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ORIGINAL RESEARCH

Expression Profiles of Long Noncoding RNAs in Mice with High-Altitude Hypoxia-Induced Brain Injury Treated with *Gymnadenia conopsea* (L.) R. Br.

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Background: The unique geographical environment at high altitudes may cause a series of diseases, such as acute altitude reaction, cerebral edema, and pulmonary edema. *Gymnadenia conopsea* (L.) R. Br. has been reported to have an effect on high-altitude hypoxia. However, the molecular mechanism, especially the expression of long noncoding RNAs (lncRNAs), is not yet clear.

Methods: The expression profiles of lncRNAs in high-altitude hypoxia-induced brain injury mice treated with *Gymnadenia conopsea* (L.) R. Br. by using a microarray method.

Results: A total of 226 differentially expressed lncRNAs, 126 significantly dysregulated mRNAs and 23 differentially expressed circRNAs were detected (>2.0-fold, $p<0.05$). The expression of selected lncRNAs, mRNAs and circRNAs was validated by qRT-PCR. KEGG analysis showed that the mRNAs coexpressed with lncRNAs were involved in inflammation and hypoxia pathways, including the HIF-1, PI3K-Akt, and NF-kappa B signaling pathways. The lncRNA-TF network analysis results indicated that the lncRNAs were regulated mostly by HMGA2, SRY, GATA4, SOX5, and ZBTB16.

Conclusion: This study is the first to report the expression profiles of lncRNAs, mRNAs and circRNAs in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. and may improve the understanding of the molecular mechanism of *Gymnadenia conopsea* (L.) R. Br. in treating high altitude hypoxia-induced brain injury.

Keywords: expression profile, noncoding RNAs, high-altitude hypoxia, *Gymnadenia conopsea* (L.) R. Br

Introduction

Acute high altitude sickness refers to a series of clinical syndromes caused by high altitude hypoxia occurring within hours or days upon exposure to high altitudes and includes acute mild altitude sickness, acute brain edema, acute high-altitude pulmonary edema, and high-altitude encephalopathy; among these conditions, high-altitude brain edema causes the most serious consequences.[1–3] In recent years, researchers have focused on the pathogenesis of acute high-altitude hypoxia mainly from the perspectives of morphology, physiology, biochemistry and related molecular biology.[4–6] High-altitude hypoxic brain injury is a complex pathophysiological process in which the cytokine network system participates in regulating and maintaining homeostasis in many aspects and stages, and the complex network system of cytokines is regulated by dynamic gene cascades.[7,8] An increasing number of studies have shown that brain injury can induce changes in the expression of multiple genes in the central nervous

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system; in addition, there are inherent regulatory relations among some specific genes, and the differential expression of these genes directly or indirectly affects the expression levels of cytokines.[9,10]

Tibetan drugs, including *Rhodiola rosea, Saussurea involucrate, Hippophae rhamnoides, Dracocephalum tanguticum, Potentilla anserina, and Gymnadenia conopsea* (L.) R. Br. have been reported to have anti-hypoxia effects.[11–16] Advanced research has shown that *Gymnadenia conopsea* (L.) R. Br. has anti-allergy and anti-silicosis fibrosis effects, promotes progenitor cell proliferation, scavenges free radicals, and inhibits hepatitis B virus surface antigen.[17] No evidence indicates that gene expression profiles, especially ncRNA expression profiles, in mice with high-altitude hypoxia-induced brain injury treated with Tibetan drugs has been investigated. Long noncoding RNAs (lncRNAs) are newly identified ncRNA transcripts of more than 200 nucleotides. A recent study showed that lncRNAs play a wide range of regulatory roles in gene expression.[18] lncRNAs were originally thought to be a byproduct of transcription. However, recent data suggest that lncRNAs can participate in gene expression by regulating transcription, posttranscriptional processing, chromatin remodeling, and the production of small ncRNAs.[19–22] However, no research has been performed on the expression profiles of long noncoding RNAs in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br.

In the present study, we aimed to investigate differential expression profiles of lncRNAs, mRNAs and circRNAs in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. using a DNA microarray.

Materials and Methods

Animals and Study Design

A total of 60 SPF, 6- to 8-week-old, male C57BL/6J mice weighing 22.5±5 g were purchased from Hunan Tianqin Biotechnology Co. Ltd. (Changsha, Hunan, China). Mice were housed at a constant temperature (21–23°C) and constant humidity (45–65%) on a 12-hour alternating light-dark cycle with free access to food and water. All animal experiments followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal work was performed in the laboratory animal center of Tibet University. The research procedures were approved by the Ethics Committee of Tibet University (EC20190512), China.

