

Exploring multiple biomarkers and constructing ferroptosis-associated competing endogenous RNA networks as dual targets in retinoblastoma

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Abstract. This study investigated the potential role of ferroptosis-associated competing endogenous RNA (ceRNA) networks in retinoblastoma (RB). In total, two independent RB gene expression datasets (GSE97508 and GSE208143) and a microRNA (miRNA) expression dataset (GSE208677) were retrieved from the Gene Expression Omnibus database. Differential expression analysis was conducted to identify ferroptosis-related (FR) differentially expressed genes (DEGs) in RB. A protein-protein interaction network for FR-DEGs was constructed using the STRING database, whereas hub genes were determined using the CytoHubba plugin. Reverse transcription-quantitative PCR (RT-qPCR) was used to verify the expression of *IDH2* and *CDKN2A*. Immune cell infiltration in RB was assessed using the CIBERSORT algorithm. Additionally, miRNA-targeting hub genes were predicted, where an FR miRNA-mRNA regulatory network was constructed. Finally, a ceRNA network and a drug-gene interaction network were constructed. A total of 584 FR-DEGs were identified from the GSE97508 dataset, revealing 135 upregulated and 449 downregulated genes. The intersection of FR-DEGs with FerrDb-extracted FRGs yielded 23 genes that were defined as core FR-DEGs for subsequent analysis. Functional and pathway enrichment analyses highlighted their involvement in iron and transition metal ion homeostasis, response to hypoxic and ischemic conditions and apoptotic processes. Hub genes (*CAVI*, *CDKN2A*, *EPAS1*, *IDH2*, *RBI* and *SLC2A3*) were identified and validated in an independent dataset, with RT-qPCR confirming *IDH2* and *CDKN2A* upregulation. Immune cell infiltration analysis indicated altered immune cell populations in RB, where an association analysis revealed associations between hub genes and immune cells. A comprehensive FR miRNA-mRNA regulatory

network and a ceRNA network were established, shedding light on additional layers of regulatory mechanisms in RB. Furthermore, the drug-gene interaction network consisted of 20 potential candidate drugs. In conclusion, the present integrated analysis offers a comprehensive understanding of the potential role in RB, providing valuable insights into the molecular mechanisms and potential therapeutic targets for this childhood ocular tumor.

Introduction

Retinoblastoma (RB) stands as the most prevalent intraocular malignant tumor in children, with an incidence ranging from 1/20,000 to 1/15,000, predominantly affecting children aged <3 years (1,2). Globally, it accounts for 2-4% of childhood malignant tumors, where there are 8,000-9,000 new cases of RB each year (3,4). RB can be typically classified into hereditary (35-45%) and non-hereditary (55-65%) types (5). The former frequently develops into bilateral or unilateral multifocal RB, characterized by mutations in the *RBI* gene, whilst the latter mainly consists of unilateral or single lesions, with leukocoria and strabismus (6). Ultrasonography is the preferred imaging method for diagnosing RB (7). Early detection and diagnosis are crucial for the management of RB in children. With the widespread implementation of genetic diagnosis for RB, novel avenues for treatment have been provided, offering potential for future clinical applications.

Ferroptosis is a type of regulated cell death that was discovered by Dixon *et al* (8) in 2012. It is characterized by intracellular iron overload, dependent on reactive oxygen species production and intracellular lipid peroxidation accumulation. Under electron microscopy, various changes, such as cell membrane rupture, decreased mitochondrial cristae and mitochondrial outer membrane wrinkling and rupture, can be observed (9). At present, pharmacological induction or inhibition of ferroptosis offers therapeutic potential for drug-resistant cancers, ischaemic organ injury and degenerative diseases linked to extensive lipid peroxidation (10). For RB, it has been reported that *TP53* gene mutations and *RBI* gene deletion are important causes of RB, both of which participate in ferroptosis (11). Therefore, ferroptosis may be involved in the pathogenesis of RB and represents a potential mechanism worthy of further investigation. Non-coding RNAs (ncRNAs) are becoming increasingly recognized for their role

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in regulating the expression of ferroptosis-related genes and influencing disease prognosis (12). They can be categorized into basic structural ncRNAs and regulatory ncRNAs based on their function (13). Amongst the ncRNAs implicated in ferroptosis regulation, regulatory ncRNAs, particularly microRNAs (miRNAs or miR), circular RNAs and long non-coding (lnc) RNAs, are the most prominent (14). Beyond directly influencing enzymes involved in iron and lipid metabolism, these regulatory ncRNAs participate in a wider regulatory network governing ferroptosis. Their dysregulation in RB also highlights their potential as novel biomarkers, paving the way for future therapeutic applications (15).

In the present study, a bioinformatics analysis of publicly available microarray datasets from RB tumor samples and control samples was performed. Differentially expressed genes (DEGs) were intersected with a list of ferroptosis-related (FR) genes (FRGs) to identify the FR-DEGs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analysis, protein-protein interaction (PPI) network and immune cell infiltration analyses were then performed to explore the potential functions and interconnections among these genes. miRNAs for these hub genes were then predicted, which were used further to predict relevant lncRNAs. An FR lncRNA-miRNA-mRNA competing endogenous (ce)RNA network was constructed, providing novel insights into the pathology and treatment of RB.

Materials and methods

Data collection. Fig. 1 illustrates the flowchart of the present study design. The microarray expression profiles for RB were obtained through the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) (16). GSE97508 represented mRNA expression profiles in GPL15207, including 6 RB tumor samples and 3 control samples. The validation dataset GSE208143 is based on GPL17077 and includes 27 RB tumor samples and 6 control samples. It was used for hub gene validation. GSE208677 represented miRNA expression profiles in GPL18358, which contains 25 RB tumors and 5 control pediatric retina samples. The FerrDb database (<http://www.zhounan.org/ferrdb/>) includes gene regulatory factors such as driver, suppressor, marker and unclassified regulatory factors.

Differential expression analyses. The raw data were background-corrected and normalized using the R package 'limma' (17). In the R software environment, the 'limma' package was utilized to identify DEGs in GSE97508, with a significance threshold set at adjusted $P < 0.05$ and $|\log_2 \text{fold change}| > 1$. As for the differentially expressed miRNAs (DEMs), the criteria were adjusted $P < 0.05$ and $|\log_2 \text{fold change}| > 2$. In addition, a Venn diagram was used to identify the overlapping sections of the DEGs and FRGs, revealing the critical FR-DEGs.

Enrichment analysis of FR-DEGs. The FR-DEGs were submitted to GO function enrichment analysis and the KEGG pathway enrichment analysis using the R package 'clusterProfiler' (18). A false discovery rate < 0.05 was considered statistically significant. The R package 'ggplot2' was adopted

to visualize the results (19). Furthermore, the FR-DEGs were submitted to Metascape (<http://metascape.org>) for supplementary KEGG analysis.

