Expression Profiles of Long Non-coding RNA and Messenger RNA in Human Traumatic Brain Injury

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Long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) play an important role in central nervous diseases; however, the exact expression and co-expressed profiles in human traumatic brain injury (TBI) are still unknown. Therefore, we investigated whole blood in 12 patients with TBI and 4 healthy controls to observe expression characteristics with different severity. We identified 3,035 lncRNAs and 1,204 mRNAs differentially expressed in the severe TBI group, 2,362 lncRNAs and 656 mRNAs in the moderate group, and 433 lncRNAs and 100 mRNAs in the mild group. Enrichment analyses showed 30 signaling pathways such as inflammatory and immune response pathways. Subsequently, a lncRNA-gene co-expression network was generated for 717 lncRNA-mRNA pairs and most of them with a positive correlation. Based on GSEA analysis, we found that TBI caused severe immune abnormality reflected on Th1, Th2, and Th17 cell differentiation deficiency. Finally, the expression of one upregulated and one downregulated lncRNA was validated in all three TBI groups, which was consistent with the microarray results. In summary, our results show that expression profiles of lncRNAs and mRNAs are significantly different in bloods from different severity TBI especially in immune response, providing novel insight for lncRNAs in human TBI.

INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of mortality and morbidity in both developed and developing countries, which exerts a huge economic and health burden on the society.1 Although a series of studies have been carried out in animal models and clinical patients with TBI, its prognosis is still poor.2 As a consequence, a growing number of research have investigated the molecular mechanisms of TBI, which are focusing on molecular, cellular, and metabolic alterations that are triggered by the primary brain injury, and it can consequently cause glial activation and neuronal death.3 In spite of this, the exact molecular and cellular alterations involved in TBI remain elusive.

Recent studies and ours have identified a series of genetic and signaling pathways that are involved in TBI, including PP2A-phosphorylated tau,4 the IGF-1–Akt pathway,4 and Wnt/β-catenin signaling.5 Nevertheless, besides protein coding genes, a lot of non-coding RNAs (ncRNAs), which are considered to be non-sense RNAs, are also having an important role in CNS.6 Actually, these ncRNAs have a direct effect on mRNA translation, splicing, and export from nucleus.7 In addition, lncRNAs are also involved in various pathophysiological mechanisms of CNS diseases and targeting these lncRNAs is able to treat most of these diseases,8–10 such as Alzheimer’s disease,11 and ischemic stroke.12 Furthermore, lncRNAs have specific expression profiles in brain tissue but also can be used as potential independent prognostic molecular markers.13 Recently, Li et al.14 identified a series of lncRNAs that are statistically altered in the injured cortex in TBI mice.

It is also found that expression profiles of lncRNAs and mRNAs are statistically different between human TBI tissue and surrounding tissue.15 However, the genetic and pathophysiological changes in brain injury are different from human patients. Therefore, TBI samples with a gene chip are more adaptable to clinical sessions. Correspondingly, to identify the exact role of lncRNAs in human TBI, we carried out a comprehensive analysis of both lncRNA and mRNAs expression in blood from human patients and compared it to healthy controls. Moreover, enrichment analyses with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were applied to find differently expressed genes (DEGs) via building a co-expression network, and the relationship between lncRNAs and mRNAs were further analyzed with cis- and trans-regulation style.

Up to now, there are very few reports regarding the lncRNA and mRNA expression profiles in whole blood of TBI patients with different severity. Therefore, we investigated the expression signatures of RNAs in human TBI with different severity by Agilent chipset. These DEGs may exert an important effect in the pathological progression in human TBI.

RESULTS

Differentially Expressed lncRNAs and mRNAs in TBI

The expression profiles of DEGs were identified in 12 TBI and 4 healthy controls. volcano figure showed 3,035 differentially
expressed lncRNAs in the severe group (p < 0.05, fold change [FC] > 2; Figure 1). Between them, 1,686 lncRNAs were upregulated and 1,349 lncRNAs were downregulated in the severe TBI group (Table 2). Among them, MSTRG.49361.3, ENST00000606282.1, NON-HSAT181489.1, ENST00000620459.1, and NONHSAT184491.1 were the five most significantly upregulated lncRNAs with a logFC value from 3.9 to 5.4, whereas ENST00000505646.1, SI-GLEC8, IFIT1, ALOX15, and PRSS33 were the five most significantly downregulated mRNAs with a logFC value from −3.9 to −6.4 (Table 4).

