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ORIGINAL RESEARCH

Analysis and Regulatory Mechanisms of Platelet-Related Genes in Patients with Ischemic Stroke

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Abstract

It was found that ischemic stroke (IS) was associated with abnormal platelet activity and thrombosis. However, the potential significance of platelet-related genes (PRGs) in IS still needs to be more thorough. This study extracted IS-related transcriptome datasets from the Gene Expression Omnibus (GEO) database. The target genes were obtained by intersecting the differentially expressed genes (DEGs), the module genes related to IS, and PRGs, where the key genes of IS were screened by two machine learning algorithms. The key genes-based diagnostic model was constructed. Gene set enrichment analysis (GSEA) and the immune microenvironment analyses were analyzed targeting key genes in IS. The co-expression, TF-mRNA, and competitive endogenous RNAs (ceRNA) regulatory networks were constructed to reveal the potential regulation of key genes. Potential drugs targeting key genes were predicted as well. Totals of eight target genes were obtained and were associated with immune-related functions. Four platelet-related key genes were acquired, which were related to immunity and energy metabolism. The abnormal expressions of DOCK8, GIMAP5, ICOS were determined by the quantitative real-time polymerase chain reaction (qRT-PCR), and the significant correlations among these key genes were identified. Notably, hsa-miR-17-3p, hsa-miR-3158-3p, hsa-miR-423-3p, and hsa-miR-193a-8p could regulate all key genes at the same time. In addition, Caffeine, Carboplatin, and Vopratelimab were the targeted drugs of these key genes. This study identified four platelet-related key genes of IS, which might help to deepen the understanding of the role of platelet-related genes in the molecular mechanism of IS.

Keywords Ischemic stroke · Platelet-related genes · Immune microenvironment · Competitive endogenous RNAs network · Nomogram

Introduction

Stroke is a leading cause of death in China, and the prevalence continues to increase (Tu et al. 2023). Two main stroke types are known as a hemorrhagic stroke and ischemic stroke (IS), and most patients are belonging to IS (80–87%) (Chen et al. 2018; Huang et al. 2022). At present, the treatment of acute ischemic stroke (AIS) remains intravenous thrombolysis (IVT) with recombinant tissue plasminogen activator (rt-PA) (Xiong et al. 2022). Timely IVT with rt-PA can recanalize the occluded vessels, thereby salvaging the ischemic penumbra zone area and ultimately reducing the rates of death and disability caused by IS (Ji et al. 2023). However, the limited lysis effect of IVT on larger, proximally located thrombi has been reported (Prabhakaran et al. 2015). Strikingly, even in patients who receive rt-PA, more than half fail to respond to the drug (Staessens et al. 2020). Given the limitations of IS treatments, there is an urgent need to explore novel targets to ameliorate the prognosis of IS, and effectively predict disease severity.

Reports shown that thrombus composition in stroke was highly heterogeneous, containing fibrin, platelets, red blood cells (RBC), von Willebrand Factor (vWF), and neutrophil extracellular traps (Jolugbo and Ariens 2021). An increasing number of studies indicated that RBC-rich thrombi were associated with better recanalization outcomes (mTICI>2b) in comparison to fibrin/platelet-rich thrombi (Staessens et al. 2021). Meanwhile, Fibrin-rich clots, composed of platelet-rich zones interspersed in dense fibrin fibers, were

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associated with increased recanalization manoeuvres, longer procedure time and less favourable clinical outcomes than RBC-rich clots (Jolugbo and Ariens 2021). Recent histological analyses revealed that platelet-rich thrombus material contains denser fibrin structures that include vWF. In addition, platelet-rich areas comprise substantial amounts of extracellular DNA. Therefore, it is tempting to speculate that vWF and DNA, together with fibrin, form the structural basis of platelet-rich thrombi, and that vWF and DNA could be responsible for the observed rt-PA resistance of platelet-rich thrombi in patients. Extracellular DNA and histones have indeed been shown to modify the structure of fibrin, making it more resistant to enzymatic degradation via rt-PA (Longstaff et al. 2013). These findings indicated that thrombus composition impacted mechanical properties, which might affect embolization and endovascular removal by thrombectomy and thrombolysis (Alkarithi et al. 2021).

Platelets are recognized as the primary cells regulating hemostasis and thrombosis. The vascular importance of platelets has been attributed to their essential role in thrombosis, mediating myocardial infarction, stroke, and venous thromboembolism (VTE) (Koupenova et al. 2018). Liu et al. found that platelet-related pathway activity and pathological pathway activity were significantly higher in IS patients than in normal control (HC) samples (Agayeva et al. 2016). However, previous reports on the relationship between high platelet reactivity and clinical deterioration did not reach a clear conclusion (Agayeva et al. 2016; Cheng et al. 2017; Oh et al. 2016; Zheng et al. 2013). Many evidences suggest that thrombus composition may contribute to the failure of blood flow recanalization in patients with acute IS (Patil et al. 2022). In summary, the exact cause of IS and the associated genes are still unclear, especially the pathogenesis involving platelet-related genes (PRGs) as well as involved genetic factors need to be further investigated (Luo et al. 2019).

