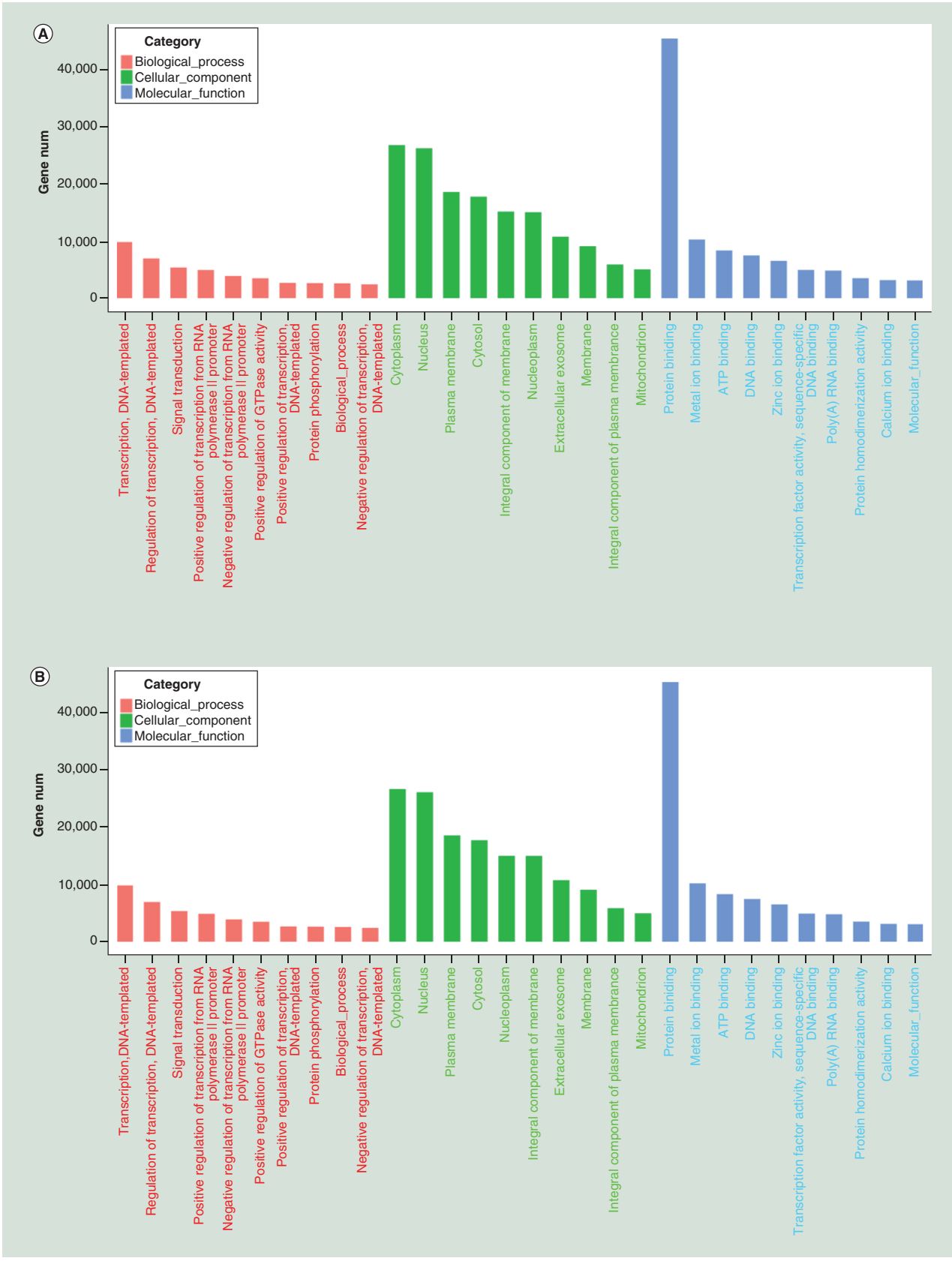

Figure 5. Gene ontology annotation analysis with the targets of differentially expressed PIWI-interacting RNAs. (A) GO annotation analysis of the targets of upregulated PIWI-interacting RNAs. According to the enrichment, the GO terms were divided into biological process, cell components and molecular function subgroups. **(B)** GO annotation analysis of the targets of downregulated PIWI-interacting RNAs. According to the enrichment, the GO terms were divided into biological process, cell components and molecular function subgroups.