Screening and validation of hub genes. The Search Tool for the Recovery of Interacting Genes and proteins (STRING) online database serves as a valuable platform for the exploration and analysis of gene or protein interactions (<https://string-db.org>). A PPI network was constructed by selecting gene pairs with confidence scores > 0.4 . To determine the hub genes, the CytoHubba (version 0.1) plug-in of Cytoscape (version 3.7.1) software was employed (20). In total, 5 algorithms [BottleNeck, Degree, Edge Percolated Component (EPC), Maximal Clique Centrality (MCC) and Maximum Neighborhood Component (MNC)] were applied to calculate scores for the FR-DEGs and generated a Venn diagram to identify hub genes.

Furthermore, a validation was conducted using GSE208143. A bar chart illustrating the expression levels of hub genes in RB and normal tissues was generated using the R package 'ggpubr'. In the ROC curve analysis, the area under the curve (AUC) for each hub gene was calculated using the R package 'pROC' to evaluate their standalone diagnostic performance. Statistical significance was assessed using an unpaired t-test with a significance threshold of $P < 0.05$.

Immune infiltration analysis. The R package 'IOBR' was utilized to employ the CIBERSORT algorithm for detecting the expression of 22 different immune cell populations in the GSE97508 dataset (21). Box plots were then generated to compare the expression of various immune cell types between the RB group and the normal group. The association between hub genes and the immune cell populations was assessed using Spearman correlation analysis, where the results were visually presented using the R package 'ggplot'.

Prediction of miRNAs and construction of FR miRNA-mRNA regulatory network. The R package 'multiMiR' was utilized to predict the miRNAs for the hub genes (22). Subsequently, the predicted miRNA was filtered by overlapping them with the identified DEMs in the GSE208677 dataset. To further refine the selection, only miRNAs showing a significant negative correlation with the expression of their target hub genes in the GSE97508 dataset were retained for network construction. In addition, an FR miRNA-mRNA regulatory network was constructed and visualized using Cytoscape.

Prediction of lncRNA and construction of ferroptosis-related lncRNA-miRNA-mRNA ceRNA network. The Starbase online tool (<https://starbase.sysu.edu.cn/>) was utilized to input the three miRNAs negatively regulating hub genes and predict potential upstream lncRNAs involved in their interactions. To refine the focus, lncRNAs that overlapped with all three identified miRNAs were focused upon.

Predication of drug-gene interaction. To explore the interaction between drugs and genes, the Comparative Toxicogenomics Database (CTD; <http://ctdbase.org/>) was utilized to identify existing or potential relevant drugs. The selected hub genes with potential therapeutic relevance were queried in the CTD

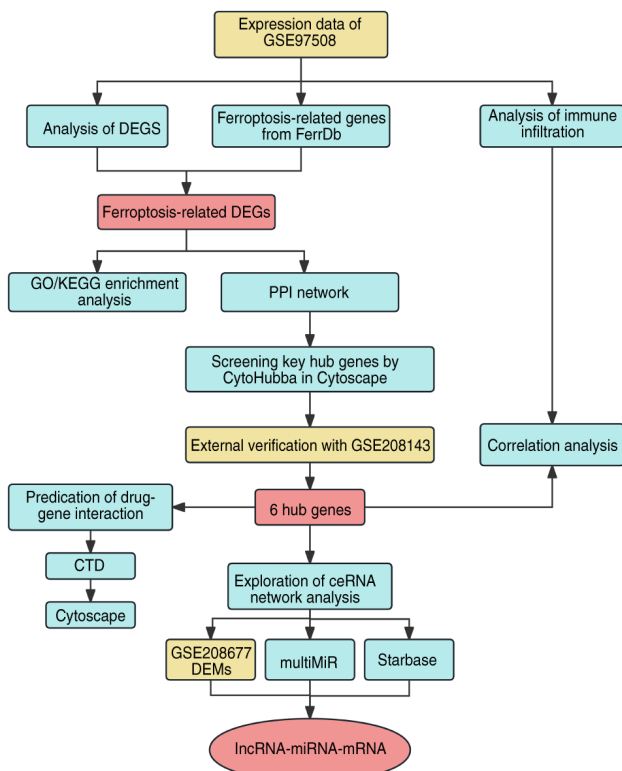


Figure 1. Workflow of the present study. DEGs, differentially expressed genes; PPI, protein-protein interaction; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; CTD, Comparative Toxicogenomics Database; ceRNA, competing endogenous RNA; DEMs, differentially expressed miRNAs.

to identify existing or potential drug-gene interactions, which were visualized using the Cytoscape software.

Cell lines and culture. The human retinal pigment epithelium cell line ARPE-19 and the human RB cell line Y79 were sourced from the American Type Culture Collection. ARPE-19 cells and Y79 cells were cultured at 37°C with 5% CO₂ in Dulbecco's Modified Eagle's Medium (cat. no. 12491015; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (cat. no. A5256701; Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml penicillin and 100 µg/ml streptomycin (cat. no. C0222; Beyotime Institute of Biotechnology).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from ARPE-19 and Y79 cells using the RNA isolater Total RNA Extraction Reagent (cat. no. R401-01; Vazyme Biotech Co., Ltd.). cDNA was synthesized with PrimeScript™ RT Master Mix (cat. no. RR036Q; TaKaRa Bio, Inc.) according to the manufacturer's instructions. qPCR was conducted using TB Green® Premix Ex Taq™ II (cat. no. RR820A; TaKaRa Bio, Inc.) according to the manufacturer's instructions. The qPCR thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. A melting curve analysis was conducted from 65 to 95°C to verify amplification specificity. Data were normalized to GAPDH expression using the 2^{-ΔΔC_q} method (23), with each experiment performed in triplicate. Primers used were as follows: *IDH2*

forward, 5'-CGCCACTATGCCGACAAAAG-3' and reverse, 5'-ACTGCCAGATAATACGGGTCA-3'; *CDKN2A* forward, 5'-GATCCAGGTGGGTAGAAAGGTC-3' and reverse, 5'-CCCCTGCAAACTTCGTCTCT-3'; and *GAPDH* forward, 5'-ACAACCTTTGGTATCGTGGAAGG-3' and reverse, 5'-GCCATCACGCCACAGTTTC-3'.

Statistical analysis. Statistical analysis was performed using R software (version 4.2.1). A two-tailed unpaired Student's t-test was used to determine the statistical significance of differences between two groups, where P<0.05 was considered to indicate a statistically significant difference. The correlation between hub genes and immune cells was assessed using the Spearman correlation coefficient, where corrections were applied using the Benjamini-Hochberg multiple testing correction method.

Results

Identification of ferroptosis-related DEGs. In total, 584 DEGs (135 upregulated and 449 downregulated genes) were obtained from the GSE97508 dataset using the R package 'limma' (Fig. 2A). Subsequently, an analysis was conducted by generating a Venn diagram to identify the intersection between the FRGs extracted from the 'FerrDb' database and the DEGs in GSE97508. The overlapping set, consisting of 23 genes, represents the FR-DEGs (Fig. 2B). The heatmap in Fig. 2C displays the standardized expression of FR-DEGs (5 upregulated and 18 downregulated). Fig. 2D displays the chromosomal positions of these 23 FR-DEGs, represented in a circular plot for visualization.

