Int J Clin Exp Med 2017;10(1):628-637
www.ijcem.com /ISSN:1940-5901/IJCEM0038548

Original Article

Gene expression profiling and identification of key genes involved in neonatal hypoxic-ischemic brain injury

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Received August 23, 2016; Accepted October 14, 2016; Epub January 15, 2017; Published January 30, 2017

Abstract: This study aimed to get a better understanding on the molecular circuitry and identify potential critical genes as therapeutic targets for neonatal hypoxic-ischemic (HI) encephalopathy. The microarray data of GSE37777, including 4 HI samples and 4 controls, was downloaded from the GEO database. Differentially expressed genes (DEGs) were screened in the contralateral cerebral cortices of mature rats (8 weeks old) after neonatal HI brain insult. Pathway clustering analysis was performed and a functionally grouped pathway network of DEGs was constructed. Besides, a protein-protein interaction (PPI) network was constructed. Total 973 DEGs (599 up- and 374 down-regulated) were identified in HI group compared with the controls. Furthermore, a functionally grouped pathway network of DEGs was constructed. Hedgehog signaling pathway was identified and pathway-related genes *SHH*, *DHH*, *WNT1*, *WNT2B*, and *WNT4* were up-regulated in HI group. Furthermore, *CCND1*, *SHH* and *RET* were hub proteins in the PPI network. Wnt signaling pathway may be activated by the hedgehog signaling pathway in the contralateral cerebral cortices of mature rats after neonatal HI injury. *CCND1* may be involved in apoptosis and cell cycle regulation in neonatal HI encephalopathy. Besides, *RET* may play a role in neonatal HI encephalopathy.

Keywords: Neonatal hypoxic-ischemic brain injury, differentially expressed genes, pathway clustering analysis, protein-protein interaction network, hedgehog signaling pathway

Introduction

Neonatal hypoxia-ischemia (HI) brain injury still remains an major issue as it is a frequent cause of acute mortality and chronic disability in newborns [1]. Neonatal HI encephalopathy can cause long-lasting morbidity, including seizure, cerebral palsy, and cognitive retardation in newborns and children [2, 3]. Currently, there is no definitive therapeutic intervention that can minimize brain disorder induced by HI except that few studies indicated the possible benefits of hypothermia in some moderate cases [4, 5]. The treatment strategies are restricted due to the incomplete understanding of the underlying pathogenesis in neonatal HI brain insult. Therefore, further investigations on the molecular mechanisms of neonatal HI brain injury remain a high priority.

Many studies have been performed to investigate the mechanisms of HI brain damage in the past years. A growing body of evidence shows that the main cause of HI brain injury is the exhaustion of glucose and oxygen, which causes a primary energy failure and initiates a cascade of biochemical events, resulting in cell dysfunction and finally cell death [6]. The secondary energy failure is characterized by superabundant entry of Ca^{2+} into cells, leading to induction of free radicals [7]. Additionally, excitotoxic amino acids are released and inflammatory responses occur, promoting cell death mostly by apoptotic mechanisms [8]. Recently, a study had demonstrated that necrostatin-1 (Nec-1), an allosteric inhibitor of receptor interacting protein (RIP)-1 kinase activity, could prevent secondary energy failure following neonatal HI via blocking early nitric oxide (NO•) accumulation, glutathione oxidation as well as attenuating mitochondrial dysfunction [9]. Mounting evidence indicates that numerous molecules were implicated to be involved in the mechanisms of neonatal HI encephalopathy [10]. For instance, Fan *et al.* reported that hypoxia-inducible factor-1α (HIF-1α) could exhibit

both neuroprotective and neurotoxic properties in HI brain injury by its participation in distinct events such as neovascularization, neuroprotection and the apoptotic process [10]. Besides, Tian *et al.* showed that cytoglobin (CYGB) was up-regulated by HI in the neonatal brain and CYGB might exhibit neuroprotective effects possibly by antioxidant and anti-apoptotic functions as well as through stimulating angiogenesis [11]. In addition, the work of Li *et al.* demonstrated that the activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway could protect the brain in the neonatal rat model with HI brain damage [12]. Moreover, Wang *et al.* suggested that the increase in neural stem cells might be regulated by the hedgehog signaling pathway in HI neonatal rats and thus alleviated brain damage [13]. However, the molecular mechanisms underlying the development and progression of HI encephalopathy deserve further research. Unravelling the complex functions of more critical genes and pathways involved in the mechanisms of HI brain injury may be important for exploring novel and effective therapies.

