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Research article

Analysis of long non-coding RNA expression profiles following focal cerebral ischemia in mice

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ABSTRACT

Long noncoding RNAs (lncRNAs) have a variety of biological functions and play key roles in many diseases. However, the knowledge of lncRNA function during a stroke is limited. We analyzed the expression profiles of lncRNAs in the brain ischemic region of mice after a 45 min middle cerebral artery occlusion (MCAO) with a 48 h reperfusion. Gene ontology and pathway analysis were used to elucidate the potential functions of the differentially expressed mRNAs. A total of 255 lncRNAs (217 up-regulated and 38 down-regulated) and 894 mRNAs (870 up-regulated and 24 down-regulated) showed significantly altered expression in the ischemic brain compared to the sham controls (fold change $\gg > 2$, $P \ll 0.05$). The gene ontology terms were mainly associated with neutrophil chemotaxis, positive regulation of inflammatory response, cell cycle, positive regulation of apoptotic process, and apoptotic process. The pathway analysis indicated that the mRNAs were mainly associated with inflammatory pathways. Additionally, the interactions between the differentially expressed lncRNAs and mRNAs are revealed by a dynamic lncRNA-mRNA network. Our findings provide an overview of aberrantly expressed lncRNAs in stroke and further broaden the understanding of stroke pathogenesis.

1. Introduction

Stroke is one of the leading causes of human death and disability worldwide. Tissue plasminogen activator (tPA) is the only pharmacological treatment that has been approved by the American Food and Drug Administration (FDA) for acute ischemic stroke [1]. In addition, mechanical devices have been approved by FDA to remove blood clot within 6 h after stroke [2–4], and this time window is even extended 24 hours for patients with mismatch between deficit and infarct [5]. Despite these exciting progresses, only a small portion of stroke patients can receive such treatment. Therefore, it is urgent to understand the underlying mechanisms of brain injury after stroke for the purpose of exploring novel stroke treatments. The important regulatory roles of microRNAs in stroke have previously been studied by us and others [6–9], however, the functional significance and molecular mechanisms of other classes of non-coding RNAs in the regulation of ischemia/reperfusion after stroke remain unknown.

LncRNAs (long non-coding RNAs) are a unique class of RNAs longer than 200 nucleotides without evident protein coding functions [10], which have been considered to be merely transcriptional noise for a long time [11]. However, it is increasingly recognized that lncRNAs are crucial in controlling the function of the homologous mRNAs [12]. The dysregulation of these lncRNAs has been associated with many human diseases, such as cancers [11], diseases of the central nervous system [13], kidney, and cardiovascular diseases [14]. A few studies have indicated that certain lncRNAs play important roles in cerebral ischemia [15–17]. Few studies to date, however, have systematically evaluated changes in lncRNA expression after stroke. In addition, it remains unclear whether lncRNA expression profiles are related to those of protein coding mRNAs in the ischemic brain after stroke.

In this study, we aim to provide insights related with lncRNAs and mRNAs in the pathogenesis of stroke. We identified the expression profiles of both lncRNAs and mRNAs in the ischemic regions of stroke in mice, and analyzed the relationships between the expression levels of lncRNAs and mRNAs by constructing a co-expression network of lncRNAs and mRNAs.

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Table 1
Primers used for RT-qPCR analysis.

Primer name	Primer sequence (5′-3′) forward	Primer sequence (5′-3′) reverse
ENSMUST00000121456	GGCAAGAACACCATGATGCA	ACAGCATGTCCCGAATCTCA
NONMMUT036055	ATGTCGGGTTCTTCTAGCGT	TCAGCAGAGGGTCATGTTGA
KnowTID_00002053	AGACTCCTCTACCCTGTGCT	AAAGCAGCGACATGAAACA
NONMMUT038744	GGGGACTCGAGATCTACTGC	ATAGCTGAACTGGGCGATCA
β − actin	CGTTGACATCCGTAAAGACC	CTAGGAGCCAGAGCAGTAATC