Function and pathway enrichment analysis of FR-DEGs. The 'clusterProfiler' package was used to conduct GO and KEGG functional analysis on the 23 FR-DEGs, aiming to gain a comprehensive understanding of their cellular functions and pathway involvement in RB. The enrichment results revealed significant involvement of FRGs in the regulation of 'iron ion homeostasis' and 'transition metal ion homeostasis', in the 'response to hypoxia' and 'response to ischemia', and in the modulation of 'epithelial cell apoptotic processes' (Fig. 3A). Furthermore, the genes exhibit enrichment in specific cellular component terms, emphasizing their roles in 'endocytic vesicles' and 'germ cell nucleus'. Molecularly, these genes demonstrated diverse transmembrane transporter activities, suggesting their influence on the transport of various ions and substances across cellular membranes. In the KEGG pathway enrichment analysis, a significant enrichment of genes related to iron death in the 'Ferroptosis' pathway was observed (Fig. 3B and C).

Identification and verification of hub genes. To investigate potential relationships among proteins encoded by FR-DEGs and identify hub genes, STRING was utilized to screen the PPI network of FR-DEGs (Fig. 4A). Subsequently, multiple topological analysis algorithms (BottleNeck, EPC, Degree, MCC and MNC) were utilized to predict and explore the top 10 significant hub genes in the PPI network. The intersection of these five algorithms led to the identification of *CAVI*, *CDKN2A*, *EPAS1*, *IDH2*, *RBI* and *SLC2A3* as the hub genes (Fig. 4B). Amongst them, *CAVI*, *EPAS1*, *RBI* and *SLC2A3* were

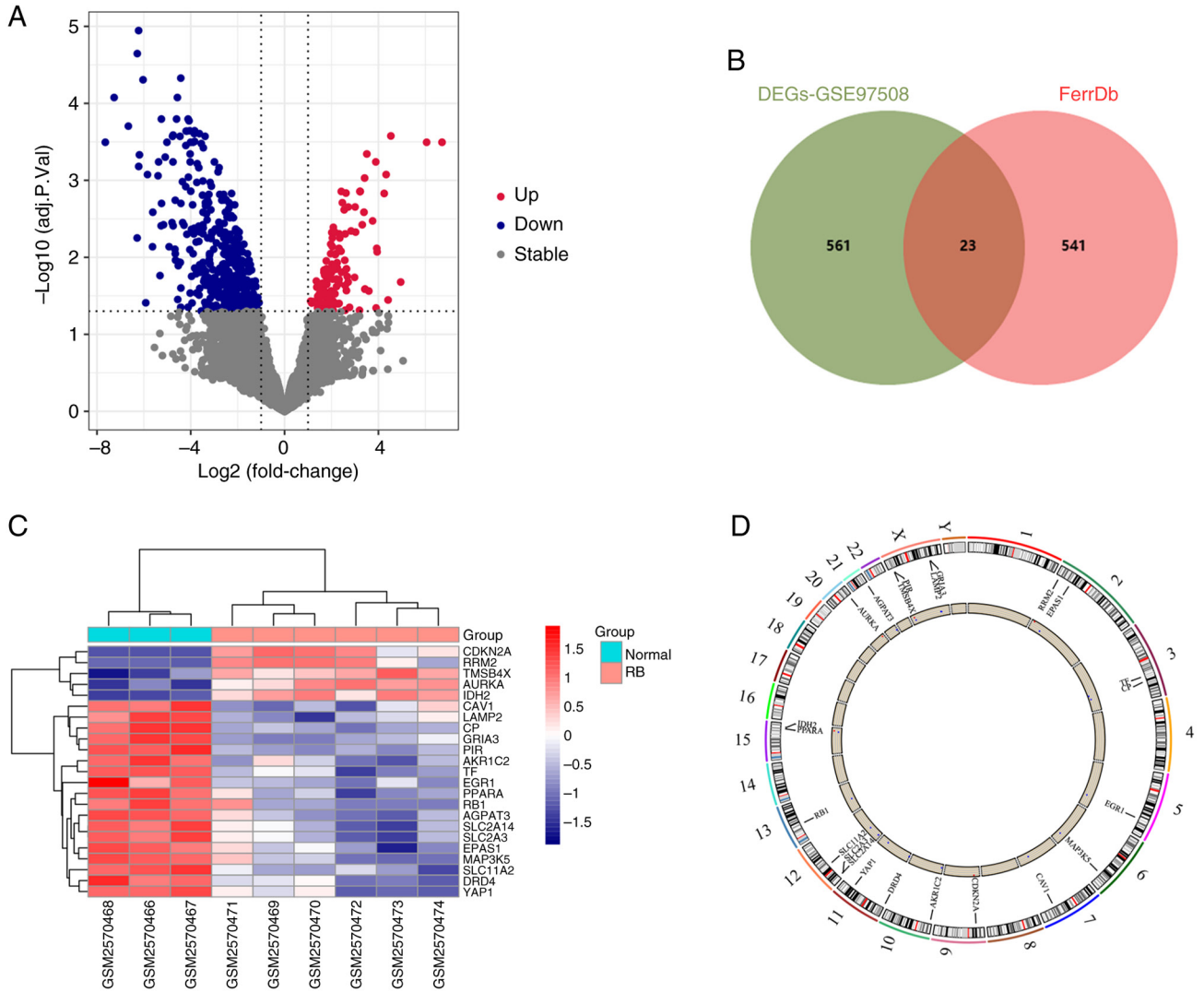


Figure 2. Identification of ferroptosis-related FR-DEGs. (A) Volcano plot showing the gene expression differences between two groups in the GSE97508 dataset. Blue nodes represent downregulated genes, red nodes represent upregulated genes and gray nodes represent genes that are stable in comparison to controls. (B) Venn diagram showing the intersection of DEGs in GSE97508 with FRGs from the 'FerrDb' database. (C) Heatmap of the identified 23 FR-DEGs in GSE97508. (D) Location of the 23 FR-DEGs on the 22 chromosomes. DEGs, differentially expressed genes; FRGs, ferroptosis-related genes; FR-DEGs, ferroptosis-related DEGs; adj.P.Val, adjusted P-value; RB, retinoblastoma

significantly downregulated in RB tumors, whilst *CDKN2A* and *IDH2* were significantly upregulated in GSE97508.