The Rice model of unilateral HI has been widely used as a useful experimental tool to study neonatal HI brain insult [14], but the resulting brain damage is reported to be mainly restricted to one hemisphere based on the histological analysis and behavioral tests [15, 16]. However, neonatal unilateral ischemia and bilateral hypoxia was identified to induce a long-term deficit in the reducing ability in the contralateral hemisphere by using the electron paramagnetic resonance imaging technique, lasting at least until the age of 8 weeks in the Rice model [17]. Recently, Kojima *et al.* performed comprehensive gene expression and network analysis using a DNA microarray system in contralateral cerebral cortices of mature rats (8 weeks old) after neonatal HI brain insult. They revealed that many up-regulated genes were related to cell death signaling even within the contralateral cerebral hemisphere [18]. In this study, we analyzed the differentially expressed genes (DEGs) in the contralateral cerebral cortex of mature rats following HI brain injury using the same gene expression profiling. Comprehensive bioinformatics analysis was used to analyze the significant pathways and to construct the protein-protein interaction (PPI) network for investigating the critical DEGs associated with HI brain injury. We aimed to get a better understanding of the molecular circuitry in neonatal HI brain insult and identify potentially critical genes as therapeutic targets for neonatal HI encephalopathy.

Materials and methods

Affymetrix microarray data

The microarray data of GSE37777 [18] deposited by Kojima *et al.* was downloaded from the Gene Expression Omnibus (GEO) database [19] in NCBI (http://www.ncbi.nlm.nih.gov/geo/). The platform information is GPL7294 Agilent-014879 Whole Rat Genome Microarray 4x44K G4131F (Probe Name version). Eight samples were included in this dataset. As described in the original study [18], pregnant Wistar rats were purchased and the pups were reared with their dams until the start of the experiment. A total of 8 rat pups from different litters were used in the experiment. Thereinto, 4 Wistar rats (7-day-old) were subjected to the Rice model construction procedure to induce HI brain injury. Briefly, these 4 rats in the HI group were anesthetized with ether, and the left carotid artery was isolated and ligated with surgical silk. These rats were allowed to recover for 1-2 hours and then exposed to 1 hour of hypoxia condition by being placed in a plastic chamber which was perfused with a mixture of humidified 8% oxygen balanced with nitrogen. While HI brain insult was not induced in the other 4 Wistar rats (7-day-old) that was used as controls. The left carotid artery of the control rats was isolated but not ligated. Animals were killed 7 weeks after the induction of HI brain insult. The cerebral cortices contralateral to the HI brain insult in the HI group and the cerebral cortices on the same side as the Rice models in the control animals were removed. Total RNA was then extracted from the tissues.

Data preprocessing and screening of DEGs

The preprocessed microarray data were obtained and then robust multi-array average (RMA) background correction [20] and quantile normalization were performed on these data using the Linear Models for Microarray data (limma) package [21]. The probes mapped to none gene were deleted. Mean expression values were analyzed when multiple probes were mapped to the same gene ID, resulting in

