Screening Potential Therapeutic Mechanisms of Houshiheisan Against Ischemic Stroke by LncRNAs and MRNAs Microarray Profiling

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Research

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Abstract

Background

Long non-coding RNAs (lncRNAs) are substantial to wide variety of biological processes and pathogenesis processes of ischemic stroke. *Houshiheisan* (*HSHS*), a typical prescription of traditional Chinese medicine, has outstanding efficacy in treating stroke, although the comprehensive molecular mechanism of its therapeutic effect has remained obscure up to now.

Methods

In this work, we induced a ischemic stroke rat model by permanent middle cerebral artery occlusion (MCAO). The microarray expression profile of lncRNAs and mRNAs was used to investigate various possible roles and molecular mechanism of *HSHS* in treating MCAO rats.

Results

HSHS improved the neurological deficit and alleviated the pathological damage after cerebral ischemia. The Clariom D Assay (rat, Affymetrix) showed that 8128 mRNAs and 3022 lncRNAs differentially expressed between sham group and model group, and 868 mRNAs and 836 lncRNAs between *HSHS* and model groups. Among the three groups, the intersections (666 mRNAs and 288 lncRNAs) were chosen and analyzed. GO and KEGG analysis disclosed that the majority of overlapping mRNAs were enriched in Axon guidance, Autophagy, PI3K-AKT signaling pathway and mTOR signaling pathway. Pathway network, protein-protein network and molecular complex detection analysis identified significant hub genes, e.g., Rock2, Rps6kb1, Wnt4, IL-6 and so on. Furthermore, we explored dynamic interactions between the dysregulated lncRNAs and mRNAs by lncRNA-mRNA network analysis. Finally, qRT-PCR verified expressions of the critical differentially expressed lncRNAs and mRNAs within the three groups.

Conclusion

Our results indicate that these differentially expressed lncRNAs may affect pathological processes of ischemic stroke by regulating co-expressed mRNAs, providing novel insight in regarding lncRNAs' involvement in the treatment of ischemic stroke.

Background

Stroke is a severe cerebrovascular disease with high mortality and morbidity worldwide, bringing significant burden and pain to the family and society. To date, 85% of stroke is due to various reasons leading to regional blood supply disorders in brain tissue, resulting in ischemic and hypoxic lesion necrosis of brain tissue and causing irreversible neurological damage^[1-2]. Currently, thrombolysis with

recombinant human tissue-type plasminogen activator (t-PA) is the only approved stroke therapy, but the window is short, which can be merely used safely and effectively within 4.5 h after symptom onset^[3]. However, due to a narrow therapeutic time window and high hemorrhagic risk, only a relatively small patient population (less than 5%) has benefitted from this treatment^[4]. Consequently, there is a critical need to find new strategies to treat stroke. Increasing evidence suggests that traditional Chinese medicine prescriptions are promising agent for treating ischemic stroke^[5].

Houshiheisan (*HSHS*), a Chinese herbal prescription with 13 herbs, identified as the first classic formula against stroke. It was created by Zhang Zhongjing, which has been clinically applied to treat stroke safely and effectively for over 2000 years in China. Our previous findings demonstrated that *HSHS* could accelerate neurovascular remodeling to improve neurological deficit via promoting angiogenesis and neural regeneration. For example, *HSHS* can reduce amyloid precursor protein deposition (APP), depress the release of inflammatory mediators, increase the expression of brain-derived neurotrophic factor (BDNF), and regulate related angiogenic factors (VEGF and Ang-1/Ang-2) thereby improving neurological function^[6–8]. However, the molecular mechanism underlying the treated effect has not been fully investigated.

Long non-coding RNAs (lncRNAs), ranging from 200bp to several kilobases in length, can regulate gene expression through many patterns, including transcriptional and post-transcriptional RNA processing, DNA methylation, and chromatin architecture through local (cis) and distal (trans) mechanisms^[9]. Meanwhile, lncRNAs can also act on various biological processes through enhancer-like activities, regulation of transcription factor activity, recruitment of histone modification complexes, and co-activation with neighboring genes^[10]. So far, an accumulating number of evidence indicates that the aberrant expression and mutations of lncRNAs are connected with various dysfunctions of the central nervous system, which makes researchers focus on the potential role of lncRNAs in brain physiology and pathology^[11–12]. Furthermore, brain RNA-seq analysis and microarray analysis found that cerebral ischemia significantly changed the expression of lncRNAs in the development of the brain disorders both in human and adult rat^[13–14].