Mice in the intervention group were given 750 mg/kg *Gymnadenia conopsea* (L.) R. Br. ethanol extract via intragastric administration once a day for 30 consecutive days. Mice in the control group were given distilled water in a similar manner. Then, mice in the intervention and control groups were housed in a decompression chamber and exposed to a simulated high altitude of 4000 m for 24 h.[23]

Sample Collection

Mice were anesthetized with pentobarbital sodium (2%, 0.1 mL/10 g) by intraperitoneal injection. Brain tissues were stored in 10% neutral formalin, and other tissues were stored at −80°C until further analysis.

RNA Extraction

Total RNA was isolated from each brain tissue sample by using an E.Z.N.A.® Total RNA Kit II (Omega, USA). Total RNA was quantified with a NanoDrop ND-2000 (Thermo Scientific, USA), and RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

Microarray Analysis

Sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was transcribed to double-stranded cDNA, transcribed into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned with an Agilent Scanner G2505C (Agilent Technologies, USA). All experiments were carried out according to the manufacturers' standard protocols. The experiments were performed by OE Biotechnology Co., Ltd. (Shanghai, China). An Agilent Mouse LncRNA Microarray V3 (4*180K, Design ID: 084388) was used in the present experiment.

Differential Expression Analysis

Raw data in the array images were analyzed with Feature Extraction software (version 10.7.1.1, Agilent Technologies). The basic analysis was performed using GeneSpring (version 14.8, Agilent Technologies, USA) with the raw data. First, the quintile algorithm was used to normalize the raw data. Fold change values (≥ 2.0), as well as *t*-test-based P values (≤ 0.05), were calculated to identify the differentially expressed genes. Then, gene ontology (GO) analysis (http://www.geneontology.org) and kyoto encyclopedia of genes and genomes (KEGG) analysis (http://www.genome.jp/kegg/) were applied to determine the roles of these differentially expressed mRNAs, as well as circRNA host mRNAs.

Finally, hierarchical clustering of differentially expressed lncRNAs, circRNAs and mRNAs between brain samples from the intervention and control groups was performed to visualize the distinguishable gene expression patterns.

Co-Expression Network Analysis

A coexpression network was constructed using the top 20 differentially expressed lncRNAs and mRNAs. Coexpression of lncRNAs and mRNAs was identified by the Pearson correlation coefficients (PCC) (≥0.7). Values of $p < 0.05$ indicated a statistically significant correlation. Additionally, via coexpression analysis of the differentially expressed lncRNAs and mRNAs, the transcription data of the top 40 lncRNAs were extracted, and a network map (lncRNAs- Transcription factors (TFs)) was drawn according to the top 5 lncRNAs enriched on the TFs. The top 20 lncRNAs were sorted according to the lncRNAs enriched on the TFs, and the top 2 lncRNAs were used to draw a network map (lncRNA-target mRNA-TF). Cytoscape software (The Cytoscape Consortium, San Diego, CA, USA) was used to construct the networks.

Quantitative Real-Time-PCR (qRT-PCR)

qRT-PCR was performed using a HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech Co., Ltd., Nanjing, China) in a QuantStudio 3 Flex Real-Time PCR System (Thermo Scientific, USA). The $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression levels of lncRNAs, circRNAs and mRNAs. The significance of the differences was assessed by Student's *t*-test. Repeated measures ANOVA was performed. Values of $p < 0.05$ were considered statistically significant. SPSS (version 19.0, SPSS, Inc., Chicago, IL, USA) was used to perform the statistical tests.

Results

Differentially Expressed LncRNAs, CircRNAs and mRNAs in Mice with High-Altitude Hypoxia-Induced Brain Injury Mice Treated with *Gymnadenia conopsea* (L.) R. BR

A total of 226 differentially expressed lncRNAs were identified in mice with high-altitude hypoxia-induced brain injury mice treated with *Gymnadenia conopsea* (L.) R. Br.