In the moderate group, 1,874 lncRNAs were upregulated and 488 lncRNAs were downregulated. In the mild group, 183 lncRNAs were upregulated and 250 lncRNAs were downregulated. Here, we can find an obvious trend that the total number of altered lncRNA increased with more severe brain injury.

Next, we compared mRNA changes between two groups. In total, we identified 1,204 differentially expressed mRNAs (also based on p < 0.05, FC > 2; Figure 1). Between them, there are 575 mRNAs were upregulated and 629 mRNAs were downregulated.

In the severe TBI group, OLAH, CD177, ADAMTS2, NECAB1, and HPR were the five most significantly upregulated mRNAs with a logFC value from 6.9 to 4.5. OLIG2, SIGLEC8, IFIT1, ALOX15, and PRSS33 were the five most significantly downregulated mRNAs with a logFC value from −4.0 to −3.3 (Table 3).

In the moderate group, 239 mRNAs were upregulated and 417 mRNAs were downregulated. In the mild group, 47 mRNAs were upregulated and 53 mRNAs were downregulated. A similar trend can also be identified in the total number of altered mRNAs increased with the more severe brain injury (Table 2).

To further investigate DEGs, we selected candidates that changed >2-fold to build a hierarchical clustering map. As demonstrated in Figure 1, the four severe TBI groups clustered together, which was definitely different from the control group. Similar results can be found in moderate and mild TBI group compared to control group (Figures S2 and S3).

On the whole, the DEGs were found to be separated by different severity (Figure 1). These findings suggest that there may be potential interactions between lncRNAs and mRNAs, which rewire the whole transcriptomic network in TBI.

**GO and KEGG Analyses**

GO analysis of DEGs was carried out to reveal the changes in cellular components (CCs), biological processes (BPs), and molecular functions (MFs). We found the differentially expressed mRNAs in...
the severe TBI group were primarily enriched for inflammatory response, innate immune response, and neutrophil degranulation (related to BPs; Figure 2A); transmembrane signaling receptor activity, carbohydrate binding, and RAGE receptor binding (related to MFs; Figure 2A); and plasma membrane, T cell receptor complex, and SHG alpha-glucan phosphorylase activity, and linear malto-oligosaccharide phosphorylase activity in MF (Figure 2B); and plasma membrane, extracellular region, and kinetochore (related to CCs; Figure 2C). We further did the GO analysis based on upregulated genes and downregulated genes in all three groups, respectively (data not shown). We also did the bubble plot to demonstrate the GO results, which are consistent with the bar graph.

Then, we carried out the KEGG analysis and found that altered mRNAs were related to 30 selected pathways, including Th1 and Th2 cell differentiation, Th17 cell differentiation, hematopoietic cell lineage, and T cell receptor signaling pathway (Figure 3). Completely, these DEGs are mostly involved in T cell and immune system. The top 10 related KEGG pathways in severe TBI group are listed in Table 5. Here, we can see the GO and KEGG pathways were relatively different in TBI groups with different severity. The inflammatory response was at the top three in both the severe TBI and moderate TBI group.

### Establishment of a lncRNA-mRNA Co-expression Network

Constructing lncRNA regulatory networks may be useful in revealing interactions between lncRNAs and related mRNAs. With NCBI software, one lncRNA-mRNA network was built according to the Pearson’s coefficient score. The Pearson correlation test was used to calculate the expression correlation between different lncRNA (less than 6,000 nucleotides [nt] in length) and mRNA expression data. We selected the correlation coefficient more than 0.8 and the p value less than 0.05. In order to display the information more intuitively, the differential comparison group is displayed in the Circos diagram by using the drawing software (Figure 4A). We identified 717 lncRNA-mRNA groups with coefficient score >0.99. Most of them (608/717) were positively associated, and the rest (109/717) of the groups were negatively associated (Figure 4A). The top 5 lncRNA-mRNA pairs with positive and negative coefficient score >0.99 are shown in Table 6. These findings indicate that there is a tight connection between lncRNAs and mRNAs.