Until now, few studies have analyzed the mechanisms of PRGs in IS. Hence, more advanced methods and studies are needed to validate the markers of IS and further reveal the pathogenesis of the disease. In this study, PRGs from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/) and IS-related transcriptome data from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/) will be used to screen and identify key PRGs in IS, and the function of key genes, the relationship between them and the immune microenvironment were further analyzed to predict their regulatory mechanisms. These results might provide an innovative insight into the therapy of IS.

Materials and Methods

Data Extraction and Pre-processing

The IS-related transcriptome datasets were downloaded from the GEO database. The GSE16561 contains 39 IS and 24 control samples, the GSE37587 contains 34 IS samples, the GSE58294 contains 23 IS and 23 control samples, the GSE158312 contains 20 IS and 4 control samples, and the GSE110993 contains 20 IS and 20 control samples. All of these samples were peripheral blood samples. Besides, the GSE16561 and GSE37587 were combined and removed batch effect by the "sva" R package (version 3.44.0) (Zhao et al. 2021). The combined dataset was used as the training dataset, the GSE58294 and GSE158312 were used as the validation datasets, and the GSE110993 was used for screening the differentially expressed miRNAs (DE-miRNAs). On the other hand, total of 692 PRGs were obtained from the MSigDB (Table S1) (Shu et al. 2022).

Functional Enrichment Analysis of Target Genes of IS

Firstly, the differentially expressed genes (DEGs) between IS and control samples in the combined dataset were compared by the "limma" R package (version 3.48.3) ($|\log_2 FC| > 1$, adj.p.value < 0.05) (Colaprico et al. 2016). Then, the co-expression network was constructed by the "WGCNA" R package (version 1.70-3) to screen relevant module genes which were associated with IS (Langfelder and Horvath 2008). Then, the target genes were obtained by intersecting the DEGs, module genes, and PRGs using "Venn". Besides, the functional enrichment analysis of target genes was conducted by the "clusterprofiler" R package (version 4.0.2) ($p < 0.05$, count ≥ 1) (Wu et al. 2021).

Identification of the Key Genes and Construction of the Diagnostic Model of IS

Firstly, the minor absolute shrinkage and selection operator (LASSO) analysis was used to screen the characteristic genes by "glmnet" R package (version 4.1-4) (Friedman et al. 2010). Secondly, the importance of target genes was evaluated. The features with low correlation were removed to find the best variable, and then, the feature genes were obtained by the "Boruta" R package (version 7.0.0). Thirdly, the key genes were obtained by crossing two sets of genes. Besides, the expression pattern of key genes in the training dataset (combined dataset) and two validation datasets (GSE58294 and GSE158312) were studied.

To study the ability of key genes to distinguish the IS, the receiver operating characteristic (ROC) curves of key genes

logic regression model in the training dataset and two validation datasets were drawn by the "pROC" R package (version 1.18.0). The area under the ROC curve (AUC) value of the logic regression model greater than 0.7 were considered the genes in the model to have the diagnostic value. Based on it, the diagnostic model (nomogram) with these critical genes was constructed by the "rms" R package (version 6.1-0). Then, the calibration and decision curves were drawn to verify the validity of the diagnostic model.

The Function Analysis of Key Genes

The median expression value of each key gene was used to divide the samples into high and low expression groups, and the gene set enrichment analysis (GSEA) was performed to study the pathways of each key gene by "clusterProfiler" R package (version 4.0.2) and "org.Hs.eg.db" R package (version 3.13.0) (|NES|> 1, NOM $P < 0.05$, FDR < 0.25), respectively (Colaprico et al. 2016).

Analysis of Immune Microenvironment

In this study, the proportions of 24 immune cells, 17 immune reactions and 26 human leucocyte antigens (HLAs) between IS and control samples were calculated by "ssGSEA" algorithm and compared by the "Wilcox test", respectively (Zhang et al. 2021). Moreover, the correlations between key genes and immune cells, the correlations between key genes and immune reactions, and the correlations between key genes and HLAs were further studied by "Spearman", respectively. Furthermore, the inflammation index of all samples was calculated by the "gsva" R package (version 1.40.1) according to 200 inflammation-related genes, and the correlations between key genes and inflammation index were analyzed by "Spearman" (Li et al. 2022).

Analysis of Potential Regulatory Mechanisms of Key Genes and Drug Prediction

The correlations among key genes were calculated by "Spearman". Then, the co-expression network of critical genes and the top 20 genes with the highest correlation with key genes were constructed in the GeneMANIA database, respectively (http://genemania.org/).

Moreover, the transcription factors (TFs) targeting key genes were predicted in the Cistrome database (http://cistrome.org/db/), and the TF-mRNA network was constructed by "Cytoscape" (Shannon et al. 2003).