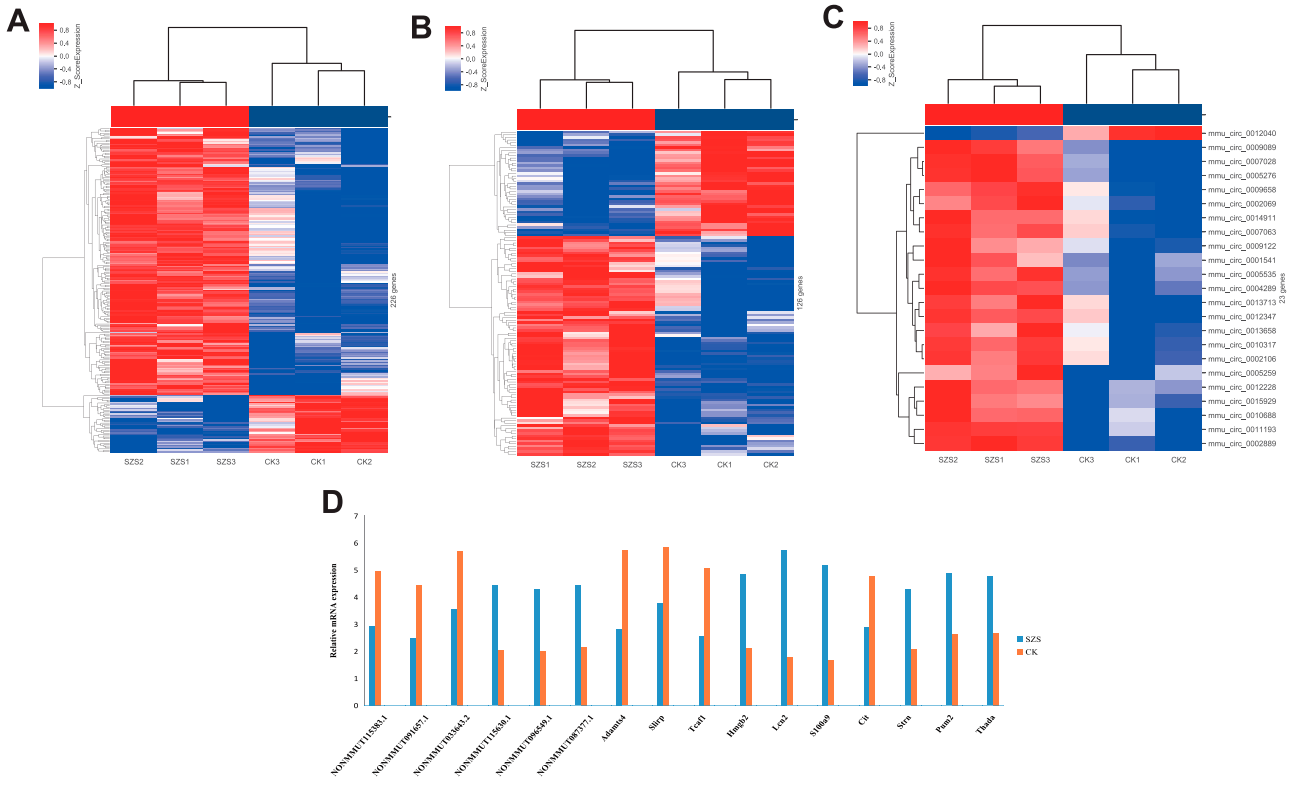

Figure 1 The hierarchical clustering of the differentially expressed lncRNAs (**A**), mRNAs (**B**) and CircRNAs (**C**) in treated group (SZS) (n = 3/group) and control group (CK) (n = 3/group) hippocampal tissues. (**D**) The quantitative real-time PCR (qRT-PCR) validated in randomly selected six lncRNAs, six mRNAs and four circRNAs. The qRT-PCR results were consistent with the microarray data. SZS: Gymnadenia conopsea (L.) R. Br.; CK: control.