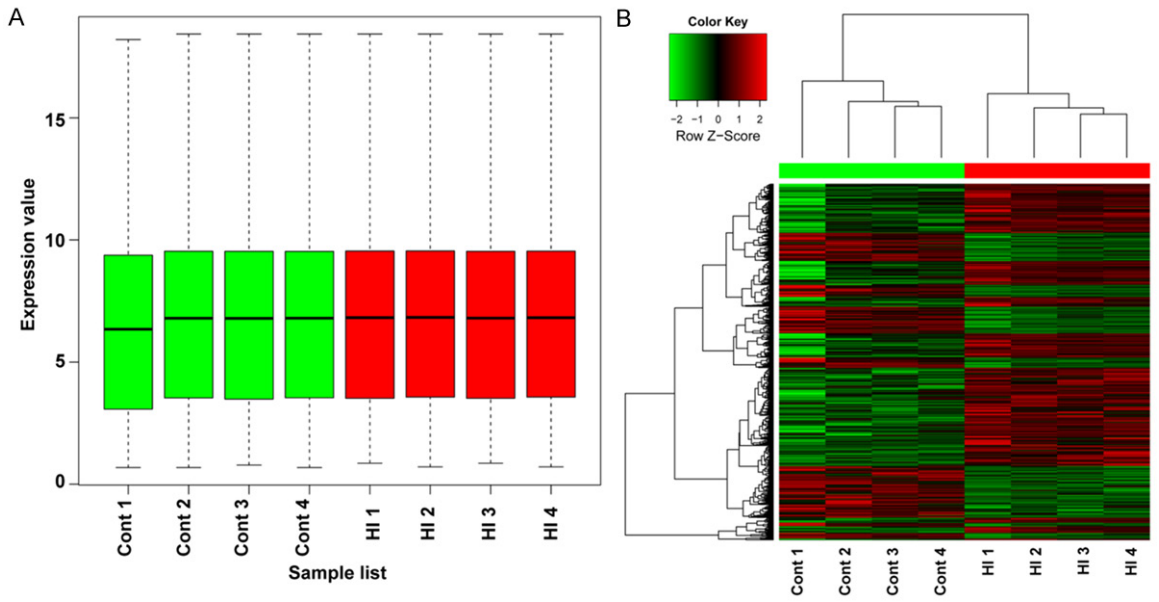

Figure 1. DEGs screening. A. Normalized expressed value data. The box in the black line means the median of each set of data, which determine the degree of standardization of data through its distribution. The green rows indicate the control samples (n = 4) and the red rows indicate samples in the HI group (n = 4). B. Heat-map overview of the DEGs between the HI group (n = 4) and the control group (n = 4). Green color indicates decreased expression and red color indicates increased expression.

22,503 sequences. Up-regulated and down-regulated genes were identified in the HI group compared with the control group using limma package [21]. Fold change (FC) was used to evaluate whether genes were differentially expressed. The FC was expressed as a $\log_2$ value in keeping with the microarray output format [22]. In order to complete the significance of a gene's differential expression estimation, we attempted to get the most robust or reliable lists of DEGs by simultaneously filtering our results on *p*-value and a minimum cutoff of absolute $\log_2$ FC. In this study, p-value < 0.05 and FC ≥ 1.5 were chosen as the threshold for identifying DEGs.

Pathway enrichment analysis

ClueGO, an easy-to-use Cytoscape plug-in, strongly improves biological interpretation of a large list of genes. ClueGO can integrate Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and create a functionally organized pathway term network [23]. For biological networks, CluePedia can calculate the correlation for DEGs based on four tests, Spearman's rank, Pearson correlation, distance correlation and maximal information coefficient, to investigate linear and non-linear dependencies between the implemented variables [24].

In this study, we used the Cytoscape [25] plug-in, ClueGO+Cluepedia [23] to integrate KEGG pathway terms to create a functionally grouped pathway network of the DEGs. KEGG pathway terms served as the clustering criterion with a two-sided (enrichment/depletion) hypergeometric test followed by Bonferroni correction (significance level of 0.05) to identify significantly affected pathways. The network represented each pathway as an individual node, while the edges between pathways denoted an approximation of biological interaction between the pathways based on the cross-pathway feature overlap. The node size corresponded to the statistical significance for each term enriched. Additionally, the most significant pathways (with bigger node size) identified in this network were selected as our focus and were subsequently carried out for DEG distribution presentation using cytoKEGG [26], a Cytoscape plug-in.