The expression of the 6 hub genes was next validated using the GSE208143 dataset. However, *SLC2A3* was not represented in this dataset—a common limitation when re-analyzing public genomic data due to the inherent differences in microarray platform designs (GSE97508 used GPL15207, while GSE208143 used GPL17077). *SLC2A3* was not solely dependent on the validation dataset for its significance. Its identification as a hub gene was based on multiple topological algorithms (BottleNeck, EPC, Degree, MCC and MNC) applied to the PPI network, and its differential expression was initially observed in the discovery dataset GSE97508. While it was not possible to validate its expression in GSE208143, its biological relevance in ferroptosis and cancer metabolism is supported by existing literature (24). Therefore, validation was possible for the remaining 5 hub genes. Despite this limitation, the successful validation of 5 out of 6 hub genes (an 83% validation rate) provides robust support for

the hub gene selection in the present study. *CDKN2A* and *IDH2* exhibited higher expression levels in the RB group, whilst *CAV1*, *EPAS1* and *RB1* showed significantly lower expression levels in patients with RB (Fig. 4C). Subsequent ROC curve analysis revealed an AUC value of 0.967 for *CAV1*, 0.8736 for *CDKN2A*, 0.9121 for *EPAS1*, 0.7033 for *IDH2* and 0.7418 for *RB1* (Fig. 4D). These results indicate that *CAV1* and *EPAS1* possess excellent diagnostic accuracy, while *CDKN2A* shows good diagnostic performance, and *IDH2* and *RB1* demonstrate moderate diagnostic value. Collectively, these 5 genes represent promising diagnostic biomarkers for RB.

Relationship between the immune cell population and hub FR-DEGs. The CIBERSORT algorithm was used to calculate the infiltration of 22 immune cell types in GSE97508 (6 RB and 3 normal samples). The differences in the immune cell infiltration ratios between the RB and normal groups are shown in Fig. 5A. Compared with those in the control group,

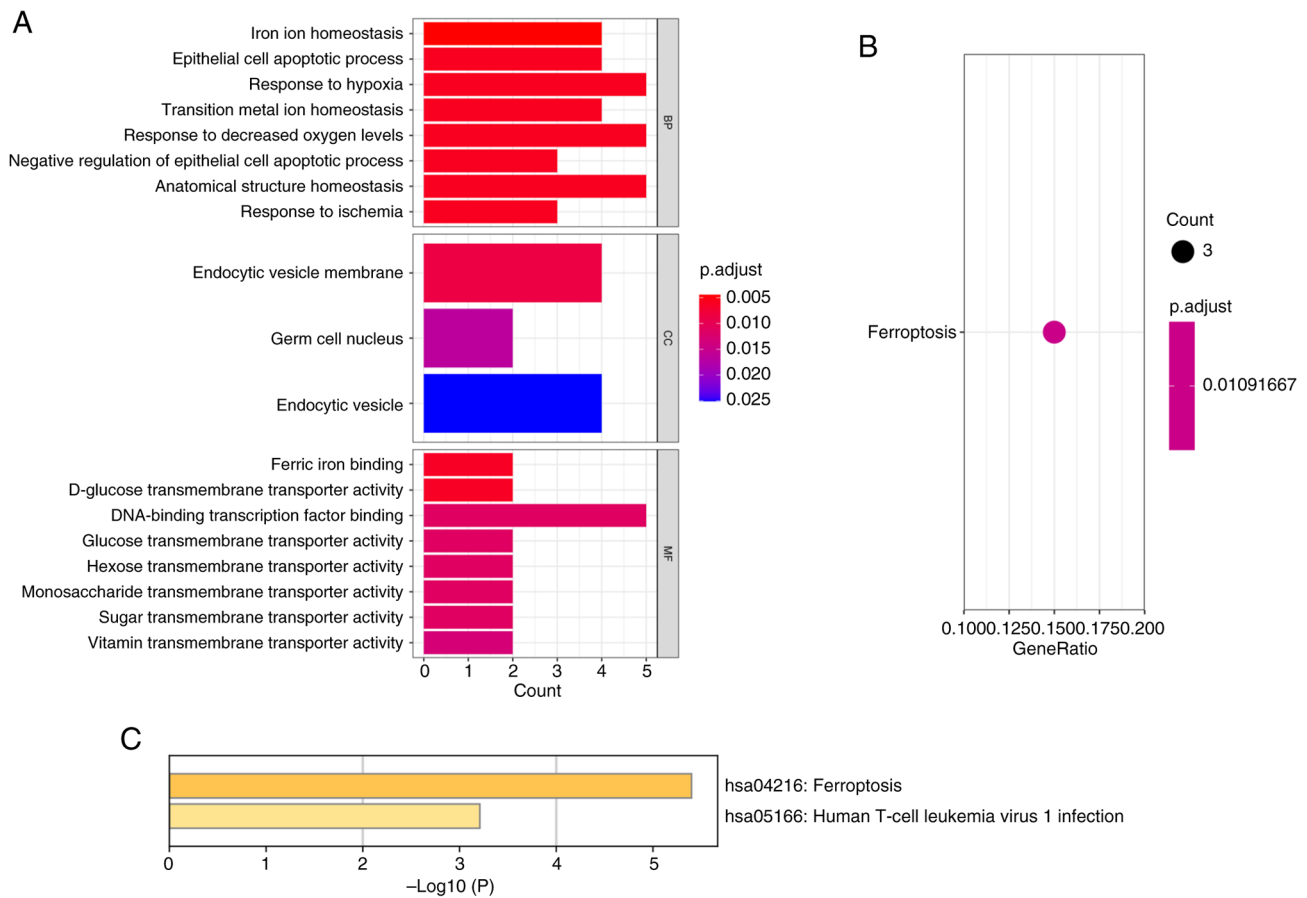


Figure 3. Enrichment analysis of FR-DEGs. (A) Gene Ontology analysis of the FR-DEGs. (B) Kyoto Encyclopedia of Genes and Genomes analyses of the FR-DEGs. (C) Metascape bar chart of top 20 non-redundant enrichment clusters. BP, biological processes; CC, cellular components; MF, molecular functions; FR-DEGs, ferroptosis-related differentially expressed genes.

the infiltration levels of naive B cells and regulatory T cells (Tregs) were higher, whilst the infiltration levels of T follicular helper (Tfh) cells and resting mast cells were significantly reduced.

Correlation analysis between hub genes and immune cell populations revealed that *EPAS1*, *RB1* and *SLC2A3* exhibited negative correlations with naive B cells and Tregs, whilst showing positive correlations with Tfh cells and activated dendritic cells (Fig. 5B). *IDH2* demonstrated significant positive correlations with Tfh cells and activated dendritic cells, whilst showing a significant negative correlation with naive B cells. Resting mast cells displayed a significant positive correlation with *CDKN2A* but a significant negative correlation with *CAVI*. Additionally, *CAVI* exhibited a significant positive correlation with naive B cells.

Construction of the miRNA-mRNA network. From the GSE208677 dataset, DEMs (32 upregulated and 59 down-regulated) were identified (Fig. 6A and B). For the six FR hub genes identified from the PPT network analysis, namely *CAVI*, *CDKN2A*, *EPAS1*, *IDH2*, *RB1* and *SLC2A3*, multiMiR was utilized to predict 351 potential miRNAs. Subsequently, an intersection analysis between these predicted miRNAs and DEMs from the GSE208677 dataset was performed, resulting in the identification of 39 FR miRNAs (Fig. 6C).

Given that miRNA expression levels are typically negatively correlated with mRNA expression, to enhance the reliability of the present findings, the predicted relationships were then integrated with the corresponding expression data. Subsequently, the miRNA-mRNA regulatory network was visualized using Cytoscape software to provide a comprehensive and insightful representation of the regulatory interactions (Fig. 6D).

