



A ferroptosis-related ceRNA network for investigating the molecular mechanisms and the treatment of neonatal hypoxic-ischemic encephalopathy

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Background: Neonatal hypoxic-ischemic brain damage (HIBD) is a clinical syndrome causing brain injury in newborns with obscure etiology. Increasing evidence suggests that ferroptosis plays a role in HIBD. This study aimed to clarify the key ferroptosis-related genes (FRGs) of HIBD, construct a long non-coding RNA-microRNA-messenger RNA (lncRNA-miRNA-mRNA) network, and further investigate the pathogenesis of HIBD.

Methods: Gene expression data were downloaded from the Gene Expression Omnibus and FerrDb databases. The differentially expressed lncRNAs and FRGs were screened, and the related miRNAs and mRNAs were predicted. The obtained mRNA was intersected with the differentially expressed FRGs (DE-FRGs) to identify the key DE-FRGs. Cell-type Identification by Estimating Relative Subsets of RNA Transcripts method was applied to analyze the immune cell infiltration level and the relationship between key genes and immune cells.

Results: Gene differential expression analysis revealed that 1,178 lncRNAs, 207 miRNAs, and 647 mRNAs were differentially expressed in the blood of HIBD patients in comparison to healthy controls. The correlations of the lncRNAs, miRNAs, and mRNAs lead to the establishment of a competing endogenous RNA (ceRNA) network associated with ferroptosis in HIBD. Further validation using an external dataset and quantitative real-time polymerase chain reaction (PCR) analysis of brain tissues from hypoxic-ischemic encephalopathy rats confirmed the expression patterns of three key genes, including *HMOX1*, *MYCN*, and *QSOX1*. Meanwhile, the three key genes were closely correlated with the infiltration of multiple immune cells and might affect the function of HIBD regulatory genes such as *CPT2* and *GCK*. In addition, drug prediction suggested that four drugs, including cephaline, emetine, mestranol, and sulmazole, might alleviate HIBD.

Conclusions: Our study established a ceRNA network, identified three key genes, and predicted four drugs that are associated with ferroptosis in HIBD, which provides new ideas for the investigation of the disease mechanisms and might facilitate the diagnosis and treatment of the disease.

Keywords: Hypoxic-ischemic brain damage (HIBD); ferroptosis; bioinformatics; newborn; competing endogenous RNA (ceRNA)

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Introduction

Neonatal hypoxic-ischemic brain damage (HIBD) represents hypoxic-ischemic brain injury in newborns due to perinatal asphyxia and is one of the most common causes of neurological disease and death in newborns, occurring in 2–3 per 1,000 births (1,2). Unfortunately, specific and effective treatments for this disease have not yet been developed (3). Despite the timely adoption of mild hypothermia treatment, a number of newborns with severe asphyxia may have adverse outcomes, such as cerebral palsy, intellectual disability, epilepsy, and even death. Moreover, adverse outcomes lead to a considerable socio-economic burden. Hence, it is crucial to discover an effective method for the treatment of HIBD (2,4).

The pathophysiological mechanism of HIBD has not fully been deciphered. According to recent data, the development of HIBD can be promoted by various factors, such as disrupted metabolism and a protracted inflammatory response (5). These factors can lead to different types of cell death, a complex process closely related to the development

and maintenance of body homeostasis and closely linked to the occurrence, development, and prognosis of various diseases (6). Iron death, also known as ferroptosis, is a unique form of programmed cell death (PCD) characterized by iron accumulation and oxidative stress with distinct morphological, biochemical, and genetic features compared to other types of PCDs such as apoptosis, necrosis, and autophagy (7). The morphology changes of ferroptotic cells mainly include mitochondrial atrophy, increased membrane density, and reduction or disappearance of mitochondrial ridge (7-10). The most relevant biochemical characteristics include increased reactive oxygen species (ROS), decreased intracellular glutathione peroxidase 4, accumulation of lipid peroxides, and perturbations of iron metabolism (9). In the fight against human diseases, including HIBD, many types of immune cells, such as neutrophils, T and B cells, macrophages, natural killer (NK) cells, and dendritic cells, operate as key factors. The function of immune cells is regulated by cell death. Some researchers have found that ferroptosis is essential for regulating the functions and numbers of immune cells, whereas ferroptosis in non-immune cells may induce the release of damage-associated molecular patterns (DAMPs), triggering an immune cell response (11). However, the function of ferroptosis in the pathogenesis of HIBD is unclear. Therefore, exploring the potential role and mechanism of ferroptosis may provide a new strategy and approach to preventing and treating such patients.

The non-coding RNA (ncRNA) is a non-protein-coding transcript containing hidden regulatory signals for gene expression and disease progression. Based on the length, the ncRNAs can be arbitrarily defined as either long non-coding RNAs (lncRNAs, at least 200 nucleotides) or smaller non-coding RNAs such as transfer RNA, microRNA (miRNA) and piwi interacting RNAs (12-14). MiRNAs and lncRNAs participate in many immune response processes based on the modulation of the immune response genes (15). The hypothesis of competing endogenous RNA (ceRNA) revealed a new mechanism of RNA interaction. lncRNA may compete for miRNA-messenger RNA (miRNA-mRNA) binding, thus affecting gene expression mediated by miRNA (16). However, little is known about the expression patterns and the interaction network of ceRNA in HIBD.

Highlight box

Key findings

- We have established a competing endogenous RNA (ceRNA) network.
- We have identified three key genes.
- We have predicted four drugs associated with ferroptosis in hypoxic-ischemic brain damage (HIBD).

What is known and what is new?

- There are studies on iron death in HIBD, but there are few studies on the mechanism of iron death.
- This manuscript mainly includes identifying key apoptosis-related genes of HIBD, constructing a lncRNA-miRNA-mRNA network, and further exploring the pathogenesis of HIBD.

What is the implication, and what should change now?

- This paper promotes the further mechanism research of HIBD. We further explored the potential molecular mechanisms of key genes that affect the progression of HIBD, bringing some new ideas for the diagnosis and treatment of HIBD.
- A large number of lncRNAs and miRNAs have been identified in this study, and their expression in HIBD and the established ceRNA network need to be further verified.

In the present study, we performed a bioinformatic-based analysis of the microarray data of HIBD samples from the Gene Expression Omnibus (GEO). We identified many differentially expressed lncRNAs, miRNAs, and mRNAs, which resulted in establishing a ceRNA network associated with ferroptosis in HIBD. Moreover, our analysis identified three key genes that may play a pivotal regulatory role in modulating ferroptosis and immune cell infiltrations via multiple signaling pathways in this disease. In addition, the drug prediction analysis indicated that four drugs might have a role in ameliorating HIBD-related brain injuries. The ceRNA network and key genes identified in our study might shed new light on the studies of molecular mechanisms and new drug development for HIBD. We present this article in accordance with the ARRIVE reporting checklist (available at <https://tp.amegroups.com/article/view/10.21037/tp-23-596/rc>).

Methods

Data download

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Data were downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/info/datasets.html>). The series matrix file of GSE121178 was obtained from the GEO database, and the annotation platform was GPL22120, which contained six samples, including a control group (n=3) and a case group (n=3). The series matrix file of GSE112137 was obtained from the GEO database, and the annotation basis was GPL20301, which contained 16 samples, including a control group (n=8) and a case group (n=8). The 431 ferroptosis-related genes (FRGs) in this study were obtained from the FerrDb V2 database.

Differential expression analysis

Limma is an R package for analyzing differentially expressed genes (DEGs) to identify genes that exhibit obvious differential expression between cases and controls. Data on the molecular mechanisms of neonatal ischemic hypoxic encephalopathy were analyzed by using limma, and DEGs were identified between the two sample groups. The DEGs screening conditions were $|\log \text{fold change (FC)}| > 1$ and $P < 0.05$. A differential gene volcano map and heat map were drawn.

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

In order to determine biological functions and pathways involved in disease development, Metascape database (<https://metascape.org/>) was applied for annotation and visualization; GO analysis and KEGG pathway analysis were conducted for specific genes.