PPI network construction

The Search Tool for the Retrieval of Interacting Genes (STRING) database provides particularly comprehensive coverage and ease of access to both predicted as well as experimental interaction information. The interaction probabilities of proteins correspond to the confidence in the

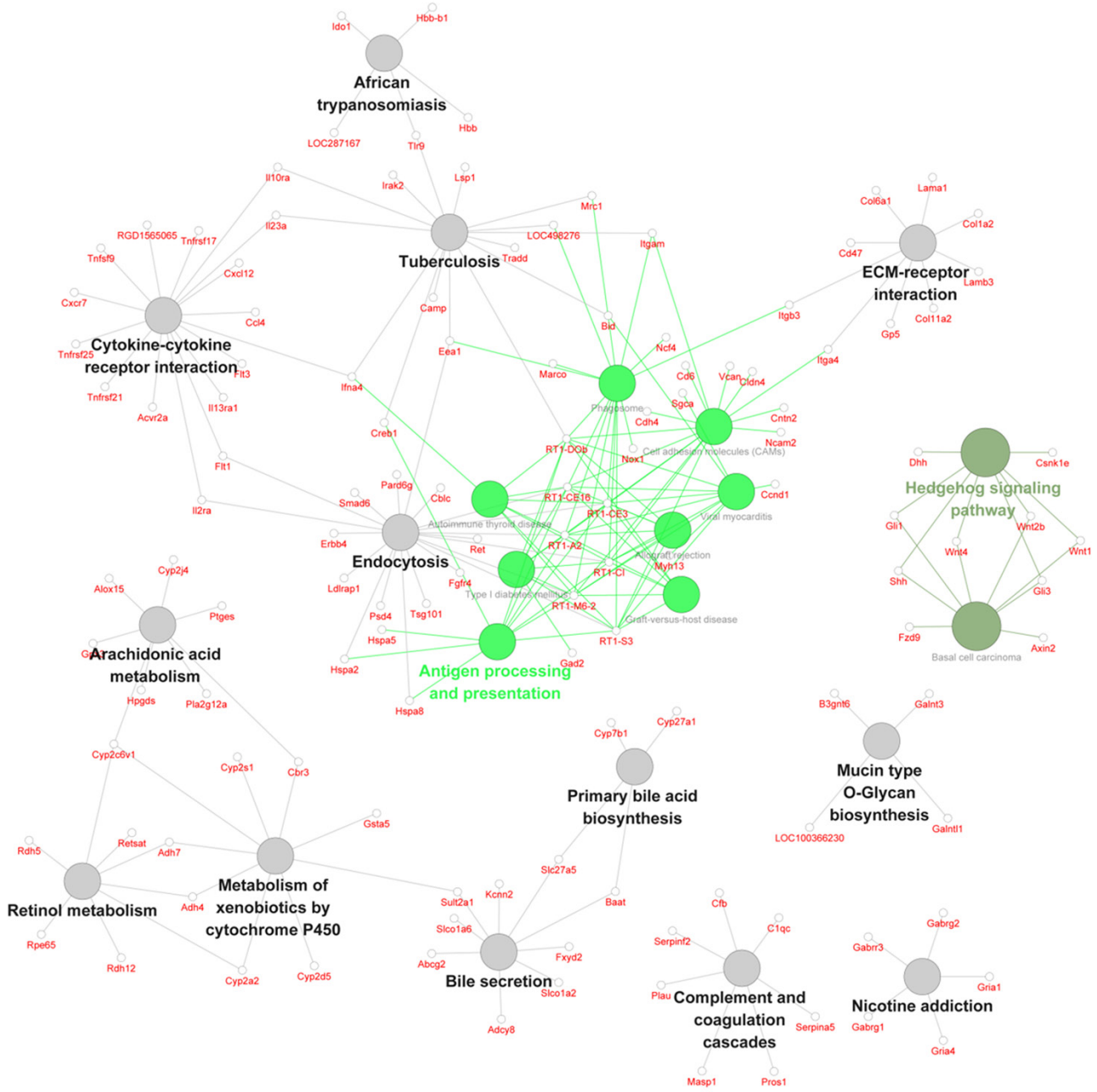
African trypanosomiasis
Ido1
Hbb-b1
Hbb
LOC287167
Tlr9
Tuberculosis
Il10ra
Irak2
Lsp1
Mrc1
LOC498276
Tradd
Itgam
Il23a
RGD1565065
Tnfrsf17
Tnfsf9
Cxcl12
Cxcr7
Ccl4
Camp
Cytokine-cytokine receptor interaction
Tnfrsf25
Tnfrsf21
Il13ra1
Acvr2a
Flt3
Flt1
Il2ra
Ifna4
Eea1
Creb1
Pard6g
Smad6
Cblc
Erbb4
Endocytosis
Ret
Ldlrap1
Fgfr4
Psd4
Tsg101
Hspa5
Hspa2
Hspa8
Gad2
Antigen processing and presentation
RT1-S3
RT1-M6-2
RT1-CI
RT1-A2
RT1-CE3
RT1-CE16
RT1-DOb
Nox1
Ncam2
Cntn2
Cdh4
Sgca
Cd6
Vcan
Cldn4
Ncf4
Bid
Marco
Phagosome
Cell adhesion molecules (CAMs)
Autoimmune thyroid disease
Viral myocarditis
Allograft rejection
Myh13
Type I diabetes mellitus
Graft-versus-host disease
Ccnd1
Itgb3
Itga4
ECM-receptor interaction
Lama1
Col6a1
Col1a2
Cd47
Lamb3
Col11a2
Gp5
Hedgehog signaling pathway
Dhh
Csnk1e
Gli1
Wnt2b
Wnt4
Wnt1
Shh
Gli3
Fzd9
Axin2
Basal cell carcinoma
Arachidonic acid metabolism
Cyp2j4
Alox15
Ptges
Hpgds
Pla2g12a
Cyp2c6v1
Cyp2s1
Cbr3
Metabolism of xenobiotics by cytochrome P450
Gsta5
Retinol metabolism
Rdh5
Retsat
Adh7
Adh4
Rpe65
Rdh12
Cyp2a2
Cyp2d5
Primary bile acid biosynthesis
Cyp7b1
Cyp27a1
Slc27a5
Baat
Bile secretion
Sult2a1
Kcnn2
Slco1a6
Abcg2
Fxyd2
Slco1a2
Adcy8
Mucin type O-Glycan biosynthesis
B3gnt6
Galnt3
LOC100366230
Galntl1
Complement and coagulation cascades
Cfb
C1qc
Serpinf2
Plau
Serpina5
Masp1
Pros1
Nicotine addiction
Gabrg2
Gabrr3
Gria1
Gabrg1
Gria4