Based on the results of differential co-expression, GO and KEGG enrichment analysis were carried out for each lncRNA that co-expressed mRNA by hypergeometric distribution algorithm. The function of lncRNAs may be closely related to the enrichment of GO or KEGG. The top three GO results are innate immune response, inflammatory response, and neutrophil degranulation in BP; plasma membrane, tertainary granule membrane, and specific granule membrane in CCs; and SHG alpha-glucan phosphorylase activity, glycogen phosphorylase activity, and linear malto-oligosaccharide phosphorylase activity in MF (Figure 4B). Most of these pathways are consistent with the altered mRNAs GO enrichment. To be noticed, the molecular function of lncRNA co-expressed mRNAs is mainly related to phosphorylase activity.

Next, we performed KEGG pathway enrichment based on first class (cellular processes [CP], environmental information processing [EIP], genetic information processing [GIP], human diseases [HDs], metabolism [Meta.], and organismal systems [OSs]). Dysregulated mRNAs were associated with the 10 top pathways in each class. For the cellular processes, the top pathways are necroptosis,
ferroptosis, peroxisome, signaling pathways regulating pluripotency of stem cells, phagosome, autophagy-other, focal adhesion, apoptosis, cellular senescence, and regulation of actin cytoskeleton, which are all very important pathologies in TBI (Figure 4C). We also did the co-expression analysis for mild and moderate TBI (Figures S4 and S5).

Therefore, these findings indicate that the candidate genes are related to 30 signaling pathways, particularly those involved in neuronal death in TBI.

**Cis and Trans-Regulation of lncRNAs in TBI**

Based on the results of differential co-expression, we used FEELnc20 software to search all the coding genes in the range of 100 kb upstream and downstream of differential expression lncRNA and intersected the differential genes with significant co-expressed (Pearson correlation) lncRNA. These genes, which are close to the genome and co-expressed in the expression pattern, are likely to be cis-regulated by the lncRNA (Figure 5A). The top 20 cis-regulated lncRNA and mRNAs in severe TBI were listed in Table 7.

Based on the results of differential co-expression, the RNA interaction software RIsearch-2.021 was used to predict the binding of candidates for co-expressed lncRNA and mRNA at the nucleic acid level. According to the number of bases for direct interaction between two nucleic acids no less than 10 and base binding free energy no more than \(-10\), the selected lncRNA and mRNA may have direct regulation. The top 500 (co-expressed p value) relationship pairs were extracted and the lncRNA-mRNA target interaction network was drawn by using network software package in R (Figure 5B).

The way that ncRNA binding transcription factor (TF) participates in the regulation is that lncRNA can recruit TFs and guide TFs to a specific position of DNA sequence (such as promoter region) to regulate the transcription activity; another way is that multiple TFs can be

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**Table 3. Top Five Upregulated and Downregulated lncRNAs in Severe TBI**

| lncRNA          | p Value  | FC    | Log2FC    | Chromosome | Strand |
|-----------------|----------|-------|-----------|------------|--------|
| Top Five Upregulated lncRNAs |          |       |           |            |        |
| MSTRG.49361.3   | 0.00052956 | 42.31602696 | 5.40313227 | Chr4       | +      |
| ENST000006606282.1 | 0.00177649 | 17.78799389 | 4.15283191 | Chr5       | −      |
| NONHSAT181489.1 | 0.00048352 | 17.68015597 | 4.1440591  | Chr2       | +      |
| ENST00000620459.1 | 0.01562149 | 15.29458798 | 3.93494334 | Chr20      | +      |
| NONHSAT184491.1 | 0.00058541 | 15.1680378  | 3.92301639 | Chr2       | −      |
| Top Five Downregulated lncRNAs |          |       |           |            |        |
| NR_024075       | 0.00122553 | −16.59895684 | −4.0530207 | chr19      | −      |
| MSTRG.8453.11   | 0.00349908 | −16.49115913 | −4.0436209 | chr10      | +      |
| Inc-CREG1-3:5   | 0.00568788 | −11.03433674 | −3.463928  | chr1       | −      |
| NONHSAT187532.1 | 0.00964318 | −9.916857214 | −3.309883  | chr2       | −      |
| ENST0000050566.1 | 0.00517046 | −9.868992636 | −3.3029028 | chr4       | +      |

