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Original Article

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,[3] 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, human 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

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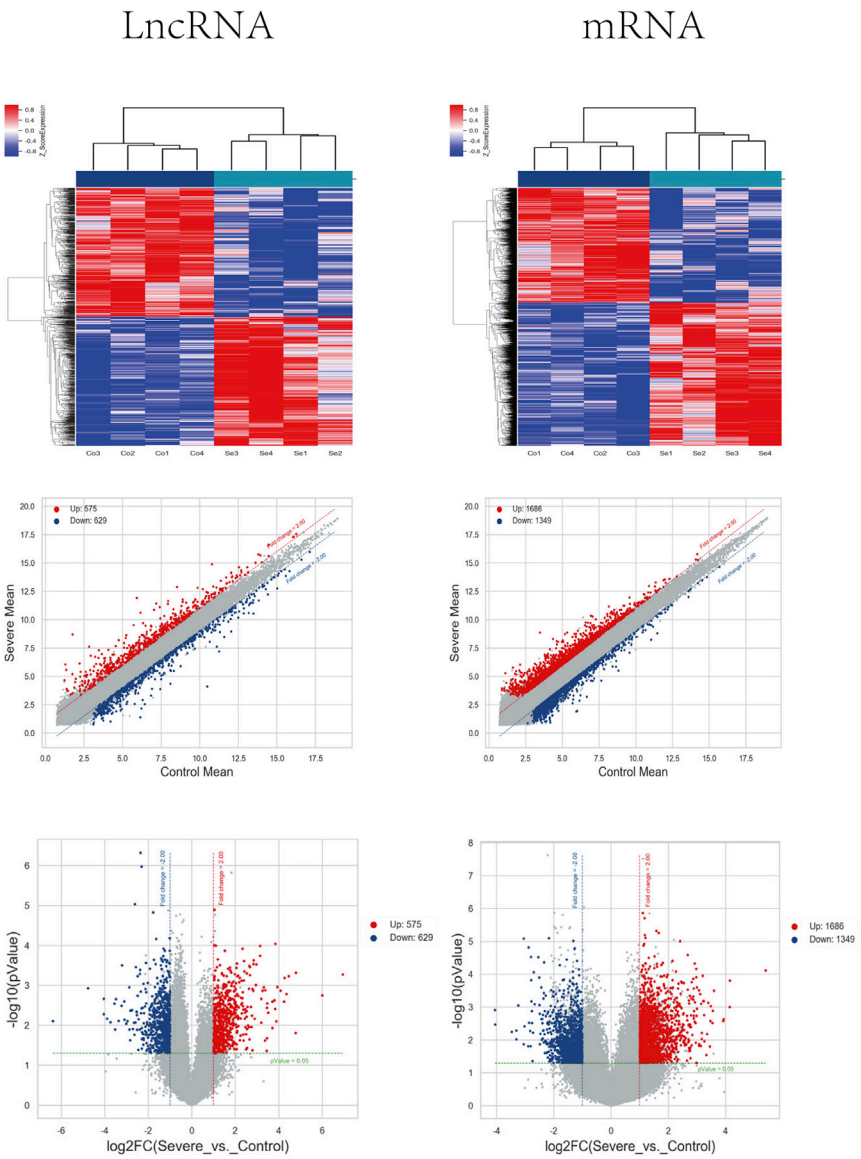

Figure 1. Volcano, Scatter, and Cluster Data for lncRNA and mRNA in Severe TBI

Volcano plot filtering identified 3,035 lncRNAs and 1,204 mRNAs differentially expressed in the severe group, and cluster map showed the four severe TBI groups clustered together in one group, which was mostly distinct from the control group.

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, NONHSAT181489.1, 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, NONHSAT187532.1, lnc-CREG1-3:5, MSTRG.8453.11, and NR_024075 were the five most significantly downregulated lncRNAs with a logFC from −4.0 to −3.3 (Table 3).

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 −3.9 to −6.4 (Table 4).

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).[16,17] We found the differentially expressed mRNAs in

Table 1. Clinical Characteristics of Patients with Traumatic Brain Injury

Patient	Severity	Age (year)	Sex	Brain Injury	GCS	GCS (EVM)
1. Mi1	mild	48	F	laceration	14	E3V5M6
2. Mi2	mild	28	M	contusion	15	E4V5M6
3. Mi3	mild	41	M	SAH	15	E4V5M6
4. Mi4	mild	47	F	SDH	15	E4V5M6
5. Mo1	moderate	29	F	laceration	12	E3V4M5
6. Mo2	moderate	49	F	SDH	12	E3V3M6
7. Mo3	moderate	29	M	SAH	12	E3V4M5
8. Mo4	moderate	50	M	SAH	12	E2V4M6
9. Se1	severe	23	M	brain stem injury	3	E1V1M1
10. Se2	severe	43	F	contusion	8	E2V2M4
11. Se3	severe	44	F	SDH	6	E1V1M4
12. Se4	severe	60	M	SDH	6	E1V1M4
13. Co1	control	40	M			
14. Co2	control	46	F			
15. Co3	control	50	F			
16. Co4	control	45	M			