Construction of the lncRNA-miRNA-mRNA network

Recently, ceRNA has attracted much academic attention. It represents a new mode of gene expression regulation. The ceRNA regulatory network is more refined, involving abundant RNA molecules; it includes mRNA, pseudogene encoding genes, miRNA, and lncRNA, among others. miRcode (<http://www.mircode.org/>) is a popular database for querying lncRNA and miRNA relationships. It covers lncRNA genes with a length of more than 10,000. In this study, we used the miRcode database to predict lncRNA-miRNA interaction pairs. Moreover, three databases, miRDB (<http://www.mirdb.org/>), miRTarBase (<https://mirtarbase.cuhk.edu.cn/>), and TargetScan (https://www.targetscan.org/vert_80/), were combined to analyze the interaction of the two kinds RNA. Targeted mRNAs identified by two of the three databases were selected for subsequent research. Then, lncRNA-miRNA interaction and mRNA-miRNA interaction were combined to establish a lncRNA-miRNA-mRNA network, which was visualized by Cytoscape (<https://cytoscape.org/>).

Analysis of immune cell infiltration

To assess the impact of genes on immune infiltration, the Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) method was applied to determine immune infiltration level, and the corrplot package in R was used to analyze the interaction association in immune cells and analyzed the influence of the interaction association. The vioplot package was used to plot the relative content of immune cells, and Pearson analysis was performed for gene expression and immune cell content.

Gene set enrichment analysis (GSEA)

The GSEA method was applied to rank genes based on how

differently they were expressed in two groups, and we then checked if the predefined gene sets were enriched according to the list. In this study, by comparing the differences in signaling pathways between the GSEA group and the low-expression group, the molecular mechanism of core genes related to such disease was discussed. The number of replacements was set to 1,000.

Transcriptional regulation analysis of key genes

In the present research, the R package “RcisTarget” was applied to predict transcription factors (TFs) based on motif. The normalized enrichment score (NES) for motifs depends on the total motifs. Except for the motifs annotated by the source data, we inferred further annotated files. The first step in estimating the overexpression of each motif was to calculate the area under the curve (AUC) of every motif-motif set pairs. This was calculated by the recovery curve of the sequence based on the gene set. The motif NES was determined according to the AUC distribution.

Connectivity Map (CMap) drug prediction

The CMap is a gene expression profile database based on intervention gene expression developed by the Broad Institute. It is mainly used to reveal functional associations of small molecule compounds, genes, and disease states. It contains gene chip data before and after 1,309 small molecule drugs are treated with five human cell lines. Treatment conditions are varied, including different drugs, different concentrations, different treatment duration, and so on. In this study, the differentially expressed genes of the disease were used to predict the targeted therapeutic drugs for the disease. The differentially up-regulated and down-regulated mRNAs were uploaded to the CMap database, and L1000 was selected as the background set to predict the potential therapeutic drugs for the disease.

Neonatal HIBD in an animal model

Our model references the Rice-Vannucci rat model (17). A protocol was prepared before the study without registration. All animal experiment procedures were approved by the Ethics Committee of the Nantong University (approval No. S20220321-008) and were conducted according to the institutional animal care and use guidelines for the care and use of animals. The newborn rats were fed freely by their mothers. The conditions were maintained with a room

temperature of 24±2 °C and a 12-hour light/dark cycle. Rats have free access to food and water. Both male and female pups received the HIBD model. Neonatal Sprague-Dawley (SD) rats (12–17 g, 7 days old) were purchased from the Experimental Center of Nantong University. Six newborn rats were randomly divided into the HIBD group and sham operation control group, with 3 rats in the HIBD group and three rats in the sham operation control group. In this study, a blind method was used to take pup brains and do polymerase chain reaction (PCR). The 7-day-old SD rats in the HIBD group were anesthetized with isoflurane, then the rats were fixed in supine position, the left common carotid artery was exposed, separated from nerves and veins, ligation with 4-0 surgical line, and finally sutured to allow the animals to recover. After surgery, the rats were placed back with their mothers for 1 hour and then placed in a closed anoxic chamber (8% oxygen, 92% nitrogen, flow rate of 4 L/min) on a heating mat at 37 °C for 2.5 hours. After hypoxia, the surviving pups were returned to their mothers (there were no dead pups in the experiment). The same procedure was performed in the sham operation control group except that the exposed carotid artery was not lapped. After 24 hours, the rats were decapitated and their brains removed for subsequent tests.

Quantitative PCR

The total RNA of rat brain tissues was prepared by TRIzol, and reverse transcription was performed using a reverse transcription kit. The primers used in quantitative real-time PCR (qRT-PCR) test were as follows: HMOX1, F: 5'-GCCTCTTCTGTCACCCTGT-3', R: 5'-TCTGCTTGGCTTCCTCCC-3'; MYCN, F: 5'-ACCCAACATCAGCGGTTCG-3', R: 5'-CGTGACTGTCGGGTT TTCCA-3'; QSOX1, F: 5'-AGCCACTGCCCTAGATGTACC-3', R: 5'-TGAGGCCTGCGTTTtagttcc-3'. The initial denaturation period of the reaction was 95 °C for 10 minutes, then 95 °C for 15 seconds, 60 °C for 60 seconds, and 95 °C for 15 seconds, for a total of 40 cycles. Fluorescence was recorded at each annealing step. After each PCR run, the system automatically analyzed the data and obtained the amplification map. The expression levels of these genes were normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase complementary DNA. The results of the experiment were represented by relative quantitative analysis of $2^{-\Delta\Delta CT}$, and all experiments were repeated three times.

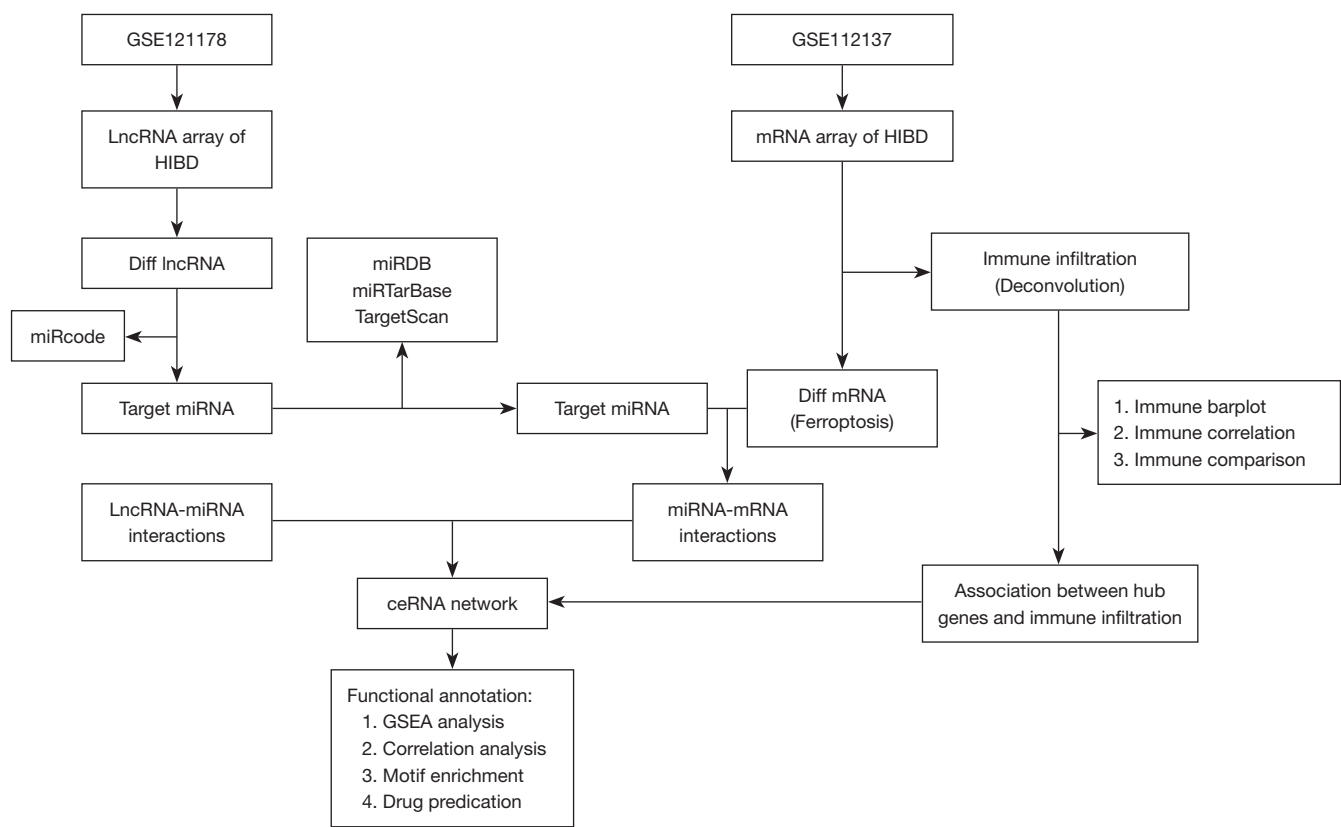


Figure 1 Experimental flowchart of this study. lncRNA, long non-coding RNA; HIBD, hypoxic-ischemic brain damage; Diff, different; miRNA, microRNA; mRNA, messenger RNA; ceRNA, competing endogenous RNA; GSEA, gene set enrichment analysis.