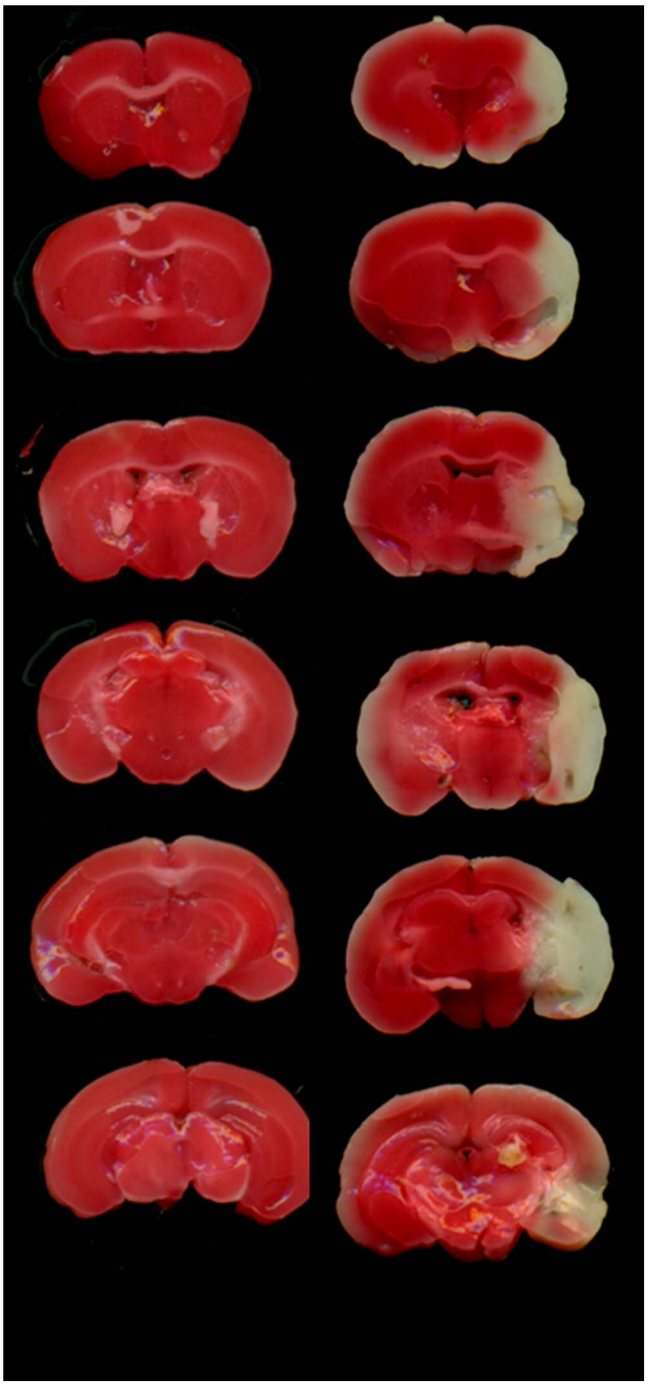

Fig. 1. Ischemia/reperfusion-induced cerebral injuries in mice. Representative TTC images from the sham and ischemia groups, n = 6 per group.

2. Materials and methods

2.1. Focal cerebral ischemia in mice

Adult male C57BL/6 mice weighing 20–22 g were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and housed in temperature-controlled rooms with 12 h light/dark cycles, where they had free access to food and water. All procedures in this study were conducted according to the guidelines set by the University Animal Care and Use Committee of Capital Medical University.

Transient focal cerebral ischemia was induced by a 45-min occlusion of the right middle cerebral artery (MCA), as has been previously described [18]. In brief, anesthesia was induced by inhalation of 5% isoflurane (Lunan Pharmaceutical Group Corporation; Shandong, China) in a 30% O_2, 68.5% N_2O mixture, and maintained with 2% isoflurane inhalation. Rectal temperature was maintained at 37 ± 0.5 ˚C with a heating pad during surgical procedures. Sham-operated animals underwent sham surgery under anesthesia, but without the right MCA occlusion. Mice were placed in a post-operative cage, and kept warm and undisturbed for a minimum of 2 h for observation.The mouse brains were removed 48 h after reperfusion. The ischemic regions were collected and immediately frozen in liquid nitrogen cans.

2.2. Measurement of ischemic infarct sizes

Mice were sacrificed 48 h after MCAO, and the brain was quickly removed and cut into 1 mm thick coronal sections. The sections were immersed in 2% TTC at 37 ˚C for 20 min and then fixed in 4% paraformaldehyde. The infarct size of the ischemic cortex was normalized to the non-ischemic cortex and expressed as a percentage [19].

2.3. LncRNA microarray and analysis

Total RNA containing small RNA was extracted using TRIzol reagent (Invitrogen, Canada) according to the manufacturer's protocol. RNA purity and concentration were determined with NanoDrop ND-1000 spectrophotometry, and the RNA integrity was determined via 1% gel electrophoresis.

The microarray analysis was performed with the Affymetrix GeneChip Mouse Transcriptome Array 1.0, which was designed with about 55,000 mouse lncRNAs and 23,000 mouse genes. The purified RNA was transcribed into complementary DNA (cDNA) according to the protocol set forth by Gminix (Shanghai, PR China). The cDNA was fragmented, labeled with fluorescent dyes and was then incubated in the GeneChip Hybridization Oven 645. After washing the chip, the GeneChip Scanner 3000 7G was used to measure the fluorescence intensity. After image data were saved, the probe summarization was performed using the software Expression Console (version 1.2.1).