In this study, to understand the biological mechanisms of *HSHS* against ischemic stroke, we obtain comprehensive expression profiles of mRNAs and lncRNAs with a microarray analysis. By identifying differentially expressed mRNAs (DEmRNAs), differentially expressed lncRNAs (DELncRNAs) and constructing co-expression networks, our data revealed a new perspective on undiscovered biomarkers and drug targets of ischemic stroke.

Materials And Methods

Preparation of *HSHS*

HSHS formular is composed of 13 herbs: Flos Chrysanthemi (Juhua), Radix Saposhnikoviae (Fangfeng), Ramulus Cinnamomi (Guizhi), Rhizoma Chuanxiong (Chuanxiong), Radix et Rhizoma Asari (Xixin), Radix

Platycodonis (Jiegeng), Rhizoma Atractylodis Macrocephalae(Baizhu), Poria(Fuling), Rhizoma Zingiberis(Ganjiang), Radix Angelicae Sinensis(Danggui), Radix et Rhizoma Ginseng(Rensheng), Radix Scutellariae(Huangqin) and Concha Ostreae(Muli). All the herbs were purchased from Beijing Tongren-Tang Chinese Medicine Co. Ltd. (Beijing, China), and verified by Pro. Li Jia in keeping with the Chinese Pharmacopoeia 2020. The herbs were extracted with 10×30% ethanol (ten fold weight of the thirteen components) for 2 h. Then, sediment was immersed with 8×30% ethanol for 1.5 h. Vacuum extraction at 50°C to take out ethanol and got the *HSHS* extract. 1.2 g herbs generated one milliliter of the extract^[7].

Animal experiments and samples collection

All male Sprague-Dawley (SD) rats (verified SPF, weighing 280-320g) were supplied by the Vital River Laboratory Animal Technology Co. Ltd., Beijing, China (license No. SCXK(Jing) 2016-0011). Rats were randomly distributed into sham group, model group and *HSHS* group with 10 rats in each group. All rats were anesthetized with a face mask inhalation of 1.5–2.5% isoflurane in a 2:1 N₂O:O₂ atmosphere during surgeries to minimize suffering. The preparation of MCAO model was performed as we described^[15]. Additionally, the clinical equivalent daily dose of *HSHS* in rats was 10.5g/kg, which was the optimal dosage against MCAO in our previous study^[16]. Seven days after MCAO, the ischemic core region of brain cortex tissues was frozen in liquid nitrogen immediately.

The above experiments were approved by the Institution Animal Care and Use Committee of Capital Medical University [Ethical License No.AEEI-2019-001], and all animal experimental manipulations conformed to the Guidance Suggestions for the Care and Use of Laboratory Animals published by the Ministry of Science and Technology of China.

Behavioral testing

Neurological assessment

The behavioral function was assessed at 1,3,5 and 7 days after MCAO. Elimination standard: rats scored 0, unable to walk or unconscious. Score scale: 0=no evident symptom; 1=can not wholly extend the left forepaw; 2=spin to the left; 3=failure to the left; 4=only walk when irritated.

Motor function assessment

The beam test was carried out at 3 and 7 days after MCAO^[17]. Trained rats in advance to ensure they could cross a bar (100 cm × 3 cm, located 60 cm above the floor). After MCAO, test was conducted with the scoring system: 0, drop from the bar; 1, stand on the bar but no moving; 2, try to pass the bar but fall off; 3, pass the bar with ≥ 50% left posterior slips; 4, pass the bar with < 50% left posterior slips; 5, pass the bar with only one left posterior slip; 6, pass the bar and no slips.

Neuropathological assessment

7 days after cerebral ischemia, 3 rats from each group were transcardially perfused with 500 mL of physiological saline, and then changed to 4% paraformaldehyde(PFA). After that, immersed the brain tissue in 4% PFA to fix them, then made them dehydrated, embedded in paraffin and sliced into sections. The sections were baked, dewaxed, dehydrated, washed and stained with hematoxylin-eosin(HE). Pathological alterations were observed by a light microscope (Nikon, Tokyo, Japan).