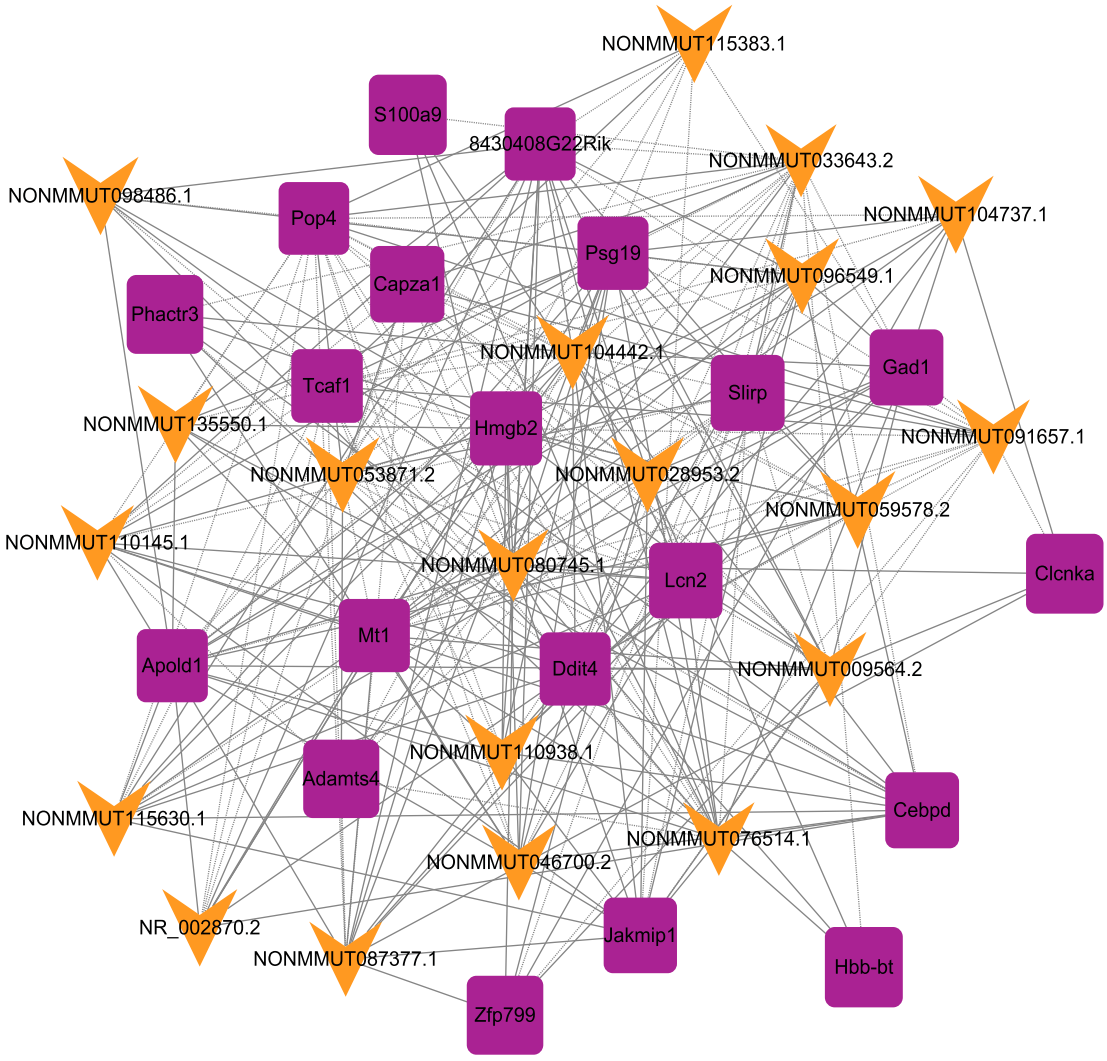

Figure 2 lncRNA-mRNA-network analysis. Purple squares represent dysregulated mRNAs, green arrows represent dysregulated lncRNAs.

compared with normal controls. Among these differentially expressed lncRNAs, 186 were upregulated and 40 were downregulated. NONMMUT033643.2, on chr19, was the most downregulated lncRNA, with a FC of 4.46. NONMMUT117581.1, on chr4, was the most upregulated lncRNA, with a FC of 8.01. In addition, 126 significantly dysregulated mRNAs (85 upregulated and 41 downregulated) were detected. Adamts4 (FC= 7.42) and Lcn2 (FC= 15.67) were the most downregulated and upregulated mRNAs, respectively. Furthermore, 23 differentially expressed circRNAs were identified. The most upregulated circRNA was Pum2 (FC= 4.71). The most downregulated circRNA was Cit (FC= 3.68) (Figure 1A–C). Six lncRNAs and mRNAs and four circRNAs were randomly selected and verified by using qRT-PCR. The results were consistent with those of the microarray chip data analysis (Figure 1D).

Identification of Coexpression Networks and Potential Functions

The correlations between the top 20 dysregulated lncRNAs and mRNAs were predicted. The p values of each lncRNA-mRNA correlation were ranked. The coexpression network was constructed with the selected lncRNA-mRNA pairs with the highest Pearson correlation coefficient. A total of 265 connections (168 positive connections and 97 negative connections) were included in the network (Figure 2).

