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Original Article

A new pyroptosis-related signature for predicting the immune status and injury of liver ischemia-reperfusion

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Abstract: Objective: Pyroptosis is a type of programmed cell death. This study aimed to explore the roles of key pyroptosis-related genes in liver ischemia-reperfusion injury. Methods: After collection and standardization of the transcriptome data from GSE12720 database, differentially expressed pyroptosis-related genes were identified. The risk genes screened by a random forest model were used to establish the line graph model. Consensus clustering was used to classify all samples according to the differentially expressed pyroptosis-related genes. Single-sample Gene Set Enrichment Analysis (ssGSEA) was performed to investigate the immune cell infiltration after hepatic ischemia-reperfusion. Cytoscape was used to visualize the regulatory network of transcription factor (TF)-microRNA (miRNA)-target genes. Results: We identified 18 significantly and differentially expressed pyroptosis-related genes between the disease and normal samples. Among these 18 genes, IL1β was positively correlated with CXCL8 (r = 0.791) and BIRC3 (r = 0.78), while ADORA3 was negatively correlated with GZMB (r = -0.567) and CXCL8 (r = -0.566). Furthermore, the random forest model constructed using the top 10 pyroptosis-related genes could predict the risk of hepatic ischemia-reperfusion. Importantly, the decision curve analysis showed that patients could benefit from the risk prediction model. Moreover, we found that the expression of TXNIP, IRF1, and GJA1 was the mostly regulated by miRNAs, while the expression of BIRC3, NFκB1, and TXNIP was regulated by the TF RELA. RELA had the most hub genes involved in the regulation. Conclusion: Our study provides an overview of the expression landscape and the functional significance of pyroptosis-related genes in liver ischemia-reperfusion. Our findings also shed light on the clinical application of pyroptosis-related genes in the treatment of hepatic ischemia-reperfusion injury.

Keywords: Pyroptosis, signature, immune, liver ischemia-reperfusion

Introduction

Liver transplantation is the primary treatment option for severe late-stage liver disease. Hepatic ischemia-reperfusion injury is a common tissue injury in clinical liver surgery, which can lead to liver dysfunction and even organ failure [1]. Current studies have shown that a strong inflammatory response and massive cell death are the typical pathological features of liver ischemia-reperfusion injury. Multiple molecular signaling pathways, including NF-κB and MAPK pathways, are involved in cell survival and inflammatory response regulation during ischemia-reperfusion [2, 3].

Pyroptosis is a new type of programmed cell death characterised by the inflammasome activation which leads to a strong inflammatory response [4]. During the process of pyroptosis, cells form various vesicles, and gasdermin (GSDMD) is polymerized and sheared, forming pores of 10-20 nm on the cell membrane. The cytosolic contents continuously leak through the membrane pore, and the cells produce apoptotic vesicle, leading to the gradual expansion and rupture of the cells [5, 6]. In recent years, inhibition of tlR4-NF-κB-NLRp3 pathway activity has been shown to suppress the inflammasome-induced pyroptosis [7], while Caspase-1 activation stimulated by ischemia-reper-

fusion injury induces pyroptosis and mediates hepatic steatosis [8]. Therefore, pyroptosis is closely related to hepatic ischemia-reperfusion. However, its specific functional significance is still poorly understood.

During gene expression, mRNA transcripts interact with a diversity of transcription co-factors to control the temporal and spatial expression of genes [9, 10]. The rapid development of precision medicine can accurately capture the dynamic characteristics of mRNA transcripts, thus providing accurate diagnosis and personalized treatment according to individual genetic features and pathogenesis [11]. In this study, we first identified pyroptosis-related genes that were differentially expressed before and after perfusion. Then, risk genes screened by a random forest model were used to construct diagnostic prediction models. Concurrently, we explored the underlying mechanisms of hepatic ischemia-reperfusion injury. We aimed to discover the important pyroptosis-related gene and their correlation with immune infiltration to provide new insight into the diagnosis and treatment of liver ischemia-reperfusion injury.

Methods

Data acquisition

Dataset GSE12720 from the GPL570 [HG-U133_PLus_2] Affymetrix Human Genome U133 Plus 2.0 Array platform was used in our study. We selected 21 pre-perfusion and 21 post-perfusion samples for subsequent analysis. The "Limma" package screened differentially expressed pyroptosis-related genes before and after reperfusion, and a total of 18 genes were identified.

Correlation analysis and machine learning modeling of pyroptosis-related genes

The "Corrplot" package was used to analyze the correlation among these 18 pyroptosis-related genes in samples extracted before and after hepatic ischemia-reperfusion. A Wilcox test was used to compare gene expression before and after reperfusion. "E1071" and "randomForest" were utilized to identify risk genes and built prodiction models via machine learning modeling. The "pROC" package plotted receiver operating characteristic (ROC) curves to evaluate the predictive performance of the model.

Nomogram model construction

We further constructed a nomogram using the 10 pyroptosis-related genes by the "RMS" package to demonstrate the predictive value of these 10 pyroptosis-related genes as the biomarkers after hepatic ischemia-reperfusion. The consistency between the predicted value and the actual value was evaluated by a calibration curve. The decision curve assessed whether pyroptosis-related genes could be used as molecular markers.

Molecular subtypes of pyroptosis-related genes

Consistent clustering is an unsupervised clustering method that uses repeated sampling to extract a certain number of datasets and specifies the number of clusters as well as calculates the rationality under different numbers of clusters. We used the "ConsensusClusterPlus" package for pyroptosis-related gene consistent clustering to obtain the sample classifications after hepatic ischemia-reperfusion.