In addition, the differentially expressed miRNAs between IS and control samples in GSE110993 were compared by the "limma" R package ($|\log_2 FC| > 1$, adj.p.value < 0.05). Then, the miRNAs targeting key genes were predicted in the miRWalk database (https://mirwalk.umm.uni-heidelberg.de/). The targeted miRNAs were obtained by intersecting the DE-miRNAs and predicted miRNAs for further analyses. Next, the lncRNAs targeting targeted miRNAs were expected in the miRTarBase database (https://miRTarBase.cuhk.edu.cn/). Finally, the competitive endogenous RNAs (ceRNA) network was constructed by "Cytoscape".

Furthermore, the targeted drugs of critical genes were expected in the Drug-Gene Interaction database (DGIdb, http://www.dgidb.org/), and the gene-drug network was constructed by "Cytoscape".

Validation of the Expression of Key Genes

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate the expression of key genes in the blood of 5 IS and 5 control samples. Total RNA was extracted using TRIZol (Thermo Fisher, Shanghai, CN), mRNA was reverse transcribed into cDNA, and the qPCR reactions were performed using SureScript-First-strand-cDNA-synthesis-kit (Servicebio, Wuhan, CN).

Results

Totals of Eight Target Genes Were Associated with Immune-Related Functions

The GSE16561 and GSE37587 were combined and removed batch effect, and the combined dataset contains 73 IS and 24 control samples (Fig. 1A, B). There were 459 DEGs (371 up-regulated and 88 down-regulated) between 73 IS and 24 control samples in combined dataset (Fig. S1A, B). Sample clustering analysis was implemented and the results showed that there were no outlier samples in combined dataset (Fig. S2A). When the optimal soft threshold value was identified as 18, the signed R^2 approached the threshold value of 0.85 (red line) and the mean connectivity was close to 0, which suggested that the network was close to scale-free distribution (Fig. S2B). Totals of 14 modules were obtained (Fig. S2C). Then, the correlations between modules and IS were evaluated, and the results showed that the greenyellow module had a significantly positive correlation with IS (cor = 0.4, $p = 5e{-}05$), and the yellow module had a significantly negative correlation with IS (cor = −0.5, $p = 2e{-}07$) (Fig. S2D). Finally, 344 genes in greenyellow module and 950 genes in yellow module were screened for subsequent analyses. Hence, totals of eight target genes, including CAT, CD81, DOCK8, GIMAP5, ICOS, ITPR3, SNX10, and USP8 were obtained by intersecting 459 DEGs, 1,294 module genes and 692 PRGs (Fig. 1C).

As the perspective of function, these eight target genes were enriched to 265 Gene Ontology (GO) functions, including immunological synapse formation, positive regulation of