Statistical analysis

Most of the statistical analyses in this study were performed using R software (version 4.2.1; The R Foundation for Statistical Computing, Vienna, Austria). The Kolmogorov-Smirnov test was applied to data obtained by the qRT-PCR analysis of the mRNA expression in brain tissues of HIBD and control rats. $P < 0.05$ was considered statistically significant.

Results

Differential expression analysis of lncRNA associated with HIBD and prediction of miRNA

Figure 1 depicts the workflow of the current study. To identify lncRNAs inducing a regulatory function in the HIBD process, we downloaded the GSE121178 dataset from the GEO database and analyzed the DEGs of 6 blood samples, including a control group ($n=3$) and case group ($n=3$). We used the limma package to calculate

differential lncRNAs in the dataset. The differential gene judgment criteria were $P < 0.05$ and $|\text{LogFC}| > 1$. Up to 1,178 differential lncRNAs were screened, including 473 up-regulated lncRNAs and 705 down-regulated lncRNAs (Figure 2A, 2B). Subsequently, we used the miRcode database to predict the miRNA targets of the 1,178 differentially expressed lncRNAs. The analysis detected 207 lncRNA-related target miRNAs, which were visualized using Cytoscape (Figure 2C).

Differentially expressed ferroptosis-related mRNAs in HIBD

To investigate if the alterations in the expression of lncRNAs and miRNAs affect ferroptosis in HIBD, we screened the differential mRNAs in the GSE112137 dataset downloaded from the GEO database using the limma package. The differential gene judgment criteria were $P < 0.05$ and $|\text{LogFC}| > 1$. Up to 647 differential mRNAs were screened, including 264 up-regulated and

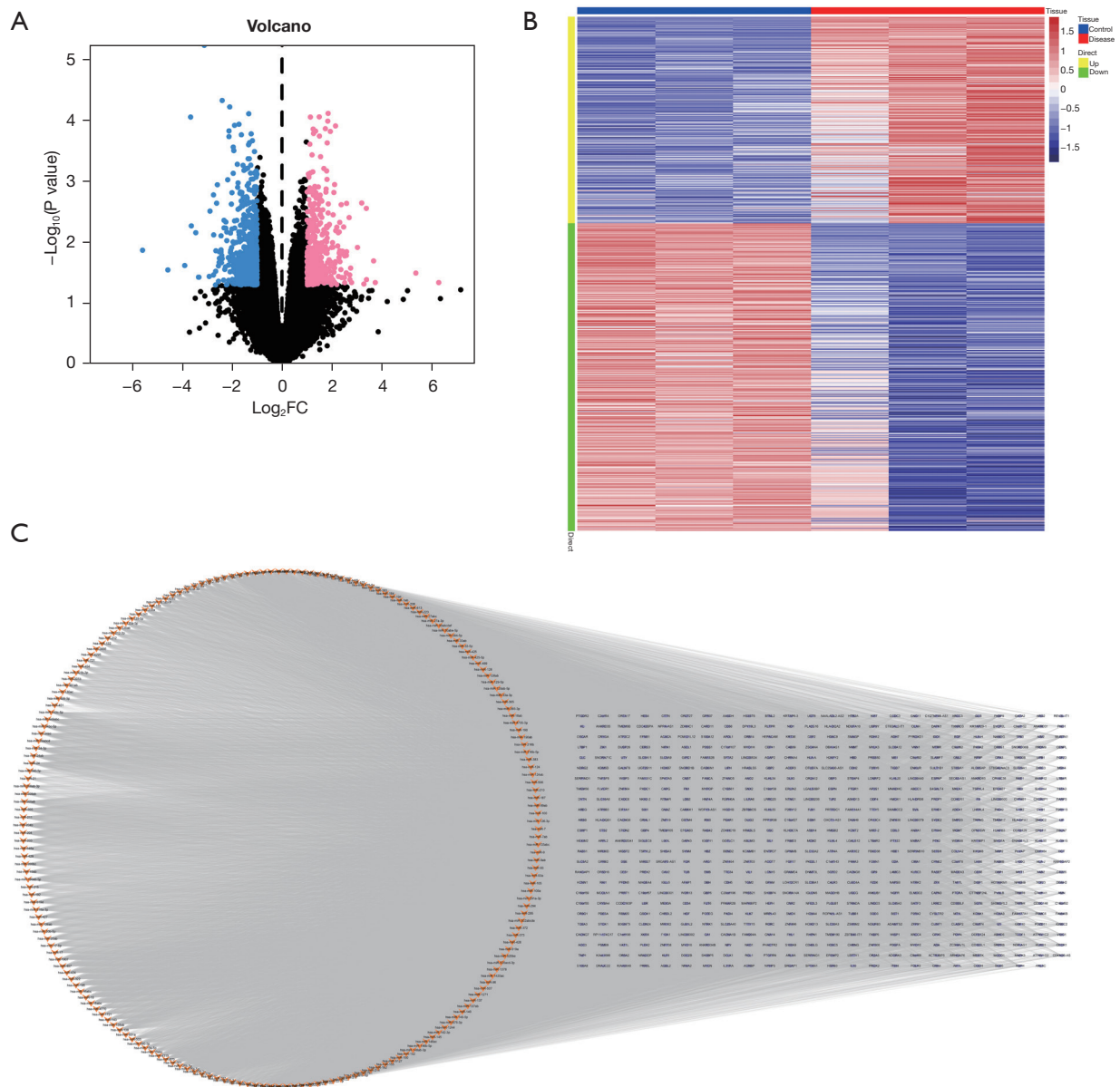


Figure 2 Differentially expressed lncRNAs and target miRNAs detected in samples of HIBD patients. (A) Volcanic plot of lncRNAs with differential expression between HIBD patients and healthy controls (the pink dots represent up-regulated genes, the blue dots represent down-regulated genes, and the black dots represent genes that are not significantly different). (B) Cluster analysis of the differentially expressed lncRNAs (blue represents the control group, red represents the disease group, yellow represents the up-regulated gene, and green represents the down-regulated gene). (C) The predicted target miRNAs of the differentially expressed lncRNAs in (A,B). FC, fold change; lncRNA, long non-coding RNA; miRNA, microRNA; HIBD, hypoxic-ischemic brain damage.

