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LncRNA-mRNA co-expression network in the mechanism of butylphthalide treatment for ischemic stroke

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Abstract

Background Butylphthalide has shown significant potential in the treatment of ischemic stroke, but its precise mechanisms of action remain unclear. Long non-coding RNAs (lncRNAs) and messenger RNAs (mRNAs) play crucial roles in the pathogenesis of ischemic stroke and may serve as potential therapeutic targets. This study investigated the effects of butylphthalide treatment on the lncRNA-mRNA co-expression network in ischemic stroke patients.

Methods Peripheral blood samples were collected from ischemic stroke patients treated with butylphthalide and from control subjects. mRNA and lncRNA expression profiles were obtained using microarray scanning, and differentially expressed lncRNAs (DElncRNAs) were validated by qRT-PCR. Target genes interacting with DElncRNAs were predicted using the miRTargetLink database. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on both DElncRNAs and differentially expressed mRNAs (DEmRNAs). A protein-protein interaction (PPI) network was constructed for proteins encoded by DEmRNAs. Co-expression analysis, based on Pearson correlation coefficients, identified the top five mRNAs and lncRNAs with high connectivity. Finally, molecular docking was performed to investigate the binding interaction between butylphthalide and key mRNAs.

Results A total of 86 differentially expressed mRNAs (69 upregulated, 17 downregulated) and 35 DElncRNAs (all upregulated) were identified. DEmRNAs were primarily associated with pathways related to cell receptors, signal transduction, cell proliferation, migration, and glucose metabolism, while DElncRNAs were involved in processes such as embryonic development, neuronal connectivity, and energy metabolism. Co-expression analysis identified key mRNA nodes (SETD9, ZNF718, AOC2, MPND, ODF1) and lncRNA nodes (IDH2-DT, CLEC12A-AS1, CARD8-AS1, LINC01275, ZNF436-AS1). Molecular docking analysis suggested that MT-CO1, SETD9, and ZNF718 could be potential targets of butylphthalide.

Conclusion Butylphthalide may exert its therapeutic effects by regulating the LncRNA-mRNA co-expression network, influencing energy metabolism and neuronal development. This provides new insights into its mechanism of action and potential therapeutic targets.

Keywords Butylphthalide, Ischemic stroke, LncRNA-mRNA co-expression network, RNA-seq

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Introduction

Ischemic stroke is a common global health issue and a significant cause of mortality and disability, accounting for 65% of all stroke cases [1]. In recent years, particularly in low- and middle-income countries, its incidence and mortality rates have been on the rise [2]. Data indicate that an ischemic stroke occurs approximately every 40 s in the United States [3], while in China, there are about 1.68 million new cases annually, with a total of over 7.56 million affected individuals [4]. Ischemic stroke results from an obstruction of cerebral blood flow, leading to oxygen deprivation and insufficient nutrient supply to nerve cells [5]. It often manifests as facial or limb numbness, cognitive impairment, blurred vision, and speech difficulties [6]. Severe cases may result in hemiplegia or even death [7]. Although traditional treatments such as thrombolysis, anticoagulation, and endovascular therapy can improve outcomes for some patients, their efficacy is limited and they carry risks, particularly bleeding complications [8]. Therefore, the development of safer and more effective therapeutic strategies is of critical importance.

Butylphthalide is a neuroprotective drug primarily used to improve brain dysfunction and cognitive decline caused by cerebrovascular diseases, and its potential in ischemic stroke treatment has garnered significant attention [9]. Studies have demonstrated that butylphthalide alleviates cerebral ischemic injury by dilating cerebral blood vessels, improving cerebral blood flow, and enhancing microcirculation [10]. Additionally, it mitigates oxidative stress by reducing the generation of reactive oxygen species [11], regulates intracellular calcium ion levels to maintain ionic balance, and suppresses inflammatory responses, thereby reducing secondary neuronal damage caused by inflammation [12]. Recent research has further elucidated its molecular mechanisms. For instance, DL-3-n-butylphthalide (NBP) enhances Sonic Hedgehog (Shh) signaling via autocrine activity in endothelial cells and paracrine activity in glial cells, activating downstream expression of Gli1 and VEGF to promote angiogenesis in ischemic regions, thereby providing molecular evidence for its role in vascular regeneration [13]. However, the precise mechanisms of butylphthalide in ischemic stroke remain incompletely understood and warrant further investigation.

Messenger RNA (mRNA), as a template for protein synthesis, plays a central role in biological processes [14]. Long non-coding RNA (lncRNA), which is longer than 200 nucleotides, does not encode proteins but plays an important role in the regulation of gene expression [15]. The co-expression network of lncRNA and mRNA reveals their potential synergistic effects in specific biological processes by analyzing the expression relationships between them [16]. Recent studies have shown that both lncRNA and mRNA play significant roles in the occurrence and development of cerebrovascular diseases [17]. For example, through bioinformatics analysis, a research constructed a lncRNA-mRNA co-expression network in Moyamoya disease patients (a cerebrovascular disease leading to vascular narrowing and increased stroke risk), identifying several core nodes related to processes such as angiogenesis, inflammation, and cell cycle regulation. Further analysis identified key lncRNA-mRNA pairs that showed significant co-expression relationships in patients, suggesting they may serve as potential biomarkers or therapeutic targets [18]. However, studies on the lncRNA-mRNA co-expression network in ischemic stroke remain limited, and exploring its role and mechanisms in ischemic stroke is of great significance.

This study aims to explore the therapeutic mechanism of butylphthalide in ischemic stroke, with a focus on analyzing the co-expression network of lncRNAs and mRNAs and their potential regulatory roles. Additionally, molecular docking technology was employed to reveal for the first time that butylphthalide may exert its effects by regulating specific mRNAs. These findings provide new insights into the molecular mechanisms of ischemic stroke and offer potential therapeutic targets for the development of lncRNA- and mRNA-based targeted treatment strategies.

Methods

Clinical sample collection and RNA isolation

These patients were specifically diagnosed with acute cerebral infarction within a week of onset. Exclusion criteria comprised individuals with concurrent hemorrhagic stroke, transient ischemic attack, cancer, trauma, arterial inflammation, cerebrovascular malformation, or aneurysm. Informed consent was obtained from all participants, who voluntarily agreed to take part in the study. Approval for the research was granted by the Yiyang Central Hospita Ethics Committee (NO.2022-046), and the study adhered to the principles outlined in the Declaration of Helsinki, ensuring compliance with relevant guidelines and regulations. The patients ultimately enrolled were categorized into two groups: the butylphthalide group (BP group, 10 cases) and the control group (control group, 10 cases), based on whether they had received butylphthalide treatment.