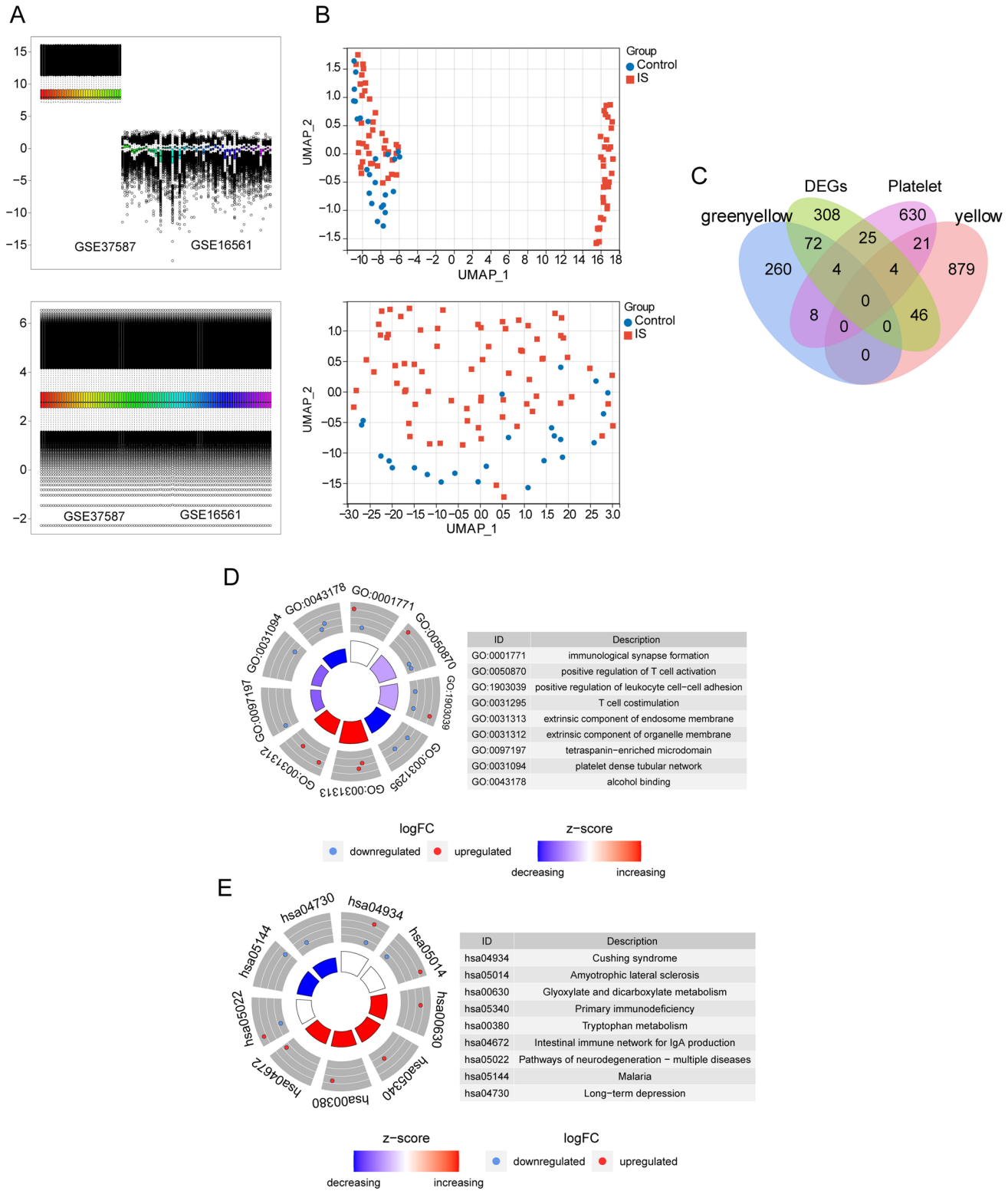


Fig. 1 Identification and functional enrichment analysis of eight target genes in the ischemic stroke (IS)-related combined dataset. **A** Boxplot for the combined dataset before and after removing batch effect based on the GSE16561 and GSE37587 datasets. **B** Uniform manifold approximation and projection (UMAP) plot between the control and IS samples in the combined dataset before and after removing batch effect. **C** Venn diagrams for eight target genes. **D** and **E** Circle plot for gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analysis of eight target genes

leukocyte cell–cell adhesion, positive regulation of T-cell activation, etc. Besides, these genes were associated with 19 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including Cushing syndrome, glyoxylate and dicarboxylate metabolism, tryptophan metabolism, primary immunodeficiency, intestinal immune network for IgA production, etc. (Fig. 1D, E; Tables S2, S3).

DOCK8, GIMAP5, ICOS and ITPR3 Were Identified as the Key Genes of IS

In this study, five characteristic genes, including CAT, DOCK8, GIMAP5, ICOS, and ITPR3 were screened by "LASSO", and six feature genes, including CD81, DOCK8, GIMAP5, ICOS, ITPR3, and SNX10 were screened by "Boruta" (Fig. S3A, B). Then, four key genes, including DOCK8, GIMAP5, ICOS, and ITPR3 were obtained by crossing above two sets of genes (Fig. 2A). The expression trends of these key genes were consistent in training dataset and two validation datasets. Among them, DOCK8 was significantly highly expressed, and GIMAP5, ICOS and ITPR3 were significantly lowly expressed in IS samples in both combined dataset and GSE58294 ($p < 0.05$) (Fig. 2B).

When all key genes were considered as a whole (logic regression model), the AUC value was greater than 0.8 in training dataset and two validation datasets (Fig. 2C). Then, the nomogram with these four key genes was constructed (Fig. 2D). The calibration curve of the nomogram showed that the disease risk prediction closed to fact, and the decision curve showed that the benefit rate of the nomogram model was higher than each individual gene, all these results were indicated that the prediction model could be used as an effective diagnostic model (Fig. 2E, F).

The Functions of Key Genes Were Related to Immunity and Energy Metabolism

The results of GSEA of these four key genes were revealed that all these key genes were related to energy metabolism functions such as mitochondrial gene expression. The function of myeloid leukocyte-mediated immunity was highly enriched in high DOCK8 expression group and both low GIMAP5 and ICOS expression groups. Besides, the function of toll-like receptor signaling pathway was highly enriched in high DOCK8 expression group. On the other hand, it was worth noting that all these key genes were associated with primary immunodeficiency. The pathways of GIMAP5 and ICOS were associated with complement and coagulation cascades, FC gamma R-mediated phagocytosis, leukocyte transendothelial migration, oxidative phosphorylation, etc. Besides, T-cell receptor signaling pathway was highly enriched in high ITPR3 expression group (Fig. 3A–D).