We used the significance analysis of microarrays (SAM) model to identify the differentially expressed lncRNAs and mRNAs between the ischemic and sham groups. After a significant analysis and false-discovery rate (FDR) analysis, a P value $\ll 0.05$ was regarded as significant. Moreover, fold changes greater than 2 or less than 0.5 were considered to be higher fold changes.

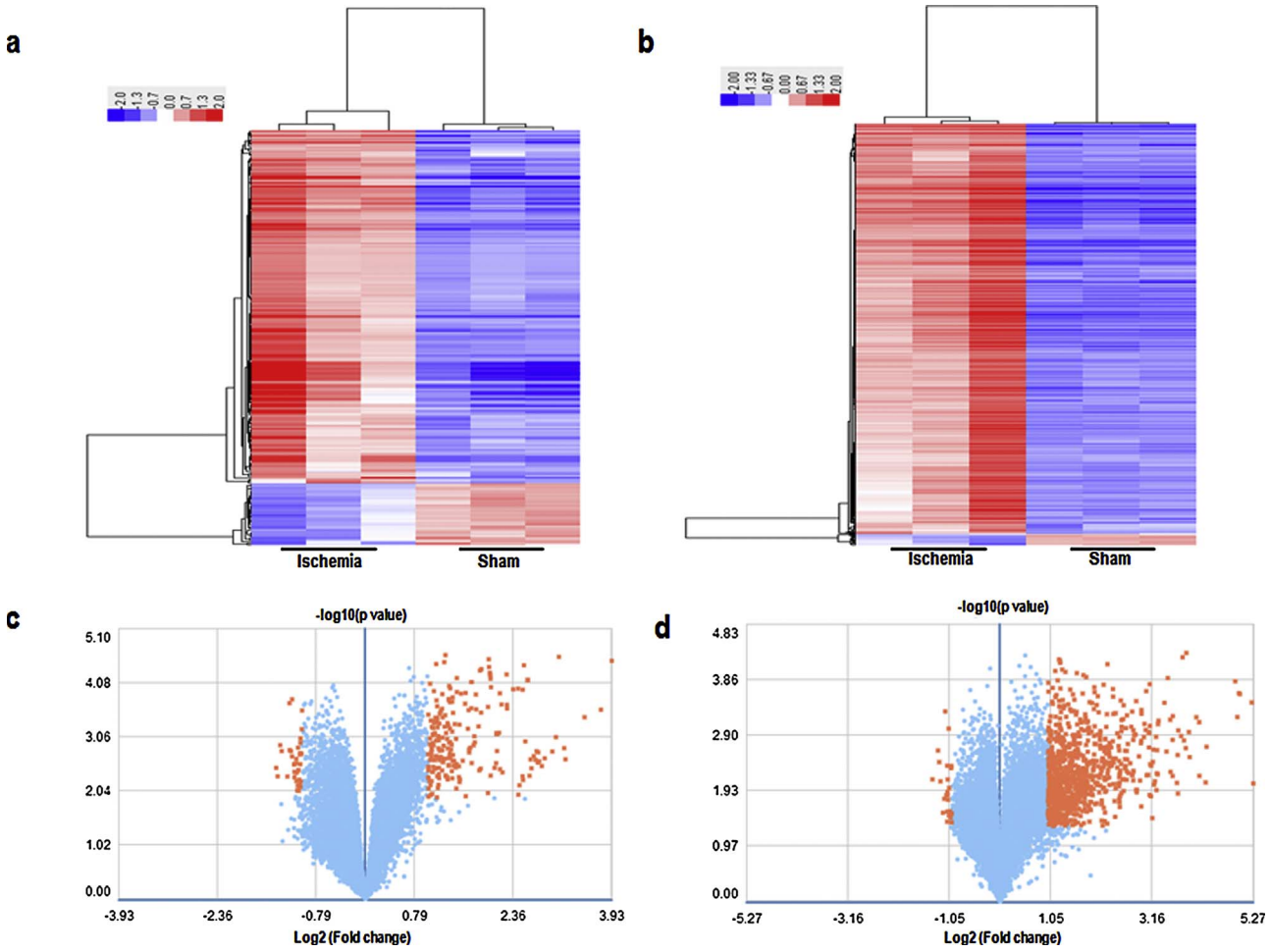

Fig. 2. The expression profiles of lncRNAs and mRNAs in mice from the sham and ischemic groups by microarray analysis. The top row: the hierarchical clusterings show a remarkably differential expression of lncRNAs (a) and mRNAs (b), n = 3 per group. Red and green colors represent high and low expression levels, respectively. Each column represents a single group, and each row represents an lncRNA or mRNA. The bottom row: the volcano plots of expression profiles of differentially expressed lncRNAs (c) and mRNAs (d) in mice from the sham and ischemic groups. Orange dots represent genes that passed the statistical and fold-change cutoffs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