Functional enrichment analysis of differentially expressed genes among pyroptosis related subtypes

The "Limma" package was utilized to identify differentially expressed genes among different subtypes, and the ANOCA test was performed for data analysis. Gene Ontology (GO) functional enrichment analysis was used to investigate the potential mechanism of differentially expressed genes after hepatic ischemia-reperfusion, and the results were presented as a bubble diagram.

Immune cell infiltration analysis

Single-sample Gene Set Enrichment Analysis (ssGSEA) was utilized to analyze the degree of immune cell infiltration after hepatic ischemia-reperfusion. A heat map was used to exhibit the difference of immune cells in the samples before and after hepatic ischemia-reperfusion, and t test was employed to analyze the scores of immune cells in the samples.

Construction of a regulatory network based on transcription factor (TF)-pyroptosis-related gene-microRNA (miRNA)

We used the prediction algorithm and experimental support from the starBase database (https://starbase.sysu.edu.cn/starbase2/index.php) to download the relevant miRNA-target genes. miRNAs that regulate key pyroptosis-related genes were extracted from these pairs. The TF-target gene data confirmed by experimental data were downloaded from the TRRUST database (https://www.grnpedia.org/trrust/). Cytoscape integrated and visualised these two sets of data.

Results

Identification of 18 differentially expressed pyroptosis-related genes

The gene expression landscape in the samples extracted before and after hepatic ischemia-reperfusion was compared by the "Limma" package, and 18 differentially expressed pyroptosis-related genes (ADORA3, BIRC3, CAMP, CEBPB, CXCL8, GBP1, GJA1, GSDMB, GZMB, IL1β, IL1RN, IRF1, JUN, NFκB, NLRP3, SERPINB1, TNF, and TXNIP) were identified. Compared with before reperfusion, the expression of ADORA3 and TXNIP was decreased after reperfusion, while the expression of other genes was increased after reperfusion (**Figure 1A**, **1B**, **1D**). **Figure 1C** showed the distribution of these 18 pyroptosis-related genes on chromosomes.

Correlation analysis of pyroptosis-related genes

We performed correlation analysis on these 18 genes in all samples to explore the association among pyroptosis-related genes before and after hepatic ischemia-reperfusion. As shown in **Figure 2**, IL1β was positively correlated with CXCL8 (r = 0.791) and BIRC3 (r = 0.78), while ADORA3 was negatively correlated with GZMB (r = -0.567) and CXCL8 (r = -0.566).

Random forest and support vector machine models

We constructed random forest and support vector machine models to evaluate the predictive power of these 18 differentially expressed pyroptosis-related genes on reperfusion injury. The ROC curve was created after cross-validation of the fold, and we found that both the random forest and support vector machine models showed high accuracy (**Figure 3A**). We further carried out a multidimensional analysis and used a random forest model for further verification. The random forest model suggested that IL1β, CXCL8, and BIRC3 were the top three genes that exhibited the effective predictive ability (**Figure 3B**). **Figure 3C** and **3D** demonstrated that the predictive value of the model.

Nomogram model construction

The nomogram model was constructed mainly based on the top 10 pyroptosis-related genes by using the "RMS" package to predict the degree of injury from hepatic ischemia-reperfusion (**Figure 4A**). The calibration curve showed the good prediction accuracy of this nomogram model (**Figure 4B**). In the decision curve analysis (DCA) curve, the red line was always above the grey and black lines, indicating that the model could effectively predict hepatic ischemia-reperfusion injury (**Figure 4C**). The clinical impact curve in **Figure 4D** suggested the significant predictive power.

Identification of pyroptosis-related subtypes and functional enrichment analysis

Based on the 18 differentially expressed pyroptosis-related genes, we employed the "ConsensusClusterPlus" package to classify the subtypes of liver ischemia-reperfusion samples. **Figure 5A** showed that all the samples could be divided into two subtypes (clusterA and clusterB), and each subtype contained 15 and 6 samples, respectively. **Figure 5B** and **5D** showed the difference in the expression level of these 18 pyroptosis-related genes between clusterA and clusterB. BIRC3, CXCL8, JUN, and NLRP3 were significantly differentially expressed, and their expression was all decreased in clusterB samples. When comparing the global gene expression landscape, we identified a total of 1718 genes that were differentially expressed between these two subtypes ($P < 0.01$). We then performed GO enrichment analysis for these 1718 differentially expressed genes (**Figure 5C**). We found that these genes