**Table 4. Top Five Upregulated and Downregulated mRNAs in Severe TBI**

| mRNA           | p Value  | FC      | Log2FC  | Chromosome | Strand |
|----------------|----------|---------|---------|------------|--------|
| Top Five Upregulated mRNAs |          |         |         |            |        |
| OLAH           | 0.00052956 | 123.177867 | 6.94459925 | chr10      | +      |
| CD177          | 0.00177649 | 64.303478  | 6.0068246 | chr19      | +      |
| ADAMTS2        | 0.00048352 | 27.7340521 | 4.79358651 | chr5       | −      |
| NECAB1         | 0.01562149 | 27.3996291 | 4.77608778 | chr8       | +      |
| HPR            | 0.00058541 | 22.0173338 | 4.46056788 | chr16      | +      |
| Top Five Downregulated mRNAs |          |         |         |            |        |
| PRSS33         | 0.00792196 | −83.209048 | −6.3786685 | chr16      | −      |
| ALOX15         | 0.00118097 | −27.257375 | −4.7685747 | chr17      | −      |
| IPIT1          | 0.00528846 | −16.490056 | −4.0345244 | Chr10      | +      |
| SIGLEC8        | 0.00217712 | −16.44025  | −4.0391604 | Chr19      | −      |
| OLIG2          | 0.00681314 | −14.876976 | −3.8950094 | Chr21      | +      |
Figure 2. GO Results with Bar Graph and Bubble Plot in Severe, Moderate, and Mild TBI Groups

Upper, middle, and down panel indicate the severe, moderate, and mild TBI groups.
combined to a lncRNA molecule. In the cell body, when multiple signal pathways are activated simultaneously, these downstream effectors (TFs) can be combined to the same lncRNA molecule to achieve information exchange and integration between different signal pathways.

Through analyzing and predicting of potential TF binding of lncRNA (based on the TF data from Jaspar database), using the gene-TF pair provided by GTRD database and the co-expression of lncRNA and mRNA, we can construct the three element regulatory network of lncRNA-TF-mRNA by extracting the top 500 pairs, and draw the three element regulatory network diagram by using the network software package in R (Figure 5C). The top 20 cis-regulated lncRNAs were used in this analysis, and the top 20 mRNAs co-expressed by lncRNA were used as well.

Figure 3. Top 30 KEGG Pathway Results with Bubble Plot in Severe, Moderate, and Mild TBI Groups
Upper panel shows all altered mRNAs related. Middle panel shows upregulated mRNAs related. Down panel shows downregulated mRNAs related. Left panel shows severe TBI, middle panel shows moderate TBI, and right panel shows mild TBI.
Effects of Brain Injury on the Immune Response Pathway

We carried GSEA (with a Signal to Noise Method) to investigate pathways that are altered in human blood post-injury. GSEA results demonstrated downregulation of multiple alterations in the immune system, including Th1, Th2, and Th17 cell differentiation, and natural killer (NK) cytotoxicity (Figure 6). According to the heatmap, Th1 and Th2 differentiation-related genes were more downregulated in severe TBI group. These results demonstrate further evidence of a severity-dependent injury-associated change in the immune response toward a pro-inflammatory state (NK-cell-mediated cytotoxicity) in TBI (Figure S6).