Figure 2. Pathway network analysis of DEGs. The network represents each pathway as an individual node, while the edges between pathways denote an approximation of biological interaction between the pathways based on the cross-pathway feature overlap. DEGs are in red text. The node size represents the term enrichment significance. Hedgehog signaling pathway with bigger node size seemed to be most significant involved in the contralateral cerebral cortex of mature rats (8 weeks old) after neonatal HI brain injury.

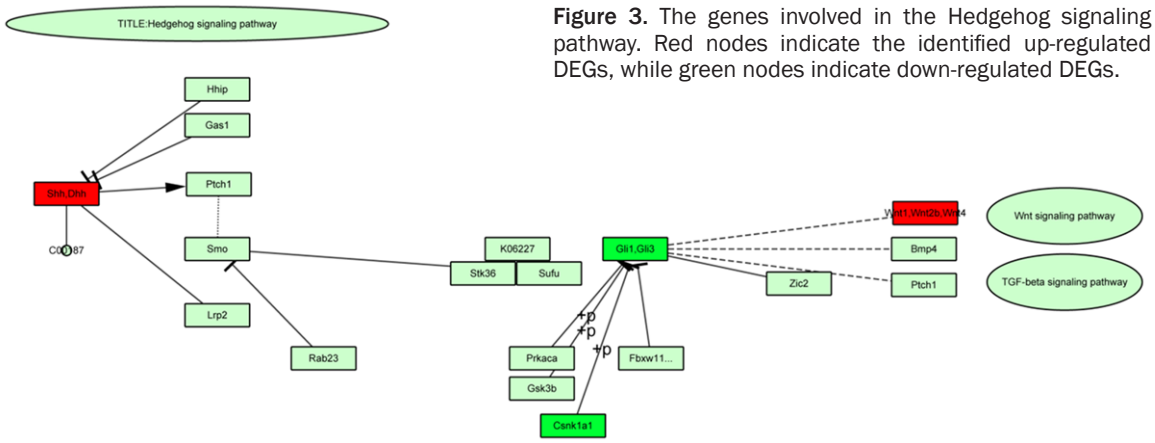

Figure 3. The genes involved in the Hedgehog signaling pathway. Red nodes indicate the identified up-regulated DEGs, while green nodes indicate down-regulated DEGs.