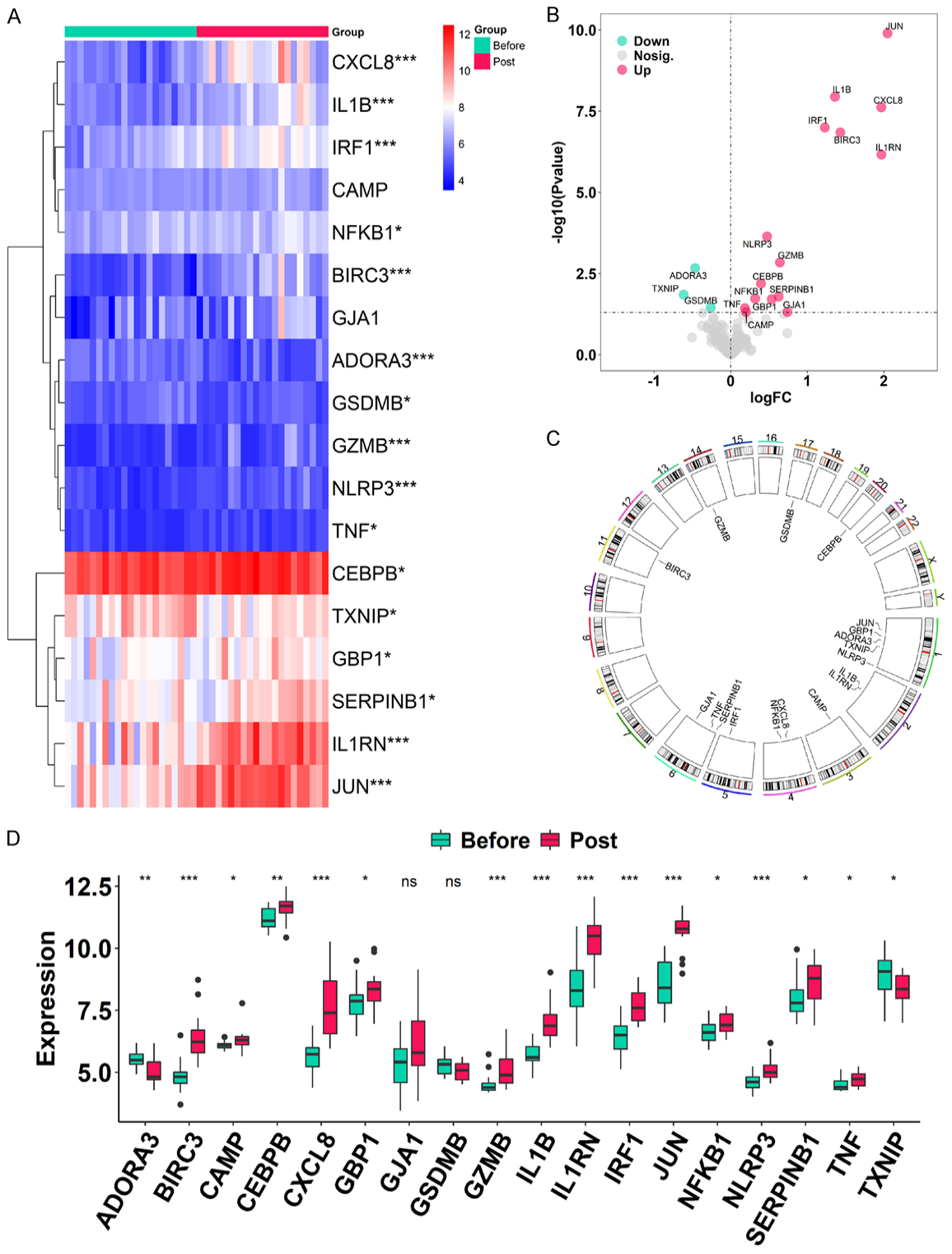

Figure 1. The differential expression and characterization of 18 pyroptosis-related genes before and after hepatic ischemia-reperfusion. A. Heat map of pyroptosis-related differentially expressed genes. B. Volcano map of pyroptosis-related differentially expressed genes. C. Location distribution of pyroptosis-related genes on chromosomes. D. Boxplot of pyroptosis-related genes differentially expressed in samples before and after ischemia reperfusion. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

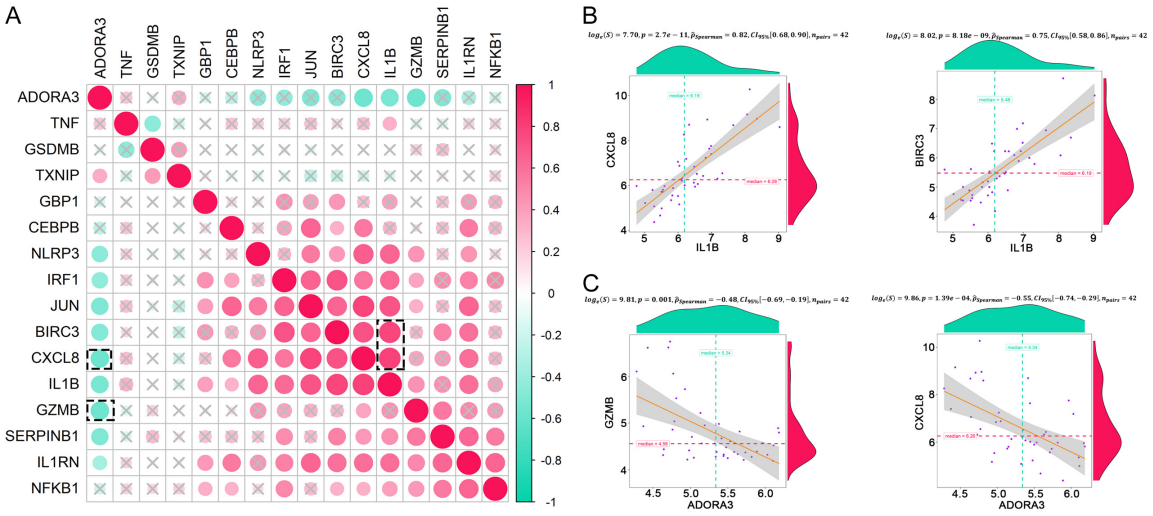

Figure 2. Correlation analysis of 18 pyroptosis-related genes in hepatic ischemia-reperfusion samples. A. Correlation of pyroptosis-related genes in all samples. B. Positive correlation genes. C. Negative correlation genes.