SAH, subarachnoid hemorrhage; SDH, subdural hemorrhage

Table 2. Number of Altered lncRNAs and mRNAs in TBI

Group	Class	Upregulated	Downregulated	Total
Severe versus control	lncRNA	1,686	1,349	3,035
Moderate versus control	lncRNA	1,874	488	2,362
Mild versus control	lncRNA	183	250	433
Severe versus control	coding	575	629	1,204
Moderate versus control	coding	239	417	656
Mild versus control	coding	47	53	100

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 GO: 0035580 (related to CCs; Figure 2A). In the moderate group, the DEGs were generally enriched for defense response to virus, inflammatory response, and response to virus and neutrophil degranulation (related to BPs; Figure 2B); carbohydrate binding, protein homodimerization activity, and chemokine activity (related to MFs; Figure 2B); and plasma membrane, extracellular region, and kinetochore (related to CCs; Figure 2B). In the mild group, the DEGs were generally enriched for leukotriene metabolic process, proline transmembrane transport, and glycine transport (related to BPs; Figure 2C); extracellular matrix structural constituent, phospholipase D activity, and L-proline transmembrane transporter activity (related to MF; Figure 2C); and apical plasma membrane, postsynapse, and excitatory synapse (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[18] 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.[19] 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, tertiary 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,

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	+
ENST00000606282.1	0.00177649	17.78799389	4.15283191	Chr5	–
NONHSAT181489.1	0.00048352	17.68015597	4.1440591	Chr2	+
ENST00000620459.1	0.01562419	15.29458798	3.93494934	Chr20	+
NONHSAT184491.1	0.00058541	15.16860378	3.92301639	Chr2	–
Top Five Downregulated lncRNAs					
NR_024075	0.00122553	−16.59895684	−4.0530207	chr19	–
MSTRG.8453.11	0.00340908	−16.49115913	−4.0436209	chr10	+
lnc-CREG1-3:5	0.00568788	−11.03433674	−3.463928	chr1	–
NONHSAT187532.1	0.00964318	−9.916857214	−3.309883	chr2	–
ENST00000505646.1	0.00517046	−9.868992636	−3.3029028	chr4	+

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 FEELnc[20] 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-2.0[21] 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 −100, 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

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

lncRNA	p Value	FC	Log2FC	Chromosome	Strand
Top Five Upregulated mRNAs					
OLAH	0.00052956	123.177867	6.94459925	chr10	+
CD177	0.00177649	64.3034778	6.00682486	chr19	+
ADAMTS2	0.00048352	27.7340521	4.79358651	chr5	–
NECAB1	0.01562419	27.3996921	4.77608778	chr8	+
HPR	0.00058541	22.0173338	4.46056787	chr16	+
Top Five Downregulated mRNAs					
PRSS33	0.00792196	−83.209048	−6.3786685	chr16	–
ALOX15	0.00118097	−27.257375	−4.7685747	chr17	–
IFIT1	0.00528846	−16.490056	−4.0435244	Chr10	+
SIGLEC8	0.00217712	−16.44025	−4.0391604	Chr19	–
OLIG2	0.00681314	−14.876976	−3.8950094	Chr21	+