Immune Microenvironment Analyses

Totals of three immune cells (macrophages, mast cells and neutrophils), seven immune reactions (antimicrobials, BCR signaling pathway, chemokine receptors, etc.) and five HLAs (HLA-DMA, HLA-DRB1, HLA-H, and so on) were significantly increased in IS individuals, and 12 immune cells (contained B cells, CD8 T cells, cytotoxic cells, etc.) and seven HLAs (HLA-DMB, HLA-DOA, HLA-DOB, and so on) were significantly decreased in IS group ($p < 0.05$) (Fig. S4A, C, E). The correlation analyses results showed that the expression of DOCK8 was positively associated with most of immune cells, immune reactions and HLAs, which were increased in IS group, and negatively associated with most of them which were decreased in IS group. It was worth noting that the situations of GIMAP5, ICOS and ITPR3 were opposite to DOCK8, which were consistent with the results of their expression and function analyses (Fig. S4B, D, F). Among them, ICOS had the strongest negative correlation with macrophages ($R = -0.74$, $p < 2.2e{-}16$), and ITPR3 had the strongest positive correlation with T-cell ($R = 0.84$, $p < 2.2e{-}16$). ITPR3 had the strongest negative correlation with chemokines ($R = -0.72$, $p < 2.2e{-}16$), and DOCK8 had the strongest positive correlation with TNF family members ($R = 0.58$, $p = 5.9e{-}10$). ICOS had the strongest negative correlation with HLA-E ($R = -0.66$, $p = 2.2e{-}13$), and GIMAP5 had the strongest positive correlation with HLA-DOB ($R = 0.72$, $p < 2.2e{-}16$) (Fig. 4A–C). In addition, there was a significantly positive correlation between DOCK8 and inflammatory index ($p < 0.05$), and GIMAP5, ICOS and ITPR were negatively correlated with inflammatory index (Fig. 4D).

Potential Regulatory Mechanisms of Key Genes and Drug Prediction

The correlation analysis results showed that there were strongly significant positive correlations among GIMAP5, ICOS and ITPR, and DOCK8 had strongly negative correlations with other three key genes. These results were consistent with the results of their expression and function analyses ($p < 0.01$). Besides, there was the strongest positive correlation between GIMAP5 and ICOS (cor = 0.77) (Fig. 5A). The co-expression network of key genes and top 20 genes with the highest correlation with the key genes was constructed, respectively. Notably, DOCK8 might be a hub gene which was associated with the functions of hemostasis, coagulation, regulation of GTPase activity, etc. (Fig. S5).

Moreover, the TF-mRNA regulatory network was constructed, where MYB and STAG1 could regulate DOCK8 and GIMAP5 at the same time (Fig. 5B). The ceRNA regulatory network was constructed as well. Among them, hsa-miR-17-3p, hsa-miR-3158-3p, hsa-miR-423-3p, and