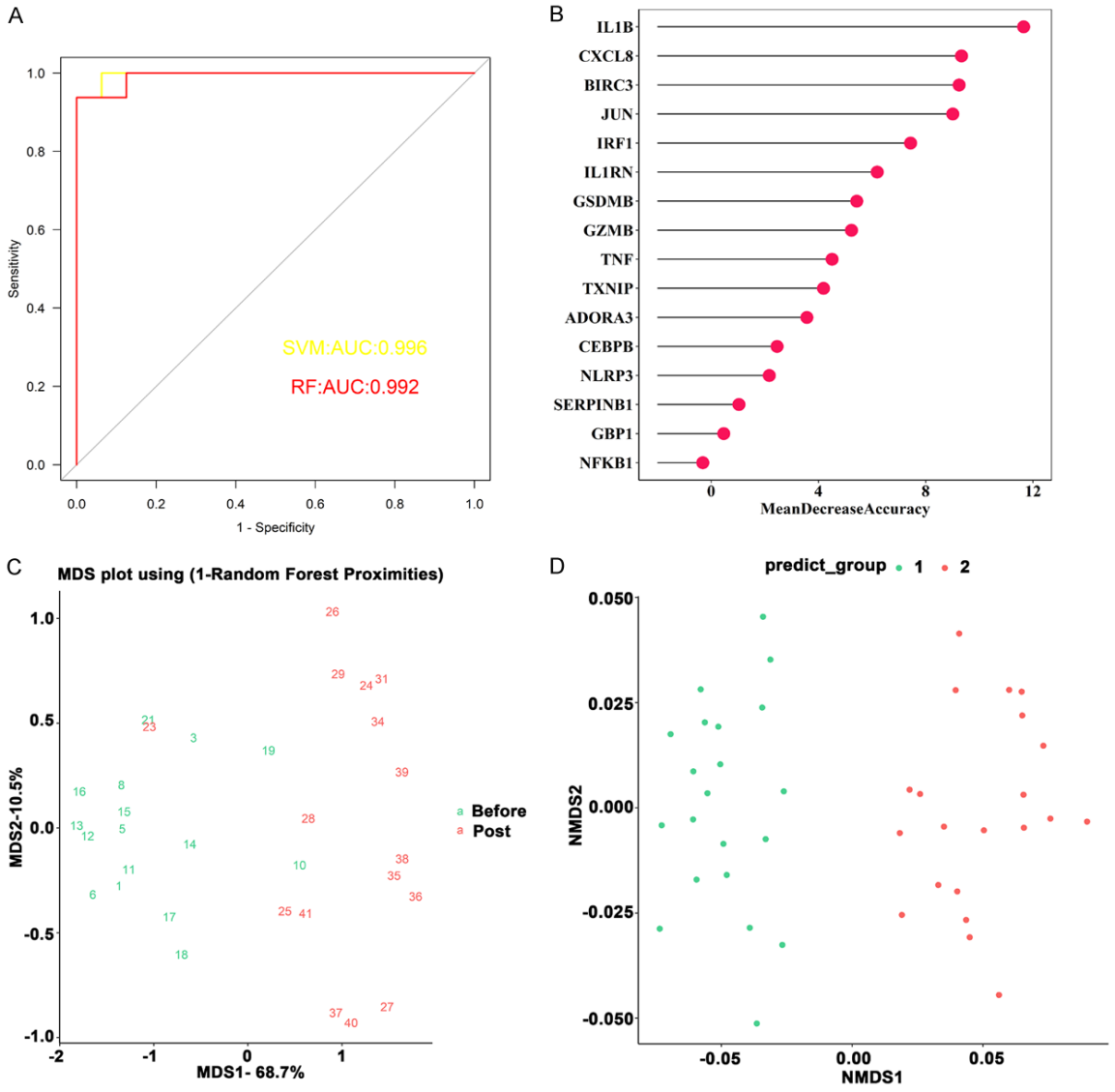

Figure 3. Construction of random forest model. A. ROC curves of SVM and RF models. B. Importance sequencing of pyroptosis-related genes based on RF modeling. C. Multi-dimensional scaling (MDS) was used to examine sample classification. D. Non-metric multidimensional scaling analysis (NMDS) was used to test the predictive power of RF models.