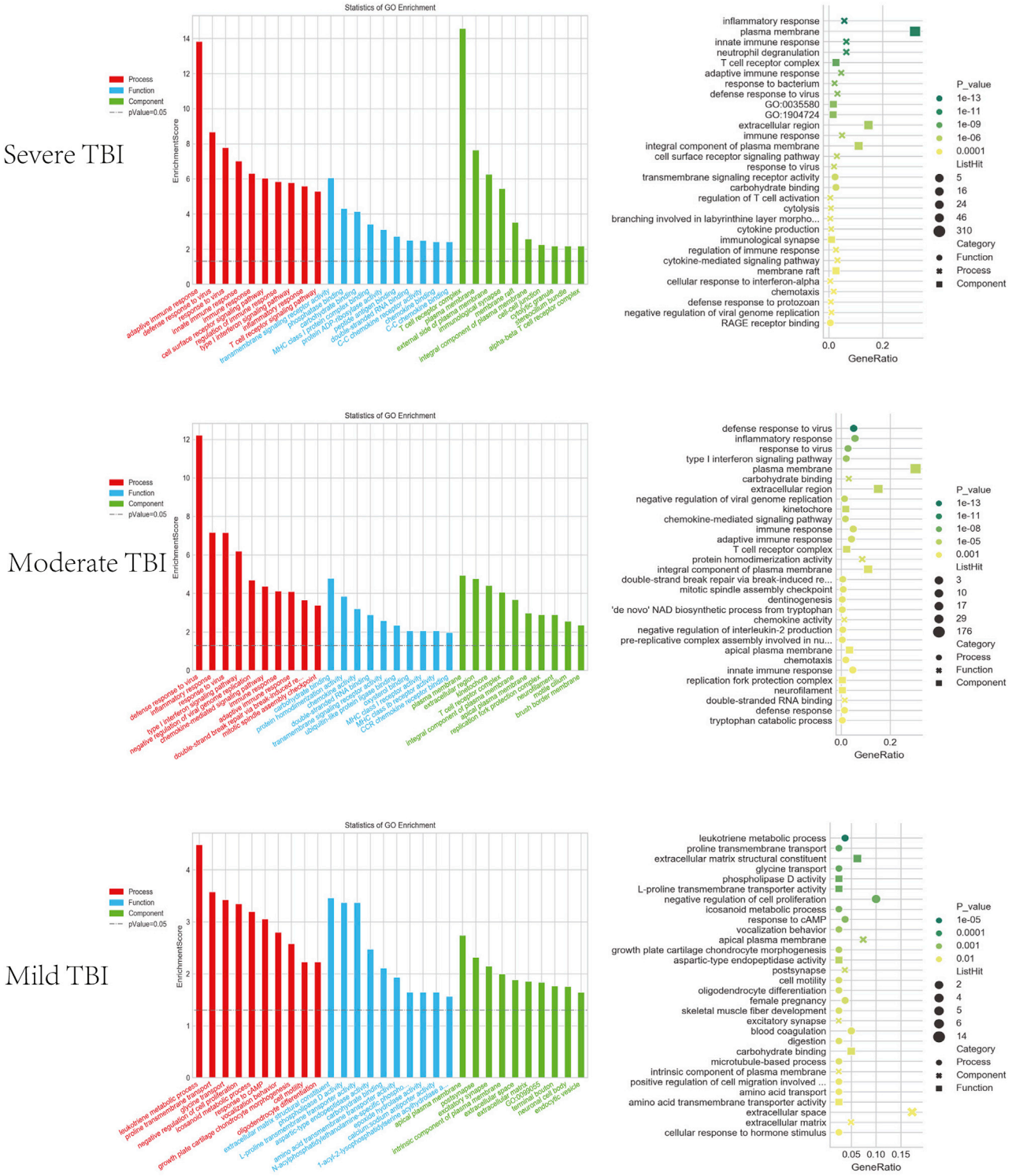

Figure 2. GO Results with Bar Graph and Bubble Plot in Severe, Moderate, and Mild TBI Groups

Upper, middle, and down panel indicate the severe, moderate, and mild TBI groups.