Microarray analysis

RNA preparation and microarray detection

Total RNA was extracted from the infarcted tissue using Trizol reagent (Life Technologies, Carlsbad, CA, USA) and purified with an RNeasy mini kit (Qiagen, Valencia, CA, USA). Biotinylated cDNA was prepared in line with the standard Affymetrix protocol from 250ng total RNA by using Ambion® WT Expression Kit. Fragmented cDNA was hybridized for 16 h at 45°C on Clariom D Assay(rat, Affymetrix). GeneChips were washed and stained in the Affymetrix Fluidics Station 450. All arrays were scanned by Affymetrix® GeneChip Command Console (AGCC) which was installed in GeneChip® Scanner 3000 7G.

DEmRNAs and DElncRNAs analysis

In microarrays, we used moderated F-statistic to select the multi-group DEmRNAs and DElncRNAs among groups (model vs sham and *HSHS* vs model) by the limma R package (version:3.36.5). Limma R Empirical Bayes moderation was used to correct the *P* values. Benjamini-Hochberg was used for multiple tests correction (FDR was used to adjust the *P* values for multiple comparisons). The threshold set for up- and down-regulated mRNAs and lncRNAs was fold change > 1.2, *P* value < 0.05 and FDR < 0.05. The intersections of DEmRNAs and DElncRNAs between model vs sham and *HSHS* vs model were determined by Venn diagrams. Hierarchical clustering was performed based on overlapped mRNAs and lncRNAs using the R package pheatmap (version:1.0.12).

Bioinformatics analysis of the coding genes

Gene Ontology(GO) and Kyoto Encyclopedia of Genes and Genomes(KEGG) pathway analysis

Based on the GO database, analyzed the primary function of the DEmRNAs from molecular function, biological process and cellular component^[18]. Moreover, according to the KEGG database, we found out the significant pathways of the DEmRNAs^[19]. Pathway enrichment analysis indicated the main metabolic and signaling pathways. Through the Cytoscape plug-in ClueGO and CluePedia, we constructed the enriched pathways interaction networks.

Protein-protein interaction (PPI) network

We used the STRING database to investigate functional interactions between proteins, including prediction and experimental interaction data^[20]. By setting the criteria with combined scores ≥ 0.9 , we generated PPIs among the DEmRNAs, and used Cytoscape to visualize the network. Furthermore, we

used the Molecular Complex Detection plug-in (MCODE) in Cytoscape to screen out significant modules from the PPI network with the conditions: degree cut-off = 2, k-core = 2, node score cut-off = 0.2, and max depth = 100. MCODE score ≥ 8 and node ≥ 10 were considered as functional enrichment analysis of the modules.

Bioinformatics analyses of the non-coding genes

LncRNA-mRNA co-expression network

LncRNAs regulate genes expression positively or negatively at the level of transcription and post-transcription. After normalizing the signal intensity between the DElncRNAs and DEmRNAs, the R function cor.test (Hmisc and corplot) was used to calculate the Pearson's correlation coefficient(PCC) of lncRNA-mRNA pairs^[21]. The significant correlation pairs and the correlation value cutoff was 0.99. The visualization of the network was built by the program Cytoscape3.8.3 software(<http://cytoscape.github.io/>). The degree was a measurement that indicates a lncRNA or mRNA centrality in a network. Core lncRNAs or mRNAs were determined by the degree differences between two groups.

LncRNA target pathway network

LncRNA target pathway network was constructed according to the relationships of significant pathways and lncRNAs^[22]. In the network, a circle means a pathway, a square means a lncRNA, and relationships between them are indicated by edges. We evaluated the regulatory status of lncRNAs and pathways with graph theory; the standard were the degrees of lncRNAs and pathways in the network. The degree of each lncRNA was the number of pathways regulated by that lncRNA, and the degree of each pathways was the number of lncRNAs which regulated that pathway. Prime lncRNAs and pathways in the network had the highest degrees.

Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from about 40 mg of cerebral ischemic cortex in rats with TRIzol(Sigma-Aldrich). RNA samples with an OD260/OD280 ratio of 1.9-2.1 and an OD260/OD230 ratio greater than 2.0 were used for analysis. The expression level of the destination mRNAs and lncRNAs was confirmed with a one-step qRT-PCR kit (Toyobo, Osaka, Japan). β -actin was used to normalize the mRNAs and lncRNAs. The expression level of mRNAs and lncRNAs was quantified with the Bio-Rad CFX real-time system and analyzed by the CFX management software v2.0 (BioRad, Hercules, CA). Relative quantification of mRNAs and lncRNAs was performed with the $2^{-\Delta\Delta C_t}$ method, and each sample was normalized.