The RNAeasy™ Blood RNA Extraction Kit (R0091S, Beyotime, China) was employed for the isolation of RNA from blood samples. Specifically, the lysate was mixed with three times its volume, followed by the addition of an equal volume of binding solution. Subsequently, the mixture was then applied to a purification column, after washing steps, the RNA was eluted using 30–50 μL of elution buffer. The purified RNA was then collected

post-centrifugation. The concentration and integrity of the total RNA were assessed using the NanoDrop ND-1000.

mRNA and lncRNA microarrays

The 28 S, 18 S, and 5 S ribosomal RNAs in total RNA, along with the 12 S and 5.8 S ribosomal RNAs from mitochondria, were selectively removed prior to microarray analysis, while the mRNA and non-coding RNA components were retained for further analysis. The Arraystar Flash RNA Labeling Kit was used to amplify and transcribe mRNA and non-coding RNA into fluorescently labeled complementary RNA (cRNA). The labeled cRNA was then hybridized to a pre-warmed microarray overnight in an incubator. Following the manufacturer's instructions, any non-specifically bound cRNA was washed away. The fluorescence signals on the microarray chip were scanned using the Agilent DNA Microarray Scanner (G2505C). Background correction and normalization of the initial fluorescence signal were performed with the Agilent Feature Extraction software (version 11.0.1.1). The Agilent Gene Expression Analysis Differential Expression (GEDAD) software was used to assess gene expression variations across different sample groups. Differentially expressed lncRNAs (DElncRNAs) and mRNAs (DEmRNAs) were identified based on fold change (FC) ≥ 2 and $p < 0.05$ criteria. Visualization of the DElncRNAs and DEmRNAs was carried out by generating heat maps and volcano plots using R pheatmap and volcano tools.

Prediction of DElncRNAs target genes

Based on the cis-regulatory and trans-regulatory effects of lncRNAs, the target genes of DElncRNAs were predicted. Cis-regulation involves the control of target gene expression by lncRNAs within their genomic vicinity, typically with a 10 kb range upstream or downstream of the lncRNA locus. Trans-regulation, on the other hand, involves the modulation of target gene expression by lncRNAs that are located distally from the target gene's genomic region. The miRTargetLink database (https://www.ccb.uni-saarland.de/mirtargetlink2) was utilized for the prediction of target genes that interact with DElncRNAs.

GO and KEGG enrichment analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed on DElncRNAs and DEmRNAs using the org.Hs.eg.db and KEGG.KGML. Homo_sapiens software tools for annotation. Following this, clusterProfiler software was used for GO and KEGG enrichment analyses to identify significant biological functions and pathways associated with DElncRNAs and DEmRNAs. The significance of pathway enrichment was assessed by adjusting the p-value using the False Discovery Rate (FDR) and Benjamini-Hochberg (BH) methods. A p-value or FDR value below 0.05 was considered statistically significant.

Protein-protein interaction (PPI) analysis

Interaction information of proteins encoded by differentially expressed genes (DEGs) was obtained from the STRING database (https://cn.string-db.org/). Subsequently, a PPI network was constructed using Cytoscape software (version 3.10.1), and key genes within the network were identified and analyzed using the Network Centrality plug-in.

The co-expression network of lncRNAs and mRNAs

The Pearson correlation coefficient was used to calculate the co-expression relationships between DElncRNAs and DEmRNAs. Based on predefined thresholds ($|r| > 0.3$ and $p < 0.05$), mRNA and lncRNA pairs with significant co-expression relationships were identified. A co-expression network was then constructed using Cytoscape software, with the Network Centrality plug-in employed to identify key nodes within the network. Finally, the top five mRNAs and lncRNAs with the most significant co-expression patterns were determined.

Molecular docking analysis

The three-dimensional structure of butylphthalide (PDB ID: 3F4M) was obtained from the Protein Data Bank (PDB) at https://www.rcsb.org/. The RNA three-dimensional structures were predicted using the RNAfold software, focusing on the top five mRNA nodes with the highest PPI network values among DEGs, as well as the top five mRNA nodes with the highest co-expression network values between DElncRNAs and DEmRNAs. After performing energy minimization and adding hydrogen atoms to both butylphthalide and mRNA structures using AutoDockTools software, molecular docking was carried out, with ligand flexibility set to rotatable and target flexibility set to fixed.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

In the aforementioned blood samples, RNA was isolated using the RNAeasy™ Blood RNA Extraction Kit (R0091S, Beyotime, China), and the total RNA's quantity and quality were assessed using NanoDrop ND-1000. Subsequently, the PrimeScript™ RT reagent Kit (RR037Q, Takara, Japan) was utilized for the detection of IDH2-DT, CLEC12A-AS1, CARD8-AS1, LINC01275, and ZNF436-AS1 expression levels. GAPDH served as the internal control, and the target gene's expression level was determined using the $2^{-\triangle\triangle ct}$ method. The primer sequences can be found in Table 1.

Table 1 Primer sequences

Gene name	Sequences (5′ to 3′)
IDH2-DT	F: GCCCTTTGTGCGCCTGA
	R: ACCTCGCAAGAGCAGCC
CLEC12A-AS1	F: GTGAGGCCAGTCAAGGGAAT
	R: AAATGTGGTCTGGCAAAGCC
CARD8-AS1	F: TCCTGACCTCAGCTGGAATG
	R: ACAATCATTCTTGGGCGGGG
LINC01275	F: CAAGGAGCGAGGAGCAGTTT
	R: ACTGGCTTCTGTACGTGGTG
ZNF436-AS1	F: GTTCTCTCAGACCTGGCGTTC
	R: GTCCTGCTGCTTCTACACAC
GAPDH	F: GTCTCCTCTGACTTCAACAGCG
	R: ACCACCCTGTTGCTGTAGCCAA

Statistical analysis

The data underwent statistical analysis utilizing SPSS16.0 software and were graphically represented through GraphPad Prism 9.2.0 software. Group differences were assessed using one-way analysis of variance (ANOVA) for comparisons among multiple independent samples. For pairwise comparisons, the independent t-test was used. Statistical significance was determined at a threshold of $p < 0.05$.