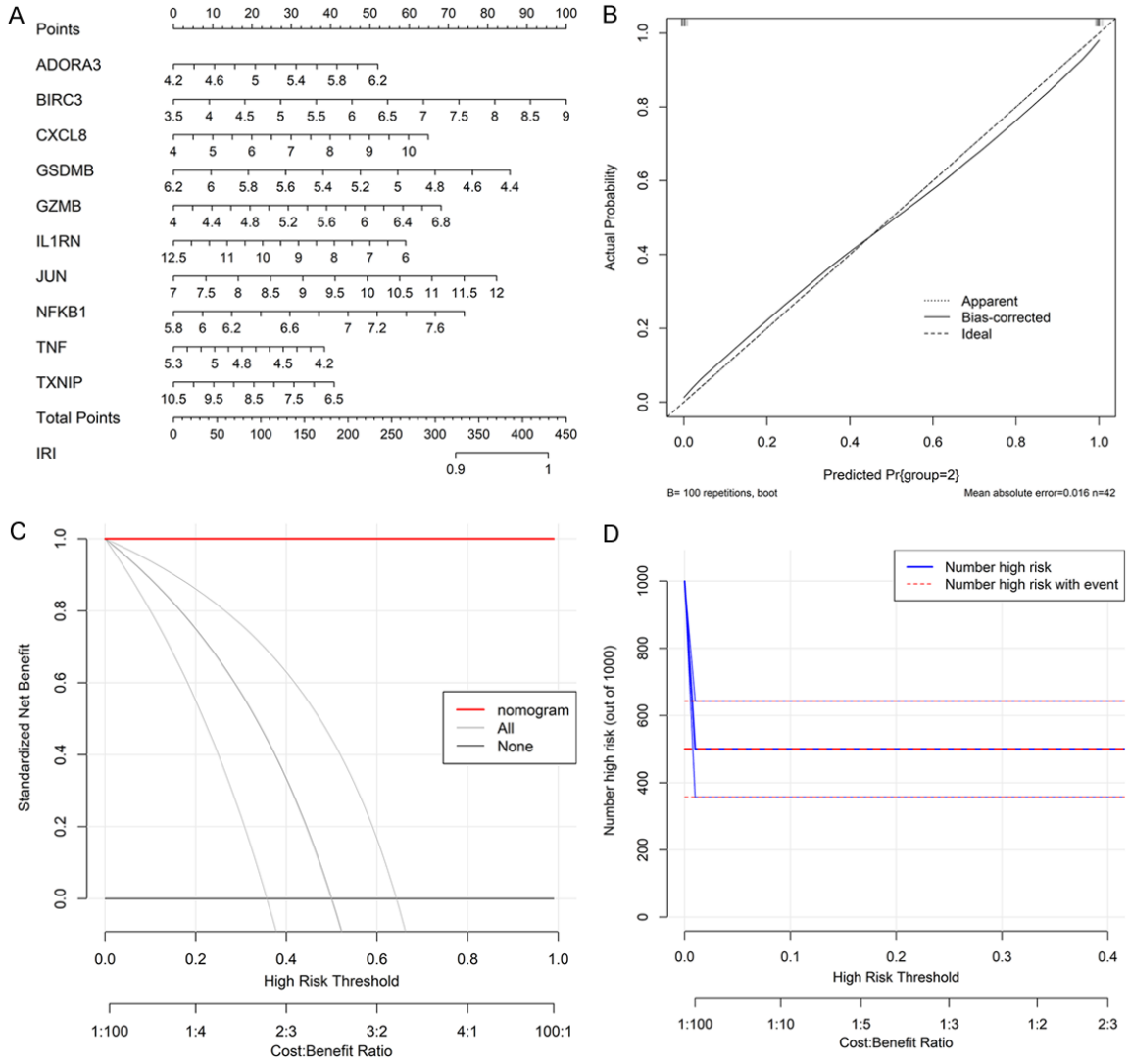

Figure 4. The construction of the Nomogram model. A. Construct the Nomogram model based on the top 10 cell pyroptosis-related genes. B. Calibration curve to demonstrate the predictive power of the Nomogram model. C. Decision curve for the benefit of the Nomogram model. D. Clinical impact curve to evaluate the clinical role of the Nomogram model.

were mainly enriched in GO: 0005515 (protein binding), GO: 0005737 (cytoplasm), GO: 0005829 (cytosol), GO: 0070062 (extracellular exosome), and GO: 0005654 (nucleoplasm). Furthermore, data from principal component analysis (PCA) suggested that the 18 pyroptosis-related genes could distinguish between these two subtypes well (**Figure 5E**).

Next, we performed ssGSEA to calculate the distribution of immune cells in hepatic ischemia-reperfusion injury samples and analyzed the correlation between the 18 pyroptosis-related genes and immune cells. Since we found that BIRC3 was positively correlated with many immune cells (**Figure 6A**), we assessed the difference in immune cell infiltra-