383 down-regulated genes (Figure 3A,3B). Subsequently, we intersected these 647 differential mRNAs with FRGs and found 11 intersection genes (Figure 3C). We further conducted a GSEA for the 11 differential ferroptosis mRNAs via Metascape, which indicated that the main

enrichment pathways of the differential iron death-related genes include ferroptosis, negative regulation of cell activation, epithelial cell proliferation, and regulation of cytoskeleton organization (Figure 4). These data indicated that the 11 FRGs might be involved in the development of

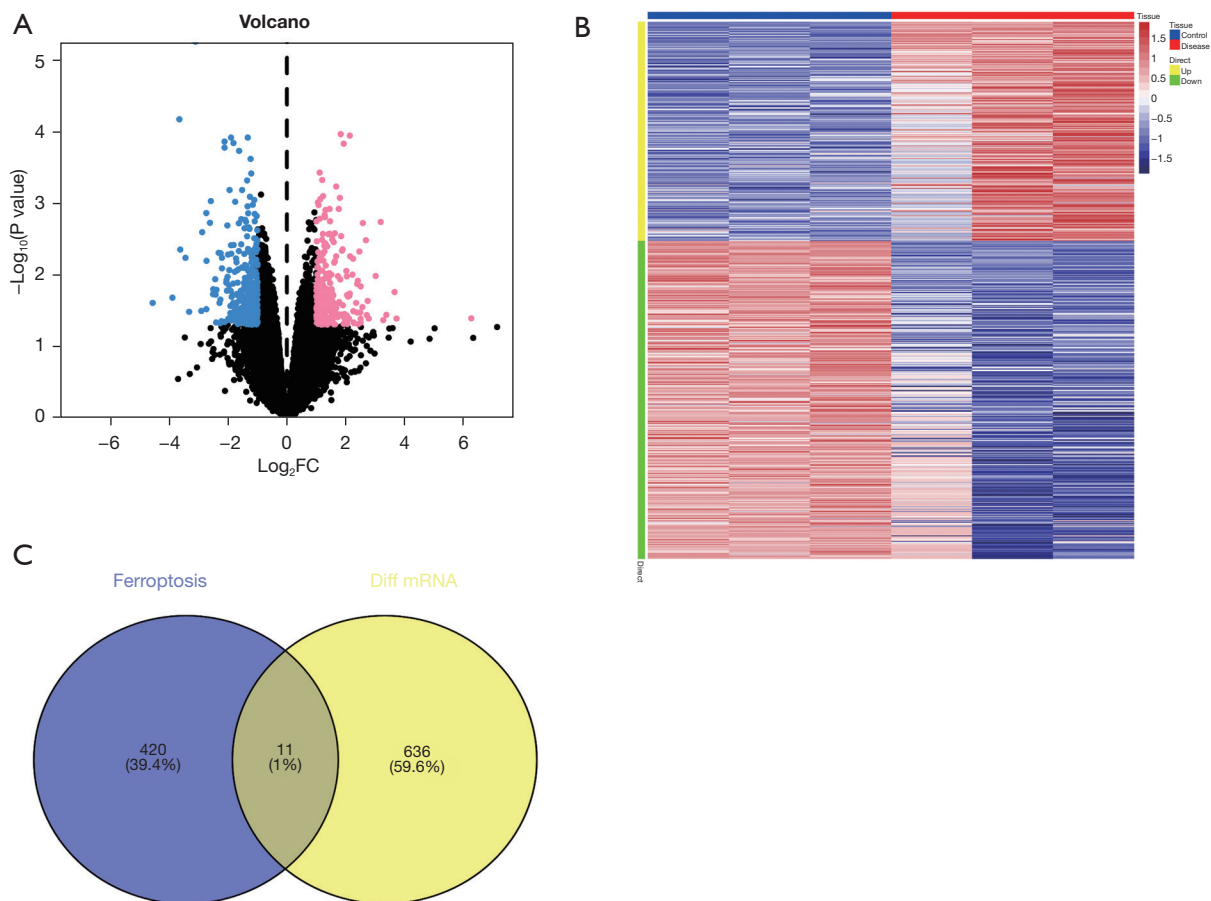


Figure 3 Differentially expressed mRNA and differentially expressed iron death genes in HIBD patient samples. (A) Volcano plot of differentially expressed mRNA (the pink dots represent up-regulated genes, the blue dots represent down-regulated genes, and the black dots represent genes that are not significantly different). (B) Cluster analysis of mRNA expression (blue represents the control group, red represents the disease group, yellow represents the up-regulated gene, and green represents the down-regulated gene). (C) The figure shows that there are 11 intersecting genes between differential mRNA and ferroptosis-related genes. FC, fold change; Diff, different; mRNA, messenger RNA; HIBD, hypoxic-ischemic brain damage.

HIBD, possibly by regulating ferroptosis via the enriched pathways.

Establishment of ceRNA network associated with ferroptosis and identification of key genes

We used the miRwalk, miRDB, and miRTarBase databases to predict the target mRNAs of the 207 miRNAs identified above and identified 8,612 mRNAs in the GSE121178 dataset. Finally, the intersection of 8,612 predicted mRNAs and 11 differential FRGs yielded four mRNAs (Figure 5A). The ceRNA network was successfully constructed and visualized via Cytoscape (Figure 5B). In addition, we downloaded the GSE112137 dataset from the GEO

database as an external dataset for subsequent analysis. The results indicated that three mRNAs, including HMOX1, MYCN, and QSOX1, were differentially expressed in HIBD samples compared to the normal controls (Figure 5C). Therefore, HMOX1, MYCN, and QSOX1 were selected as the key genes for subsequent analysis.

Validation of key genes in an animal model

To further verify the expression levels of the three key genes in HIBD, we constructed an animal model of the disease and collected brain tissue samples from the HIBD rats 24 hours after hypoxia treatment. The mRNA levels of the three genes (HMOX1, MYCN, and QSOX1) in these

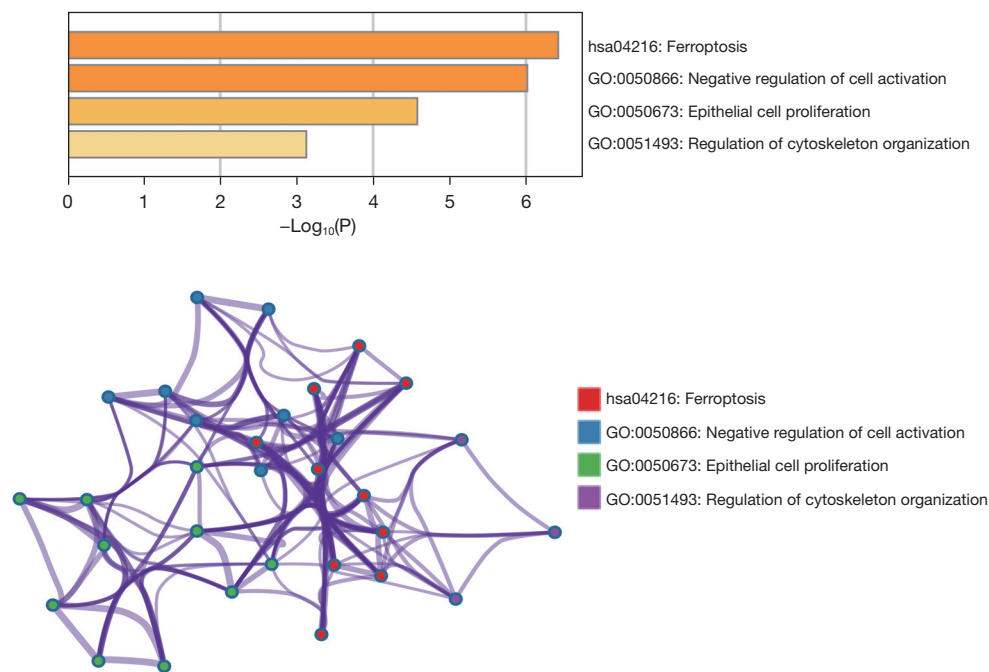


Figure 4 GSEA analysis of DE-FRGs. GO, Gene Ontology; GSEA, gene set enrichment analysis; DE-FRGs, differentially expressed ferroptosis-related genes.

samples were tested using qRT-PCR and compared with those in the brain tissues of the control rats. The results showed that the levels of *HMOX1* and *QSOX1* in HIBD rats were significantly higher than those in control rats, whereas the expression of *MYCN* in HIBD rats was significantly reduced compared to that in controls. These results were consistent with findings obtained in the above bioinformatic analysis (Figure 6A-6C).