Gene.Symbol	forward	reverse
IL-6	5'-AGGATACCAACCCACAACAGACC-3'	5'-TTGCCATTGCACAACCTCTTTTC-3'
Wnt4	5'-ATCCTGACACACATGCGGGT-3'	5'-ATCCGTATGTGGCTTGAAGTGC-3'
Rock2	5'-TGCTATTGGATAAACACGGACA-3'	5'-ACCAATCACATTCTCGTCCATAG-3'
Rps6kb1	5'-GAAATGCTGCTTCTCGTCTTGG-3'	5'-AGACCTGGTTGGCACTTTCACT-3'
GAPDH	5'-CTGGAGAAACCTGCCAAGTATG-3'	5'-GGTGGGAAGAATGGGAGTTGCT-3'
LOC102555751	5'-GGAAGTTGGGTCTGGAGGTATT-3'	5'-TAGCCCAGTTATGAGCCTCTGT-3'
NONMMUG011611	5'-AACCTACTACTGTTATTCTGTGCGAG-3'	5'-GCTCCCTTAAAGGCCATCTTCT-3'

Data analysis and statistics

All data were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA), and presented as mean±SEM. Differences among two groups was analyzed by Student's t-test. Statistics in microarray and bioinformatic analysis were performed by One-way analysis of variance (ANOVA) using Affymetrix® Expression Console™ TAC (Affymetrix® Expression Console™), followed by Student's t-test. Using Spearman correlation analysis to analyze co-expression relationships between lncRNAs and mRNAs^[23]. $P < 0.05$ was considered statistically significant.

Results

HSHS improved neurological function impairment and pathological injury in MCAO rats

The Zea Longa deficit score indicated that, unlike model group, the neurological deficit score in *HSHS* group was sharply decreased from 3-7 days after MCAO (Fig. 1A).

The beam walking test was used to evaluate the motor function of rats. Repeated measures of ANOVA showed considerable effects of time and treatment on the scores. Notably, a time treatment interaction was found on the beam walk scores of baselines. These resulted indicated a better motor function in treated rats compared with model rats. It also showed that MCAO decreased the scores compared with sham group from the 3rd day to the 7th day. Specifically, rats treated with *HSHS* increased scores on the 3rd and the 7th day after MCAO compared with model group (Fig. 1B).

HE staining revealed that the rats of model group had severe necrosis, infiltration of inflammatory cells and vascular edema in the ischemic cortex. By contrast, the *HSHS* treatment showed more neurons and less inflammation and vascular edema.(Fig. 1C).

Analysis of DElncRNAs and DEmRNAs between model vs sham and *HSHS* vs model

For lncRNA and mRNA profiles, we set a filtering criteria ($P < 0.05$, fold change > 1.2) and obtained 8128 DEmRNAs and 3022 DElncRNAs were identified in the model vs sham group (Additional file 1: Table S1). There were 868 DEmRNAs and 836 DElncRNAs in the *HSHS* vs model group (Additional file 2: Table S2). We got 666 overlapping DEmRNAs and 288 DElncRNAs for further analysis by Venn diagram (Fig. 2A, B; (Additional file 3: Table S3). Hierarchical clustering was performed to obtain an overview of the expression pattern of DEmRNAs and DElncRNAs among groups (Fig. 2C, D). Most of the DEmRNAs and DElncRNAs exhibited opposite expression patterns between model vs sham and *HSHS* vs model. For instance, the DEmRNAs *Hspa5*, *IL-6*, *Pon1* and *Jam2* were up-regulated in model vs sham, but opposite in *HSHS* vs model. The DEmRNAs *Fus*, *Rab3b*, *Ntn4*, *Amer1* and *Rps6kb1* were down-regulated in model vs sham, but opposite in *HSHS* vs model. The DElncRNAs LOC100911343 and LOC102554834 were increased in the model vs sham but decreased in the *HSHS* vs model. These results suggested that *HSHS* could influence the gene expression of MCAO rats.