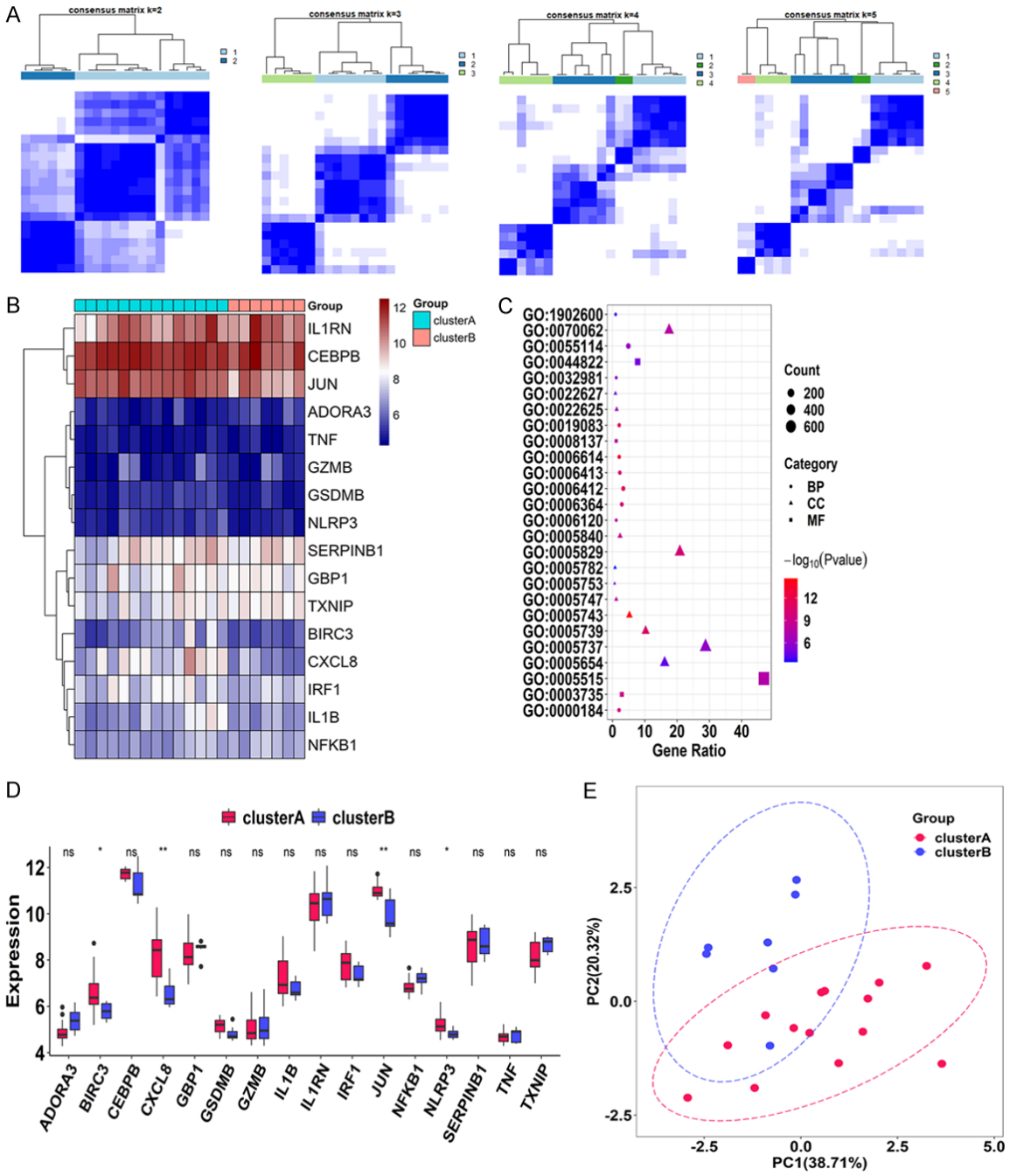

Figure 5. Consistent cluster analysis of 16 genes in ischemia-reperfusion. A. K = 2-5 consistent clustering. B. Heat map of pyroptosis-related genes in 16 cells. C. Enrichment analysis of differentially expressed genes (DEGs) in clusterA and clusterB samples after ischemia-reperfusion. D. Box plot of the expression of 16 pyroptosis-related in clusterA and clusterB. E. Principal component analysis of clusterA and clusterB. *$P < 0.05$, **$P < 0.01$.

tion between these two subtypes according to the high and low expression of BIRC3. The results suggested that samples with high BIRC3 expression had a high level of immune cell infiltration (**Figure 6B**). Finally, we examined the difference in the distribution of immune cell infiltration between these two subtypes. **Figure 6C** showed that clusterA was mainly related to Type 1 helper cells, while clusterB was related to immature B cells.