The three key genes correlate with the infiltration of multiple immune cells

The immune microenvironment is mainly composed of immune cells, extracellular matrix (ECM), growth factors, and inflammatory factors, which affect the prognosis and the sensitivity of the disease therapy. Based on the association of key genes and immune infiltration, we further explored key genes' potential molecular mechanisms in affecting the progression of neonatal hypoxic encephalopathy. The distribution of the infiltrating immune cells and their correlations are shown in Figure 7A, 7B. Significant differences were observed regarding the infiltration of the memory B cells, monocytes, and resting mast cells between the disease and control

groups (Figure 7C). Subsequently, the association of key genes and immune cells was analyzed, and it was found that the three key genes were closely related to several immune cells. *HMOX1* was significantly positively related to gamma delta T cells and negatively related to resting mast cells and memory B cells (Figure 7D). *MYCN* was positively related to activated NK cells and resting mast cells and negatively correlated with monocytes (Figure 7E). *QSOX1* was positively correlated with monocytes and negatively related to resting mast cells (Figure 7F). In addition, we obtained the association between these key genes and immune factors from the TISIDB (<http://cis.hku.hk/TISIDB/>), including immunosuppressors, immunostimulators, and receptors (Figure 8). These analyses suggest that key genes are significantly associated with the level of immune cell infiltration and play a key role in the immune environment in HIBD.

The key genes regulate multiple signaling pathways

The three key genes may be involved in the development of HIBD by regulating some signaling pathways. Thus, we performed GSEA to analyze the specific signaling pathways that may be regulated by *HMOX1*, *MYCN*, and *QSOX1* and

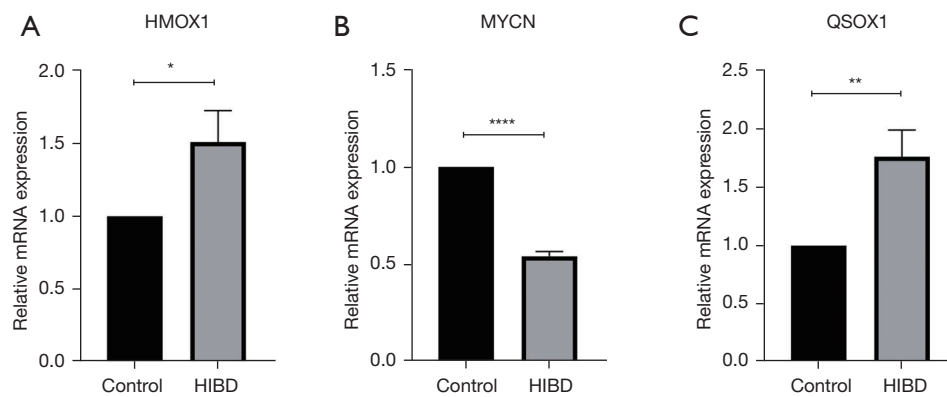


Figure 6 qRT-PCR was used to detect the expression levels of 3 key genes in HIBD tissues and normal controls 24 hours after hypoxia treatment ($n=3$, $P<0.05$). (A) *HMOX1*; (B) *MYCN*; (C) *QSOX1*. *, $P<0.05$; **, $P<0.01$; ****, $P<0.0001$. mRNA, messenger RNA; HIBD, hypoxic-ischemic brain damage; qRT-PCR, quantitative real-time polymerase chain reaction.

cisbp_M0451. All the enriched motifs and relevant TF results are displayed in *Figure 10*.

Analysis of the correlation between key genes and disease-regulating genes

To investigate the possible relationship between the key genes and the HBID-regulating genes, we obtained the disease-regulating genes from the GeneCards database (<https://www.genecards.org/>). We then analyzed the expression levels of the top 20 genes with the highest relevance score in different groups and found that the expressions of *SCN1A*, *PCDH19*, *CPT2*, *INS*, *GCK*, *CASR*, and *SCTAN1* were different between cases and controls (*Figure 11A*). As a result, it can be concluded that the expression levels of the three essential genes were closely related to those of several disease-related genes (*Figure 11B*). *QSOX1* and *CPT2* were positively correlated with significance ($r=0.976$), whereas a significant negative correlation between *HMOX1* and *GCK* was observed ($r=-0.993$). These data demonstrated that the key genes had significant correlations to HBID-regulating genes.

Potential drug prediction

Based on the above analysis, we attempted to predict the effectiveness of drugs for HBID treatment. The top 150 up-regulated mRNAs and the top 150 down-regulated mRNAs in HBID were used for drug prediction through the CMap database. The results showed that perturbations of gene expression induced by the drugs, including cephaeline,

mestranol, and sulmazole, were inversely related mainly to those triggered by the disease (*Figure 12*), suggesting that these drugs could alleviate the disease.

Discussion

The current treatment of HIBD is poorly effective, and great efforts are needed to improve its efficacy for this significant threat to newborn life (18). The current state of the art is mainly due to the unclear and incomplete understanding of the disease pathogenesis. A recent study has revealed that the development of HIBD is associated with ferroptosis, a unique form of cell death depending on iron and lipotoxicity (19). Studies have also shown that inhibiting ferroptosis can improve HIBD in neonates (20,21). It has been reported that ferroptosis is regulated by many cellular metabolic events, such as REDOX homeostasis, iron processing, mitochondrial activity, metabolism of lipids, sugars, and many disease-associated pathways (22). Increasing evidence demonstrates that ferroptosis is associated with cell damage in a variety of diseases, such as cancer, ischemia-reperfusion injury, stroke, neurodegeneration, myocardial infarction, acute kidney injury, and liver injury (23-26). However, the mechanism of ferroptosis in HIBD has not been deeply studied.

Our research seeks to identify ferroptosis-related biomarkers affecting neonatal HIBD, establish ceRNA networks, and explore their roles in HIBD immune cell infiltration. We screened 647 differentially expressed mRNAs through the GSE112137 dataset intersected them with FRGs, and identified 11 DEGs. Enrichment