Result

Differential expression profiles of lncRNA and mRNA in peripheral blood of ischemic stroke patients after butylphthalide treatment

Peripheral blood samples were collected from ischemic stroke patients with and without butylphthalide treatment, followed by RNA-seq analysis. Volcano plot analysis revealed that 35 lncRNAs were significantly upregulated after butylphthalide treatment, with no significantly downregulated lncRNAs observed (Fig. 1A). Additionally, 86 DEmRNAs were identified, including 69 upregulated and 17 downregulated mRNAs (Fig. 1B). Heatmap analysis demonstrated a marked increase in the expression levels of specific lncRNAs such as IDH2-DT, CLEC12A-AS1, CARD8-AS1, LINC01275, and ZNF436-AS1 (Fig. 1C), while certain mRNAs such as AOC2, ARL15, and CEBP2 were upregulated, and RPL23A, STAT6, and JAK2 were significantly downregulated (Fig. 1D). These findings provide initial insights into the regulatory effects of butylphthalide on the expression profiles of lncRNAs and mRNAs in ischemic stroke patients.

GO and KEGG enrichment analysis of DEmRNAs

To elucidate the biological functions of DEmRNAs, GO and KEGG enrichment analyses were conducted. The GO analysis indicated significant enrichment of DEmRNAs across three categories: biological process, cellular component, and molecular function. In the biological process category, enriched processes included transmembrane receptor protein signaling pathways, serine/threonine kinase signaling pathways, gastrulation, and cellular responses to xenobiotic stimuli (Fig. 2A). For the cellular component category, DEmRNAs were significantly enriched in the endoplasmic reticulum lumen, intercellular bridge, and tertiary granule lumen (Fig. 2B). In the molecular function category, enriched functions encompassed serine hydrolase activity, histone deacetylase binding, and disordered domain-specific binding (Fig. 2C). KEGG analysis revealed significant enrichment of DEmRNAs in metabolic pathways such as glycolysis/gluconeogenesis, steroid hormone biosynthesis, and the pentose phosphate pathway (Fig. 2D). These results suggest that butylphthalide may exert its therapeutic effects by regulating genes associated with carbohydrate metabolism, signaling pathways, and cellular functions.

GO and KEGG enrichment analysis of DElncRNAs

To elucidate the functions of DElncRNAs, we utilized the miRTargetLink database to predict their interacting target genes and performed GO and KEGG enrichment analyses on these target genes. GO analysis revealed that the biological processes associated with DElncRNAs include pattern specification processes, embryonic organ development, and morphogenesis (Fig. 3A). The enriched cellular components were neuron synapses and postsynaptic density (Fig. 3B). The molecular functions were significantly enriched in RNA polymerase II-related transcription activator and repressor activities (Fig. 3C). KEGG analysis demonstrated that DElncRNAs were enriched in metabolic pathways such as oxidative phosphorylation, glycolysis/gluconeogenesis, and fatty acid degradation (Fig. 3D). These findings suggest that butylphthalide treatment may induce changes by regulating the expression of metabolism-related lncRNAs.

Butylphthalide regulates the lncRNA-mRNA co-expression network

Constructing a co-expression network of lncRNA and mRNA is a critical approach for understanding the molecular mechanisms of butylphthalide in the treatment of ischemic stroke and facilitating the identification of potential therapeutic targets. In this study, a lncRNA-mRNA co-expression network was constructed based on correlation coefficients $|r| > 0.3$ and $p < 0.05$ (Fig. 4A), identifying the top five key nodes with the highest connectivity. The results showed that the top five mRNA nodes were SETD9, ZNF718, AOC2, MPND, and ODF1 (Fig. 4B), while the top five lncRNA nodes were IDH2-DT, CLEC12A-AS1, CARD8-AS1, LINC01275, and ZNF436-AS1 (Fig. 4C). Butylphthalide may exert its therapeutic effects by modulating these key nodes within the lncRNA-mRNA co-expression network.