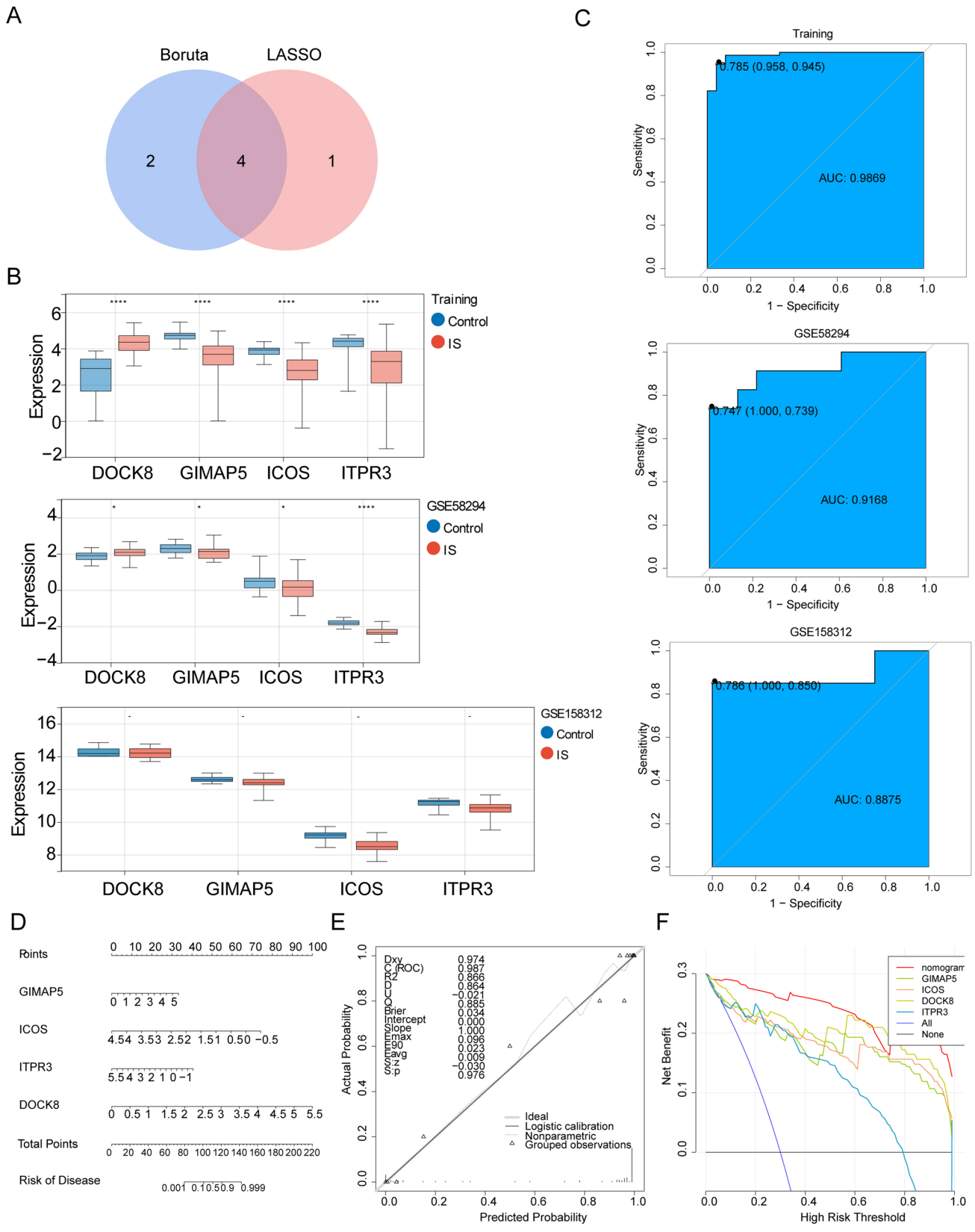


Fig. 2 Collection and exploration of four key genes of IS. **A** Venn diagrams for four common key genes. **B** Boxplots for the expressions levels of four key genes between the IS and control samples in three cohorts, respectively. *$p < 0.05$, ****$p < 0.0001$. **C** Receiver operating characteristic (ROC) curves of four key genes for distinguishing the IS and control samples in three cohorts. **D** Nomogram was constructed based on four key genes. **E** Calibration curve of nomogram. **F** Decision curve analysis (DCA) of nomogram and four key genes

hsa-miR-193a-8p could regulate all of these four key genes at the same time. LINC00598 could regulate hsa-miR-20a-5p, hsa-miR-17-5p, and hsa-miR-3158-5p at the same time. Besides, LncRNA HOX transcript antisense RNA (HOTAIR) and C17orf77 could impact hsa-miR-148b-3p and further regulate DOCK8, ICOS and ITPR3 at the same time (Fig. 5C).

Furthermore, totals of five targeted drugs of key genes (DOCK8, ICOS and ITPR3) were predicted, which contains *Caffeine*, *Carboplatin*, *Gemcitabine*, *MEDI-570* and *Vopratelimab* (Fig. 5D).

Expression Verification

The results of qRT-PCR were showed that DOCK8 was significantly highly expressed (Fig. 6D), ICOS and ITPR3 were significantly lowly expressed in IS samples (Fig. 6B, C) ($p < 0.05$), GIMAP5 was lowly expressed in IS samples but not significant ($p > 0.05$) (Fig. 6A). These results were consistent with the results of our previous analyses in both combined dataset and GSE58294.