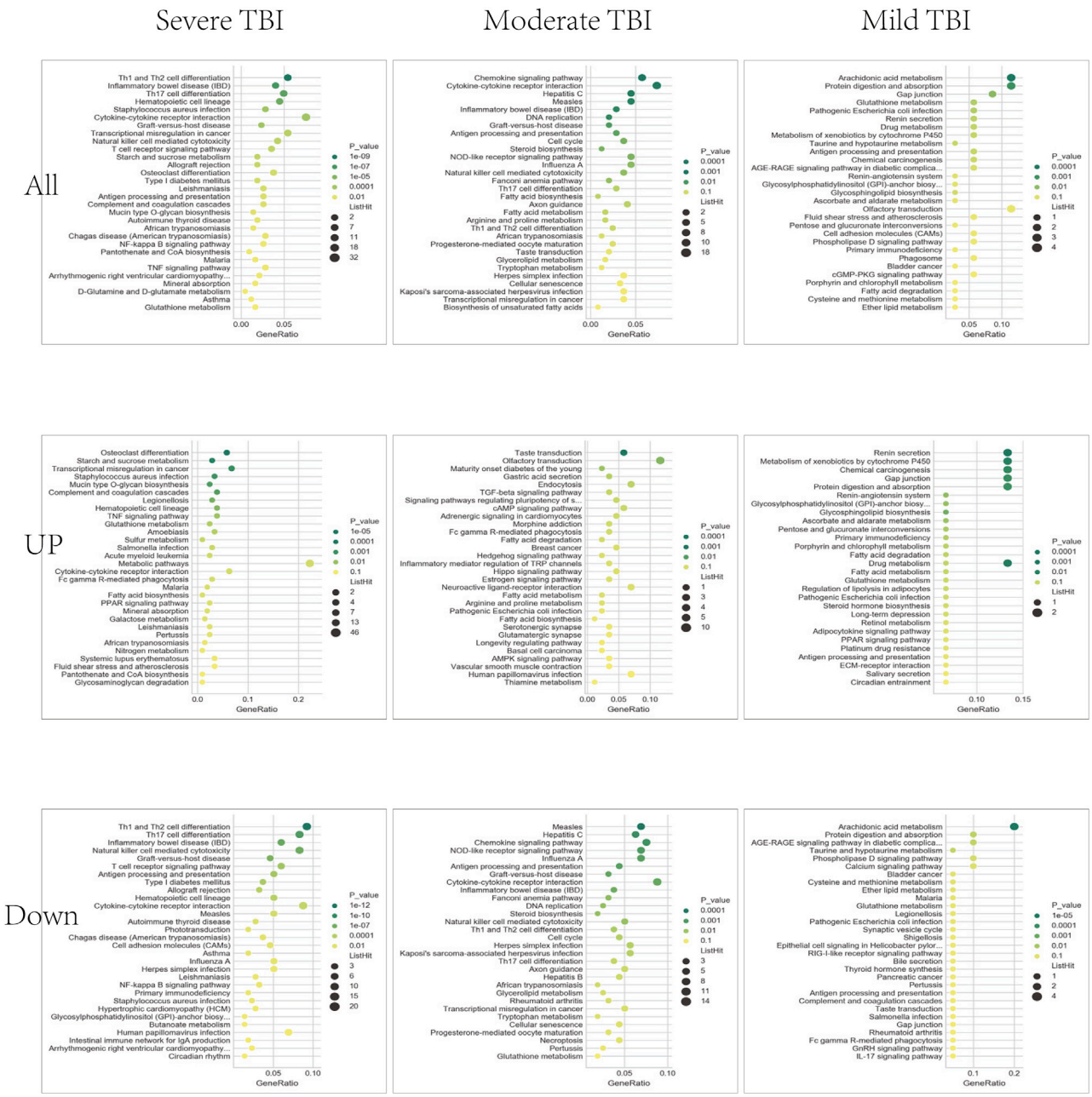

Figure 3. Top 30 KEGG Pathway Results with Bubble Plot in Severe, Moderate, and Mild TBI Groups

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.

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),[22] using the gene-TF pair provided by GTRD[23] 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.

Table 5. Top 10 KEGG Pathways Related to Target Genes in Severe TBI

Signaling Pathway	Count	Gene Ratio	Fold Enrichment	p Value	FDR_bh
Th1 and Th2 cell differentiation	23	0.05424528	4.313089623	1.35E-09	3.88E-07
Inflammatory bowel disease (IBD)	17	0.04009434	4.512155298	9.67E-08	1.39E-05
Th17 cell differentiation	21	0.0495283	3.3859769	6.20E-07	5.96E-05
Hematopoietic cell lineage	19	0.04481132	3.379327952	2.20E-06	0.000158746
Staphylococcus aureus infection	12	0.02830189	3.696933962	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	0.000620327	0.019850456
T cell receptor signaling pathway	15	0.03537736	2.512479392	0.00080569	0.022420497

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 lncRNAs Measured by Quantitative Real-Time PCR

To confirm our microarray data, we selected three upregulated lncRNAs (lnc-ANKRD34B-3:1, lnc-ICOSLG-1:4, and ENST00000423149.1) and seven downregulated lncRNAs (MSTRG.22548.1, MSTRG.67532.1, NONHSAT210656.1, MSTRG.41651.1, NONHSAT177352.1, lnc-NGEF-2:3, and NONHSAT168122.1) in all groups of TBI with a Venn Graph and further verified the expression level of lnc-ANKRD34B-3 and lnc-NGEF-2 by quantitative real-time PCR. Computed tomography (CT) values were normalized to actin. The candidate lncRNAs demonstrate different expression levels according to different severity of TBI (Figure 7), confirming our microarray results. In addition, the expression of lnc-ANKRD34B-3 had a negative correlation with Glasgow Coma Scale (GCS) of TBI patients, while the expression of lnc-NGEF-2 was fairly reduced in TBI patients compared to control groups (Figure 7).

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 Ptch-1 pathway.[24] A series of lncRNAs in the injured cortex in TBI mice were altered, which may be involved in different pathologies.[14] 3A recent study found that lncRNAs play an important role in gene regulation and take part in various CNS diseases.[9] Nevertheless, the exact role of lncRNAs in TBI is still elusive, and the exploration of their role is limited as well. Herein, we investigated lncRNA 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 434 lncRNAs (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 lncRNAs were upregulated and 488 lncRNAs were downregulated, while in the severe group, 1,686 lncRNAs were upregulated and 1,349 lncRNAs were downregulated. Expression profiles of these DEGs could be clustered hierarchically. There is an obvious trend that the total number of altered lncRNA 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 lncRNAs were MSTRG.49361.3, ENST00000606282.1, NONHSAT181489.1, ENST00000620459.1, and NONHSAT184491.1. Furthermore, ENST00000505646.1, NONHSAT187532.1, lnc-CREG1-3:5, MSTRG.8453.11, and NR_024075 were the five most significantly downregulated 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 (BLCA), 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 lncRNAs have very low expression in the brain and it increased after brain injury, it indicates that these lncRNAs might be involved in pathological mechanisms in TBI. Some lncRNAs are thought to become transcriptional regulators[25] that regulate coding gene expression by *cis* or *trans* mechanisms. Unfortunately, there are very few studies that have