GO and KEGG pathway analysis

We used the 666 overlapping DEmRNAs to perform GO and pathway enrichments for further exploring their biological functions. The results indicated some terms against ischemic stroke, such as extracellular exosome, neuron projection, axon guidance, autophagy, PI3K-Akt signaling pathway and mTOR signaling pathway. The top 30 enriched GO terms and 26 KEGG pathways were showed (Fig. 3A,B). To identify the crucial genes in these pathways, we constructed enriched KEGG pathway interactions, indicating some hub genes such as *Kras*, *Rps6kb1*, *Eif4e2*, *Wnt4*, *Pdpk1*, *Rock2*, and *IL-6* (Fig. 3C). The results indicated that these genes were associated with stroke.

PPI network

We used the 666 DEmRNAs co-regulated by sham, model and *HSHS* groups to construct a PPI network, which consisted of 209 nodes and 396 edges (Fig. 4A). By restricting the score > 8 , we filtered out two significant modules with MCODE (Fig. 4B, C).

Construction of lncRNA-mRNA network

Although lncRNAs are essential to regulate gene expression, we still know little about their biological functions. To uncover the functions of DElncRNAs, we screened out differentially expressed lncRNAs and mRNAs, which had PCC values ≥ 0.99 . Then using the Cytoscape program to construct the lncRNA-mRNA co-expression network between model vs sham and *HSHS* vs model groups, which contained 94 lncRNA nodes, 377 mRNA nodes and 1220 edges (Fig. 5). According to the node degree and correlation between ischemic stroke, we selected four significant lncRNAs (LOC102555751, LOC100911903, NONMMUG011611 and *barglo*). In MCAO rats with *HSHS* treatment, LOC102555751 and LOC100911903 were up-regulated and NONMMUG011611 and *barglo* were down-regulated.

lncRNA target pathway network

To investigate the lncRNAs functions in ischemic stroke after treatment of *HSHS*, we established a lncRNA target pathway network, finding out lncRNAs were essential in the pathogenesis of ischemic stroke. They had relations to many signaling pathways including the cAMP signaling pathway, axon guidance, autophagy-animal, oxytocin signaling pathway, EGFR tyrosine kinase inhibitor resistance, other glycan degradation and so on (Fig. 6).

QRT-PCR validation of key mRNAs and lncRNAs

These results from qRT-PCR proved the microarray data was reliable and confirmed the regulation effect of *HSHS* on cerebral ischemia. For example, *HSHS* reduced the expression of IL-6, Rock2 and lncRNA NONMMUG011611 in comparison with model group ($P < 0.05$, $P < 0.01$; Fig. 7A, B, F), while the expression of Wnt4, Rps6kb1 and lncRNA LOC102555751 were increased in *HSHS* group compared with model group ($P < 0.05$, $P < 0.01$; Fig. 7C, D, E).

Discussion

In this study, we showed that *HSHS* improved neurological deficit and ameliorated brain pathological injury in MCAO rats. Moreover, we systematically elucidated more hidden mechanisms of *HSHS* in treating ischemic stroke from the protein-coding mRNA and non-coding lncRNA expression profile through microarray analysis for the first time. A total of 666 DEmRNAs and 288 DElncRNAs were identified among sham group, model group and *HSHS* group. With further bioinformatics analysis including GO analysis, KEGG pathway analysis, pathway network analysis, PPI network analysis, lncRNA-mRNA co-expression network analysis and lncRNA target pathway network analysis, we studied the potential role of these identified genes associated with ischemic stroke following *HSHS* treatment.