Construction of a predicted FR lncRNA-miRNA-mRNA ceRNA network. Among the 39 ferroptosis-related miRNAs identified in the miRNA-mRNA network, those that were predicted to target both *CDKN2A* and *IDH2* and that showed negatively correlated expression patterns with these mRNAs were further selected. A total of 3 miRNAs met these criteria: hsa-let-7b-5p, hsa-let-7g-5p and hsa-miR-124-3p. These were selected as the core miRNAs for subsequent ceRNA network construction. To explore potential lncRNAs in RB, the Starbase tool was used to computationally predict the target lncRNAs of these miRNAs. Applying a stringent filtering criterion, only 4 lncRNAs predicted to have binding sites for all miRNAs were retained for further analysis. These lncRNAs were long intergenic non-coding RNA (LINC)00963, nuclear enriched abundant transcript 1 (NEAT1), small nucleolar RNA host gene 16 (SNHG16) and X-inactive specific transcript (XIST).

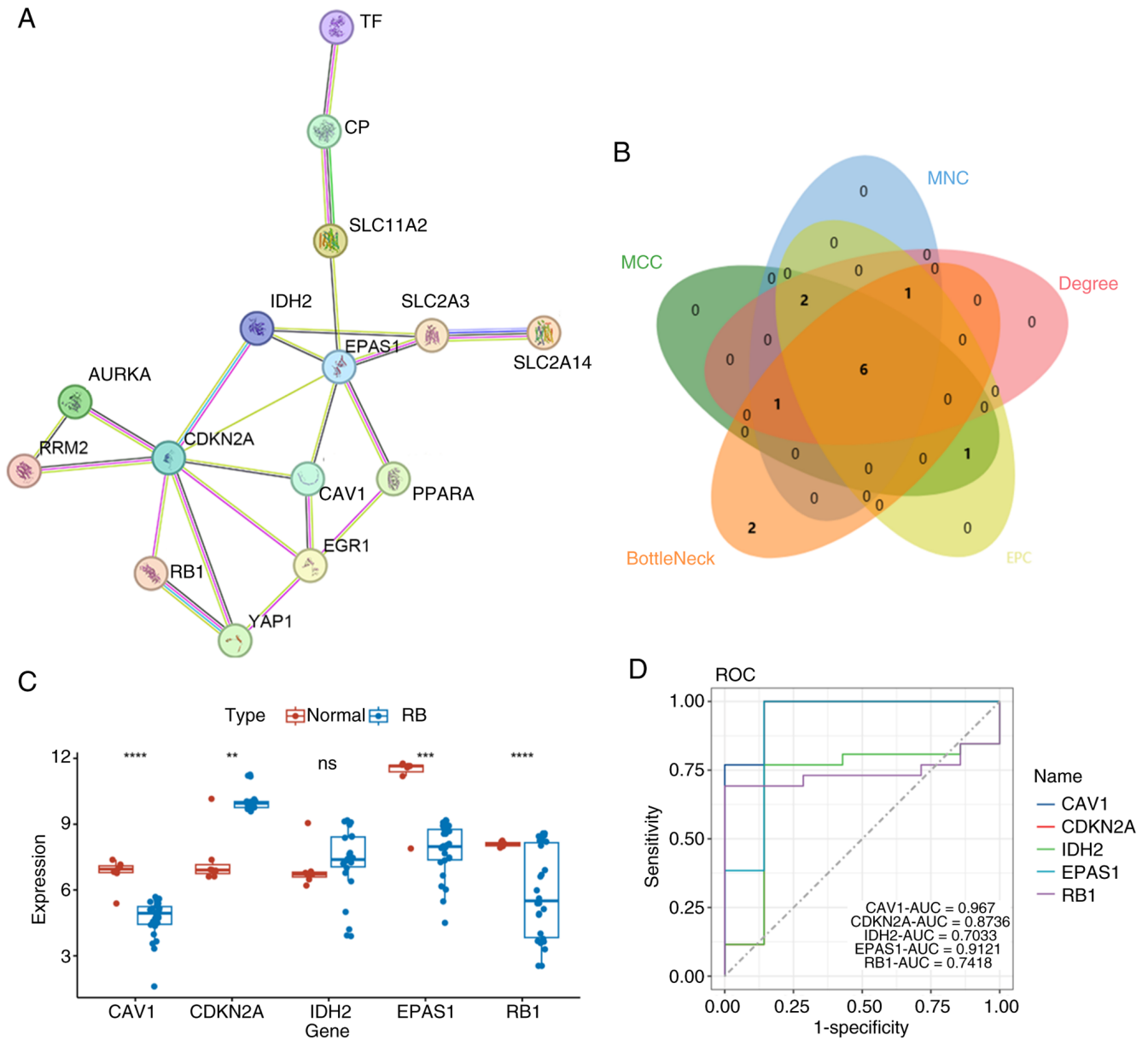


Figure 4. Construction of PPI network and identification of hub genes. (A) PPI network of ferroptosis-related differentially expressed genes. Each node in the network represents a protein. The small 3D structural models inside the circles are schematic representations of the protein conformations. TF, Transferrin; CP, Ceruloplasmin; SLC11A2, Solute Carrier Family 11 Member 2; SLC2A3, Solute Carrier Family 2 Member 3; SLC2A14, Solute Carrier Family 2 Member 14; IDH2, Isocitrate Dehydrogenase 2; EPAS1, Endothelial PAS Domain Protein 1; PPARA, Peroxisome Proliferator-Activated Receptor Alpha. (B) Venn diagram illustrating the hub genes identified by five algorithms (BottleNeck, Degree, EPC, MCC and MNC) using Cytoscape software and CytoHubba. (C) Validation of hub genes in the GSE208143 dataset. The box represents the interquartile range, the line inside the box denotes the median and the dots represent individual sample data points. (D) ROC curve of hub genes in the GSE208143 dataset. ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$. ns, no significance; PPI, protein-protein interaction; EPC, Edge Percolated Component; MCC, Maximal Clique Centrality; MNC, Maximum Neighborhood Component; ROC, receiver operating characteristic; AUC, area under the ROC curve; RB, retinoblastoma.

Based on these computational predictions, a putative lncRNA-miRNA-mRNA ceRNA network for RB was successfully established. This predicted network includes 2 mRNAs (CDKN2A and IDH2), 3 miRNAs (hsa-let-7b-5p, hsa-let-7g-5p and hsa-miR-124-3p) and 4 lncRNAs (LINC00963, NEAT1, SNHG16 and XIST). It is important to note that these interactions are based solely on bioinformatic predictions and have not been experimentally validated. This ceRNA network may be closely associated with the progression of RB, where some of these molecules may serve as key therapeutic targets for RB (Fig. 7A), warranting further experimental investigation.