KEGG and GO Analyses

As presented in Figure 3A, GO enrichment analysis indicated that the differentially expressed lncRNAs were mostly enriched in the hemoglobin complex cellular component, the peroxidase activity molecular function, and the

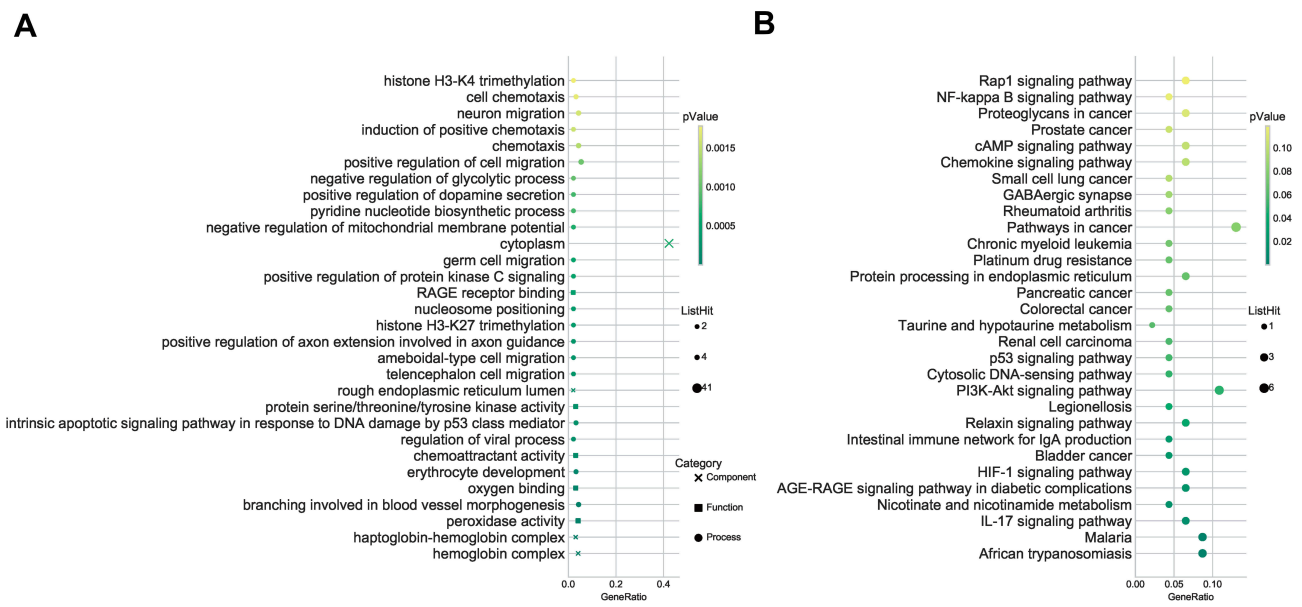

Figure 3 Kyoto encyclopedia of genes and genomes (KEGG) pathway and gene ontology (GO) enrichment analysis of differentially expressed lncRNAs and mRNAs. (**A**) Go enrichment analysis; (**B**) KEGG pathway.

Abbreviations: RAGE, Advanced glycosylation end product-specific receptor; NF-kappa-B, nuclear factor- kappa B; cAMP, Cyclic Adenosine monophosphate; GABA, γ-aminobutyric acid; PI3K, Phosphoinositide 3-kinase; IgA, immunoglobulin A; HIF-1, Hypoxia-inducible factor 1; IL-17, interleukin-17.

branching involved in blood vessel morphogenesis biological process. The differentially expressed circRNAs were mostly enriched in the protein complex cellular component, the ATP binding molecular function, and the dendrite development biological process. Furthermore, KEGG analysis indicated that the lncRNAs were involved in the following pathways: IL-17 signaling pathway, HIF-1 signaling pathway, PI3K-Akt signaling pathway, NF-kappa B signaling pathway, TNF signaling pathway, Apoptosis, and VEGF signaling pathway. The circRNAs were involved in the following pathways: TNF signaling pathway, Apoptosis, Neurotrophin signaling, and MAPK signaling pathway (Figure 3B).

LncRNA-TF Network Analysis

Transcription data for the top 40 lncRNAs were extracted. Then, the top 5 enriched lncRNAs on TFs were used to construct the lncRNA-TF network. The results showed that a total of 87 TFs with 1354 lncRNA-TF pairs were predicted. The top 5 TFs enriched with lncRNAs were HMGA2, SRY, GATA4, SOX5, and ZBTB16 (Figure 4).

LncRNA-Target-TF Network Analysis

Transcription data for the top 20 lncRNAs were extracted. Then, analysis was conducted on the top 2 related lncRNA-mRNA and lncRNA-TF pairs ranked according to the p values. A total of 80 TFs and 68 mRNAs were predicted to regulate or be the target of these lncRNAs, respectively. Among these TFs, HMGA2 and SRY were shown to regulate most of the lncRNAs. For example, HMGA2 and SRY were predicted to regulate NONMMUT033643.2 and NONMMUT115383.1 (Figure 5).