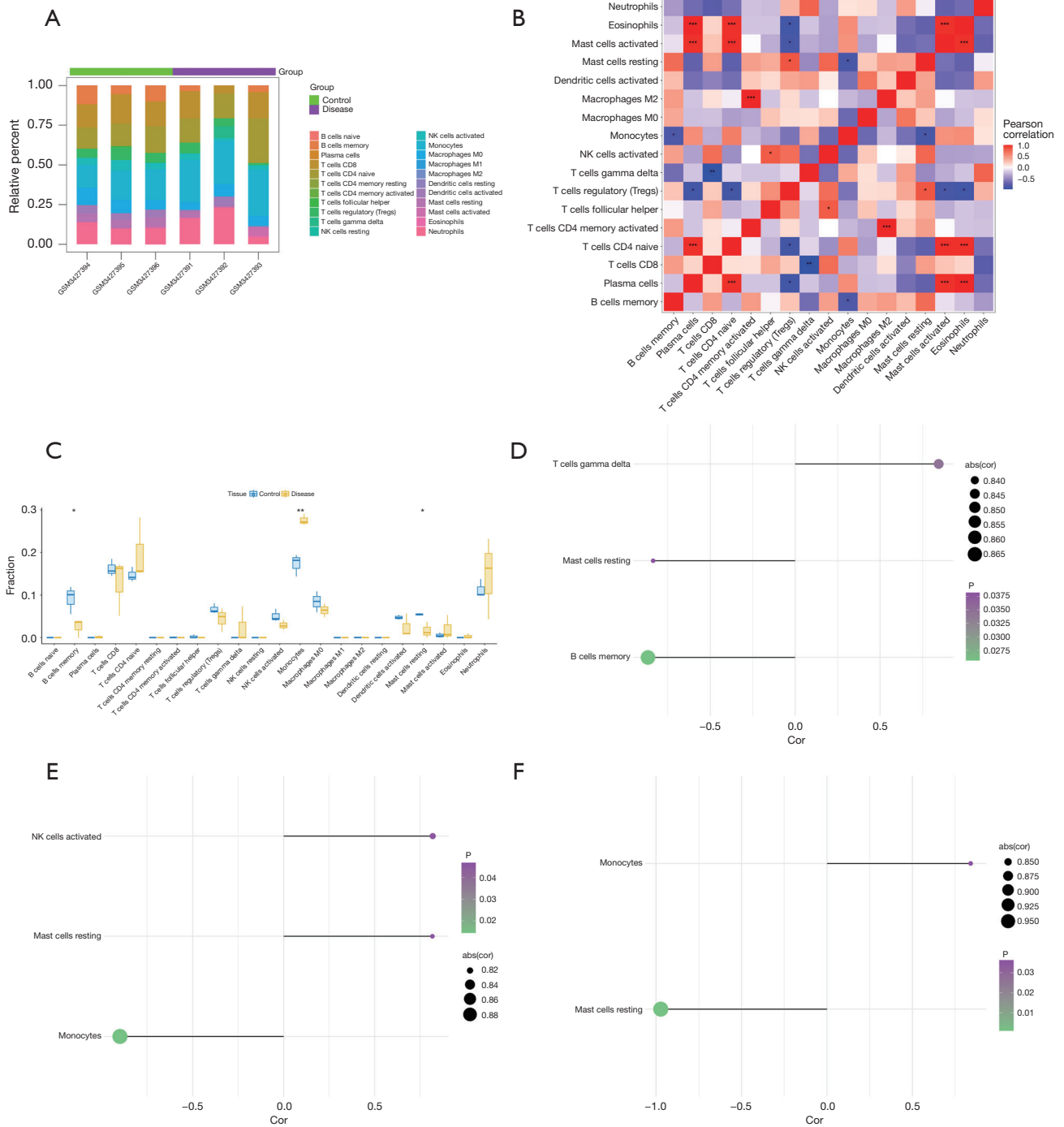


Figure 7 The 3 key genes correlated with the infiltration of multiple immune cells. (A) The ratio of 22 immune cells detected in each sample. (B) Correlation analysis between infiltrating immune cells, with red and blue colors indicating positive and negative correlation, respectively. (C) The different rates of immune cells between the HIBD group and control group. (D-F) The correlations between *HMOX1*, *MYCN*, or *QSOX1* with infiltrated immune cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NK, natural killer; abs(cor), absolute value (correlation); cor, correlation; HIBD, hypoxic-ischemic brain damage.

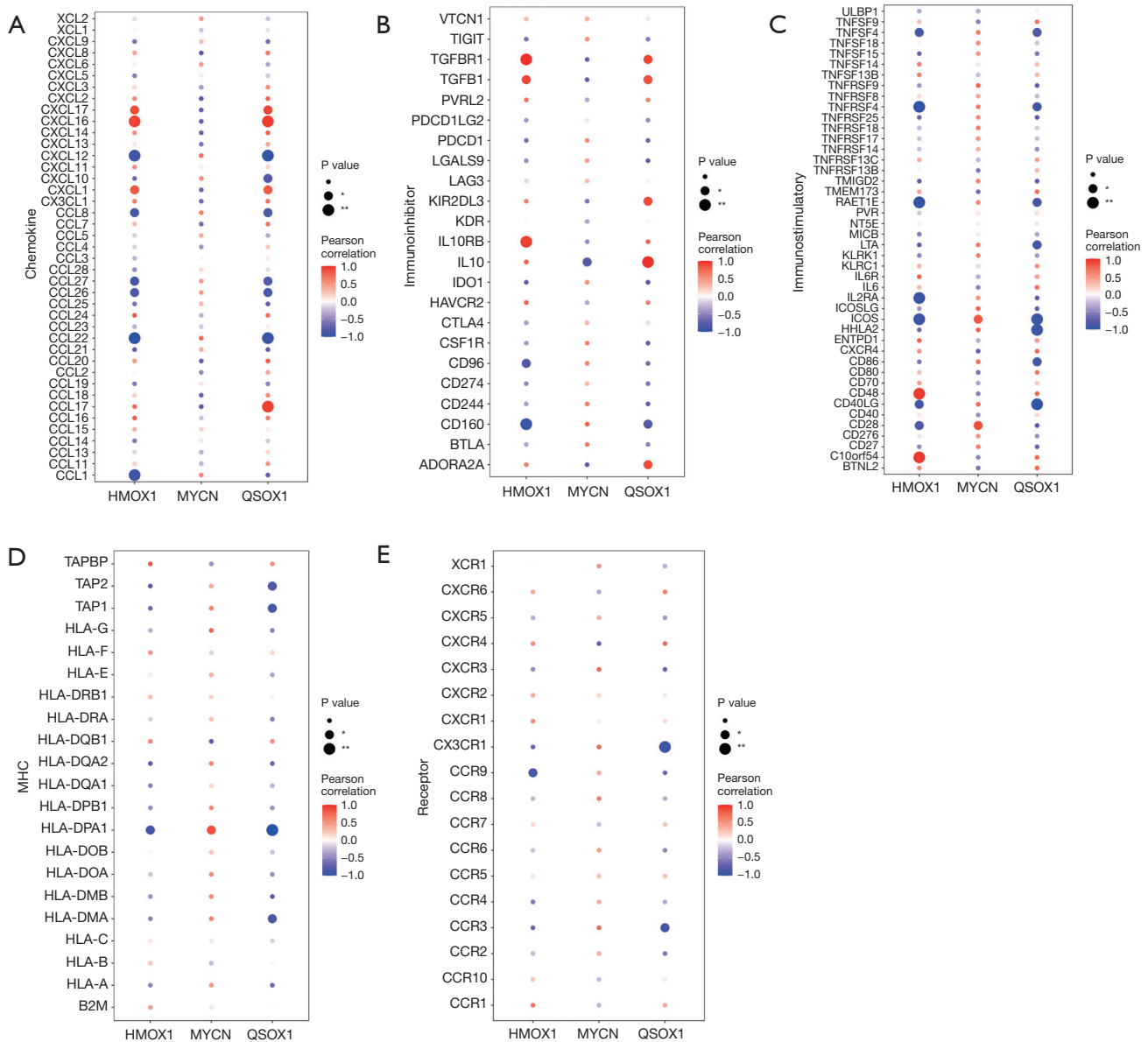


Figure 8 The correlations between the 3 key genes and different immune factors. (A) Chemokines; (B) immunosuppressive factors; (C) immunostimulatory factors; (D) MHC; (E) receptors. *, $P < 0.05$; **, $P < 0.01$. MHC, major histocompatibility complex.

analysis showed that the main enrichment pathways of these 11 genes were ferroptosis, negative regulation of cell activation, cytoskeletal tissue regulation, and epithelial cell proliferation. Moreover, we successfully constructed the ceRNA network through the GSE121178 dataset and intermixed the predicted mRNA with 11 differential ferroptosis genes to obtain four mRNAs. Through external verification, three key genes showed significant differences. Compared with the control group, the expression of

HMOX1 and *QSOX1* was higher in the HIBD group, whereas the expression of *MYCN* in the HIBD group was lower than that in the control group. We constructed an animal model of HIBD in which these three key molecules were validated using qRT-PCR, and the results were consistent with those of microarray analysis.

HMOX1, also known as heat shock protein 32, is an enzyme involved in the catabolism of heme induced by various pro-oxidation and inflammatory stimuli (27).