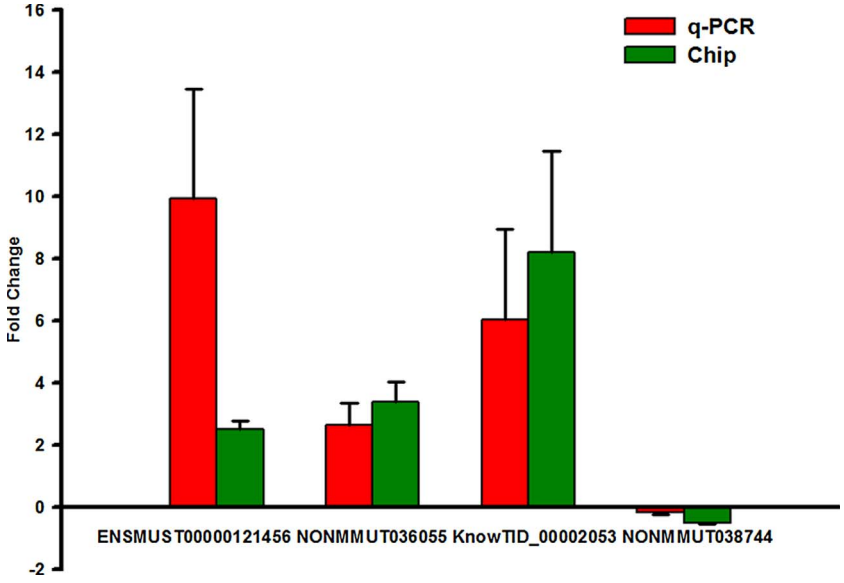

Fig. 3. Validation of the reliability of microarray data via quantitative real-time PCR. The fold-changes represent ratios by comparing the expression values of the ischemia to sham results, as detected by microarray or qRT-PCR, n = 3 per group. The positive refers to up-regulation, and the negative refers to downregulation.

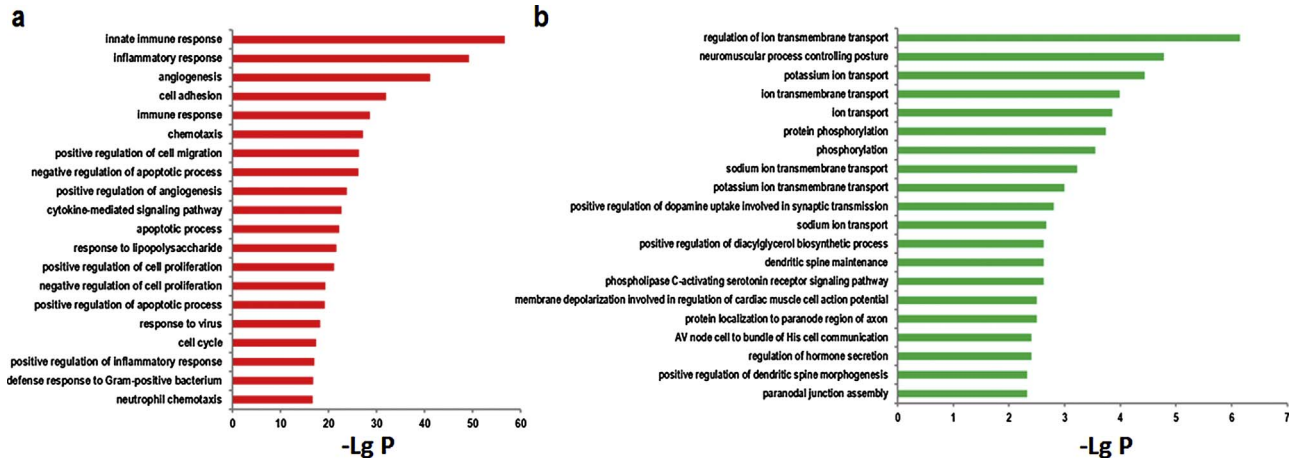

Fig. 4. GO analysis of differentially expressed mRNAs in ischemia mice. (a) The top 20 GO terms associated with upregulated mRNAs. (b) The top 20 GO terms associated with downregulated mRNAs. The value of −lg (p value) was calculated to reflect the significance of GO terms.