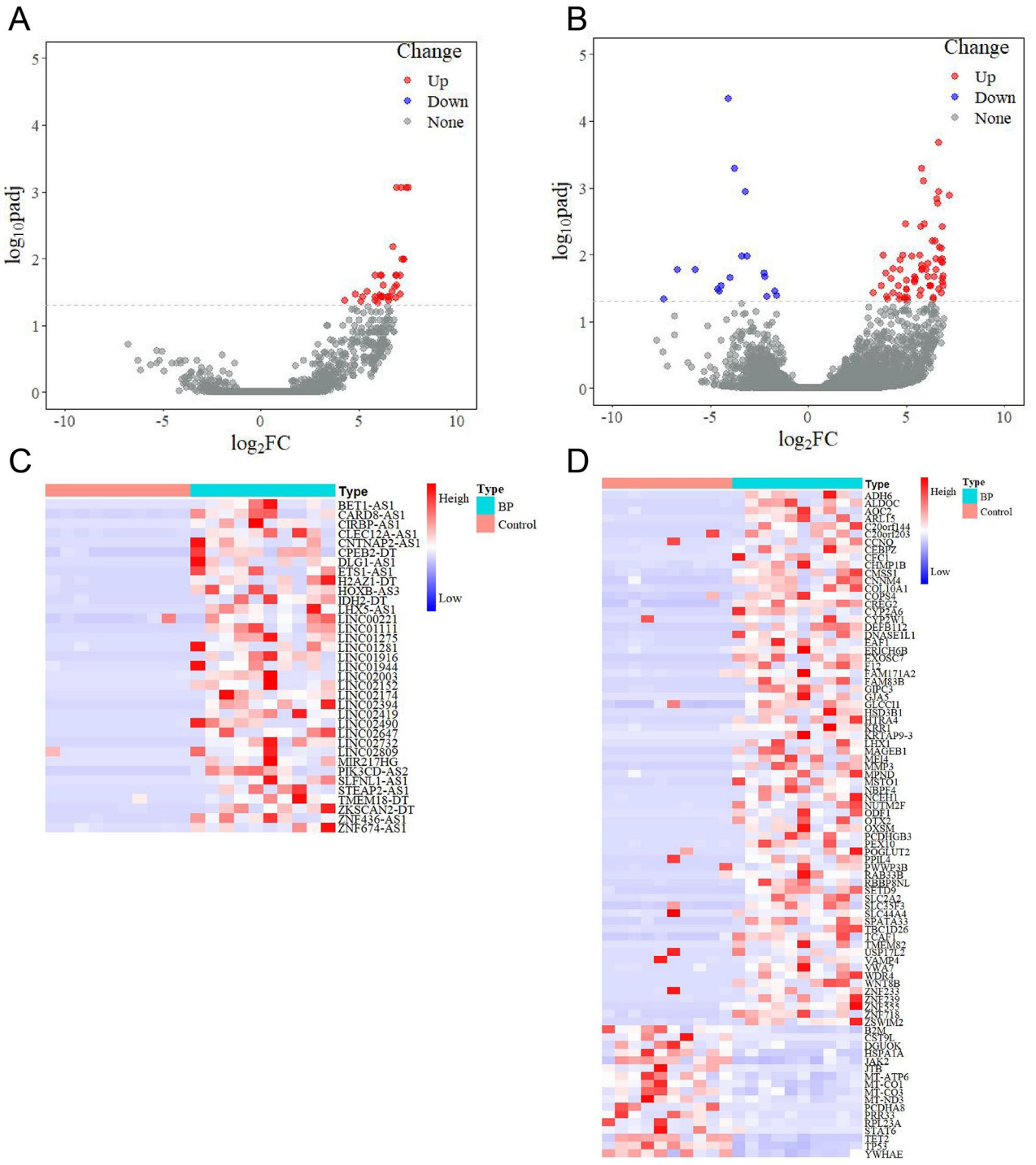


Fig. 1 Differential expression profiles of lncRNA and mRNA in peripheral blood of ischemic stroke patients after butylphthalide treatment. (**A-B**), Volcano plots showing DElncRNAs and DEmRNAs. (**C-D**), Heatmaps illustrating expression patterns of DElncRNAs and DEmRNAs

qRT-PCR validation of key lncRNA expression levels

To validate the RNA-seq sequencing results, we measured the expression levels of key lncRNAs by qRT-PCR. The results showed that the expression levels of IDH2-DT (Fig. 5A), CLEC12A-AS1 (Fig. 5B), CARD8-AS1 (Fig. 5C), LINC01275 (Fig. 5D), and ZNF436-AS1 (Fig. 5E) in the peripheral blood of patients treated with butylphthalide were significantly higher compared to the control group ($P < 0.01$). These findings are consistent with the trends observed in the RNA-seq data.

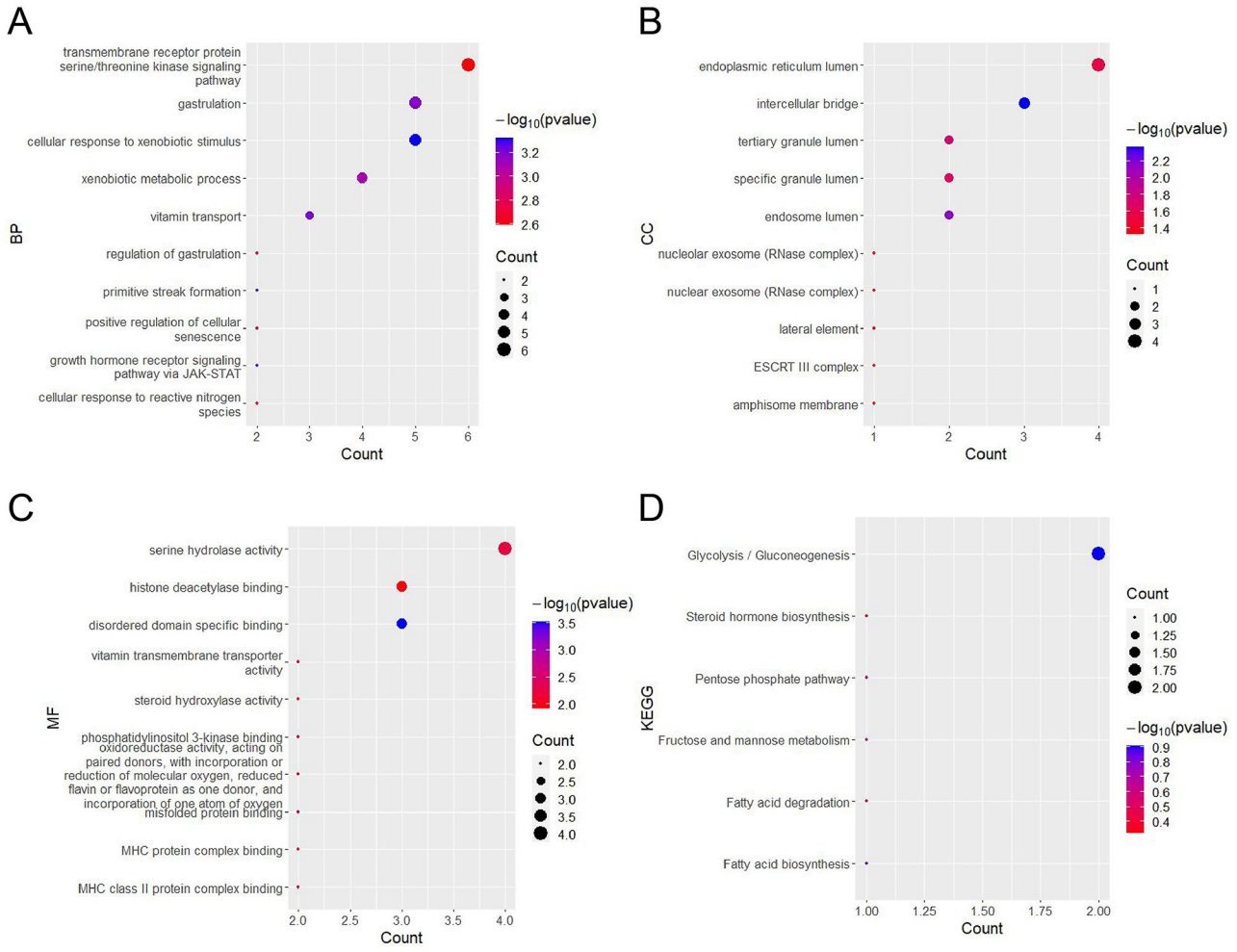


Fig. 2 GO and KEGG enrichment analysis of DEmRNAs. (**A**-**C**) GO analysis of DEmRNAs includes (**A**) Biological Process (BP), (**B**) Cellular Component (CC), and (**C**) Molecular Function (MF). (**D**) KEGG pathway enrichment analysis was performed on the DEmRNAs

PPI network analysis of DEGs

We performed PPI network analysis of the DEGs (Fig. 6A). The constructed PPI network revealed that interactions were primarily concentrated around key nodes such as TP53, MT-ATP6, MT-CO3, MT-CO1, and PWWP3B. Further degree centrality analysis showed that TP53 had the highest connectivity, followed by MT-ATP6, MT-CO3, MT-CO1, and PWWP3B, which were ranked as the top five core nodes in the network (Fig. 6B). These results suggest that these key genes may play an important role in the mechanism of butylphthalide treatment for ischemic stroke.