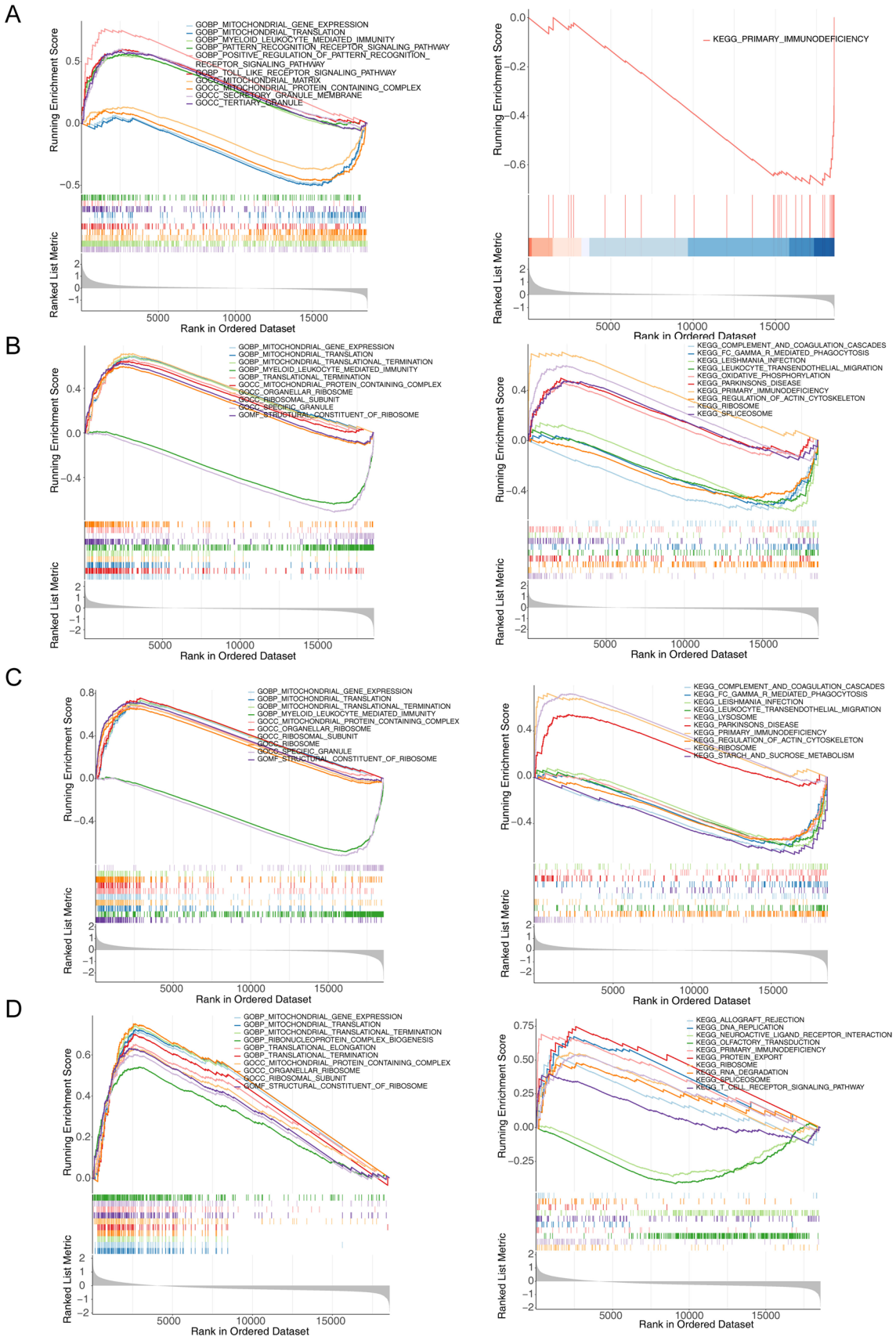
Discussion

Many human diseases are associated with platelets, including coronary artery disease, diabetes, stroke, renal disease, and autoimmune diseases (Qian et al. 2008). In this study, we found out four key genes, including GIMAP5, ICOS, ITPR3, and DOCK8. Among them, GTPase of immunity-associated protein 5 (GIMAP5) was associated with lymphocyte survival, immune homeostasis and auto-immune disease, but the mechanism of which in IS was still not clear (Patterson et al. 2018). Inducible costimulator (ICOS) was a co-stimulating molecule expressed by activated conventional T cells and regulatory T cells (Nakamura 2019). Changes in type 3 inositol 1, 4, 5-triphosphate receptor (ITPR3) was highly expressed in a variety of diseases, such as cholestasis, non-alcoholic fatty liver disease, autism spectrum disorders, and etc., which might be associated with mitochondrial dysfunction (Trinchese et al. 2022; Khamphaya et al. 2018; Shibao et al. 2003). The dedicator of cytokinesis 8 (DOCK8), a well-studied member of the DOCK protein family, was an atypical guanine nucleotide exchange factor (GEF). DOCK8 has been reported mainly expressed in the immune system, kidney, lung, placenta, pancreas, and microglia of the central nervous system (CNS) (Zhang et al. 2022).

Our findings indicate that the DOCK8 gene expression is upregulated in IS and downregulated in GIMAP5, ICOS, and ITPR3. However, AlKhater described a young woman with a DOCK8 deletion who was diagnosed with CNS vasculitis and later developed stroke as well (AlKhater 2016). The Rayasam study found that ICOS, one of the T follicular helper cells (TFH) markers, enters ischemic brain tissue and involves the JAK/STAT pathway as a mechanism of injury during the reperfusion phase of stroke (Rayasam et al. 2022). Cheng suggested that elevated ICOS genes in smokers may lead to an increased risk of stroke and that some genes expressed in blood leukocytes and platelets after stroke may lead to worse stroke outcomes in smokers (Cheng et al. 2019). Dueker et al. identified several candidate genes for the pathogenesis of carotid plaque, including ITPR3 (Dueker et al. 2020). Although this differs from our study, it can be found that ICOS, ITPR3, and DOCK8 are all involved in the development of stroke, but the exact mechanism is unclear.

In terms of immunization of IS, mounting evidence has demonstrated that peripheral innate immune cells such as neutrophils, macrophages, and natural killer cells are recruited to the infarct site after IS in patients (even the T-cell infiltration persists for several years) as well as experimental IS models (Xie et al. 2019; Yilmaz et al. 2009; Iadecola and Anrather 2011). Consistent with these studies, we also found that the myeloid leukocyte-mediated immune functions were highly enriched in high DOCK8 expression and low GIMAP5 expression groups. In addition, the functions of TLR signaling pathways were highly enriched in the high DOCK8 expression group. TLR, as part of the innate immune system, is a bridge between innate and acquired immunity and has been shown to be closely associated with the inflammatory cascade observed after cerebral ischemia (Fadakar et al. 2014; Yang et al. 2022). After hypoxic-ischemic events, TLRs present partially in the endothelial cell membrane are involved in endothelial dysfunction and play an integral role in the activation of the inflammatory cascade response (Ashayeri Ahmadabad et al. 2021). Therefore, we speculate that DOCK8 plays a critical role in the TLR signaling pathway and further affect the progression of IS. Besides, the T-cell receptor signaling pathway was highly enriched in the high ITPR3 expression group. These results suggested that the key genes could impact the IS by myeloid leukocyte-mediated immune function, toll-like receptor signaling pathway, and T-cell receptor signaling pathway. In addition, our results also proved that these key genes were related to energy metabolism functions such as mitochondrial gene expression, ITPR3 might play an important role in this.