Drug-gene interactions and expression levels of CDKN2A and IDH2 in RB. RT-qPCR analysis confirmed that CDKN2A and IDH2 mRNA expression levels were significantly higher in Y79 retinoblastoma cells than in ARPE-19 cells (Fig. 7B). Based on these findings, their potential clinical implications were further explored by constructing a drug-gene interaction network using the CTD database. As shown in Fig. 7C, this analysis identified 111 potential therapeutic agents targeting CDKN2A and 89 targeting IDH2. Notably, several drugs, including bisphenol A (code: C006780), sodium arsenite (code: C017947), docetaxel

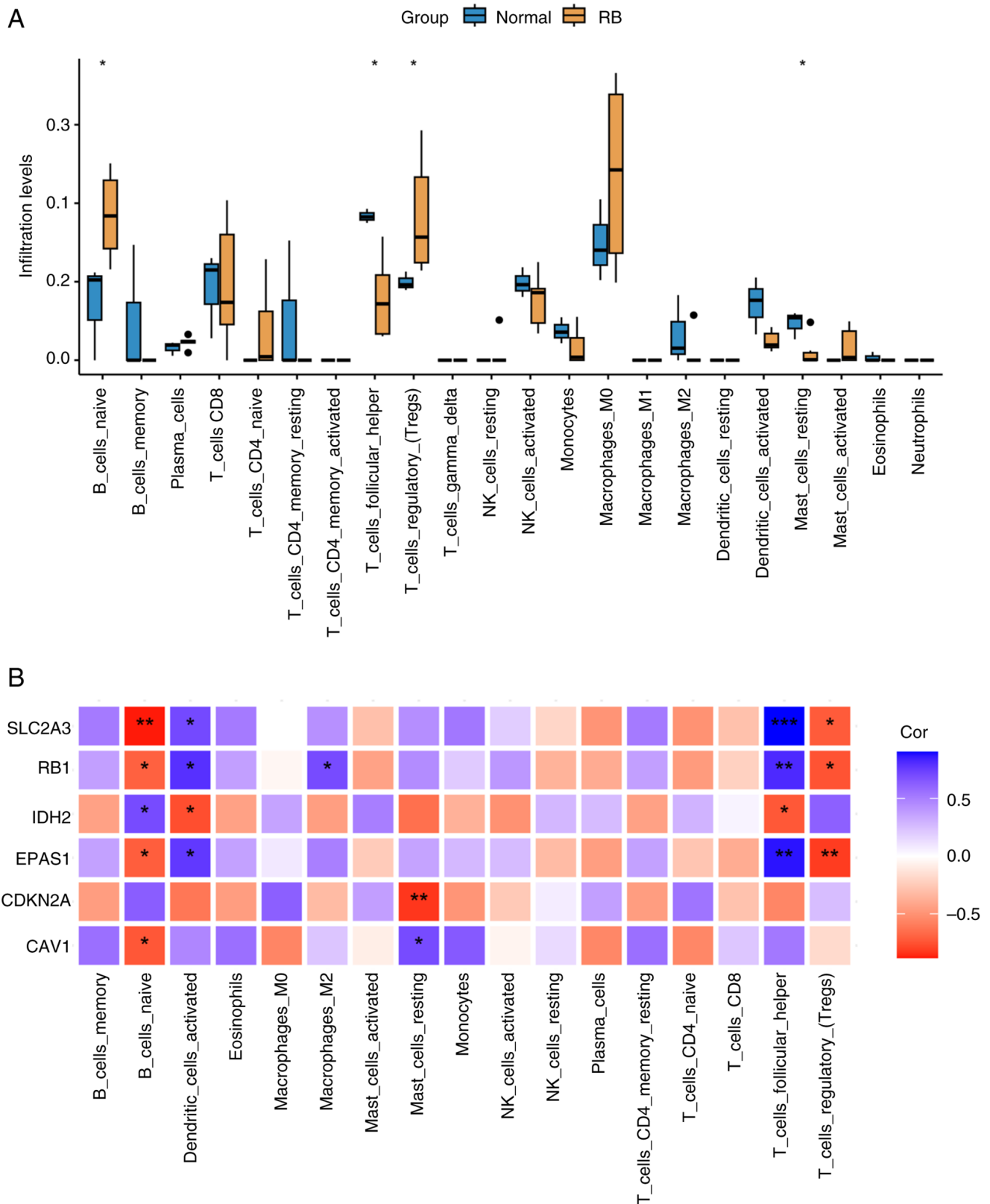


Figure 5. Landscape of immune cell infiltration. (A) Infiltration levels of each immune cell type between control and RB samples. (B) Correlation between hub genes and immune cells in RB. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RB, retinoblastoma; cor, correlation coefficient.

(code: D000077143) and hexabromocyclododecane (code: C089796), were found to target both genes, suggesting they may serve as candidate compounds for further investigation in RB therapy.

Discussion

RB treatment requires early detection, diagnosis and treatment. However, severe outcomes, such as blindness, proptosis