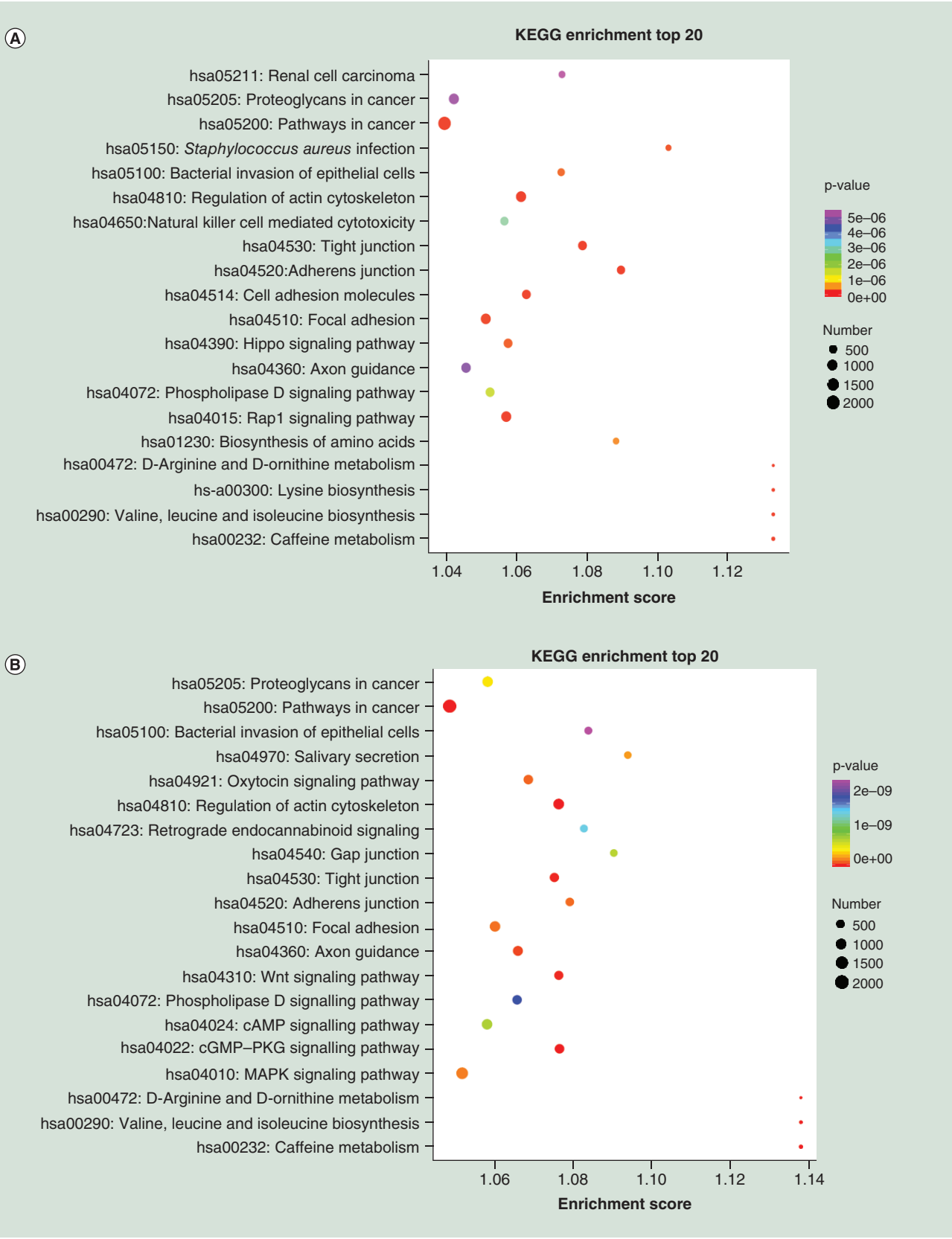

Figure 6. Kyoto Encyclopedia of Genes and Genomes enrichment analysis with the targets of differentially expressed PIWI-interacting RNAs. (A) KEGG enrichment analysis of the targets of upregulated piRNAs. **(B)** KEGG enrichment analysis of the targets of downregulated piRNAs. Graph size represents the numbers of genes and graph color represents the p-value. According to the multiple test of Benjamini & Hochberg, the top 20 enriched KEGG pathways were used to shown on the enrichment picture.