Expression Levels of IncRNAs Measured by Quantitative Real-Time PCR

To confirm our microarray data, we selected three upregulated IncRNAs (Inc-ANKRD34B-3:1, Inc-ICOSLG-1:4, and ENST00000423149.1) and seven downregulated IncRNAs (MSTRG.22548.1, MSTRG.67532.1, NONHSAT210656.1, MSTRG.41651.1, NONHSAT177352.1, Inc-NGEF-2:3, and NONHSAT168122.1) in all groups of TBI with a very low expression in brain. The exosomes from both invasive and non-invasive pituitary adenomas also have higher expression of Inc-NGEF-2:3, and NONHSAT168122.1. Furthermore, ENST00000505646.1, NONHSAT177352.1, Inc-CREG1-3:5, MSTRG.8453.11, and NR_024075 were the five most significantly expressed lncRNAs. For MSTRG.49361.3, it is enriched in whole blood and spleen based on RefLnc 2017. In lower-grade lung cancer (LUAD & LUSC), high-grade bladder cancer (BCG), glioblastoma (GBM), and low-grade glioma (LGG), its expression is increased. NONHSAT181489.1 has found to be highly expressed in testes and with a very low expression in brain. The exosomes from both invasive and non-invasive pituitary adenomas also have higher expression of NONHSAT181489.1. NONHSAT184491.1 is specifically expressed in the heart according to the Noncode database, and there is no expression in the exosomes. As most of these IncRNAs have very low expression in the brain and it increased after brain injury, it indicates that these IncRNAs might be involved in pathological mechanisms in TBI. Some IncRNAs are thought to become transcriptional regulators that regulate coding gene expression by cis or trans mechanisms. Unfortunately, there are very few studies that have

| Table 5. Top 10 KEGG Pathways Related to Target Genes in Severe TBI |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Signal Pathway   | Count | Gene Ratio | Fold Enrichment | p Value | FDR_bh |
|------------------|-------|------------|-----------------|---------|--------|
| Th1 and Th2 cell differentiation | 23 | 0.05424528 | 4.513089623 | 1.35E-09 | 3.88E-07 |
| Inflammatory bowel disease (IBD) | 17 | 0.0400934 | 4.512155298 | 9.67E-08 | 1.39E-05 |
| Th17 cell differentiation | 21 | 0.0495283 | 3.8589769 | 6.2E-07 | 5.96E-05 |
| Hematopoietic cell lineage | 19 | 0.04481132 | 3.73927952 | 2.0E-06 | 0.000158746 |
| Staphylococcus aureus infection | 12 | 0.02830183 | 3.69693362 | 6.67E-05 | 0.003495847 |
| Cytokine-cytokine receptor interaction | 32 | 0.0754717 | 2.044723969 | 7.91E-05 | 0.003495847 |
| Graft-versus-host disease | 10 | 0.02358491 | 4.207892315 | 8.50E-05 | 0.003495847 |
| Transcriptional misregulation in cancer | 23 | 0.05424528 | 2.133356157 | 0.000436783 | 0.0157242 |
| Natural killer cell mediated cytotoxicity | 18 | 0.04245283 | 2.33490566 | 0.00620327 | 0.019850456 |
| T cell receptor signaling pathway | 15 | 0.03537736 | 2.512479392 | 0.00080569 | 0.02240497 |

DISCUSSION

Recently, several studies on TBI have turned the focus to non-coding RNAs, as the results are able to prevent neuronal damage caused by brain injury and improve the outcome of TBI. In an *in vitro* TBI model, neuronal apoptosis was decreased by increasing the expression of miR-9-5p with miR-9-5p agomir via the Ptc-1 pathway. A series of IncRNAs in the injured cortex in TBI mice were altered, which may be involved in different pathologies.

3A recent study found that IncRNAs play an important role in gene regulation and take part in various CNS diseases. Nevertheless, the exact role of IncRNAs in TBI is still elusive, and the exploration of their role is limited as well. Herein, we investigated IncRNA and mRNA expression profiles in blood from 12 human TBI and 4 healthy controls by Agilent chipset. DEGs were filtered by the volcano plot and a total of 183 IncRNAs (183 upregulated and 250 downregulated) and 100 mRNAs (47 upregulated and 53 downregulated) were considered to be differentially expressed between mild TBI and control groups. In the moderate group, 239 mRNAs were upregulated and 417 mRNAs were downregulated, 1,874 IncRNAs were upregulated and 488 IncRNAs were downregulated, while in the severe group, 1,686 IncRNAs were upregulated and 1,349 IncRNAs were downregulated. Expression profiles of these DEGs could be clustered hierarchically. There is an obvious trend that the total number of altered IncRNA increased with the more severe brain injury, which indicated that the gene editing was more damaged in severe TBI.