existence of an interaction and the interactions are provided with a confidence score [27]. Different cut-off score was calculated to distinguish three confidence categories (low-, medium- and high-confidence) [28]. A protein with an evidence score greater than 0.4 is considered to have medium confidence of interaction with the other proteins [27]. In our study, the PPIs were identified by using data extracted from the STRING database. The PPIs with a confidence score > 0.4 (medium confidence) were selected to construct the final PPI network [29]. In addition, the Cytoscape software [25] was applied to create the network visualizations, where nodes represented proteins and edges represented physical interactions. The extended degree of each node in the network was calculated. The nodes with higher degree were identified to be hub proteins.

Results

Data preprocessing and DEGs screening

The gene expression profile after normalization was shown in **Figure 1A**. A t test in limma package with a significance level set at p-value < 0.05 was carried out. Moreover, the DEGs were selected using the other criterion of FC ≥ 1.5 in expression between the HI group and the control group. As a result, a total of 973 DEGs were identified in the contralateral cerebral cortex of mature rats (8 weeks old) in the HI group compared with those in the control group, including 599 up-regulated genes and 374 down-regulated genes. Heat-map of the DEGs was shown in **Figure 1B**.

Pathway enrichment analysis

In this study, KEGG pathway terms served as the clustering criterion with a two-sided hypergeometric test followed by Bonferroni correction (significance level of 0.05) to identify significantly affected pathways. An annotation network based on the identified KEGG pathways of the DEGs was constructed as a group of functionally organized pathway terms network. The term network information was described in detail as **Figure 2**. The network comprised several significantly overrepresented terms, such as Cytokine-cytokine receptor interaction, Endocytosis, Antigen processing and presentation, Complement and coagulation cascades, and Hedgehog signaling pathway. Besides, since the node size represented the term enrichment significance as described above (**Figure 2**), we found that the hedgehog signaling pathway with bigger node size seemed to be most significantly involved.

Additionally, further sub-network analysis of the pathway network was visualized specifically to understand the significant pathway involved in the contralateral cerebral cortex after HI brain damage. Genes that were involved in the

Table 1. The changes of DEGs that were involved in the Hedgehog signaling pathway

Gene change	Gene symbol	$\log_2$ FC	AveExpr	*t*	*P* value
Up-regulated	SHH	1.154606177	2.775207603	3.383100326	0.009597145
	DHH	1.28357223	2.493177681	2.529444484	0.035285281
	WNT1	0.699840738	2.872075512	3.744009275	0.005672438
	WNT2B	0.595632766	6.178388842	2.307698713	0.049867151
	WNT4	0.590357916	11.67261831	2.685038669	0.027708207
Down-regulated	GLI1	-0.767154065	6.496797209	-2.649059689	0.0292979
	GLI3	-0.713182912	3.444415873	-3.024741562	0.016439953
	CSNK1E	-0.730348807	7.214532323	-2.357035441	0.046170654

AveExpr, average expression value.