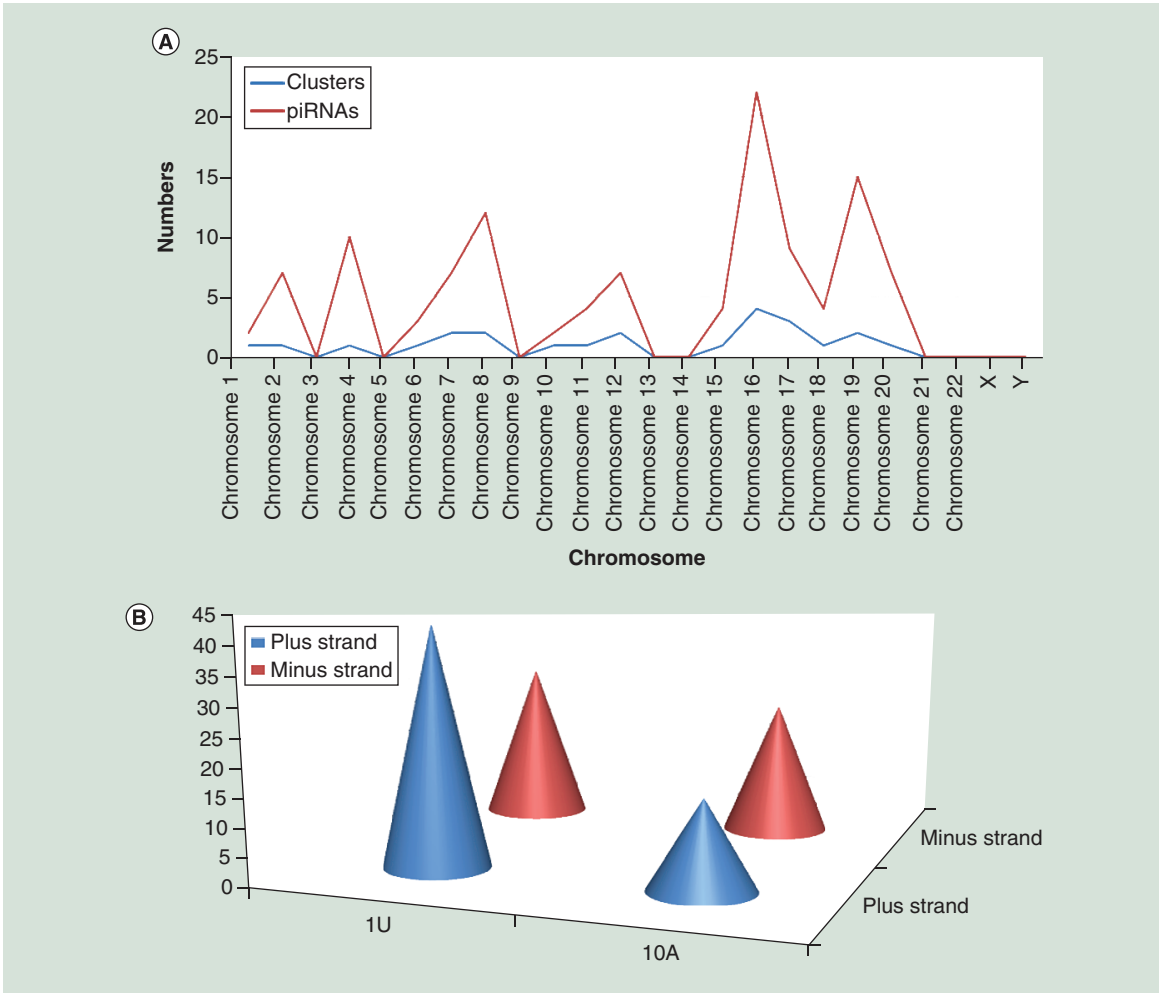

Figure 7. PIWI-interacting RNA clusters distribution on the chromosomes and in the peripheral blood of pulmonary tuberculosis patients. (A) Number of piRNA clusters and piRNAs across various chromosomes. **(B)** 1 U and 10 A bias among piRNAs encoded from plus and minus of the identified piRNA clusters from the pulmonary tuberculosis patients.

were expressed in the peripheral blood. The genes related to piRNA metabolism were also expressed in peripheral blood. Expression of PIWI proteins, piRNA-related genes and piRNAs in the peripheral blood indicates that the PIWI–piRNA system is working, and this system is not only present in the germline, but is also active in the peripheral blood.