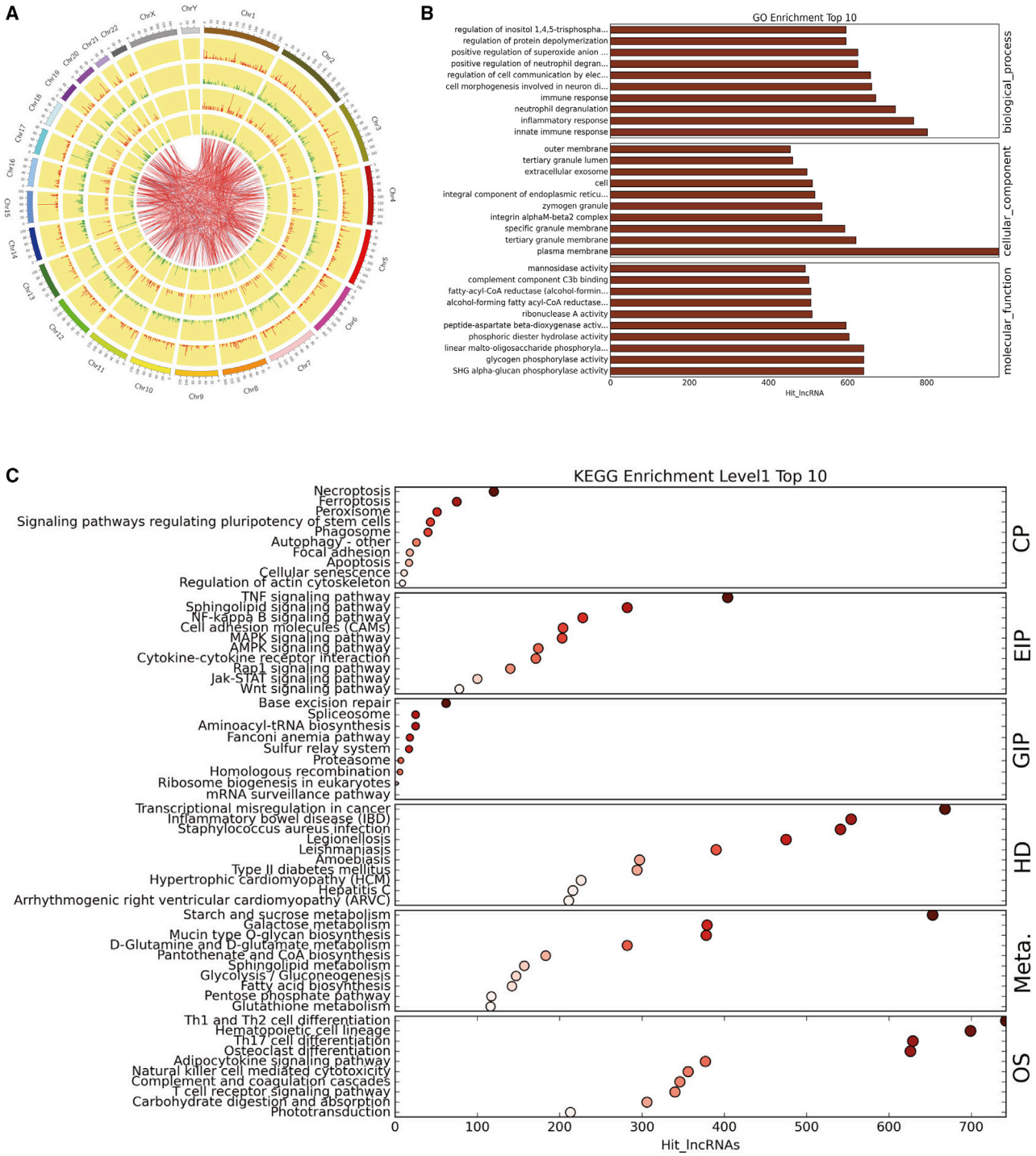

Figure 4. A lncRNA-mRNA Co-expression Network in Severe TBI

(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 that 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.