Discussion

In this study, we explored the expression profiles of lncRNAs, mRNAs and circRNAs in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. In total, our study identified 226 lncRNAs, 126 mRNAs and 23 circRNAs. The differentially expressed lncRNAs were involved in several hypoxia- and inflammation-related pathways, including the HIF-1 signaling pathway, PI3K-Akt signaling pathway, and NF-kappa B signaling pathway.

Initially, 186 lncRNAs upregulated and 40 lncRNAs downregulated relative to their control expression levels were detected. To the best of our knowledge, this is the first identification of differentially expressed lncRNAs in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. Accumulating evidence has indicated that lncRNAs may participate in multiple pathological processes, such as the occurrence of cancer,[24] neurodegenerative lesions,[25] and cardiovascular diseases.[26] Recently, two lncRNAs, nuclear-enriched abundant/autosomal transcript 1 (NEAT1) and metastasis-associated lung

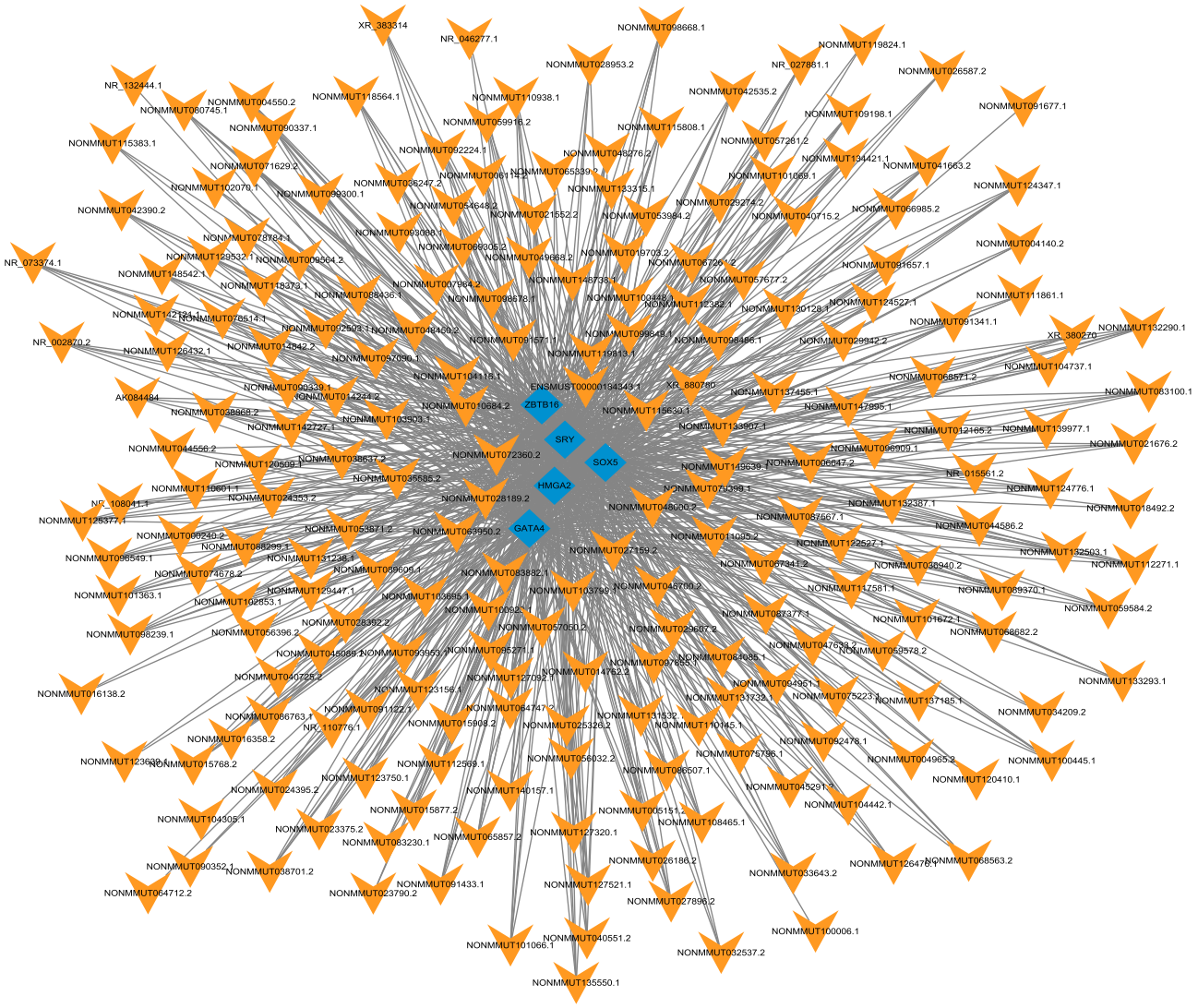

Figure 4 Network of the top 40 most related LncRNA-Transcription factors (TFs) pairs (the most 5 related lncRNA-TFs pairs according to the P value). Orange arrow: TFs; Blue diamonds: lncRNAs.