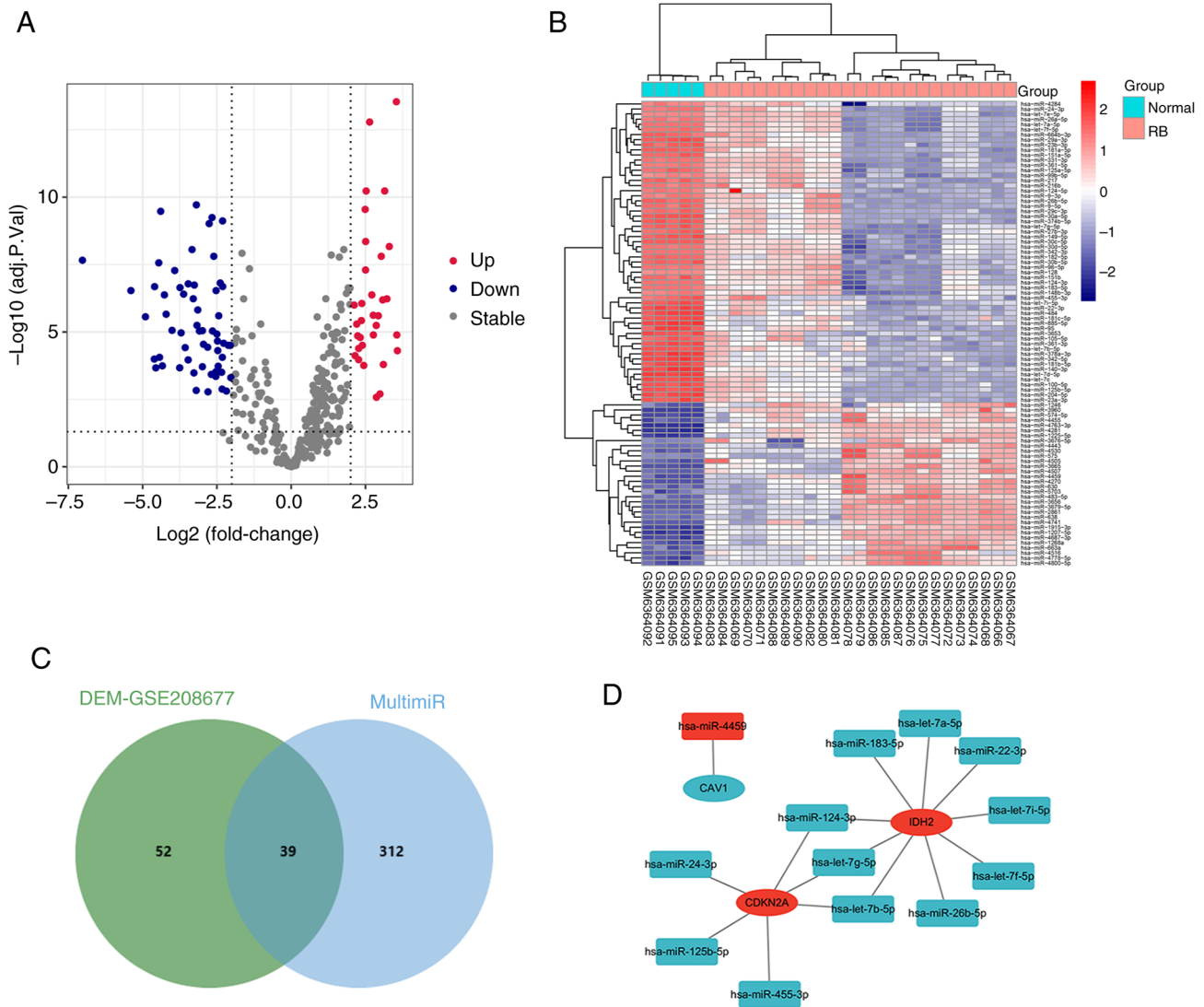


Figure 6. Construction of the miRNA-mRNA network. (A) Volcano plot showing the gene expression differences between two groups in the GSE208677 dataset. (B) Heatmap of the identified 23 ferroptosis-related differentially-expressed genes in GSE97508. (C) Venn diagram showing the intersection of DEMs in GSE208677 with predicted miRNAs from multimir. (D) miRNA-mRNA regulatory network. miRNA, microRNA; DEM, differentially expressed miRNAs; adj.P.Val, adjusted P-value.

and intracranial metastasis, can occur (25). The present study delved into the intricate molecular landscape of RB, focusing on FR ceRNA networks. Ferroptosis is a form of regulated cell death that is driven by iron-dependent lipid peroxidation. It has gained prominence in cancer research (26). In the context of RB, a set of FRGs that exhibited differential expression was identified, forming a unique FR-DEGs signature.

Malignant tumor characteristics encompass persistent proliferative signals, immune evasion and resistance to programmed cell death (PCD) (27). Among these, PCD resistance is a significant feature. Ferroptosis has been associated with the development of various tumors and drug resistance (28,29). Coordinated control over iron levels, lipid oxidation and antioxidants in tumors can prevent ferroptosis, fostering uncontrolled proliferation and tumor expansion. Abnormalities in FR proteins can cause intracellular iron accumulation, leading to increased iron-associated reactive oxygen species and lipid peroxidation, ultimately disrupting cellular membrane integrity and triggering ferroptosis (30).

The *RB1* gene mutation is closely associated with the development of RB and liver carcinogenesis. It has been demonstrated that *RB1* loss can increase cancer cell susceptibility to ferroptosis inducers by elevating acyl-CoA synthetase long chain family member 4, a key enzyme that promotes lipid peroxidation and triggers ferroptosis (31). p53 serves a bidirectional role in regulating ferroptosis, either through transcriptional or posttranslational mechanisms (32). These findings suggest that intervening in the occurrence of ferroptosis may provide a potential avenue for influencing the development and progression of RB.

GO and KEGG pathway analyses were conducted on the 23 FR-DEGs. KEGG analysis revealed significant enrichment only in the ferroptosis pathway. Apart from iron ion homeostasis, GO analysis revealed that these 23 FR-DEGs are also involved in other biological processes, including epithelial cell apoptosis process. Epithelial cell apoptosis shares similarities with other cell types, but the unique characteristics of epithelial cells equip them with specific determinants for survival (33).

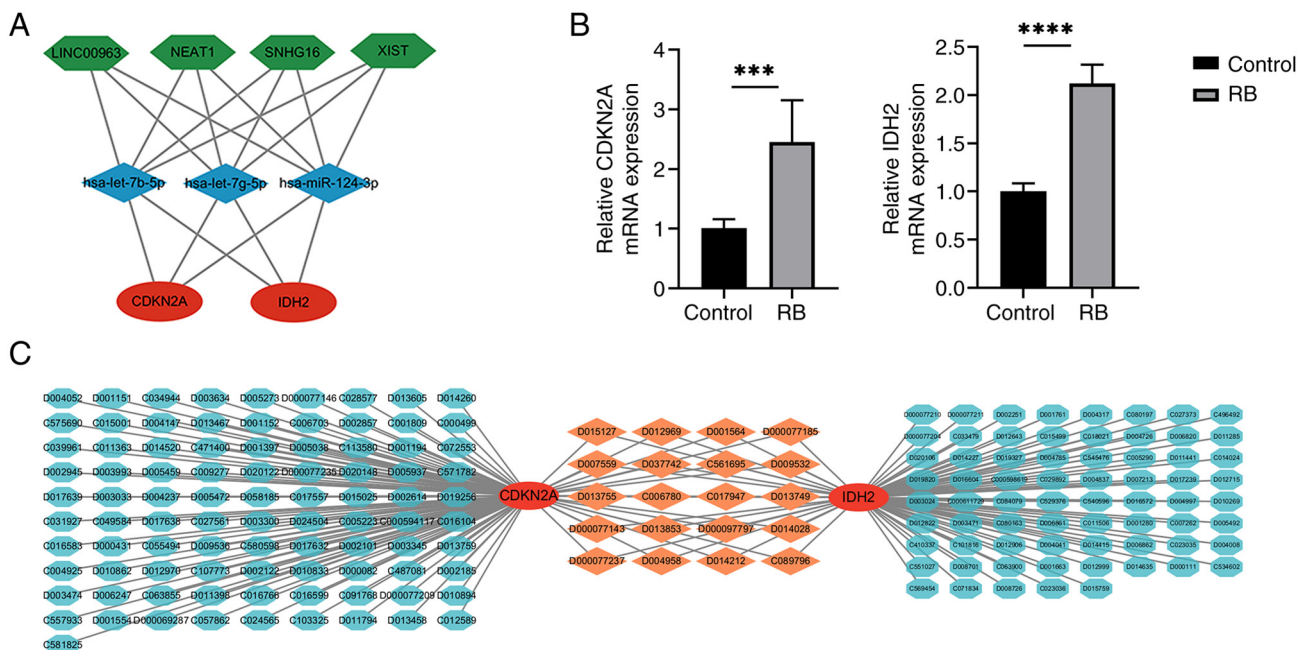


Figure 7. ceRNA networks, drug-gene interactions and PCR validation. (A) The ceRNA-regulating networks illustrate protein-coding genes (red circles), miRNAs (blue diamonds) and lncRNAs (green hexagons), with black lines indicating interactions among lncRNA, miRNA and mRNA, where ceRNA refers to ceRNAs. (B) Validation through reverse transcription-quantitative PCR analysis confirms that *CDKN2A* and *IDH2* expression levels are significantly higher in Y79 cells compared to ARPE-19 cells. Data were normalized to GAPDH expression using the $2^{-\Delta\Delta C_q}$ method, with each experiment performed in triplicate. *** $P < 0.001$ and **** $P < 0.0001$. (C) The drug-gene interaction analysis shows the linkage map of two hub genes, *CDKN2A* and *IDH2*, along with their potential target drugs, including bisphenol A (code: C006780), sodium arsenite (code: C017947), docetaxel (code: D000077143) and hexabromocyclododecane (code: C089796). ceRNA, competing endogenous RNAs; miRNA, microRNA; lncRNA, long non-coding RNA; RB, retinoblastoma.