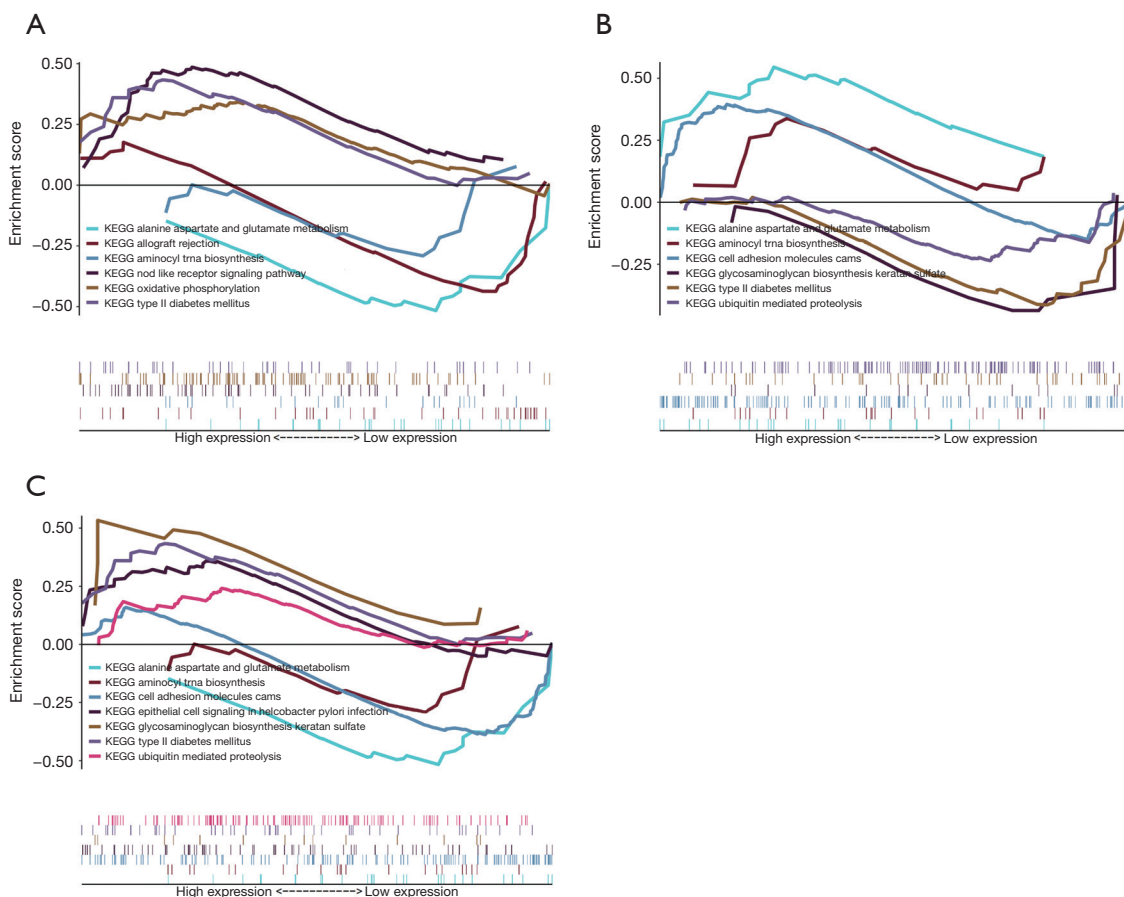


Figure 9 The 3 key genes enriched in multiple signaling pathways. (A) *HMOX1* enrichment signaling pathways; (B) *MYCN* enrichment signaling pathways; (C) signal pathways of *QSOX1* enrichment. KEGG, Kyoto Encyclopedia of Genes and Genomes.

It is protective in anti-oxidation, anti-apoptosis, anti-inflammation, and vascular relaxation (28). The expression of *HMOX1* contributes to mediating the resolution of inflammation, including neuroinflammation (29). In our study, the enrichment analysis revealed that *HMOX1* may be involved in various signaling pathways that influence the development of HIBD, such as the NOD-like receptor signaling pathway and oxidative phosphorylation. Notably, the most studied NOD-like receptor, NLRP3, has significantly higher activity in hypoxic-ischemic encephalopathy (HIE) rats (30). Using drugs to inhibit the inflammasome activity can significantly inhibit HIE-related brain damage (31). Thus, whether *HMOX1* can affect HIBD via regulating NOD-like receptors such as NLRP3 deserves further study.

MYCN is a TF of the MYC proto-oncogene family. It is involved in the basic process of controlling embryonic development (32). *MYCN* promotes cell proliferation,

growth, and apoptosis (33-35). A previous study has shown that *MYCN* is over-expressed in various pediatric cancers, including neuroblastoma, rhabdomyosarcoma, medulloblastoma, nephroblastoma, and retinoblastoma (36). However, until now, no study has described the relationship between this gene and HIBD. *QSOX1* is an enzyme that oxidizes mercaptan during protein folding, reducing molecular oxygen to hydrogen peroxide (37). Similarly, no study has shown its role in HIBD. The roles and relative contributions of *MYCN* and *QSOX1* in HIBD need further clarification in future studies. Our study also analyzed the upstream regulation and found that *HMOX1*, *MYCN*, and *QSOX1* are regulated by common mechanisms such as multiple TFs. Hence, we conducted an enrichment analysis to research these TFs and found that *GFI1* is the primary regulator of these three molecules.

Accumulating evidence shows that immune cells are closely related to the pathogenesis of HIBD (38). By using

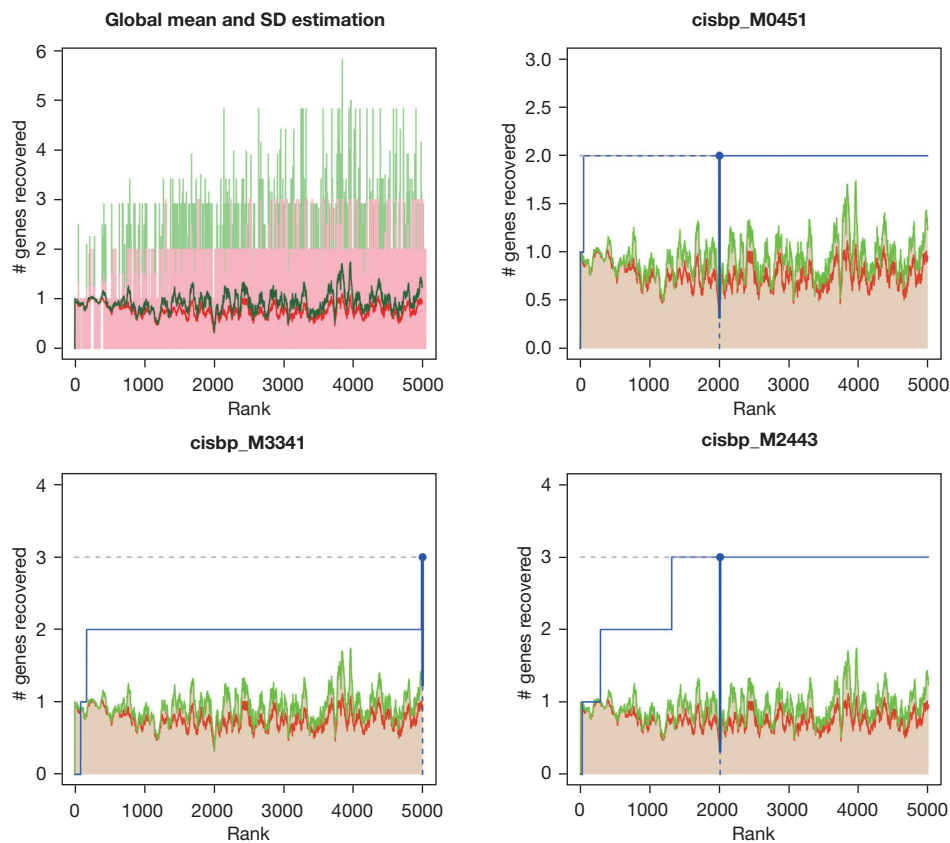


Figure 10 Analysis of transcriptional regulation of *HMOX1*, *MYCN*, and *QSOX1*. SD, standard deviation.

the CIBERSORT, we identified 22 immune cell types infiltrating the brains of HIBD patients. The proportion of monocytes in the HIBD group was high, whereas that of memory B cells and resting mast cells was low. Monocytes belong to the mononuclear phagocyte system and are involved in the immune responses following tissue injury and infection. A study has shown that monocytes promote acute inflammatory response, and monocyte infiltration has significant plasticity, pathological function, and lasting harmful effects in neonatal brain injury (39). At the same time, we verified the association between the three core genes (*HMOX1*, *MYCN*, *QSOX1*) and immune cell infiltration. Our results show that these three core genes correlate with immune cell infiltration and are closely related to gamma delta T cells, resting mast cells, memory B cells, and activated NK cells. These results further demonstrate the key function of the core genes and immune infiltration in the HIBD. Moreover, GSEA also revealed the specific signaling pathways of three core genes that may be involved in the HIBD process; this finding may be

considered highly beneficial for a deeper understanding of the pathogenesis of HIBD.