adenocarcinoma transcript 1 (MALAT1), were found to be strongly induced by hypoxia in cultured cells.[27] According to a study by Jiménez et al,[28] hypoxia could contribute to the metastatic spread of breast cancer via HIF-mediated induction of EFNA3 lncRNAs and subsequent Ephrin-A3 protein accumulation in human breast cancer cells. The above conclusions may support the hypothesis that lncRNAs play an important role in hypoxia. However, there has been no research on the relationship between the expression of lncRNAs and high-altitude hypoxia. Although the results were identified randomly by RT-PCR, we should perform more studies to obtain more concise results in the future.

In addition, 85 upregulated and 41 downregulated mRNAs were detected. We identified Adamts4 and Lcn2 as the most differentially expressed mRNAs in the treated group compared with the control group. ADAMTS-4 is one of the members of the polyprotein polysaccharide family and has been reported to cleave aggrecan, which may contribute to cartilage breakdown during arthritis.[29,30] In addition, a previous study clarified that hypoxia may inhibit the expression of ADAMTS-4 and ADAMTS-5 through HIF-1α, maintain the homeostasis of the intervertebral disc environment, and delay the occurrence of disc degeneration.[31] ADAMTS-4 is also present in the central nervous system (CNS), including the cortex, hippocampus, striatum, and spinal cord.[32] Cross et al revealed that the expression of ADAMTS-4 was significantly increased in human astrocyte cultures induced by TNF-a.[33] Ashlin et al[34] provided evidence that the expression of ADAMTS-4 in macrophages increased after treatment with TNF-α, IFN-g,

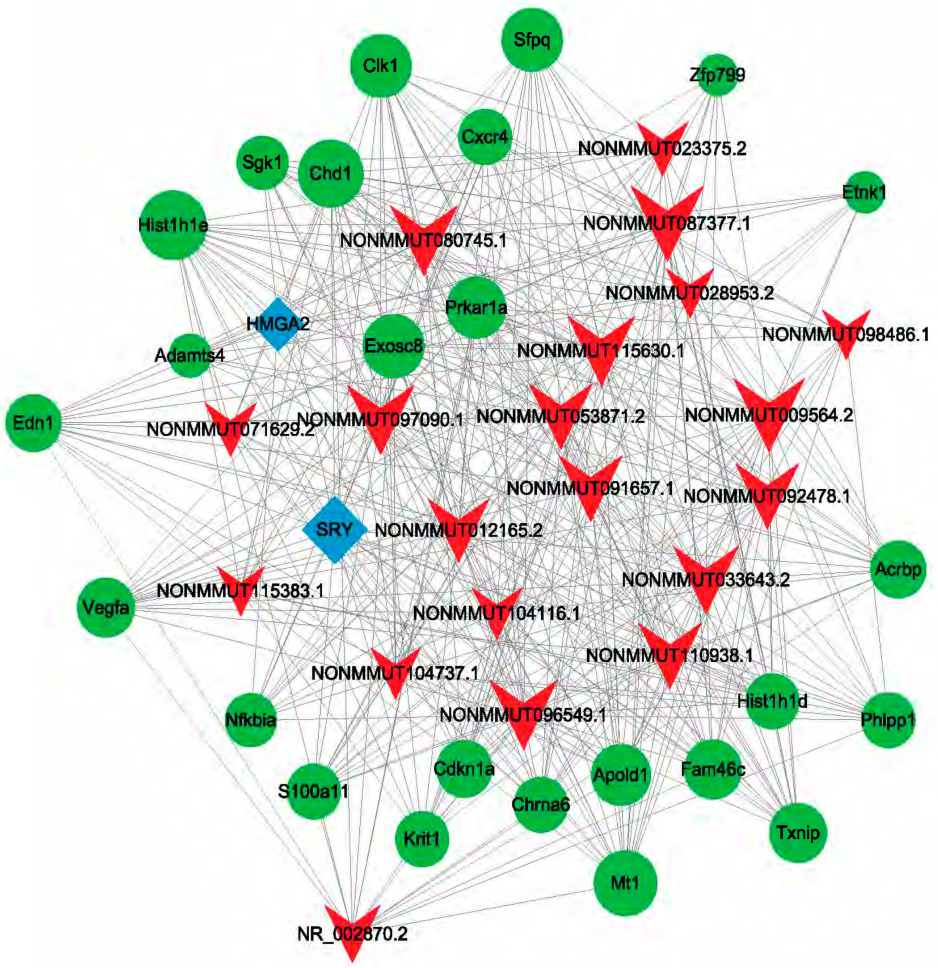

Figure 5 lncRNA-target-Transcription factors (TFs) network of 20 most differentially expressed lncRNAs. Red arrow: lncRNAs; Green round: target mRNAs; Blue diamond: TFs.