The expression profiles of piRNAs were investigated to gain insights into their possible functions in PTB pathogenesis. Screening of patients with PTB and healthy individuals ($\geq$ twofold change and FDR <0.05) identified 777 piRNAs with significant levels of differential expression, with 428 significantly unregulated and 349 significantly downregulated. This clearly demonstrates piRNA dysregulation in PTB patients, indicating that many genes involved in piRNA pathways are also dysregulated in PTB. Dysregulated piRNAs in the peripheral blood have also been reported in gastric cancer patients, and expression of piR-651 and piR-823 is significantly lower than in healthy individuals [47]. piRNAs are stably expressed in human serum or plasma samples [48]. Multiple sequences consistent with piRNA and snoRNA species have been identified in human plasma [61]. Therefore, we propose that these dysregulated piRNAs in the peripheral blood of PTB patients may be associated with the development and pathogenesis of diseases with different roles played in the germline. It is known that the role of piRNA machinery is to mediate epigenetic silencing of target diverse mRNAs, and act as siRNAs to degrade specific mRNAs [62]. Maternal mRNAs are destabilized in the early *Drosophila* embryo, and they are targeted by abundant piRNAs and their deadenylation depends on the piRNA pathway [63]. Functional analysis of RNA targeted by dysregulated

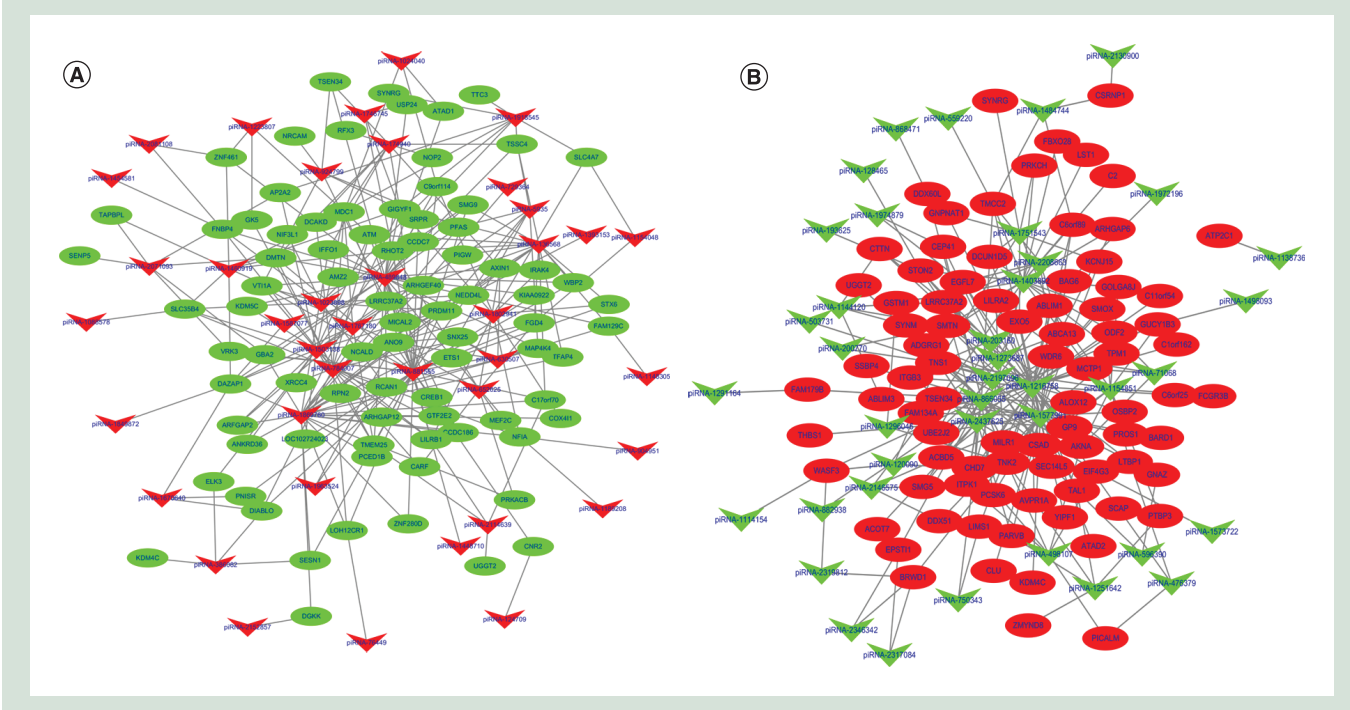

Figure 8. Correlation analysis of PIWI-interacting RNAs and mRNAs in peripheral blood of pulmonary tuberculosis patients. (A) Correlation analysis of upregulated piRNAs and downregulated mRNAs with top 100 piRNAs according to p-value. Red triangular represents piRNA, green ellipse represents mRNA. **(B)** Correlation analysis of downregulated piRNAs and upregulated mRNAs with top 100 piRNAs according to p value. Green triangular represents piRNA, red ellipse represents mRNA.

piRNAs expressed in PTB patients reveals a significant correlation with several major important pathways that are dysregulated in PTB, including signaling pathway in cancer, Rap1, Wnt and cGMP-PKG. The piRNA targets include protein binding, metal ion binding, ATP binding, DNA binding, zinc ion binding and transcript factor activity, which are all known to be active in the process of PTB pathogenesis.