Molecular docking analysis of potential targets of butylphthalide

To explore the binding sites of butylphthalide, we performed molecular docking analysis between butylphthalide and the top five mRNA nodes ranked by degree centrality in the DEGs PPI network, as well as the top five mRNA nodes in the lncRNAs-mRNAs co-expression network. The results revealed that the binding energies of MT-CO1, SETD9, and ZNF718 were the lowest at -7.1, -7.5, and −7.1, respectively (Fig. 7A), indicating strong binding affinity with butylphthalide. Subsequently, we visualized the binding sites of butylphthalide with these three proteins in three dimensions (Fig. 7B-D). The visualization results showed that MT-CO1, SETD9, and ZNF718 can tightly bind with butylphthalide, suggesting that they may serve as potential target proteins for butylphthalide action.

Discussion

This study analyzes the regulatory effects of butylphthalide on the expression of lncRNA and mRNA in patients with ischemic stroke, and combines lncRNA-mRNA co-expression networks with molecular docking techniques. It reveals that butylphthalide may regulate the expression of key lncRNAs and mRNAs, thereby influencing critical pathological processes such as energy metabolism, neural function, and inflammation. This

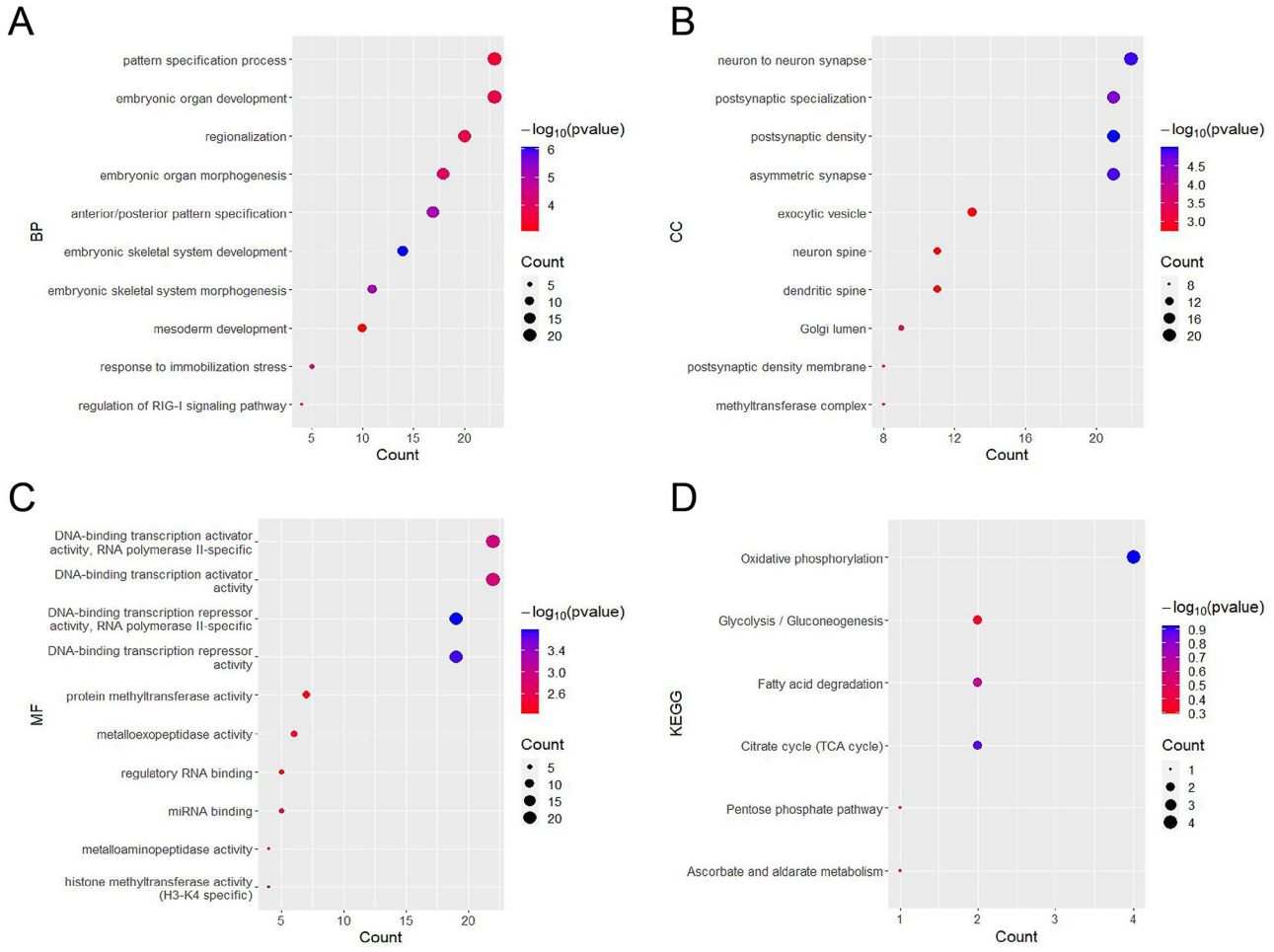
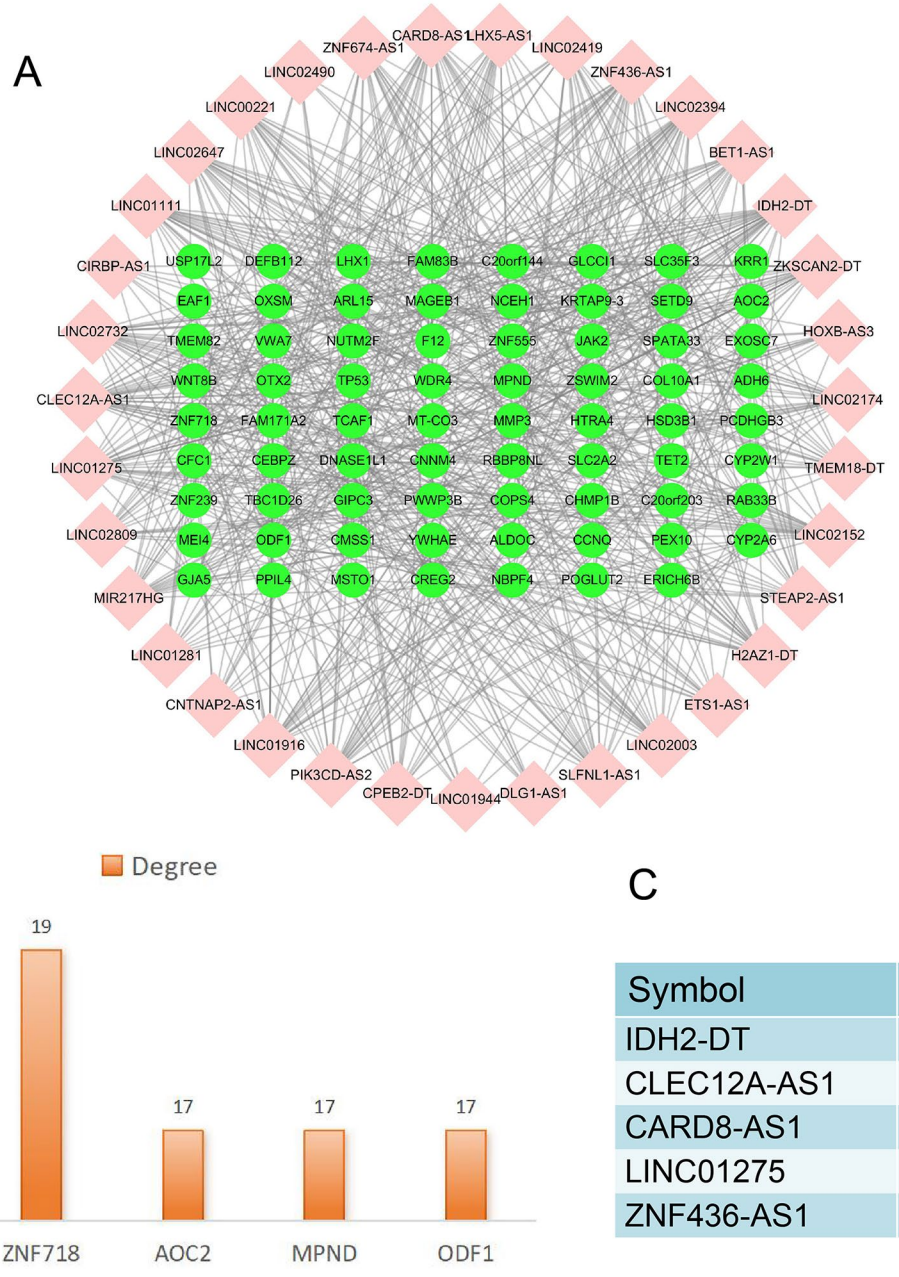