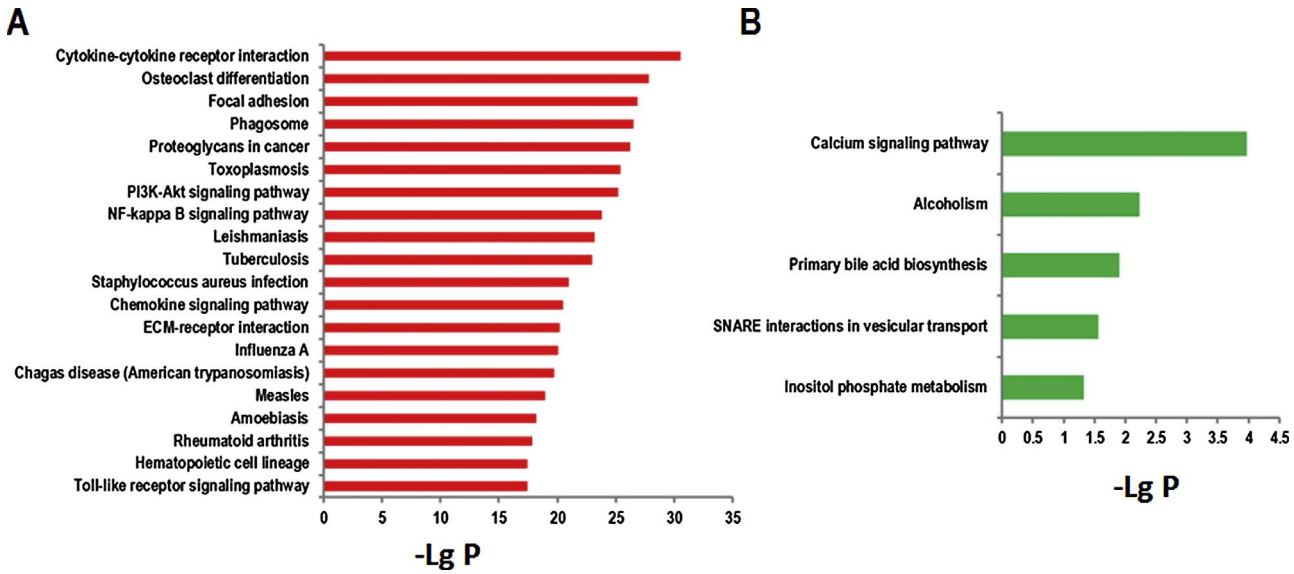

Fig. 5. The KEGG analysis of differentially expressed mRNAs in ischemic brains. (a) The top 20 pathway terms associated with upregulated mRNAs. (b) The pathway terms associated with downregulated mRNAs. The value of −lg (p value) was calculated to reflect the significance of pathway terms.

2.4. Quantitative real-time PCR (qRT-PCR) assay

Total RNA was isolated using the Trizol reagent and reverse transcribed into cDNA using the Thermo First cDNA Synthesis Kit (SinoGene). The qRT-PCR was conducted to test the expression of four lncRNAs using the 2 × SG Green qRT-PCR Mix. All of the reagents were obtained from SinoGene. The expression levels were analyzed based on the cycle threshold (CT) values and normalized to internal β-actin. The primer sequences are listed in Table 1.

2.5. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis

GO analysis was used to investigate the roles of all differentially expressed mRNAs (http://www.geneontology. org). A pathway analysis was used to determine the significant pathways related with the differentially expressed mRNAs, according to KEGG (http://www.genome.jp/kegg/). We used Fisher's exact test and the χ^2 test to select the significant GO categories and pathways. The threshold of significance was a P value ≪0.05, and false discovery rate (FDR) was calculated to correct the P value.

2.6. Construction of the lncRNA-mRNA co-expression network

The mRNA-lncRNA co-expression network, which was used to identify interactions between the differentially expressed lncRNAs and mRNAs, was constructed based on Pearson's correlation analysis. For each pair of genes, correlation coefficients of 0.95 or greater were selected to construct the network through the software Cytoscape. In the network, each gene/lncRNA corresponds to a node, and the nodes are connected by an edge. The more adjacent genes or lncRNAs to which a gene is connected, the larger its degree and the more important it is.

3. Results

3.1. Overview of lncRNA and coding gene profiles in the brains of mice following MCAO

The TTC staining results showed a large area of infarction in the cerebral cortex and striatum in the ischemic group (Fig. 1). The ischemic regions in the brains were dissected for the lncRNA microarray assay.

In order to characterize the lncRNA expression profile in the ischemic brains, we performed a lncRNA and mRNA microarray assay to

