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MICROARRAY EXPRESSION PROFILE ANALYSIS OF LONG NONCODING RNAs IN PREMATURE BRAIN INJURY: A NOVEL POINT OF VIEW

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Abstract—Long noncoding RNAs (lncRNAs) are abundant in the central nervous system and have a key role in brain function as well as many neurological disorders. However, the regulatory function of lncRNAs in the premature brain has not been well studied. This study described the expression profile of lncRNAs in premature mice using microarray technology. 1999 differentially expressed lncRNAs and 955 differentially expressed mRNAs were identified. Gene Ontology (GO) and pathway analysis showed that these lncRNAs were involved in multiple biological processes, including the nervous system development and inflammatory response. Additionally, the lncRNA-mRNA-network and TF-gene-lncRNA-network were constructed to identify core regulatory lncRNAs and transcription factors. The sex-determining region of Y chromosome (SRY) gene may be a key transcription factor that regulates premature brain development and injury. This study for the first time represents an expression profile of differentially expressed lncRNAs in the premature brain and may provide a novel point of view into the mechanisms of premature brain injury.

Key words: premature, brain injury, microarray, long noncoding RNA.

INTRODUCTION

Preterm birth is a serious public health issue throughout the world. According to the reports from World Health Organization (WHO) in 2012, about 15 million preterm babies are born each year (Howson et al., 2012). Brain injury is one of the most severe problems seen in preterm infants and often leads to poor neurodevelopmental outcomes. Adverse intrauterine environment such as hypoxia, infection and inflammation may affect normal process of fetal brain development or even induce fetal brain injury including white matter injury (Yuan et al., 2010). The molecular mechanisms of premature brain injury are complicated and currently unknown. Multiple genes and pathways involved in brain development and injury process have been identified in previous studies, such as Wnt/β-catenin signaling, Cdk2-p27^{Kip1} pathway (Jablonska et al., 2012; Fancy et al., 2014; Salmaso et al., 2014), GPR56 (Giera et al., 2015) and others. Nevertheless, growing evidence suggests that despite the protein coding genes, a large amount of noncoding RNAs (ncRNAs) which were previously considered as "junk RNAs" are also involved in a variety of biological processes and diseases. The ncRNAs are usually classified as short or long ncRNAs, except for the most investigated miRNAs and siRNAs which belong to short RNAs, the regulatory function of long noncoding RNAs (lncRNAs) in the nervous system has been under active investigation.

LncRNAs are transcripts longer than 200 nucleotides in length that play complex and vital roles in cell function, multiple organs development, inflammatory process and disease progression (Guennewig and Cooper, 2014). There is increasing recognition that lncRNAs are crucial members of gene regulatory networks, they exert regulatory functions by binding with noncoding RNAs or genes, including transcription factors (Ng et al., 2013). In addition, lncRNAs play their regulatory role via a variety of mechanisms, for example, chromatin modification, transcription, splicing and translation (Guennewig and Cooper, 2014). Therefore, analyzing the relationship between lncRNAs and protein coding genes as well as their interacting patterns will undoubtedly contribute to identify their potential functions and the mechanisms of their action.

Brain development requires accurate temporal and spatial regulation of gene expression in numerous neurons and glia cells. Even though a majority of studies focused on the neurological roles of miRNAs, recent studies have started to illustrate the novel function of lncRNAs. LncRNAs exhibit strong abundance of expression in developing and adult brains (Irfan et al., 2012; Hangauer et al., 2013) and a growing number of researches have shown their key roles in brain development and organization. For example, the lncRNA Sox2OT (sex-determining region Y-box 2 overlapping transcript) and Sox2DOT (sex-determining region Y-box 2 distal

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Abbreviations: AD, Alzheimer's disease; BACE1-AS, β-secretase-1 antisense transcript; cDNA, complementary DNA; FDR, false-discovery rate; GO, Gene Ontology; HE, hematoxylin-eosin; lncRNAs, long noncoding RNAs; LPS, lipopolysaccharide; ncRNAs, noncoding RNAs; PBS, phosphate-buffered saline; PCC, Pearson correlation coefficients; RVM, random variance model; SRY, sex-determining region of Y chromosome; Sox, SRY-related HMG box; TFs, transcription factors.