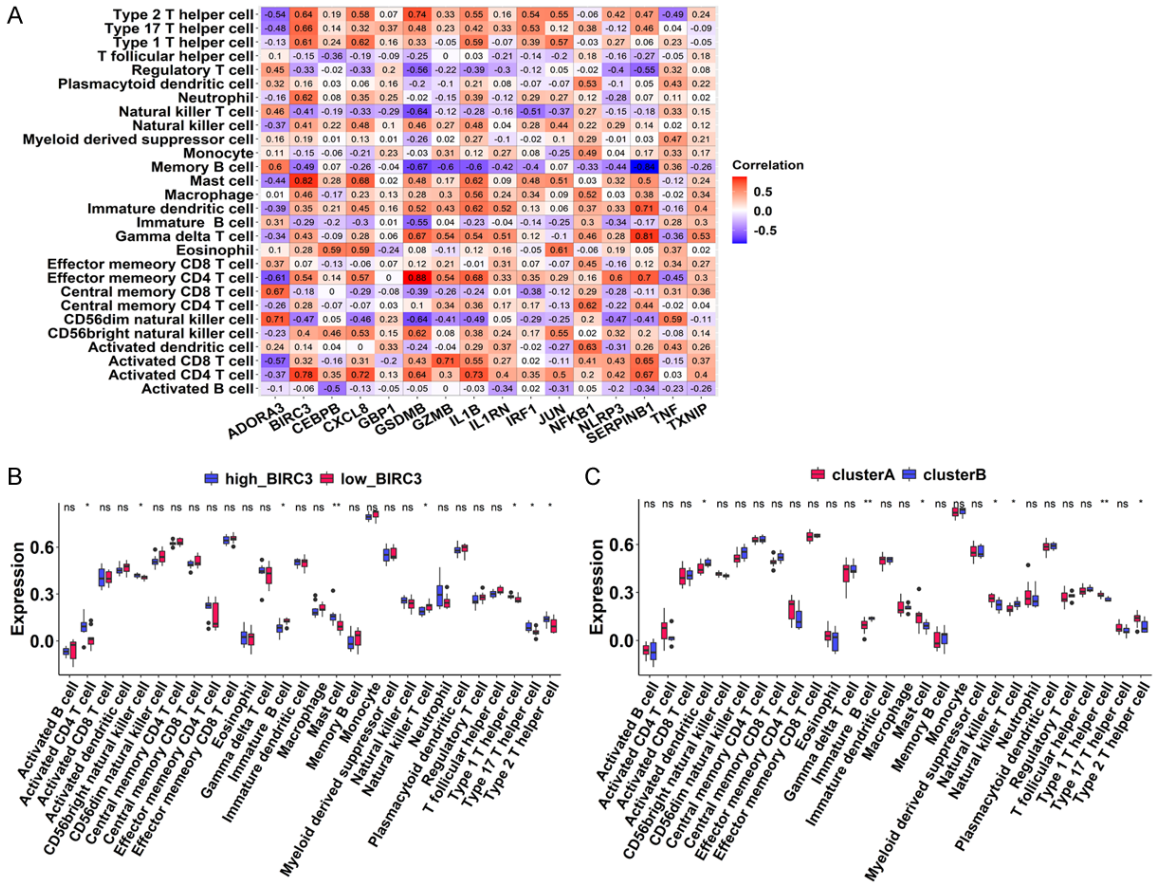

Figure 6. SsGSEA analysis. A. Correlation analysis of 16 pyroptosis-related genes and immune infiltration cells. B. Differences in the abundance of immune infiltration cells between high and low expression of BIRC3 protein. C. Differences in immune infiltration cells between clusterA and clusterB. *P < 0.05, **P < 0.01.

Construction of a TF-miRNA-hub gene regulatory network

We first downloaded the miRNA-target gene relationship from the starBase database and the TF-target gene relationship from the TRRUST database. Then, we integrated these two datasets to construct the regulatory network of the disease (**Figure 7**). We found that TXNIP, IRF1, and GJA1 were mainly regulated by miRNA, while BIRC3, NFκB, and TXNIP were modulated by the TF RELA. RELA had the most hub genes involved in the regulation.

Discussion

In this study, we revealed the significant difference in the expression of 18 pyroptosis-related genes before and after hepatic ischemia-reperfusion. The nomogram model was established based on these 18 genes, and the top 10 genes in the model were selected to construct the line graph model. The DCA curve showed that this model could accurately predict the degree of liver injury in patients. Finally, TFs and miRNAs that regulate pyrophoric-related genes were utilized to construct a TF-miRNA-pyroptosis-related gene network and explore the potential mechanism of the disease development. To date, no studies have systematically investigated the functional significance of pyroptosis-related gene in hepatic ischemia-reperfusion injury. Therefore, the results of our study may shed light on the relationship between pyroptosis and liver ischemia-reperfusion injury and identify valuable biomarkers for the prognostic prediction of liver injury.

The development of high-throughput sequencing technologies has enabled scientists to discover new biomarkers and therapeutic targets and understand the molecular mechanisms

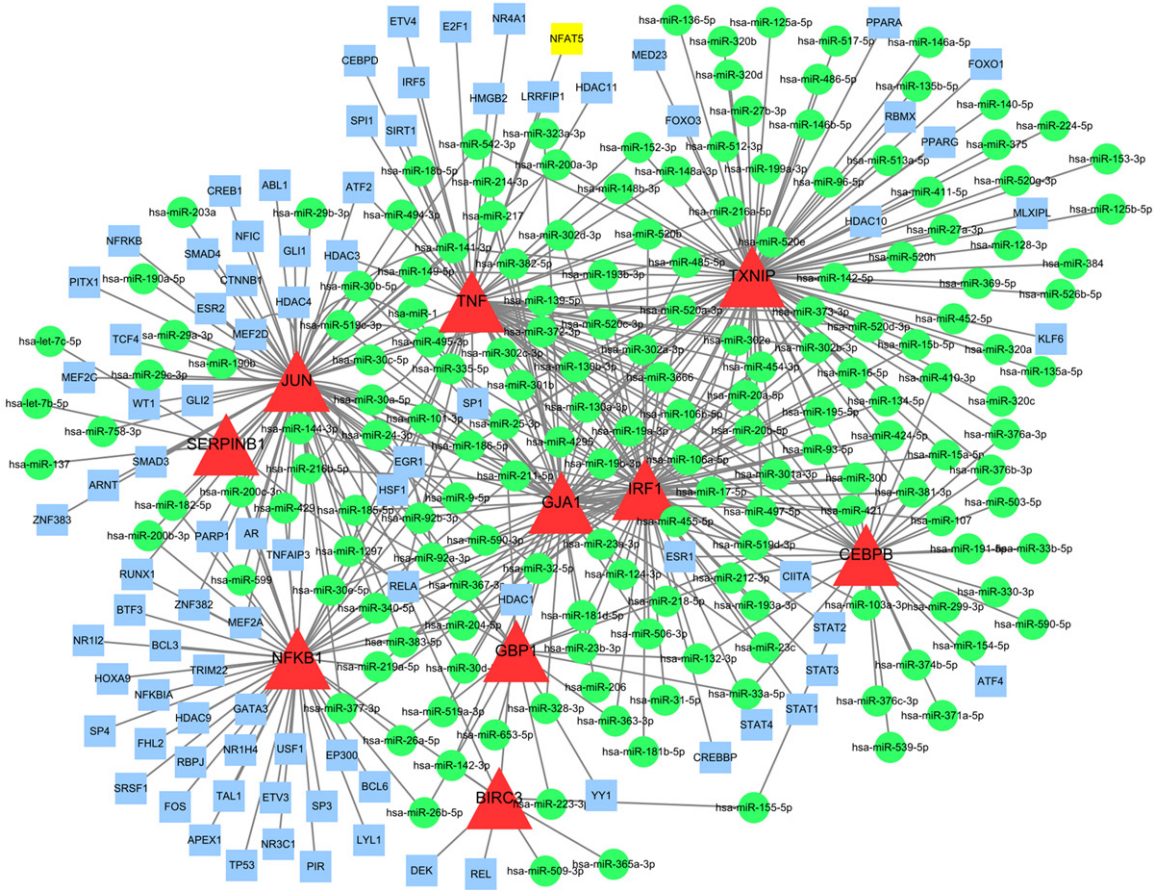

Figure 7. Constructing a TF-miRNA-hub gene regulatory network.