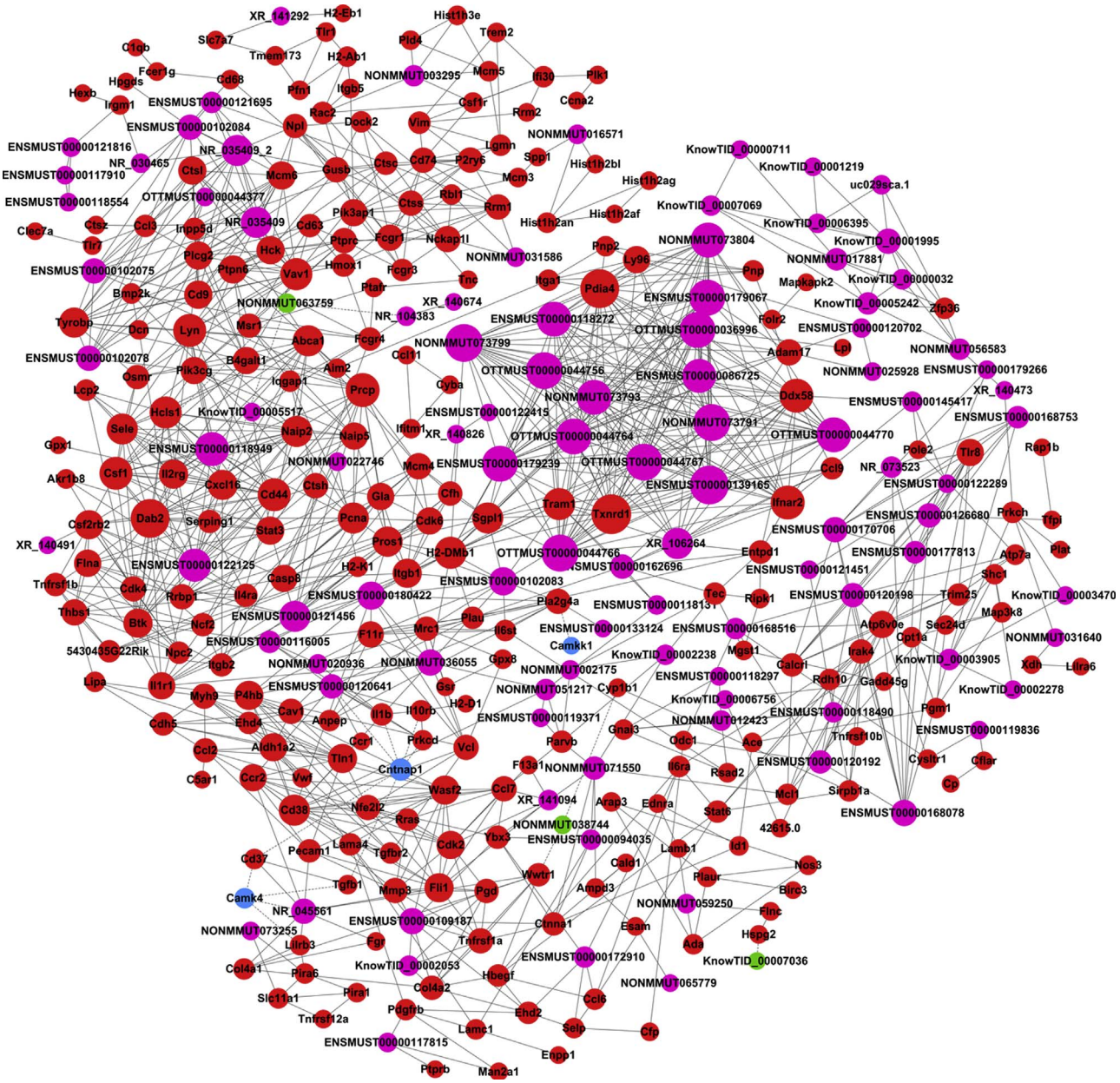

Fig. 6. Co-expression networks of all differentially expressed lncRNA-mRNA in the ischemic brains. The lncRNAs are shown in purple (upregulated) and green (downregulated), while mRNAs are presented in red (upregulated) and blue (downregulated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

determine their respective expression levels. The results showed that the expression of a total of 255 lncRNAs changed dramatically with at least 2 or 0.5-fold changes. Among the 255 differentially expressed lncRNAs, 217 were up-regulated, while 38 were down-regulated in the ischemic regions, compared with those in the corresponding non-ischemic brains of the sham group (Fig. 2).

3.2. Validation of deregulated lncRNAs in ischemic brains

Four lncRNAs were randomly selected from the microarray analysis data for validation by using quantitative RT-PCR. As shown in Fig. 3, the qRT-PCR analysis revealed that the expressions of the lncRNAs ENSMUST00000121456, NONMMUT036055, and KnowTID_00002053 were up-regulated, whereas NONMMUT038744 expression was down-regulated in the ischemic brains (Fig. 3). These results are consistent with the microarray data.

3.3. GO and pathway analyses of differentially expressed mRNAs

GO analysis was performed to determine the function of the differentially expressed mRNAs after a stroke. As shown in Fig. 4a and b, the highest enriched GOs of upregulated mRNAs were those involved in neutrophil chemotaxis, the inflammatory response, the cell cycle, and apoptosis. In contrast, the highest enriched GOs of downregulated mRNAs were those regulating ion transmembrane transport, potassium ion transport, ion transmembrane transport, and protein phosphorylation.