Neurons, endothelial cells, astrocytes and basal lamina in brain make up the neurovascular unit (NVU), a multicellular complex, maintaining a homeostatic neuronal microenvironment^[24]. During cerebral ischemia, the complex contact of NVU is badly damaged, leading to neurological dysfunction. Promoting neurogenesis and angiogenesis effectively is beneficial to the recovery of ischemic stroke. Neurological prognosis and axonal regeneration after ischemic stroke are tightly regulated by many extracellular factors^[25]. Previous studies have showed several neurovascular-protective mechanisms of *HSHS*, for example, promoting the secretion of BDNF, reducing the accumulation of APP, promote neuronal recovery and axonal regeneration via Nogo and Netrin signals^[6–7,26]. In this study, GO enrichment analysis has suggested that the DEmRNAs in MCAO rats with *HSHS* treatment are involved in neural biological process, such as neuron projection, focal adhesion, regulation of neuron apoptotic process and Rho protein signal transduction. It has revealed that axon guidance has significant difference in KEGG pathway analysis, and Rock2 ranked high in axon guidance. Rock2 plays an important role in multiple biological processes, such as inflammation, vascular reconstruction, cell adhesion and apoptosis^[27–29]. Importantly, Rock2 is particularly abundant in brain tissues, performing an essential function in brain pathophysiology processes^[30]. RhoA is a negative regulator of axonal growth that causes growth cone collapse and axonal retraction. Rock2 is a main downstream target of RhoA and the inhibition of

RhoA/Rock2 can induce axonal regeneration after injury^[31]. The study has demonstrated that *HSHS* can down-regulate the Rock2 expression and may promote axonal repair and regeneration, which are consistent with the previous results^[7].

Another culprit of nerve damage in ischemic brain tissue is inflammation. The inflammatory process increases the adhesion of white blood cells to blood vessels, then penetrates into the brain, destroys endothelial cells, and causes ischemic damage of nerves^[32]. Interleukin 6(IL-6) is a major pro-inflammatory cytokines that is up-regulated after cerebral ischemia^[33]. The increased expression of IL-6 will mediate the inflammatory cascade, damage vascular endothelial cells, increase BBB permeability, which leads to impaired cerebral autoregulation and further aggravates cerebral ischemic injury^[34]. We have shown that the effective ingredients of *HSHS* can regulate the secretion of inflammatory response cytokines after cerebral ischemia and reduce IL-6 expression^[35]. In this study, the qRT-PCR results also have showed that IL-6 expression in *HSHS* group was lower than model group, which is consistent with the microarray analysis, suggesting that *HSHS* may help alleviate the inflammatory response after cerebral ischemia, thereby reducing nerve damage.

In addition to axon guidance-mediated nerve regeneration, effective angiogenesis after ischemia can promote collateral circulation, help to restore blood supply in the ischemic tissues, and reduce ischemic necrosis^[36]. Previous results have showed that *HSHS* could activate BDNF/PI3K/AKT pathway to resist ischemic injury, regulate SDF-1/CXCR4 pathway to prime and improve endothelial cells migration, and further promote the angiogenesis through HIF-1 α /VEGF and Ang-1/Ang-2 pathway^[7-8]. In this study, we have found PI3K/AKT signaling pathway has significant difference in KEGG pathway analysis, and Rps6kb1 ranks high in this pathway. The PI3K/AKT pathway has been extensively studied because of its neuroprotective effect in ischemic stroke. A large number of studies have shown that AKT activation plays a vital role in neuron survival after cerebral ischemia-reperfusion injury^[37]. Besides, the PI3K pathway is substantial to the formation of normal blood vessels during development. VEGF stimulates endothelial cells to activate the PI3K pathway, which helps endothelial cells migrate and promote angiogenesis^[38]. Rps6kb1 is a mitogen-activated serine/threonine protein kinase related to mRNA processing, protein synthesis, glucose homeostasis, cell growth, and cell survival^[39]. Affecting Rps6kb1/HIF-1 α signaling pathway can inhibit the process of migration and proliferation of cardiac microvascular endothelial cells in ischemic conditions, which finally suppresses the angiogenesis^[40]. The qRT-PCR results have showed that *HSHS* could up-regulate the expression of Rps6kb1, which is consistent with the microarray analysis. Besides, the Wnt signaling pathway is also critically involved in angiogenesis through the coordination of endothelial cell proliferation, migration, vascular sprouting, remodeling, and vascular system maturation^[41-42]. Wingless-type MMTV integration site family, member 4(Wnt4) has been another differentially expressed mRNA in this analysis. By activating Wnt4-dependent pathway after ischemia, hypoxia preconditioned mesenchymal stem cells promote vascular regeneration^[43]. Human umbilical cord mesenchymal stem cells exosomes can enhance angiogenesis by transmitting Wnt4 to activate Wnt/ β -catenin in endothelial cells^[44]. The qRT-PCR results have showed

that *HSHS* could up-regulate the expression of *Wnt4*, which is also consistent with the microarray analysis.