We reported that the five most upregulated IncRNAs were MSTRG.49361.3, ENST0000060282.1, NONHSAT181489.1, ENST00000620459.1, and NONHSAT184491.1. Furthermore, ENST00000505646.1, NONHSAT178732.1, Inc-CREG1-3:5, MSTRG.8453.11, and NR_024075 were the five most significantly downregulated IncRNAs. For MSTRG.49361.3, it is enriched in whole blood and spleen based on RefLnc 2017. In lower-grade lung cancer (LUAD & LUSC), high-grade bladder cancer (BCG), glioblastoma (GBM), and low-grade glioma (LGG), its expression is increased.
Figure 4. A lncRNA-mRNA Co-expression Network in Severe TBI

(A) Circos map: the outer ring is the distribution diagram of the autosomal of the species; the second and third circles show the distribution of differentially expressed genes on the chromosome, with red lines indicating upregulation and green lines indicating downregulation. The higher the column, the more number of DEGs in this region. The fourth and fifth circles show the distribution of differentially expressed lncRNAs on chromosomes, and the expression pattern is the same as that of gene. The internal lines indicate the top 500 co-expressed lncRNA and mRNA. (B) The go enrichment results in the top 10 co-expressed lncRNAs. (C) KEGG enrichment results of total top 10 lncRNA co-expressed mRNA genes: x axis enrichment score. The larger the bubble, the more differential genes are included. The color of bubbles changes from gray to red, and the enrichment value of p value gradually decreases, indicating that the significance degree increases gradually.
Table 6. Top 10 Co-expressed IncRNA and mRNA

| IncRNA          | mRNA | FC      | p Value |
|-----------------|------|---------|---------|
| Top Five Positive Correlation IncRNA-mRNA |       |         |         |
| Inc-PP1R3D-8:1  | SYCP2| 0.99905871| 2.08E-09|
| Inc-EFG5-4:1    | PSTPIP2| 0.998923975| 3.07E-09|
| MSTRG.62207.1   | KCN15| 0.998907515| 3.26E-09|
| NONHSAT200618.1 | ZNF438| 0.998897741| 3.35E-09|
| NONHSAT151637.1 | NMT2 | 0.998850793| 3.79E-09|
| Top Five Negative Correlation IncRNA-mRNA |       |         |         |
| LINC00337.6     | SMIM40| −0.997434606| 4.21E-08|
| Inc-LHPL4-5:1   | ETS2 | −0.997370493| 4.54E-08|
| NONHSAT150222.1 | HSD17B8| −0.997189557| 5.54E-08|
| NONHSAT184393.1 | CD101| −0.996791018| 8.24E-08|
| T030864         | ZBP1 | −0.996789661| 8.25E-08|

investigated the association of these differentially expressed IncRNAs with TBI especially on cis or trans mechanisms. Furthermore, linking the expression of mRNAs, we consider that these altered IncRNAs are also affecting the expression of mRNAs and it is worthwhile to explore the relationships between them.

GO and KEGG analyses were used for comprehensive exploration of differentially expressed mRNAs and showed that the mostly enriched mRNAs are located in the plasma membrane and related to inflammatory response and innate immune response. Based on KEGG results, the most enriched pathways were Th1 and Th2 cell differentiation, Th17 cell differentiation, hematopoietic cell lineage, and T cell receptor signaling pathway, which indicates that the immune deficiency is very critical in TBI.

Therefore, we have established the IncRNA-mRNA co-expression network, including differently expressed IncRNAs and their neighboring mRNAs (distance < 300 kb), and the Pearson correlation is significant (p ≤ 0.05) with Pearson’s correlation coefficients more than 0.7. We listed the top 20 cis-regulated IncRNAs and mRNAs in severe TBI in Table 7 with FEELnc software based on the co-expressed expression. On the other hand, trans-regulated IncRNAs can regulate their target genes at a relatively longer distances. The functions of these trans-regulated IncRNAs were further predicted based on the TFs that might regulate their expression with a RNA-DNA style. According to the result of IncRNA–TF–mRNA analysis, we identified c-Myc and lncRNA ENST00000518570.2. C-Myc is considered to encode a nuclear phosphoprotein that participates in cell-cycle progression and cellular survival (Figure 5C).