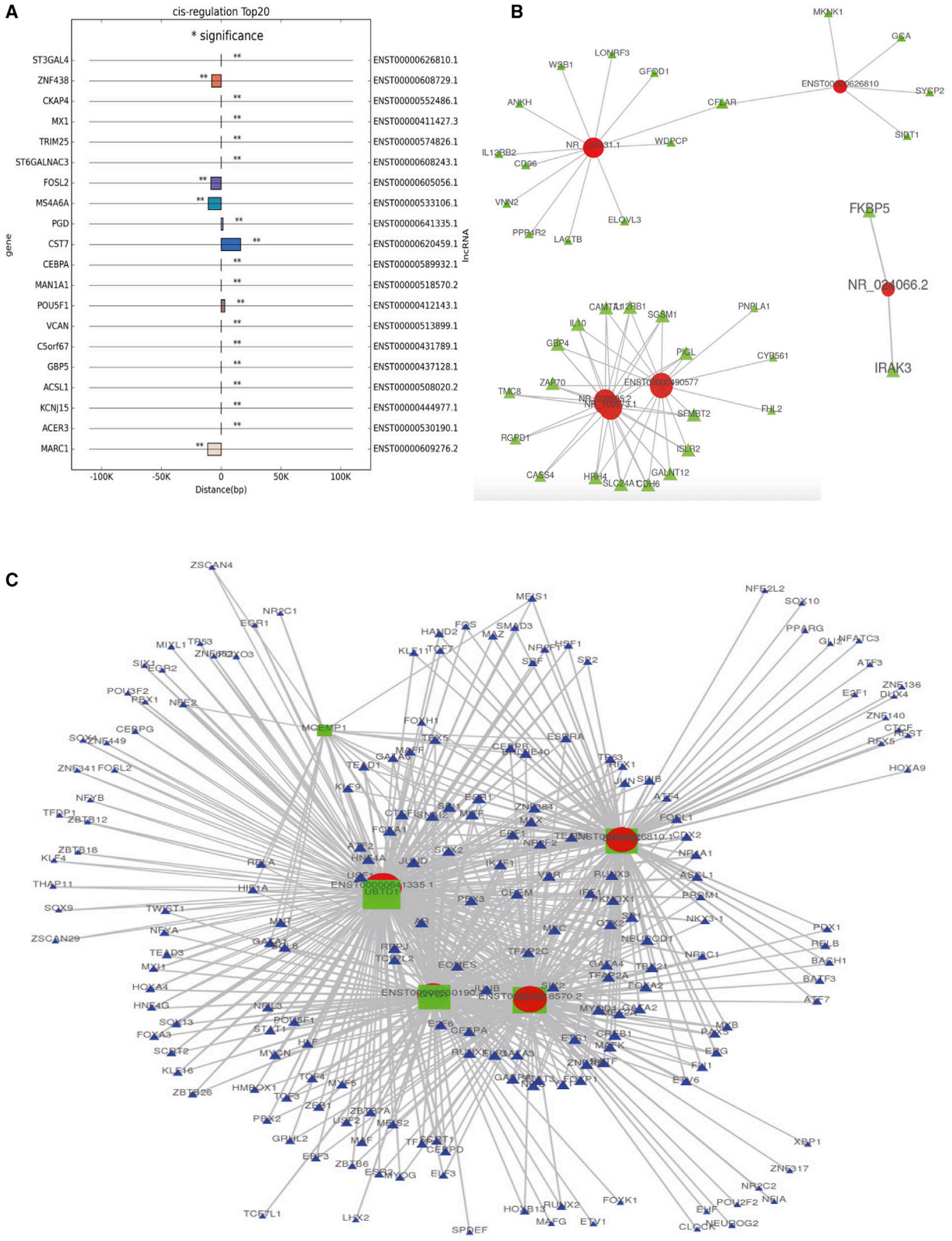

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Table 6. Top 10 Co-expressed lncRNA and mRNA

lncRNA	mRNA	FC	p Value
Top Five Positive Correlation lncRNA-mRNA			
lnc-PPP1R3D-8:1	SYCP2	0.99905871	2.08E-09
lnc-EPG5-4:1	PSTPIP2	0.998929375	3.07E-09
MSTRG.62207.1	KCNJ15	0.998907515	3.26E-09
NONHSAT208618.1	ZNF438	0.998897741	3.35E-09
NONHSAT151637.1	NMT2	0.998850793	3.79E-09
Top Five Negative Correlation lncRNA-mRNA			
LINC00337:6	SMIM40	−0.997434606	4.21E-08
lnc-LHFPL4-5:1	ETS2	−0.997370493	4.54E-08
NONHSAT150228.1	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 lncRNAs with TBI especially on *cis* or *trans* mechanisms. Furthermore, linking the expression of mRNAs, we consider that these altered lncRNAs 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 lncRNA-mRNA co-expression network, including differently expressed lncRNAs and its neighboring mRNAs (distance < 300 kb), and the Pearson correlation is significant ($p \leq 0.05$) with Pearson's correlation coefficients more than 0.7. We listed the top 20 cis-regulated lncRNAs and mRNAs in severe TBI in Table 7 with FEELnc software based on the co-expressed expression. On the other hand, *trans*-regulated lncRNAs can regulate their target genes at a relatively longer distances.[26] The functions of these *trans*-regulated lncRNAs were further predicted based on the TFs that might regulate their expression with a RNA-DNA style. According to the result of lncRNA–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 lncRNAs and mRNAs are significantly different in blood from different severity TBI especially in immune response, providing novel insight for lncRNAs 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.[27] 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 lncRNA 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 cDNA, 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 lncRNAs in Severe TBI

(A) In the graph, **p < 0.01. Left and right of y axis are mRNA and lncRNA, respectively; x axis is the distance between mRNA and lncRNA, negative value is the upstream, positive value is the downstream, and the same color bar graph is the same lncRNA. (B) The red node represents lncRNA, the green node represents mRNA, and the node size represents the number. (C) The red node represents lncRNA, the green node represents mRNA, the blue node indicates TF and the node size represents the number. Only nodes with degree >2 were shown.