Fig. 3 GO and KEGG enrichment analysis of DElncRNAs. (**A**-**C**) GO analysis of DElncRNAs includes (**A**) Biological Process (BP), (**B**) Cellular Component (CC), and (**C**) Molecular Function (MF). (**D**) KEGG pathway enrichment analysis was performed on the DElncRNAs

research provides new insights into the molecular mechanisms of butylphthalide and lays an important foundation for the development of therapeutic strategies based on these mechanisms.

Ischemic stroke, caused by the blockage of cerebral blood flow, leads to neuronal cell hypoxia and energy deficiency, triggering apoptosis, inflammation, and metabolic disorders [19]. Butylphthalide, known for its anti-inflammatory, antioxidant, and microcirculation-improving properties, is widely used in the treatment of brain injuries [20]. However, its precise mechanism of action in ischemic stroke remains not fully understood. In our study, RNA-seq analysis revealed significant changes in the expression profiles of mRNA and lncRNA in the peripheral blood samples of ischemic stroke patients treated with butylphthalide. Further GO and KEGG enrichment analyses were conducted to explore the biological functions of these genes. GO analysis indicated that the DEGs were primarily involved in key biological processes, including transmembrane receptor signaling pathways, cellular metabolism, and inflammation regulation. These genes were also enriched in cellular components related to synaptic structures and molecular functions associated with RNA polymerase. KEGG analysis revealed that these genes were significantly enriched in metabolic pathways such as glycolysis/gluconeogenesis, fatty acid degradation, and oxidative phosphorylation. These findings suggest that butylphthalide may improve the pathological conditions of ischemic stroke by regulating the expression of metabolism-related genes.

By constructing the lncRNA-mRNA co-expression network, we identified key regulatory modules consisting of lncRNA nodes such as IDH2-DT and CLEC12A-AS1, which interact with mRNA nodes like SETD9 and ZNF718. These nodes may play crucial roles in metabolic regulation, signal transduction, and neurofunctional repair [21–23]. Additionally, PPI network analysis revealed that proteins such as TP53 and MT-CO1 exhibit high connectivity. Notably, TP53 gene, as a key tumor suppressor gene, is closely associated with the development of various cancers. In particular, abnormalities in TP53 are linked to poor prognosis in patients with



Symbol	Degree
IDH2-DT	33
CLEC12A-AS1	31
CARD8-AS1	30
LINC01275	29
ZNF436-AS1	29

Fig. 4 LncRNA-mRNA co-expression network analysis. (**A**) Co-expression network of DElncRNAs and DEmRNAs. (**B**) Top five mRNA nodes based on connectivity degree. (**C**) Top five LncRNA nodes based on connectivity degree