It's worth noting that we have also found the effect of *HSHS* in treating ischemic stroke may be produced through autophagy. Autophagy is a crucial biological mechanism involved in regulating many basic cellular processes, which are mainly related to cell growth and differentiation. The autophagy promotes the turnover of non-functional proteins and organelles, which is crucial to maintain the stability of the intracellular environment^[45]. In stress response, autophagy can play a neuroprotective effect, but it can also induce cell death during cerebral ischemia^[46]. Therefore, moderate autophagy can induce the neuroprotection of ischemic brain tissue, while excessive autophagy may lead to the death of nerve cells, which plays a harmful role in the occurrence and development of ischemic stroke^[47]. Mechanistic target of rapamycin(mTOR) signaling pathway has been another representative pathway in our study, which may be closely related to autophagy. mTOR is a serine/threonine kinase, a major regulator of cell metabolism, and promotes cell growth when prompted by the environment^[48]. mTOR is considered to be the main regulator of autophagy in yeast and mammalian systems and can inhibit autophagy induction, which is a critical component of two multiprotein signaling complexes named mTOR complex 1 (mTORC1) and 2(mTORC2)^[49]. Ribosomal protein S6 kinase B1(Rps6kb1) is the downstream effector of mTORC1, which phosphorylates rapamycin-insensitive companion of mTOR, thereby affecting mTORC2 activity to regulate autophagy^[50]. Studies have shown that changes in the mTOR signaling pathway and the stimulation of productive autophagy can participate in endogenous neuroprotection^[51]. The regulation of autophagy by mTOR inhibitors, in short, provides a new therapeutic angle for neurodegenerative diseases, diabetes, cancer, etc.

Recent studies have found that lncRNAs play a critical role in regulating the expression of genes and actively participate in the progress of brain disorders^[52]. lncRNAs are considered to be potential targets of ischemic stroke^[53]. Research has shown that lncRNAs may promote functional recovery by activating angiogenesis, which is closely involved in the prognosis of patients with ischemic stroke. Moreover, lncRNAs participate in angiogenesis through acting as miRNAs sponge or directly targeting downstream pathways^[54]. For example, small nucleolar RNA host gene 12 (SNHG12) can regulate the expression of VEGF and HIF-1 α after cerebral ischemia, promote angiogenesis and reduce cerebral ischemic damage^[55]. lncRNAs are also related to the regulation of neurogenesis after ischemic stroke. lncRNA MEG3 is upregulated in ischemic brain tissue, of which silencing promotes neurogenesis via activating the Wnt/ β -catenin signal and reduces nerve damage after cerebral ischemia^[56]. Moreover, lncRNAs are also connected with the occurrence of autophagy after cerebral ischemia. lncRNA MALAT1 can regulate the expression of miR-30a during cerebral ischemia to inhibit autophagy and reduce neuronal death^[57]. lncRNA H19 activates the autophagy process through the DUSP5-ERK1/2 axis, reduces neuronal activity, and induces ischemia-reperfusion injury^[57]. Another important result in our study might be to screen out some differentially expressed lncRNAs such as lncRNA LOC102555751, LOC100911903, NONMMUG011611 and barglo. Further qRT-PCR has showed that lncRNA LOC102555751 has been

significantly down-regulated and lncRNA NONMMUG011611 up-regulated in *HSHS* group compared with model group, which are consistent with microarray analysis. The lncRNA-mRNA co-expression network indicates that lncRNA NCBI_Gene:100861650, lncRNA KnowTID_00004708, lncRNA NONMMUG027934, lncRNA NONMMUG000892 and lncRNA NONMMUG031413 could be co-expressed with Rock2, and lncRNA LOC102554076, LOC102555751, and NCBI_Gene:100861902 could be co-expressed with Wnt4. We could infer from these results that differentially expressed lncRNAs may be the regulatory targets of *HSHS* in cerebral ischemia. It cannot be ignored that due to the limited number of samples, significant pathways are unable to be enriched completely. At present, the research on the function of lncRNAs is not comprehensive enough. Based on limited understanding, only two representative lncRNAs have been selected for verification in this study. In the future, it is necessary to further explore the role of lncRNAs, and analyze the mechanism with co-expressed mRNAs in ischemic stroke.