Some limitations need to be addressed. First, the sample size in our study was limited (four patients in each TBI group and four healthy controls). Second, the altered expression of DEGs in TBI patients might be different from spatiotemporal patterns in brains, and this condition is not further verified in our analysis, which needs to be explored in future studies. Generally, our results show that expression profiles of IncRNAs and mRNAs are significantly different in blood from different severity TBI especially in immune response, providing novel insight for IncRNAs in human TBI.

MATERIALS AND METHODS

Sample Collection
Peripheral human blood was prospectively collected into PAXgene Blood RNA Tubes (QIAGEN, Shanghai, China) with RNA protect reagent less than 24 h after TBI. Patients with TBI from September to December 2019 were enrolled in this study according to the initial head CT, which showed brain damage and obvious changes in vital signs. Blood samples were immediately stored at −80°C at once. In total, 12 patients and 4 healthy controls were randomly recruited for this study.

The study protocol was approved by the Medical Ethics Committee of Shanghai Pudong New Area People’s Hospital (approval number: 20170223-001; time of ethics approval: 2017-03-07). TBI patients were further classified with Glasgow Coma Scale (GCS) into mild group (GCS 13-15), moderate group (GCS 9-12), and severe group (GCS 3-8). We excluded patients with the following points: (1) severe complication with thoracic or abdominal injury, (2) serious previous diseases (such as thrombocytopenia and cancer), and/or (3) the family refused to undergo the blood collection. Clinical information for patients is listed in Table 1.

Microarray Information
The Agilent Human IncRNA Microarray 2019 (4*180k, design ID: 086188) was used in this experiment, and data analysis of the 16 samples was conducted by OE Biotechnology (Shanghai, China).

Gene Microarray
Total RNA was quantified by the NanoDrop ND-2000 (Thermo Fisher Scientific, CA, USA) and the RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). The blood RNA manipulation was according to the manufacturer’s protocols. In brief, total RNA was transcribed to cRNA, synthesized into cRNA, and labeled with cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray chipset. After being washed, the chipset was scanned by the Agilent Scanner G2505C (Agilent Technologies, CA, USA).

Figure 5. Cis, Trans, and TF Regulated IncRNAs in Severe TBI
(A) In the graph, “*p < 0.01. Left and right of y axis are mRNA and IncRNA, respectively; x axis is the distance between mRNA and IncRNA, negative value is the upstream, positive value is the downstream, and the same color bar graph is the same IncRNA. (B) The red node represents IncRNA, the green node represents mRNA, and the node size represents the number. (C) The red node represents IncRNA, the green node represents mRNA, the blue node indicates TF and the node size represents the number. Only nodes with degree >2 were shown.
Data Analysis
Feature extraction software (version 10.7.1.1, Agilent Technologies) was used to analyze array images to get raw data. Genespring (version 14.8, Agilent Technologies) was employed to finish the basic analysis with the raw data. To begin with, the raw data was normalized with the quantile algorithm and further classified by principal-component analysis (PCA) and correlational study between groups (Figure S1). The probes that at least 1 condition out of 2 conditions have flags in "Detected" were chosen for further data analysis. DEGs were then selected with a FC >2.0 and a p value <0.05. Next, GO and KEGG enrichment were carried out to investigate the roles of these DEGs followed by co-expressed analysis with cis and trans regulation.

GSEA in TBI Groups
We performed GSEA on the normalized gene expression dataset with GO and KEGG datasets from c2.all.v6.2.cymbols.gmt curated gene sets using 1,000 permutations. Pathways with a false discovery rate (FDR) < 0.05 were selected as significant. We plotted heatmap for the gene sets from immune regulated genes as defined by MSigDB Immune. Genes in heatmap were clustered by k-means clustering methods with k selected using the gap statistics.

Quantitative Real-Time PCR Analysis
Total RNA from whole blood was extracted with a one-step method from Trizol (Invitrogen, Carlsbad, CA, USA). The mRNA was subjected to a reverse transcription reaction by using a TaKaRa PrimeScript RT reagent Kit (Perfect Real Time) reverse transcription kit. Primer sequences are listed in Table S1. Relative quantification of gene expression was calculated using the formula: $2^{-\Delta\Delta C_t} = (C_{t\text{target~gene}} - C_{t\text{actin}})^TBI - (C_{t\text{target~gene}} - C_{t\text{actin}})^\text{control}$. Three independent experiments were performed for each condition.