Cell-matrix and cell-cell interactions are crucial to prevent epithelial cells from entering apoptosis. In RB tumors, the destruction of these structures may occur alongside the apoptotic process of epithelial cells (34). This enrichment analysis provides valuable insights into the functional implications of DEGs in ferroptosis in RB, enhancing the understanding of the molecular mechanisms involved in these processes. The importance of the immune system in tumor progression and therapy has been widely recognized. Immune cells interact with malignant tumor cells through a complex network (35). Therefore, the present study aimed to identify immune cell infiltration as a biomarker for diagnosis and prognosis. Analysis revealed a significant upregulation of naive B cells and Tregs, along with a decrease in Tfh cells and resting mast cells in RB tumor tissues. Tumor cells may recruit naive B cells into the microenvironment, potentially transforming them into Breg cells through various mechanisms (36). Tregs have been shown to impede anticancer immune responses, suppressing the beneficial immunosurveillance of tumors and reducing effective anti-tumor immune protection (37). By contrast, Tfh cells are generally associated with a superior prognosis in tumor entities, whereas resting mast cells tended to be enriched in patients with cancer with a low risk of recurrence (38). These findings suggest that immune checkpoint inhibitors may offer a novel possibility for the treatment of RB.

Central to the present findings are the hub genes *CAVI*, *CDKN2A*, *EPAS1*, *IDH2*, *RBI* and *SLC2A3*, identified through PPI network analysis. These genes not only serve as key players in the FR-DEG signature but also exhibit significant dysregulation in RB. Validation in an independent dataset (GSE208143) confirmed the expression patterns of these hub

genes and their AUC values highlight their diagnostic potential as RB biomarkers. The sensitivity and specificity of these hub genes can be further evaluated in larger independent cohorts in the future to better assess their clinical diagnostic utility. It is noteworthy that *IDH2* and *RB1* exhibited only moderate diagnostic performance as individual biomarkers, suggesting limited standalone clinical utility. However, diagnostic performance is not the only measure of gene significance. *IDH2* upregulation was validated by RT-qPCR and its role in ferroptosis resistance supports its potential as a therapeutic target. *RB1* remains central to RB pathogenesis despite its moderate AUC. Therefore, these genes may be more valuable for functional studies or multi-gene panels than as standalone diagnostic markers. *CDKN2A* is a tumor suppressor gene encoding p16 and p14, the deletion of which can lead to melanoma progression and can cause alterations in the way lipids are metabolized and distributed in the cancer cells, thereby triggering ferroptosis in glioblastoma cells (39,40). *IDH2* is the most frequently mutated metabolic gene in human cancers, interfering with cellular metabolism and epigenetic regulation to promote cancer development (41). Hsa-let-7b-5p serves several different roles in inhibiting tumor cell proliferation, migration, invasion and progression by targeting hexokinase 2, high mobility group AT-hook 2, IGF1R and KIAA1377 in various tumor types (42). Hsa-let-7g-5p is a circulating miRNA and may serve as a biomarker for Alzheimer's disease (43). As a tumor-associated miRNA, hsa-miR-124-3p inhibits lung cancer by regulating the ITGB1/PI3K/AKT axis (44). Abnormal LINC00963 expression frequently promotes oncogenic activity by regulating key cellular processes, such as proliferation, migration, invasion, epithelial-mesenchymal

transition and apoptosis (45). The lncRNA NEAT1 facilitates glioma progression through stabilization of PGK1 (46). LncRNA XIST participates in the development of tumors and other human diseases, underscoring its role as an important regulator of cell growth and development (47). In the present study, an FR ceRNA network based on the aforementioned mRNAs, miRNAs and lncRNAs was established, providing a novel therapeutic approach for the treatment of RB. It should be noted that the ceRNA network is based entirely on computational predictions. While these predicted interactions offer valuable hypotheses for regulatory mechanisms in RB, they require relevant experimental validation to confirm direct binding and their roles in ferroptosis regulation.

The present study suggests a potential link between RB and ferroptosis, constructed an lncRNA-miRNA-mRNA network associated with ferroptosis and verified the expression of hub genes by RT-qPCR, which was consistent with predicted results. These findings provide a novel perspective for studying the role and mechanism of ferroptosis in RB. However, several limitations should be acknowledged. First, the sample sizes of GSE208143 and GSE97508 gene sets are relatively small, with 33 and 9 samples. Second, the lack of clinical and prognostic data precluded clinical association studies or prognostic analyses. Third, while normal human retinal progenitor cells or mature retinal neurons (such as photoreceptors) would be ideal controls, obtaining and culturing primary human retinal neuronal or progenitor cells is technically challenging due to their limited availability and difficulty in maintaining them *in vitro* (48,49). Therefore, ARPE-19 cells—one of the most commonly used and well-characterized ‘normal’ retinal cell lines in ophthalmic research—were employed as a surrogate control. ARPE-19 cells are readily available, stable in culture and enable reproducible experimental conditions (50,51). Although derived from a different retinal lineage, they represent the best available immortalized cell line from the human retina and have been widely used as controls in numerous published RB studies (52–57). Finally, the present ceRNA network construction employed a stringent filter requiring lncRNAs to bind all 3 hub gene-targeting miRNAs. While this approach minimized false positives and identified core regulatory axes, it may have omitted biologically relevant lncRNAs that interact with only one or two of these miRNAs. Future experimental studies should explore these partial interactions to fully elucidate the regulatory landscape.

In conclusion, the present study unraveled the ferroptotic landscape in RB, shedding light on the molecular intricacies that govern this aggressive eye cancer. The identification of key hub genes, functional insights and immune modulation provide a foundation for future research directions and therapeutic interventions targeting ferroptosis in RB.

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Availability of data and materials

Publicly available datasets were analyzed in this study, which can be found in the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov>), FerrDb database (<http://www.zhounan.org/ferrdb/>) and StarBase database (<http://StarBase.sysu.edu.cn/>). The data generated in the present study may be requested from the corresponding author.

Authors' contributions

ZK contributed to the data collection and analysis, as well as drafting and writing of the manuscript. GL conceived and designed the manuscript, and critically reviewed the manuscript for important intellectual content. Both authors have read and approved the final manuscript. ZK and GL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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