After identifying the differentially expressed mRNAs, we further performed a pathway analysis according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The results indicate that the upregulated mRNAs are involved in the regulation of a cytokine–cytokine receptor interaction, the NF-kappa B signaling pathway, the PI3K-Akt signaling pathway, and the Toll-like receptor signaling pathway (Fig. 5a). The downregulated mRNAs are involved in inositol phosphate

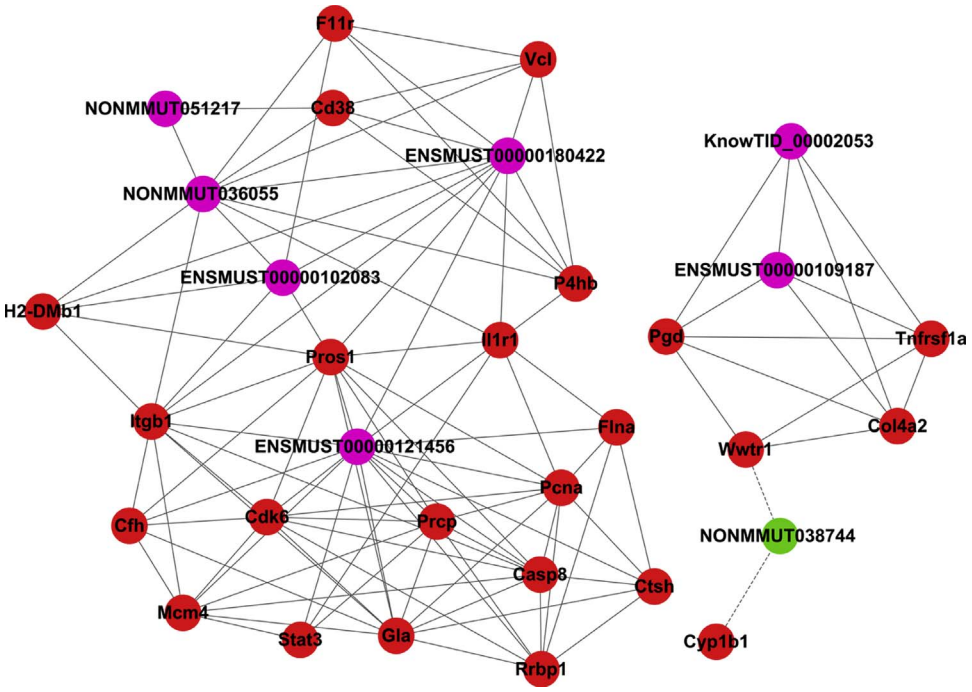

Fig. 7. Co-expression networks of the verified lncRNA-mRNA in ischemia mice. The lncRNA-mRNA network containing the four verified aberrantly expressed lncRNAs. The lncRNAs are shown in purple (upregulated) and green (downregulated), while mRNAs are presented in red (upregulated). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

metabolism and the Calcium signaling pathway (Fig. 5b).

3.4. Construction of the functional lncRNA–mRNA network

Based on the correlations of the differentially expressed lncRNAs and mRNAs, mRNA-lncRNA co-expression networks were constructed to discover the significant molecular lncRNA mechanisms in stroke (Fig. 6). The co-expression network shows that one lncRNA was associated with one to dozens of mRNAs, suggesting that there is an interregulation of lncRNAs and mRNAs in stroke. We further focused on co-expression networks centering on the four confirmed lncRNAs, ENSMUST00000121456, NONMMUT036055, KnowTID_00002053, and NONMMUT038744 (Fig. 7). For example, ENSMUST00000121456 expression is positively correlated with Casp 8 and Stat 3 mRNA expression levels, which are important for neuronal death, neuroinflammation, cell proliferation, and differentiation. The network suggests that lncRNA expression may regulate the corresponding mRNA expression levels to simultaneously participate in ischemic injures.

4. Discussion

In the present study, we provide novel data showing that 255 lncRNAs and 894 mRNAs were differentially expressed in ischemic brains, and that those differentially expressed mRNAs are mainly involved in the processes of neutrophil chemotaxis, the inflammatory response, the cell cycle, and apoptosis. The KEGG pathway analysis indicates that these mRNAs are involved in the regulation of cytokine–cytokine receptor interactions, the NF-kappa B signaling pathway, and the PI3K-Akt signaling pathway. Since our lncRNA-mRNA network analyses suggest that lncRNA alterations are closely related with mRNA changes, we provide strong evidence that altering lncRNA expression might affect brain injury after stroke via regulating the expression of these differentially mRNA, which are involved in inflammation, apoptosis, and cell survival pathways.

Most previous studies have been devoted to exploring the role of small ncRNAs such as microRNAs (~21-25 nucleotides) [20]. With the development of lncRNA microarray techniques, thousands of lncRNAs have been identified. The dysregulation of lncRNAs has been associated with many human diseases, including different types of cancers and diseases of the central nervous system [21,22]. Nevertheless, research on lncRNAs related to cerebral ischemic disease is limited.