Table 7. Top 20 *Cis* Regulated lncRNA and mRNA in Severe TBI

lncRNA	mRNA	Correlation Coefficient	p Value
ENST00000609276.2	MARC1	0.9877434247690321	4.560858586483991e-06
ENST00000530190.1	ACER3	0.9708489942461856	6.058384106755055e-05
ENST00000444977.1	KCNJ15	0.9693117147366813	7.060054020570408e-05
ENST00000508020.2	ACSL1	0.9689329615176981	7.322594891664935e-05
ENST00000437128.1	GBP5	0.9660128783945497	9.566355678315587e-05
ENST00000431789.1	C5orf67	0.9639411808219259	0.00011406560519370034
ENST00000513899.1	VCAN	0.9584008232822541	0.00017439939293255682
ENST00000412143.1	POU5F1	0.9579824266868007	0.00017965750313653578
ENST00000518570.2	MAN1A1	0.9573840286284421	0.00018735780893628086
ENST00000589932.1	CEBPA	0.9561988210278122	0.00020324508717588813
ENST00000620459.1	CST7	0.9545495759206558	0.0002267935866199957
ENST00000641335.1	PGD	0.9542375602230561	0.00023144147290796475
ENST00000533106.1	MS4A6A	0.9529278609670567	0.000251635461211397
ENST00000605056.1	FOSL2	0.9476717505939852	0.00034430741152138654
ENST00000608243.1	ST6GALNAC3	0.9471381279310238	0.00035480383441703167
ENST00000574826.1	TRIM25	0.9448100711668063	0.00040305777265801034
ENST00000411427.3	MX1	0.941652934982293	0.00047511165815773725
ENST00000552486.1	CKAP4	0.9350148559925798	0.0006530871366397738
ENST00000608729.1	ZNF438	0.9345016416834262	0.0006684194116870712
ENST00000626810.1	ST3GAL4	0.9339696129123375	0.0006845607135972268

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: $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{\beta\text{-actin}})\ TBI - (Ct_{target\ gene} - Ct_{\beta\text{-actin}})$ 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

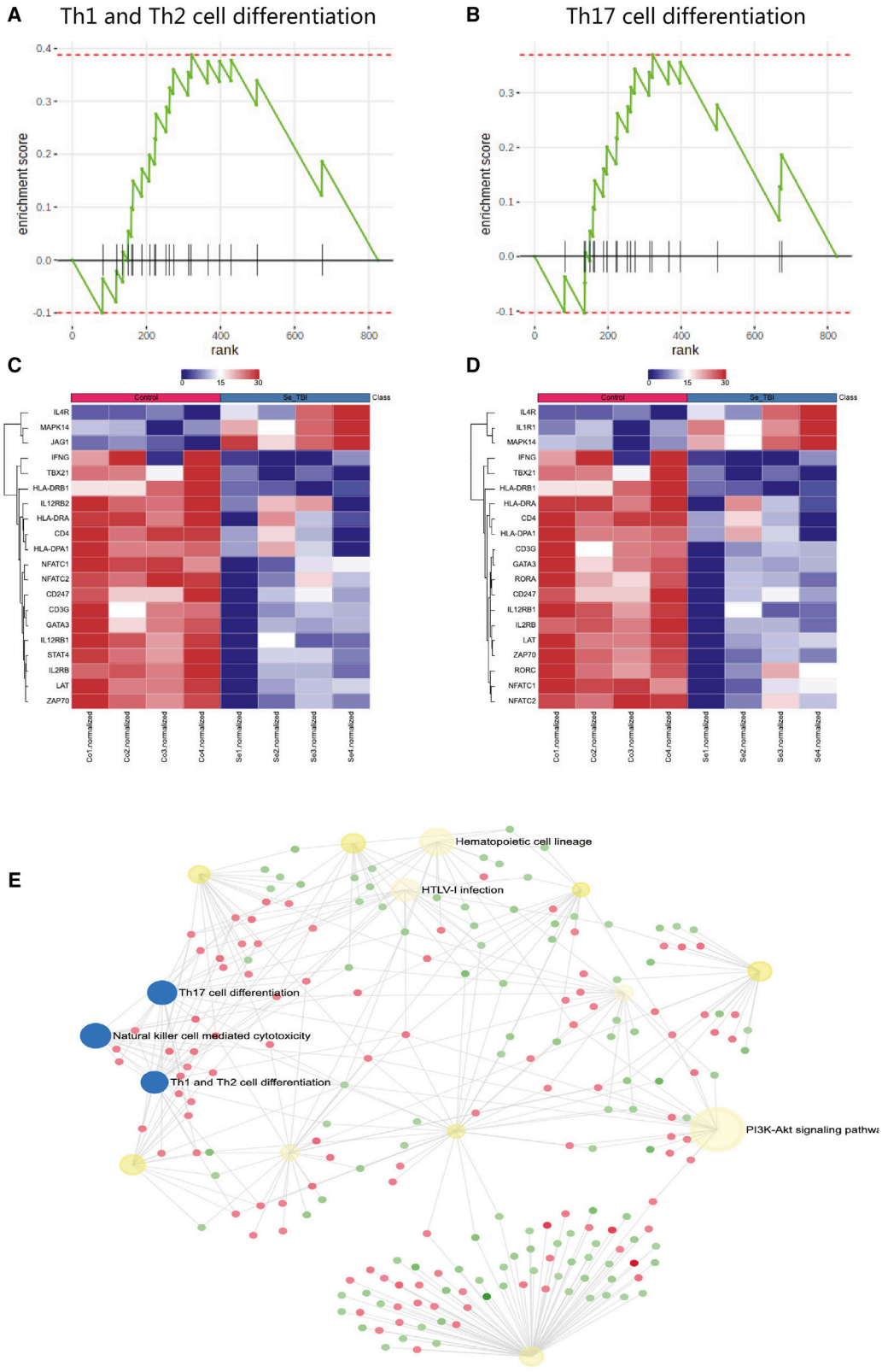
The datasets supporting the conclusions of this article are available from the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.08.012.