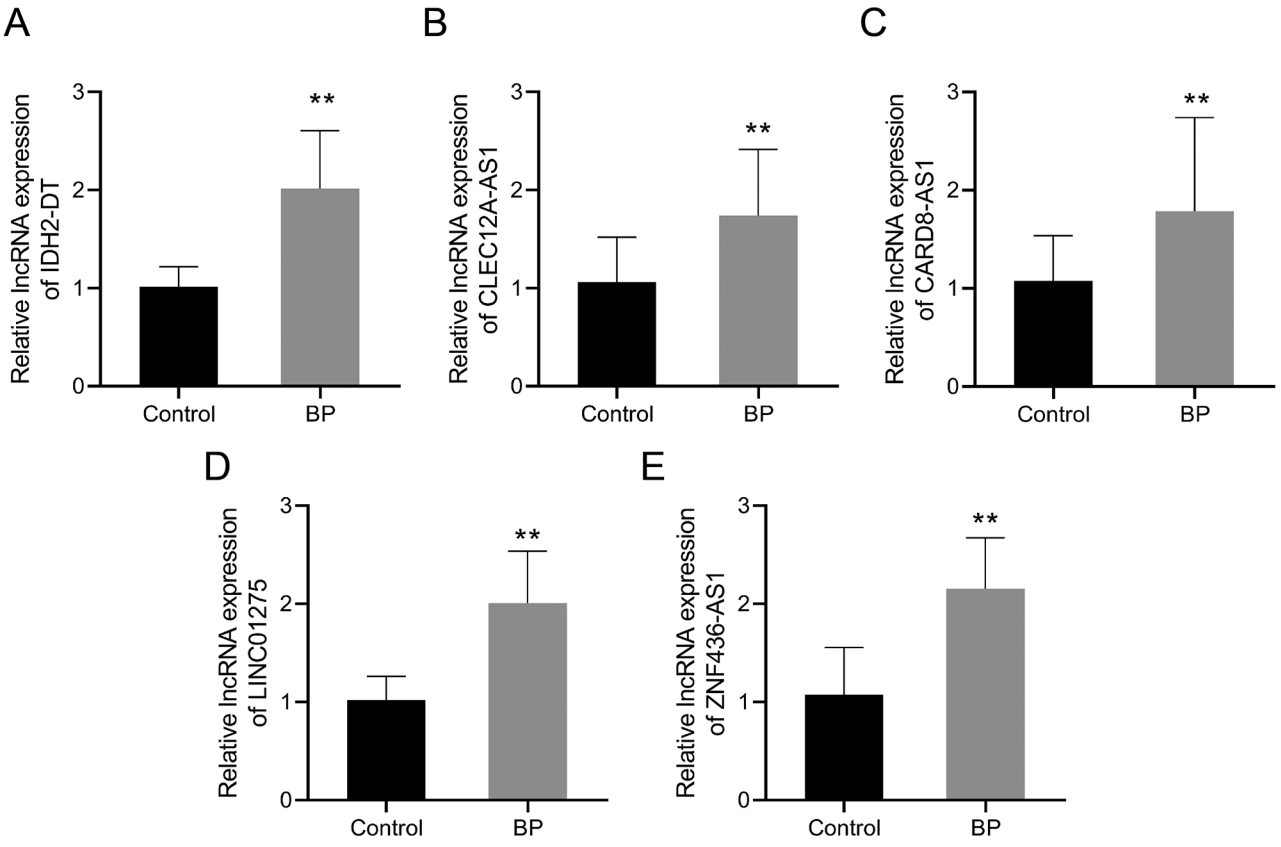


Fig. 5 Validation of RNA-seq results by qRT-PCR. Expression levels of (**A**) IDH2-DT, (**B**) CLEC12A-AS1, (**C**) CARD8-AS1, (**D**) LINC01275, and (**E**) ZNF436-AS1 were significantly upregulated in the butylphthalide-treated group (BP) compared to the Control group. $^{**}P < 0.01$ vs. Control group

malignant peripheral nerve sheath tumors [24]. Further molecular docking analysis confirmed the high binding affinity of butylphthalide to MT-CO1, SETD9, and ZNF718, suggesting these key proteins not only play a central role in signaling pathway regulation but may also serve as potential targets for the action of butylphthalide. However, the specific mechanisms by which butylphthalide regulates the expression of lncRNA and mRNA remain to be further elucidated.

Although this study reveals the potential molecular mechanisms of butylphthalide in ischemic stroke, there are still limitations. First, the functional validation of key lncRNAs and mRNAs through in vitro and in vivo experiments is lacking. Second, RNA-seq was conducted solely on peripheral blood samples, and the relatively small sample size may not fully reflect the molecular changes in brain tissue. Additionally, the scope of the molecular docking analysis is limited, potentially overlooking other potential targets. Future research should integrate functional experiments, expand sample sources, and broaden the molecular exploration to further validate and refine the underlying mechanisms.