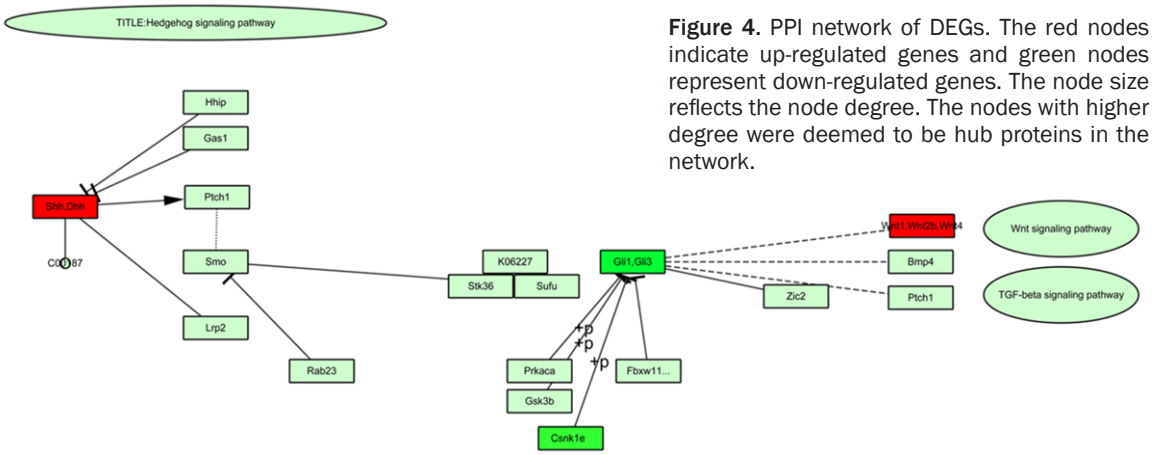

Figure 4. PPI network of DEGs. The red nodes indicate up-regulated genes and green nodes represent down-regulated genes. The node size reflects the node degree. The nodes with higher degree were deemed to be hub proteins in the network.

Hedgehog signaling pathway were shown in **Figure 3**. Besides, the changes of DEGs involved in the Hedgehog signaling pathway were represented in **Table 1**. The results showed that the hedgehog signaling pathway-related genes sonic hedgehog (*SHH*), desert hedgehog (*DHH*), wingless-type MMTV integration site family, member 1 (*WNT1*), *WNT2B*, and *WNT4* were up-regulated in HI samples.

PPI network construction

The PPI network (a confidence score > 0.4) based on the DEGs was created, consisting of 395 nodes and 528 interactions (edges) (**Figure 4**). Among these 395 nodes, 257 up-regulated genes and 138 down-regulated genes were included. After analysis of the node degrees, we found that the degrees were exponentially distributed and the network was scale-free. The top 10 hub proteins with higher node degrees in the PPI network were presented in **Table 2**, such as cyclin D1 (*CCND1*) (degree = 18), *SHH* (degree = 14), ret proto-oncogene (*RET*) (degree = 13), glioma-associated (GLI) family zinc finger 3 (*GLI3*) (degree = 13), and cell division cycle associated 5 (*CDCA5*) (degree = 11).

Discussion

Neonatal HI brain injury can lead to serious brain damage and it is a common cause of neurological handicaps in adulthood [30]. Major efforts are needed to understand neonatal HI brain injury at a molecular level. Kojima *et al.* suggested that progressive neuronal damage may occur in the contralateral cerebral cortex of mature rats [18] though previous studies mostly focus on the investigations of the ipsilateral side of the brain at early stages of neonatal HI encephalopathy. In the current study, a total of 973 DEGs were identified in cerebral cortices of mature rats (8 weeks old) after neonatal HI brain insult compared with the corresponding controls. Furthermore, a functionally grouped pathway network of DEGs was constructed and hedgehog signaling pathway was identified. The

Table 2. Top 10 hub genes of the PPI network

Gene symbol	Expression changes	Degree
CCND1	Down	18
SHH	Up	14
RET	Up	13
GLI3	Down	13
CDCA5	Down	11
TTK	Down	11
ERBB4	Down	10
AURKC	Down	10
COL1A2	Up	10
ITGB3	Up	10

hedgehog signaling pathway-related genes *SHH*, *DHH*, *WNT1*, *WNT2B*, and *WNT4* were up-regulated in HI group. Furthermore, *CCND1*, *SHH*, *RET* and *GLI3* were hub proteins in thescale-free PPI network.