and TGF-β, which may represent an external source of ADAMTS-4 in CNS injury. Additionally, ADAMTS-4 was shown to reduce neuroinflammation in vitro in microglia or astrocyte cultures and in vivo in mice after ischemic stroke.[35] Collectively, these studies suggest that the increased expression of endogenous ADAMTS-4 during neuronal injury may be an adaptive change to protect the brain against neuroinflammation. Interestingly, our findings indicated that ADAMTS-4 mRNA expression was upregulated in brain tissue from mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. compared with brain tissue from control mice, suggesting that ADAMTS-4 may play an important role in the neuroprotective process during high altitude hypoxia.

Lipid transport protein-2 (LCN-2) is also called neutrophil gelatinase-related lipid transport protein (neutrophelase neutrophelase).[36] It is a secretory protein that is related to inflammation, the apoptosis of immune response cells, and the development of various tumors.[37] In the central nervous system, the LCN-2 protein is secreted by small glial cells and astrocytes and is related to the inflammatory response of the central nervous system.[38] As Niu et al reported, the release of LCN-2 has an obvious correlation with cell injury caused by oxygen-sugar deprivation/poly-oxy sugar.[39] In addition, as an anti-inflammatory mediator, adenosine can significantly ameliorate the cell damage caused by LCN-2.[39] Moreover, LCN2 can promote the inflammatory activation of astrocytes via the RAS homologous gene-Rho related spiral curling protein kinase (Rho-ROCK)-GFAP pathway, and inflammatory activated astrocytes can selectively promote neuronal apoptosis.[40] Therefore, LCN2 plays an important role in the inflammatory activation and neuronal apoptosis of glial cells under various pathological conditions in the CNS. We found that the expression level of LCN2 was significantly decreased in

the group treated with *Gymnadenia conopsea* (L.) R. Br. compared to the control group, which may indicate that *Gymnadenia conopsea* (L.) R. Br. may provide neuroprotection by inhibiting LCN2 expression in brain tissue. However, the mechanism requires further research.

According to KEGG analysis, the dysregulated lncRNAs were involved in several pathways, such as the IL-17 signaling pathway, HIF-1 signaling pathway, PI3K-Akt signaling pathway, NF-kappa B signaling pathway, TNF signaling pathway, apoptosis, and VEGF signaling pathway. HIF-1 is an important transcription factor involved in the hypoxia regulation process. Zhang et al demonstrated that the relative expression of HIF-1α mRNA increased significantly in the hypoxia group compared to the normoxia group.[41] Furthermore, Cao et al reported that Twenty flavor Shen Xiang Pill can protect against hypoxia by interfering with the protein levels of HIF-1α and EPO.[42] In the oxygenated state, HIF-1α expression is maintained at a low level mainly through the activity of proteasome degradation pathways. However, the HIF-α degradation pathway is inhibited in hypoxia.[43] In hypoxia, HIF-α also interacts with HIF-1β to form a heterodimer and is transferred to the nucleus; is the heterodimer then interacts with hypoxia response elements (HREs) and activates the transcription of downstream target genes such as heme oxygenase-1 (HO-1), erythropoietin (EPO), vascular endothelial growth factor (VEGF), and a variety of glycolysis enzymes.[44] Recent studies have found that the expression of HIF-1α under normoxic or hypoxic conditions can be mediated by phosphatidylinositol 3-kinase (PI3 kinase, PI3K) and regulated by the Akt signaling pathway.[45,46] Su et al reported that rosamultin can ameliorate functional damage to vascular endothelial cells induced by acute hypoxia by activating the PI3K/Akt signaling pathway and then increasing HIF-α expression.[47] Thus, *Gymnadenia conopsea* (L.) R. Br. may protect against high-altitude hypoxia via these signaling pathways.

Conclusion

We identified a number of dysregulated lncRNAs, mRNAs and circRNAs that might be potential biomarkers or targets in mice with high-altitude hypoxia-induced brain injury treated with *Gymnadenia conopsea* (L.) R. Br. Further investigation is needed to elucidate the detailed mechanisms underlying the regulation of these differentially expressed lncRNAs.

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Disclosure

The authors declare no conflicts of interest.

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