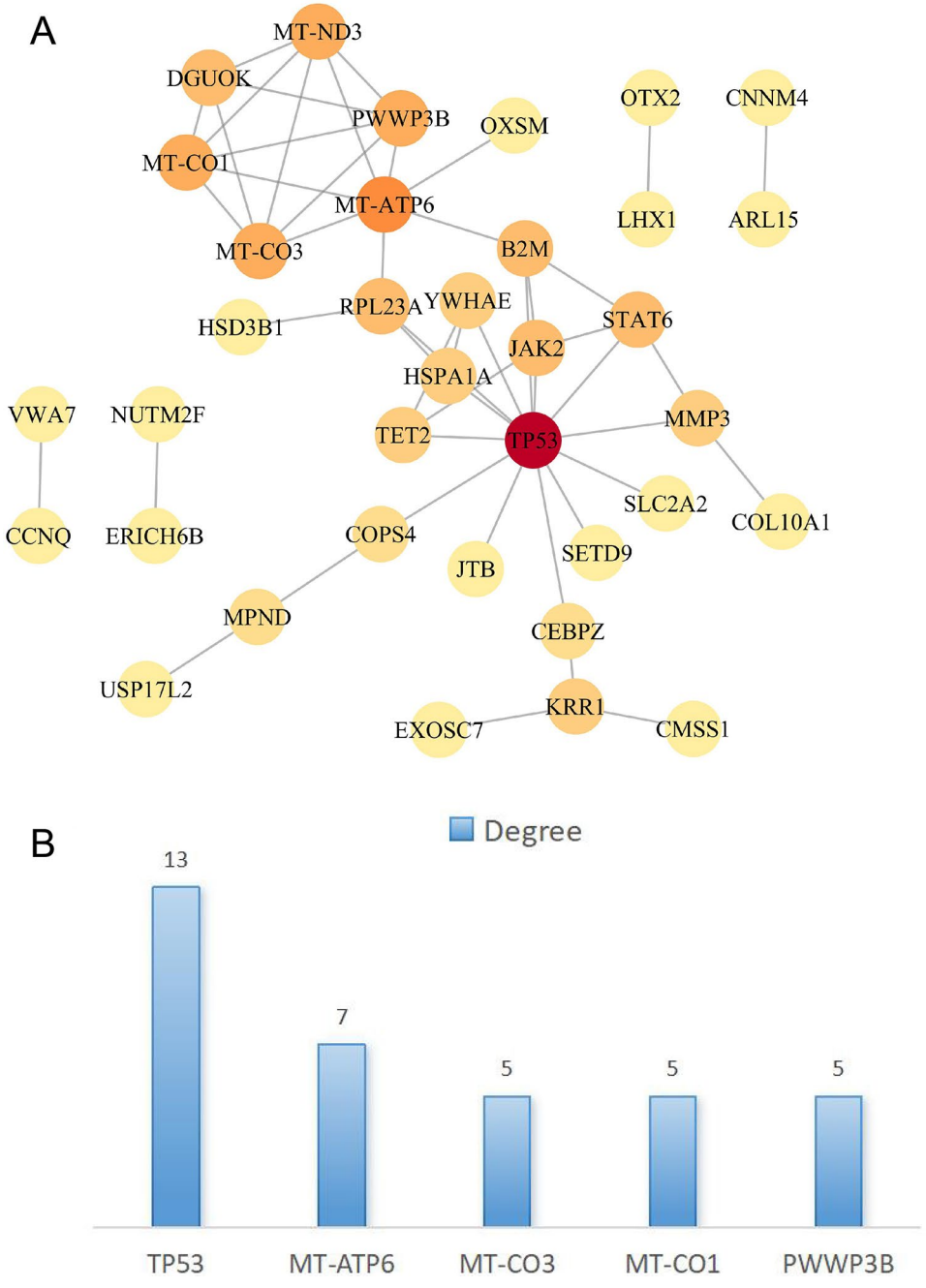


Fig. 6 PPI network analysis of DEGs. (**A**) PPI network highlighting interactions among key genes. (**B**) Top five hub genes ranked by degree centrality

A

	TP53	MT-ATP6	MT-CO3	MT-CO1	PWWP3B	SETD9	ZNF718	AOC2	MPND	ODF1
Butylphthalide	-5.1	-6.2	-6.4	-7.1	-5.6	-7.5	-7.1	-6.6	-6.7	-5.8

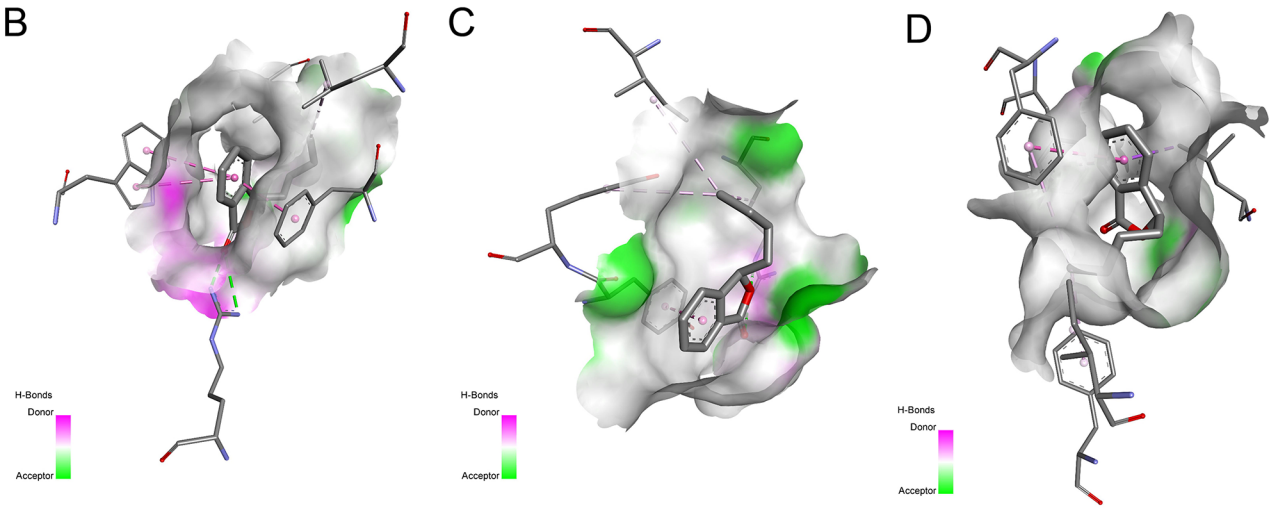


Fig. 7 Molecular docking analysis of butylphthalide. (**A**) Binding energies of butylphthalide with the top five mRNA nodes. (**B**-**D**) Visualization of docking between butylphthalide and (**B**) MT-CO1, (**C**) SETD9, and (**D**) ZNF718

Conclusion

This study reveals the potential molecular mechanisms of butylphthalide in ischemic stroke through RNA-seq analysis and molecular docking techniques, constructing an lncRNA-mRNA co-expression network and identifying key targets. Butylphthalide may exert therapeutic effects by regulating pathways related to energy metabolism and neuronal development. These findings provide novel insights into the mechanism of butylphthalide and its potential therapeutic targets.

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Author contributions
Study concept and design: Y.A., L.H.; Analysis and interpretation of data: J.L., Z.C.; Drafting of the manuscript: Y.A., L.H., J.L.; Critical revision of the manuscript for important intellectual content: J.C., B.W., Q.Z.; Statistical analysis: Z.C., J.C., B.W., Q.Z.; Study supervision: all authors; all authors have read and approved the manuscript.

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Data availability
The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethical approval and consent to participate
This study was approved by the Yiyang Central Hospita Ethics Committee (NO.2022-046), and the study adhered to the principles outlined in the Declaration of Helsinki, ensuring compliance with relevant guidelines and regulations. Informed consent was obtained from all participants, who voluntarily agreed to take part in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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