Two piRNA biogenesis pathways are in germline cells, which are the primary maturation pathway and a secondary pathway (also termed 'ping-pong' cycle) [64]. The primary maturation pathway of piRNAs shows a strong preference for U at the 5′ end and no nucleotide bias at position 10. The secondary pathway show a bias for A at position 10 and no 5′ end bias [65]. The primary pathway is in the germline and ovarian somatic follicle cells, and the secondary pathway is only in active only in the germline [66,67]. The piRNAs identified from the peripheral blood of PTB patients showed a strong preference for U at the 5′ end and no nucleotide bias at position 10, suggesting that their synthesis is likely to occur through the primary maturation pathway.

Mammalian embryonic piRNAs are involved in regulating endogenous gene expression [68], and we investigated significant negative correlations between piRNAs targeting mRNAs and piRNAs. To understand the potential molecular mechanisms of piRNAs in the peripheral blood of PTB patients, the targets (mRNA) of DE piRNAs were predicted from our whole transcriptome data, and many important genes with immune roles were identified. According to the negative regulation of piRNAs to the targets, the correlation analysis network between piRNAs and mRNAs was constructed. Here, we propose that many genes were targeted by piRNAs and which may through the siRNA-like function of the piRNA machinery in the peripheral blood of PTBs.

Future perspective

This study is believed to be the first to evaluate comprehensively the differential expression of piRNAs between PTB patients and healthy individuals by small RNA sequencing. The piRNA expression patterns and pathways analysis presented here offer insights for the elucidation of detailed functions of piRNAs in PTB pathogenesis. In the future, all of these DE piRNAs and target genes of piRNAs will be validated with other methods. The MFs of

these dysregulated piRNAs will be investigated in specific immune cells. Whether piRNAs can be used as biomarker for early diagnosis of PTB, this will be done in large number of clinical samples.

Summary points

- Dysregulated piRNAs were first indentified from the peripheral blood of pulmonary tuberculosis (PTB) patients.
- A total of 428 upregulated and 349 downregulated piRNAs were identified from PTB patients.
- Target genes of piRNAs were mainly involved in transcription and protein binding.
- Dysregulated piRNAs were enriched in many pathways related with immunity.
- piRNAs in peripheral blood may be formed from the primary biogenesis pathway.
- piRNAs regulate the target genes by siRNA-like mechanism.
- piRNAs may be as an important noncoding RNAs in pathogenesis of PTB.
- piRNAs were first identified in the peripheral blood of PTB patients.

Financial & competing interests disclosure

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