underlying various diseases [12]. Extensive cell death and a strong inflammatory response are the typical pathological features of tissue ischemia-reperfusion injury. Necrotic hepatocytes release many cytokines and chemokines, which aggravates hepatocyte injury, resulting in hepatocellular disorder and, ultimately, leading to poor prognosis [13, 14]. Therefore, it is imperative to identify key regulatory molecules in the signaling pathways that regulate/inhibit the inflammatory response in order to prevent and treat organ ischemia-reperfusion injury [15]. Pyroptosis represents an inflammation-associated cell death pathway which produces IL-1β, IL-18, and other inflammatory cytokines to form an inflammatory microenvironment [16]. The pyroptosis process is accompanied by an inflammatory response and immune system activation. Some studies have found that pyroptosis is closely related to hepatic ischemia-reperfusion injury [17]. The expression and activity of Caspase-1-GSDMD was significantly changed in liver tissue during hepatic ischemia-reperfusion. In addition, caspase-1 inhibitors can significantly mitigate ischemia-reperfusion associated liver injury and inflammation. Zhu et al. demonstrated that NLRP3 knockout reduced aseptic inflammation mediated by ischemia-reperfusion injury and prevented inflammasome activation and IL-1β and IL-18 release [18, 19]. Therefore, pyroptosis may represent a new biomarker and target for the diagnosis and treatment of ischemia-reperfusion-associated liver injury and inflammation. In this study, we selected 10 pyroptosis-related genes to establish a model that can better predict the occurrence of liver injury. DCA curves suggested that patients might benefit from this prediction model.

IL-1β and CXCL8 are important cytokines that play crucial roles as intermediates in regulating the pathogenesis of inflammation. During inflammation, infected cells can release adenosine triphosphate (ATP), which can be hydrolyzed by nucleotidase such as CD73. CD73-

mediated hydrolysis of extracellular ATP (eATP) can affect CXCL8 secretion and IL-1β induction [20]. Adenosine activates or inhibits downstream signaling pathways through regulating cyclic adenosine monophosphate (cAMP) level [21]. The function of adenosine is mediated by G protein-coupled receptors (GPCRs), including ADORA3. Interestingly, the local level of adenosine is significantly enhanced in tissue inflammatory condition to inhibit multiple inflammatory processes, including the production of toxic oxygen metabolites, cytokine release, cell adhesion, and phagocytosis [22]. In this study, we used bioinformatics analysis and revealed that ADORA3, CXCL8, and IL-1β might interact with each other. Nevertheless, further experimental studies are needed to clarify the mechanism by which IL1β and ADORA3 exert different effects on CXCL8.

Among the 18 pyroptosis-related genes, BIRC3 showed the most significant difference before and after hepatic ischemia-reperfusion. BIRC3 (cell IAP2) is one of the eight members of the human inhibitors of apoptosis (IAP) family. IAPs are characterized by the presence of baculovirus IAP repetition (BIR) domains that are involved in protein-protein interaction. In addition to the BIR domains, IAPs also contain other important domains, such as the C-terminal ubiquitin-binding (UBC) domain, caspase recruitment (CARD) domain, and the C-terminal RING zinc-finger domain [23]. BIRC3 is a negative regulatory protein that prevents apoptotic cell death by binding to the tumor necrosis factor α and preventing the formation of the tumor necrosis factor receptor pro-apoptotic signaling pathway. BIRC3 can promote epithelial-mesenchymal transformation, migration, and metastasis of HCC [24]. Specific BIRC3 inhibitor AT-406 can inhibit the proliferation of hepatocellular carcinoma cells. BIRC3 was significantly increased in the liver tissue of patients with non-alcoholic fatty liver disease compared with normal healthy samples [25]. However, the relationship between BIRC3 and hepatic ischemia-reperfusion injury has not been reported. Our study was the first to provide a theoretical basis for future experimental research.

Furthermore, we explored the underlying mechanisms of hepatic ischemia-reperfusion injury. We constructed an mRNA-miRNA-TF regulatory network to screen miRNAs and TFs potentially involved in the disease. It is well known that miRNA expression and TFs are important factors in the transcriptional and post-transcriptional regulation of gene expression. In addition, TFs are also involved in regulating miRNA signaling pathways [26]. A previous study identified several miRNAs associated with liver ischemia-reperfusion injury, such as mir-155 and mir-497 [27, 28], and TFs YAP and Nrf2 were found to predict liver ischemia-reperfusion injury [29, 30]. Nevertheless, further studies are needed to determine the regulatory mechanisms of miRNA, TFs, and target genes in liver ischemia-reperfusion.

This study also had some limitations. First, this was a retrospective analysis, and a prospective approach is necessary to confirm our results. Second, there was a lack of experimental validation to support the bioinformatics analysis results. Future experimental studies will be carried out to investigate the detailed functional mechanism of the pyroptosis-related genes in liver injury.

In summary, we found that pyroptosis was closely associated with hepatic ischemia-reperfusion injury. We used 10 pyroptosis-related genes to construct a prognostic model to accurately predict the outcomes of liver ischemia-reperfusion. Our findings shed light on the clinical application of pyroptosis-related genes in the treatment of hepatic ischemia-reperfusion injury.

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Disclosure of conflict of interest

None.

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