A recent microarray profiling study showed that stroke significantly influenced the cerebral lncRNAome in rats [23]. Bioinformatic analysis has shown that these stroke-responsive lncRNAs have ≫ > 90% sequence homology with protein-coding genes. In addition, stroke-induced lncRNAs were suggested to be associated with the chromatin-modifying protein Sin3A and corepressors of the RE-1 silencing transcription factor, in order to modulate the post-ischemic epigenetic landscape [24]. In addition, Zhao, *et al.,* demonstrated that hypoxic-ischemic injury altered lncRNA expression profiles in the neonatal rat brain [25]. In particular, the lncRNA BC088414 was upregulated after hypoxic-ischemic injury and its knockdown attenuated cell apoptosis and promoted cell proliferation by reducing the mRNA levels of Casp6 and Adrb2 in PC12 cells subjected to oxygen and glucose deprivation. Few studies to date, however, have evaluated lncRNA changes in the mouse brain after ischemia-reperfusion injury, and none have systematically studied the relationship between lncRNA and mRNA expression levels using microarray techniques.

In the current study, most of these lncRNAs identified by us have not yet been functionally characterized, while most of the identified mRNAs are well known. Therefore, a bioinformatic analysis of the aberrantly expressed mRNAs was used to gain a better understanding of the putative function of the differentially expressed lncRNAs.

First, GO enrichment analyses were used to analyze the differentially expressed protein-coding genes associated with the lncRNAs. The results revealed that the upregulated genes were associated with neutrophil chemotaxis, positive regulation of inflammatory response, the cell cycle, positive regulation of the apoptotic process, and the apoptotic process. The highest enriched GO terms for downregulated mRNAs were associated with the regulation of ion transmembrane transport, potassium ion transport, ion transmembrane transport, and protein phosphorylation.

Second, the pathway analysis was conducted by KEGG database. It is not surprising that the inflammatory response was found to hold an important position in these genes, as it is well known that the immune and inflammatory responses are major contributors to acute ischemic brain injuries [26,27]. The NF-kappa B signaling pathway has been reported to play a vital role in the inflammation response after ischemic

stroke-induced brain injuries [28,29]. In addition, the PI3K/Akt pathway has been proven to regulate neuronal survival and also participates in the inflammatory response [30]. Furthermore, the toll-like receptor has also been demonstrated to contribute to the development and progression of inflammatory and autoimmune diseases [31]. Our results suggest that these pathways may harbor significance and/or may contribute to the pathogenesis and biochemical characteristics of ischemia/reperfusion-induced cerebral injuries in mice.

Third, we constructed a co-expression network by combining differentially expressed lncRNAs with differentially expressed mRNAs, based on their locational distributions and sequence correlations. The results suggested that the differentially expressed lncRNAs interact with the differentially expressed genes. Our novel results provide strong evidence that lncRNAs may participate in brain injury by altering mRNA expression levels.

Finally, the co-expression networks centering on four lncRNAs: suggested that ENSMUST00000121456 expression is positively correlated with Casp 8 and Stat 3 mRNA expression levels, which are important for neuronal death, neuroinflammation, cell proliferation, and differentiation. In addition, another pair with a positive correlation was NONMMUT036055 with Il1r1mRNA, which is interleukin 1 receptor, type I. Previous studies have suggested that antagonism at the IL-1R induces neuroprotection in several rodent models of neuronal injury [32]. Nevertheless, NONMMUT038744 expression was negatively correlated with Wwtr1 mRNA expression. WWTR1, also knowns as TAZ, is a transcriptional coactivator. Hepatocyte TAZ/WWTR1 has been indicated to promote inflammation and fibrosis in nonalcoholic steatohepatitis [33].

We are aware of some limitations of the current study. For example, given our research methods, we merely predicted the function of differentially expressed lncRNAs and were unable to determine exactly how these lncRNAs regulate the target gene expression. In addition, we used whole brain tissues, and thus the functions of lncRNAs in specific cells types cannot be identified. Furthermore, the brain tissues were collected 48 h after stroke, and the time course of the expression of different genes was not studied. In the future, the functions of lncRNAs in different cell types should be studied, and their expression patterns at multiple time points should be identified. Furthermore, the functions of some key lncRNAs can be validated by modulating their expression levels via overexpression or inhibition.

In summary, we provide novel evidence that lncRNAs are differentially expressed in the ischemic brain after stroke, and that alteration to this expression is closely related with changes in gene expression, which are involved in neuroinflammation, apoptosis, cell differentiation, and proliferation. We conclude that lncRNAs may regulate brain injury by altering the expressions of associated genes and proteins.

Conflict of interest

None declared.

Acknowledgments

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