This study identified regulatory genes associated with HIBD through the Genecard database. Seven genes (*SCN1A*, *PCDH19*, *CPT2*, *INS*, *GCK*, *CASR*, and *SCTAN1*) differed between the two groups. *CASR*, a G-protein-coupled receptor cloned initially from the bovine parathyroid gland, maintains calcium homeostasis by regulating the secretion of parathyroid hormone (40,41). The results of Pak *et al.* showed that selective and persistent astrocyte-induced CaSR expression is a common feature of ischemic injury, suggesting a role of CaSR in ischemia-induced astrocyte response (42). We analyzed the correlation between three core genes and HIBD regulatory genes and revealed the close relationships between the genes. The mechanism of the three core genes and HIBD regulatory genes in HIBD is still unclear and needs further exploration.

The CMap database was used for drug prediction, and the results showed that the expression profiles of

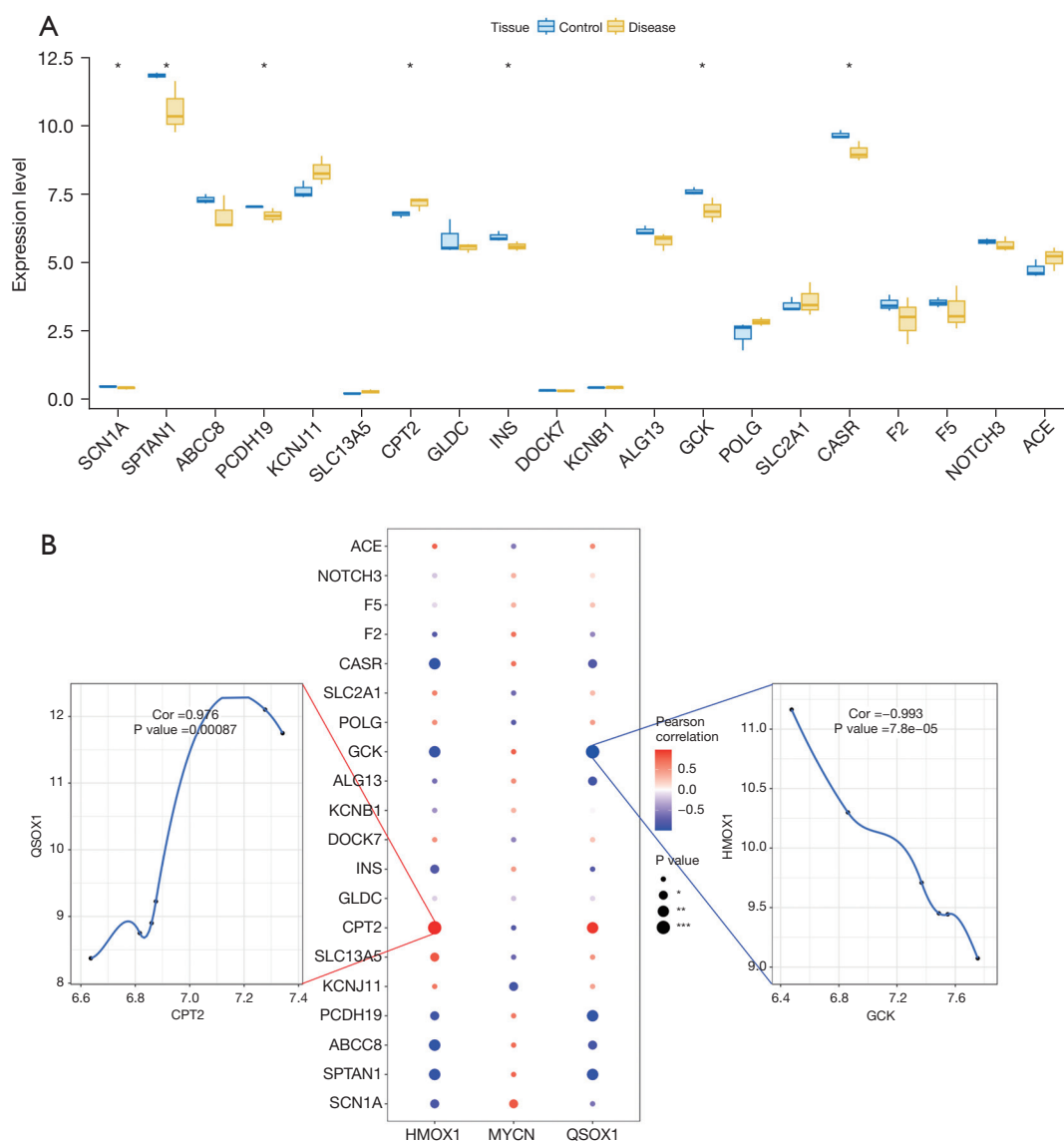


Figure 11 The key genes had significant correlations with HBID-regulating genes. (A) The differential expression of the disease-regulating genes between the HBID group and the controls. (B) Correlations between the 3 key genes and disease-regulating genes. *, P<0.05; **, P<0.01; ***, P<0.001. Cor, correlation; HBID, hypoxic-ischemic brain damage.

drug disturbances in cephaline, emetine, mestranol, and sulmazole were negatively related to the expression result of disease disturbances, suggesting that these drugs could alleviate the disease state. These findings provide new boosts for developing innovative, more effective drugs to treat HBID.

As described above, our study provides new insights for further investigation of the molecular mechanisms of HBID, even though the study has some limitations. Firstly, although we identified numerous lncRNAs and miRNAs,

their expression and the established ceRNA network in HBID should be confirmed by further studies. Secondly, there is a lack of data about the related clinical features and prognostic data in HBID patients; clinical relevance studies, prognostic analyses of the ceRNA network, and the key genes should be conducted in future studies. Thirdly, the number of patients in the database used in this study is relatively small, and thus, the results need to be further validated in a study involving a larger sample of HBID patients. Despite these limitations, our findings contribute

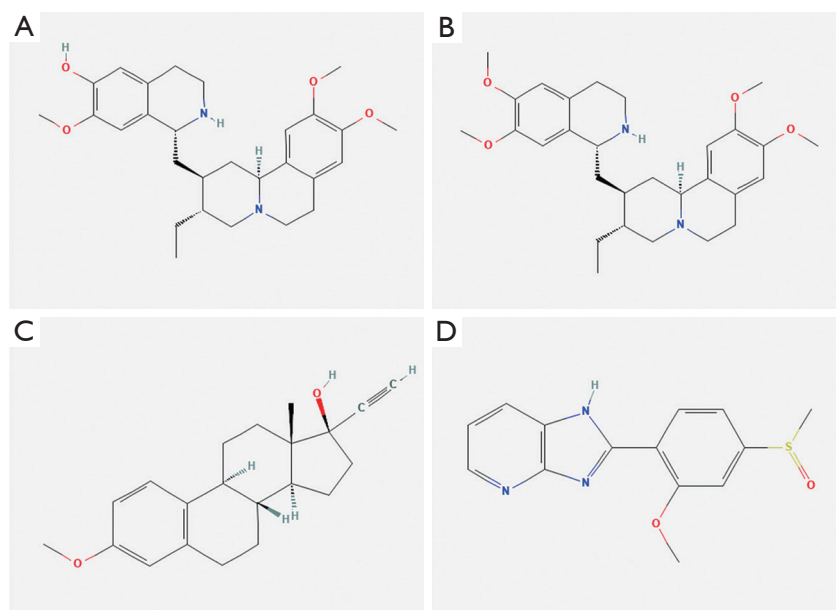


Figure 12 The predicted drugs associated with ferroptosis in HIBD. (A) Cephaeline; (B) emetine; (C) mestranol; (D) sulmazole. HIBD, hypoxic-ischemic brain damage.

to elucidating the function and mechanism of ferroptosis in HIBD.

Conclusions

In this study, after screening out 11 differential FRGs in HIBD, bioinformatics analysis identified three key genes associated with ferroptosis and the development of HIBD, including *HMOX1*, *MYCN*, and *QSOX1*. The study also established a ceRNA network in HIBD, which might promote further mechanistic studies of this disease. The potential molecular mechanism of key genes affecting the progression of HIBD was further explored and yielded some new ideas regarding the diagnosis and treatment of HIBD.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://tp.amegroups.com/article/view/10.21037/tp-23-596/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tp.amegroups.com/article/view/10.21037/tp-23-596/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All animal experiment procedures were approved by the Ethics Committee of the Nantong University (approval No. S20220321-008) and were conducted according to the institutional animal care and use guidelines for the care and use of animals.

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