Statistical Analysis
All data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 8.0 (USA). Differences between the control group and TBI group were analyzed using one-way ANOVA and further LSD test. Spearman correlation analysis was utilized to analyze co-expression relationships between lncRNAs and mRNAs and lncRNA expression with GCS. A value of p <0.05 was considered as statistically significant.

Availability of Supporting Data
The datasets supporting the conclusions of this article are available from the corresponding author.

Author Contributions
Z.W. and P.Z. designed the study. D.R. and W.C. collated the data and designed and developed the database. K.C. and P.Z. carried out data analysis.

| Table 7. Top 20 Cis Regulated lncRNA and mRNA in Severe TBI |
|-----------------|----------------|----------------|
| lncRNA          | mRNA           | Correlation Coefficient | p Value  |
| ENST0000069276.2| MARC1          | 0.9877434247690321     | 4.56085886483991e-06 |
| ENST00000530190.1| ACER3         | 0.9708489942461856     | 6.05834106755555e-05 |
| ENST00000444977.1| KCNJ15        | 0.9693117147366813     | 7.06054020570488e-05 |
| ENST00000508020.2| ACSL1         | 0.969329615176981      | 7.3225949166435e-05 |
| ENST00000437128.1| GBP5          | 0.9660128783945497     | 9.566355678315587e-05 |
| ENST00000431789.1| CSorf67       | 0.9639411808219259     | 0.00011406560519370034 |
| ENST00000513899.2| VCAN          | 0.9584008232822541     | 0.00017439939293255682 |
| ENST00000412143.1| POU5F1        | 0.9578824266868007     | 0.00017965750313655758 |
| ENST00000518570.2| MAN1A1        | 0.9573840826284421     | 0.00018735780893628086 |
| ENST00000589932.1| CEBPA         | 0.9561988210278122     | 0.0002032450871588813 |
| ENST00000620459.1| CST7          | 0.9545495759206558     | 0.0002267935866199957 |
| ENST00000641355.1| PGD           | 0.9542375602230561     | 0.0002314414729076475 |
| ENST00000533106.1| MS4A6A        | 0.9529278869670567     | 0.000251635461211397 |
| ENST00000650561.1| FOSL2         | 0.9476717565939852     | 0.00034430741152138654 |
| ENST000006808243.1| ST6GALNAC3    | 0.9471381279031038     | 0.0003548038341703167 |
| ENST00000574826.1| TRIM25        | 0.944810071668063      | 0.00040305777265801034 |
| ENST0000041427.3| MXI           | 0.941652934982293      | 0.00047111165817573275 |
| ENST00000552286.1| CKAP4         | 0.9350164559925795     | 0.0006530871366397738 |
| ENST00000608729.1| ZNF438        | 0.9350164559925795     | 0.000668419411687012 |
| ENST00000626810.1| ST3GAL4       | 0.9339696129123375     | 0.0006845607135972268 |

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A  Th1 and Th2 cell differentiation

B  Th17 cell differentiation

C

D

E

(legend on next page)
analyses. D.R. and P.Z. produced the initial draft of the manuscript. All authors have read and approved the final submitted manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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A. Up-regulated lncRNAs

Severe TBI
Moderate TBI

1529
146
1681

3
44

128

Mild TBI

B. Down-regulated lncRNAs

Severe TBI
Moderate TBI

1177
115
346

50
20

173

Mild TBI

C. Lnc-ANKRD34B-3

D. Lnc-NGEF-2

E. Lnc-ANKRD34B-3-GCS

R squared: 0.3796
P value: 0.0110

F. Lnc-NGEF-2-GCS

R squared: 3.297e-005
P value: 0.9859

(legend on next page)
Figure 7. Quantitative PCR of lncRNA Expression in Whole Blood of Patients with TBI
(A and B) The upregulated and downregulated lncRNAs in severe, moderate, and mild TBI. (C and D) Expression levels of the two lncRNAs shown are consistent with microarray data. (E and F) All results are expressed as the mean ± SEM of three independent experiments (*p < 0.05, one-way ANOVA, compared to other groups). The correlation between the expression level of lncRNAs and GCS.