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overlapping transcript) correlate with the Sox2 gene which has an important function in brain development (Irfan et al., 2012). Dysregulation of lncRNAs have already been reported to be associated with neurodegeneration and diverse neurological disorders (Qureshi et al., 2010). For example, the lncRNA β-secretase-1 antisense transcript (BACE1-AS) has been found to be linked to Alzheimer's disease (AD). Knockdown of BACE1-AS in the mouse brain decreased the expression of beta-amyloid, which is vital to the occurrence of AD (Modarresi et al., 2011). The loss function of lncRNA Evf2 was reported to influence the GABAergic interneurons and synaptic inhibition (Chodroff et al., 2010; Irfan et al., 2012). Other lncRNAs such as HAR1F, HTTAS and TUG1 were also believed to be associated with the central nervous system (Wu et al., 2013; Roberts et al., 2014; Goff et al., 2015). Thus, studying the lncRNA expression profiles in the developing brain will provide some new thoughts for premature brain injury.

In the present study, the lipopolysaccharide (LPS) was used as an inflammatory factor to build a premature animal model and the premature brain injury was confirmed via hematoxylin-eosin (HE) staining and immunostaining. Then a microarray technology was applied to identify the differentially expressed mRNAs and lncRNAs in premature mice and constructed the network for mRNA-lncRNA and transcription factor-lncRNA to investigate the core factors playing key roles in premature brain injury.

EXPERIMENTAL PROCEDURES

Animals

Female Balb/c mice were mated with male C57 mice at the age of 10 weeks. The day of detection of vagina plug of females was designated as day 0 of gestation, the females were kept in a specific pathogen-free environment individually after pregnancy. On day 17, pregnant dams were infused intraperitoneally with either lipopolysaccharide (LPS, *E. coli*, serotype 055:B5; Sigma L-2880) or phosphate-buffered saline (PBS) at a dose of 50 μg/kg twice, this dose was selected based on our preliminary studies. The LPS-injected mice gave birth to live pups at day 18, while the PBS-injected mice were sacrificed at day 21 if they were not delivered. After the infant mice were born, thirteen offspring of each group were sacrificed by decapitation and the fetal brains were removed.

Ethics statement

The experimental procedures and animals used in this study were approved by the Ethics Committee of First Affiliated Hospital of Xi'an Jiaotong University. The surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize suffering.

HE staining and immunohistochemistry

The brain tissues were fixed in 4% paraformaldehyde (pH 7.4) for 24 h and embedded in paraffin. The prepared brains were dissected coronally by 10 μm and stained with HE and CD68 (a marker of activated microglia). The primary antibody was rabbit anti-mouse CD68 (1:100, Abcam, UK), the secondary antibody was goat anti-rabbit IgG. HE sections were observed under a light microscope at magnifications of 20 × to assess brain morphology. Immunostaining sections were observed under a light microscope at magnifications of 40 × to assess the activation of microglia.

RNA isolation and purification

Brain tissues were kept in liquid nitrogen. Total RNA was isolated using Trizol reagent according to the manufacturer's guideline. The extracted RNA was validated by gel electrophoresis and purified by an RNeasy mini kit (Qiagen, Valencia, CA, USA).

Microarray analysis

The microarray analysis was performed by Gminix (Shanghai, PR China) with Affymetrix Mouse Transcriptome (MT) Array 1.0. 55000 lncRNAs and 23000 protein-coding transcripts (genes) can be detected by the microarray. Each transcript was represented by a specific exon or splice junction probe which can identify individual transcripts precisely.

Procedures to analyze the microarray are as follows: firstly, purified RNA was transcribed into complementary DNA (cDNA) applying a random priming method. Secondly, cDNA was fragmented and labeled with fluorescent, then hybridized onto the Affymetrix MT Array. Thirdly, after hybridization and washing the slides, the arrays were scanned by the GeneChip®Scanner 3000 7G (Affymetrix). After image data were saved, the probe summarization was performed in Expression Console (version 1.2.1).

Two Class Dif method and random variance model (RVM) *t*-test were used to identify the differentially expressed lncRNAs and genes between premature mice and controls, for the RVM *t*-test can increase degrees of freedom efficiently in the case of small samples (Wright and Simon, 2003). After the significant analysis and false-discovery rate (FDR) analysis, only the P value <0.05 was regarded as significant difference (Clarke et al., 2008). The fold change $\geqslant 1.2$-fold was considered as higher fold change. Hierarchical clustering was performed to show the expression pattern of differentially expressed lncRNAs and mRNAs between the two groups.

GO and pathway analysis

Gene Ontology (GO) analysis and pathway analysis were applied to investigate the roles of all differentially expressed mRNAs. GO analysis was applied to analyze their biological process and molecular function, which can form genes into hierarchical categories and discover the genes regulatory network (http://www.geneontology.org). Pathway analysis was performed to explore the significant pathway of the differentially expressed genes according to Kyoto Encyclopedia of Genes and Genomes (KEGG), Biocarta and Reactome (http://www.genome.jp/kegg/).