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



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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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Figure 6. GSEA Results for TBI

(A) Th1 and Th2 cell differentiation pathway is enriched in TBI. (B) Th17 cell differentiation pathway is enriched in TBI as well. (C) Heatmap shows that Th1 and Th2 related pathway genes are impaired in TBI. (D) Heatmap also shows a deficit in Th17 related pathway genes in TBI. (E) KEGG pathway analysis demonstrates that Th1, Th2, and Th17 cell differentiation and NK-cell-mediated cytotoxicity are involved in TBI.

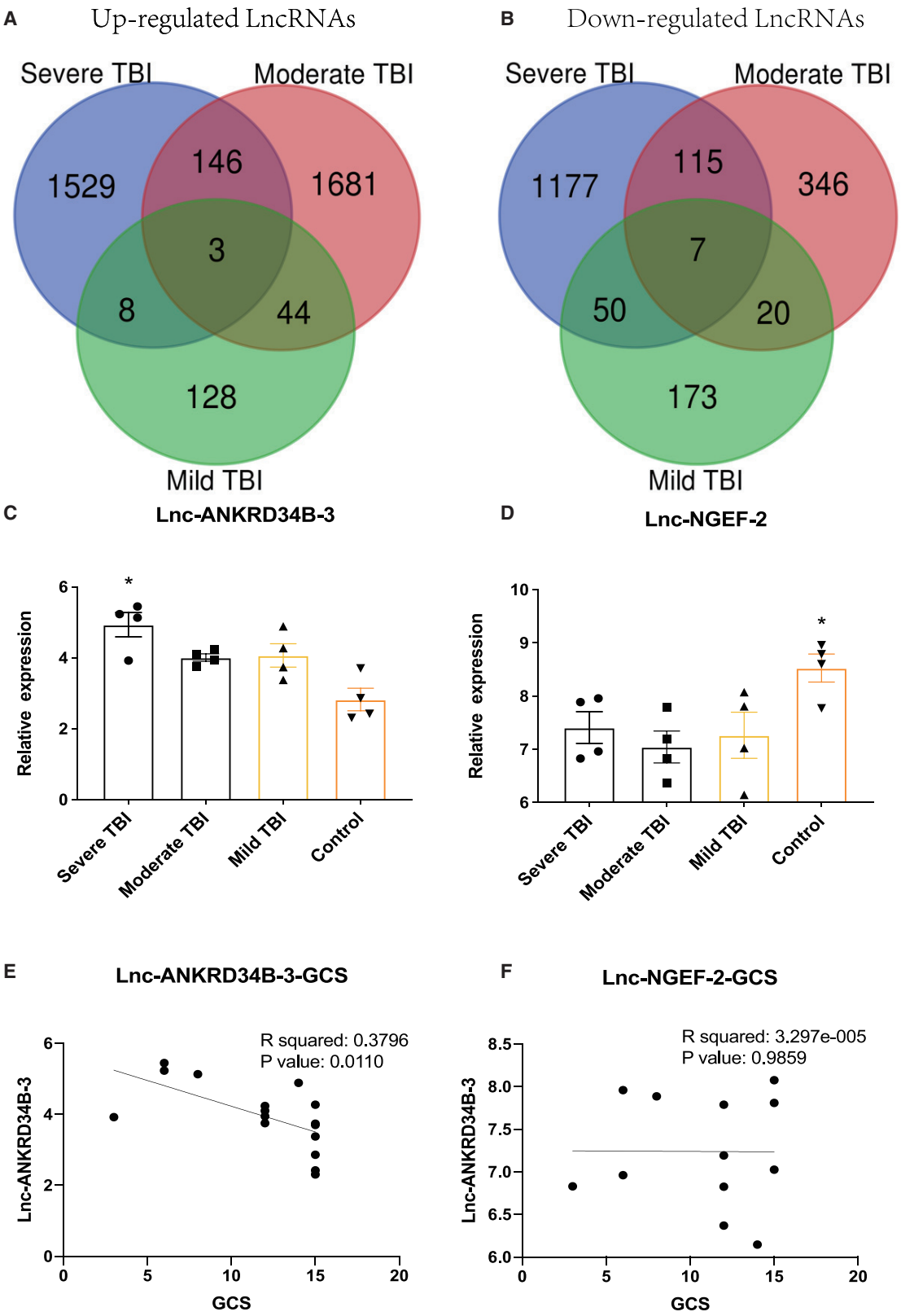

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Figure 7. Quantitative PCR of lncRNA Expression in Whole Blood of Patients with TBI

(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.