The hedgehog signaling pathway is highly conserved and crucial for the development of the normal embryo [31]. Hedgehog signaling can regulate both the patterning and polarity events in early embryogenesis and the morphogenesis of specific tissues and organs in mammals [32]. The pathway is then silenced in most adult tissues but can be reactivated after injury to accelerate repair and regeneration [32]. Recently, Wang *et al.* had demonstrated that umbilical cord blood mononuclear cells (UCBMC) could promote neuronal differentiation and reduce glial differentiation in neonatal HI rats via the hedgehog signaling pathway [13]. In the present study, we found that the hedgehog signaling pathway with bigger node size in the pathway network of DEGs was significantly identified (**Figure 2**), suggesting that hedgehog signaling pathway might be involved in the contralateral cerebral cortex of mature rats (8 weeks old) after neonatal HI brain insult to accelerate repair and regeneration. Besides, the study of Ferent *et al.* revealed that SHH transcripts were not detected in control rats but were up-regulated at a time-dependent manner in the oligodendroglia lineage within the central nervous system (CNS) lesion [33]. In accordance with previous findings, our results showed that *SHH* was up-regulated in the HI group, indicating the activation of hedgehog signaling pathway in the contralateral cerebral cortex of mature rats in neonatal HI brain insult. On the other hand, the downstream molecules *WNT1*, *WNT2B*, and *WNT4* were up-regulated in HI group (**Figure 3**). *WNT1*, *WNT2B*, and *WNT4* were members of WTN gene family which had been implicated in some developmental processes, such as regulation of cell fate and patterning during embryogenesis [34]. Wnt signaling also activates non-canonical pathways which regulate planar cell polarity via stimulating cytoskeletal reorganization and can also result in calcium mobilization [34]. Furthermore, excessive entry of Ca^{2+} into cells was recognized as an important mechanism of HI brain injury. In this context, we suggested that the Wnt signaling pathway might be activated by the hedgehog signaling pathway and might play an essential role in the contralateral cerebral cortex of mature rats after neonatal HI encephalopathy via participating in the compensatory processes.

CCND1 is a member of the highly conserved cyclin family whose members are characterized by a dramatic periodicity in protein abundance throughout the cell cycle [35]. Cai *et al.* had reported that *CCND1* contributed to cell proliferation and repression of *CCND1* could induce apoptosis and cell cycle arrest in osteosarcoma [36]. Moreover, Northington *et al.* had demonstrated that apoptosis could significantly contribute to delayed cell death in perinatal HI [37]. In line with previous study, our study showed that *CCND1* was a hub protein in the PPI network and was down-regulated in HI group (**Figure 4**), suggesting that *CCND1* may play a significant role via involving in apoptosis and cell cycle regulation in the contralateral cerebral cortex of mature rats after neonatal HI encephalopathy.

In addition, *RET*, a member of the cadherin superfamily, encodes one of the receptor tyrosine kinases which are involved in many cellular mechanisms including cell proliferation, neuronal navigation, cell migration, and cell differentiation on binding with glial cell derived neurotrophic factor (GDNF) family ligands [38]. Duarte *et al.* had revealed that the neuroprotective effect of exogenous GDNF could be observed in different experimental models of brain ischemia [39]. Furthermore, Xu *et al.* showed that increased expression of GDNF family receptor RET was identified in the ischemic cortex after electro acupuncture on HI brain injury, which at least in part was attribut-

ed to the activation of PI3-K/Akt signaling pathway [40]. Additionally, our study identified that RET was a hub protein in the PPI network (**Figure 4**). Thus, we suggested that up-regulated *RET* might play an essential role in the progression of neonatal HI encephalopathy.

In conclusion, the critical genes (*CCND1*, *SHH*, *RET, WNT1*, *WNT2B* and *WNT4*) in the contralateral cerebral cortex of mature rats following neonatal HI encephalopathy had been identified based on the gene expression profile. The Wnt signaling pathway may be activated by the hedgehog signaling pathway and play an essential role in the contralesional cerebral cortex of mature rats after neonatal HI encephalopathy via participating in the compensatory processes. Besides, *CCND1* may play a significant role via involving in apoptosis and cell cycle regulation in the contralesional cerebral cortex after neonatal HI brain injury. Moreover, the up-regulated *RET* may play an essential role in the progression of neonatal HI encephalopathy. Because of the relatively small number of samples in the current study, further studies with a larger sample size to validate and determine the role of the DEGs identified are needed. Further investigations on the molecular mechanism of neonatal HI encephalopathy may facilitate the development of novel diagnostic and therapeutic applications.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant number: 81000263) and National Key Development Program of Clinical Specialties (neonatology) (grant number: 1311200003303), Applied Basic Research Programs of Science and Technology Department of Sichuan Province (grant number: 2016JY0180).

Disclosure of conflict of interest

None.

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