Conclusion

We have demonstrated that *HSHS* could improve neurological function and reduce ischemic injury. By microarray analysis, we have profiled the expression of lncRNAs and mRNAs among the sham, model and *HSHS* groups for the first time. In addition, GO analysis and KEGG pathway analysis have revealed the main changes in genes and related pathways in MCAO rats after the treatment of *HSHS*. We also have tested and verified four mRNAs and two lncRNAs, which may be the target of *HSHS* in ischemic stroke. Based on these results, we may conclude that *HSHS* might work against ischemic stroke via the regulation of the lncRNAs and mRNAs.

Abbreviations

HSHS: *Houshiheisan*; MCAO: middle cerebral artery occlusion; DEmRNAs: Differential expression mRNAs; DElncRNAs: Differential expression lncRNAs; PPI: Protein–protein interaction; SEM, standard error of the mean; IL-6: interleukin 6; Rps6kb1: Ribosomal protein S6 kinase B1; Rock2: Rho associated protein kinase 2; Wnt4: wingless-type MMTV integration site family, member 4; mTOR: mechanistic target of rapamycin.

Declarations

Acknowledgements

Not applicable.

Author's contributions

HFC, YWZ, HYW, and XW conducted the experiments. HFC, YWZ, and XYG analyzed the data and prepared the paper. HFC and YWZ wrote the paper. QXZ and HZ designed the study and revised the paper. LW and HYZ revised the paper. All the authors have approved the final version of the manuscript.

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Availability of data and materials

The data set supporting the results of this article are included within the article and Additional files.

Ethics approval and consent to participate

The study protocol was approved by the Institution Animal Care and Use Committee (Approval number: AEEI-2019-001).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

HSHS alleviates neurological deficit and pathological change after MCAO. A Neurological deficit scores from 1,3,5 to 7 days after MCAO (n=6). B Balance beam scores at 3 and 7 days after MCAO (n=6). Higher scores in (A) mean more serious neurological damage, while higher scores in (B) represent better neurological function. C HE staining of surviving neurons (arrows) in the peri-infarct cortex at 7 days after MCAO (original magnification: 400 $\times$; Scale bars=20 μ m). Data are shown as mean $\pm$ SEM. ^{##}P < 0.01 vs sham group; ^{*}P < 0.05, ^{**}P < 0.01 vs model group.

Figure 2

Hierarchical cluster analysis of overlapped DEmRNAs and DElncRNAs among the sham, model and HSHS groups. A Venn diagram depicted overlapping DEmRNAs. B Venn diagram depicted overlapping DElncRNAs. C Heatmap of overlapping DEmRNAs(666 mRNAs total). D Heatmap of overlapping DElncRNAs(288 lncRNAs total). Red means up-regulation, and green means down-regulation. $P < 0.05$, $n=3$.

Figure 3

Functional enrichment analysis of overlapping DEmRNAs. A The first 30 enriched GO terms, y-axis indicates GO terms, and x-axis indicates enrichment factor. The size of the bubble represents the amount of DEmRNAs enriched in GO terms, and the color represents the significance. B The first 26 enriched KEGG pathways, y-axis shows pathway names, and x-axis shows enrichment factor. Size and color of the bubbles represent the amount of DEmRNAs enriched in the pathway and significance, respectively. C

Representative pathway interaction, each node is a KEGG pathway item; the node size reflects pathway significance, the edges between nodes reflect common genes; and different node colors represent different functional groups. The P value assessed by a two-sided Fisher's exact test.

Figure 4

PPI networks analysis of DE mRNAs resulting from HSHS treatment of MCAO. A PPI network with 209 nodes and 396 interactions. B and C Two modules identified by MCODE(B Score =10; C Score =8.25).

Figure 7

QRT-PCR analysis of the key DEmRNAs and DElncRNAs. A Expressions of interleukin 6 (IL-6). B Expressions of Rho-associated coiled-coil containing protein kinase 2(Rock2). C Expressions of wingless-type MMTV integration site family, member 4(Wnt4). D Expressions of Expression of ribosomal protein S6 kinase B1(Rps6kb1). E Expressions of lncRNA LOC102555751. F Expressions of lncRNA

NONMMUG011611. Results were presented as mean $\pm$ SEM (n = 4). #P < 0.01, ##P < 0.01 vs sham group; *P < 0.05, **P < 0.01 vs model group.

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