Furthermore, we found that various immune cells were significantly lower and the macrophages, mast cells and neutrophils were significantly higher in IS patients. Related studies have shown that T cells have adverse effects on stroke by promoting leukocyte adhesion to the cerebrovascular system and triggering thrombotic inflammation (Miro-Mur et al. 2016). Wang et al. found that peripheral blood T and NK cell levels were significantly lower in stroke patients than in normal peripheral blood, and that T and B cell levels were negatively correlated with stroke severity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GOCC_ORGANELLAR_RIBOSOME
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GOCC_SPECIFIC_GRANULE
GOMF_STRUCTURAL_CONSTITUENT_OF_RIBOSOME
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES
KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS
KEGG_LEISHMANIA_INFECTION
KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION
KEGG_OXIDATIVE_PHOSPHORYLATION
KEGG_PARKINSONS_DISEASE
KEGG_PRIMARY_IMMUNODEFICIENCY
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON
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KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION
KEGG_LYSOSOME
KEGG_PARKINSONS_DISEASE
KEGG_PRIMARY_IMMUNODEFICIENCY
KEGG_REGULATION_OF_ACTIN_CYTOSKELETON
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KEGG_OLFACTORY_TRANSDUCTION
KEGG_PRIMARY_IMMUNODEFICIENCY
KEGG_PROTEIN_EXPORT
KEGG_RIBOSOME
KEGG_RNA_DEGRADATION
KEGG_SPLICEOSOME
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY
Running Enrichment Score
Ranked List Metric
Rank in Ordered Dataset

◂**Fig. 3** Gene set enrichment analysis (GSEA) of four key genes, including GO (left) and KEGG (right) enrichment analyses. **A** DOCK8. **B** GIMAP5. **C** ICOS. **D** ITPR3

(Wang et al. 2017). Liu et al. also found that the IS group had significantly lower CD8+ Tcm, CD8+ Tem and Th1 cell scores were significantly lower in the IS group than in the control group (Liu et al. 2021). Our results were partially consistent with these findings. In addition, seven immune responses, as well as, inflammation-related pathway activity and platelet-related pathway activity were increased after IS. Immune-related characteristics analysis showed the notable correlations of various immune reactions and four target genes. Shao L have pointed that the pathways enriched in the IS group are complemented and coagulation cascades, lysosomes, PPAR signaling pathway, autophagy regulation and toll-like receptor signaling pathway (Shao et al. 2022), and we found a high functional enrichment of TLR signaling pathway in the high DOCK8 expression group, these evidences further indicating our conjecture that DOCK8 plays a critical role in the TLR signaling pathway and further affect the progression of IS.

In addition, we identified two regulatory networks, TF-mRNA regulatory network and ceRNA regulatory network, to further explain the molecular mechanism of key genes. It is worth noting that MYB and STAG1 were found to regulate both DOCK8 and GIMAP5, implying that MYB and STAG1 may exert similar biological functions through these two genes. Anand found that MYB/HIF1α axis is a key regulator of metabolic plasticity and hypoxic survival in pancreatic cancer cells (Anand et al. 2023). Giannini et al. demonstrated that STAG1 is overexpressed in various tumors of reproductive system cancer and is associated with aberrant activation of the epidermal growth factor (EGF) pathway (Giannini et al. 2003). Among the ceRNA regulatory network, hsa-miR-17-3p, hsa-miR-3158-3p, hsa-miR-423-3p and hsa-miR-193a-8p were found to simultaneously regulate four key genes, implying that these four miRNAs might play key regulatory roles in this biological system. In addition, LINC00598 could simultaneously regulate hsa-miR-20a-5p, hsa-miR-17-5p and hsa-miR-3158-5p, suggesting that LINC00598 might be an important lncRNA with the ability to regulate multiple miRNAs. Finally, HOTAIR and C17orf77 can affect hsa-miR-148b-3p, which further affects the expression of DOCK8, ICOS and ITPR3, suggesting that HOTAIR and C17orf77 may have important regulatory roles in this biological system. Very interestingly, Huang et al. found that lncRNA HOTAIR uptakes miR-148a-3p through a ceRNA mechanism, upregulates KLF6 expression, inhibits STAT3 pathway, promotes apoptosis and inflammation after IS, and exacerbates nerve

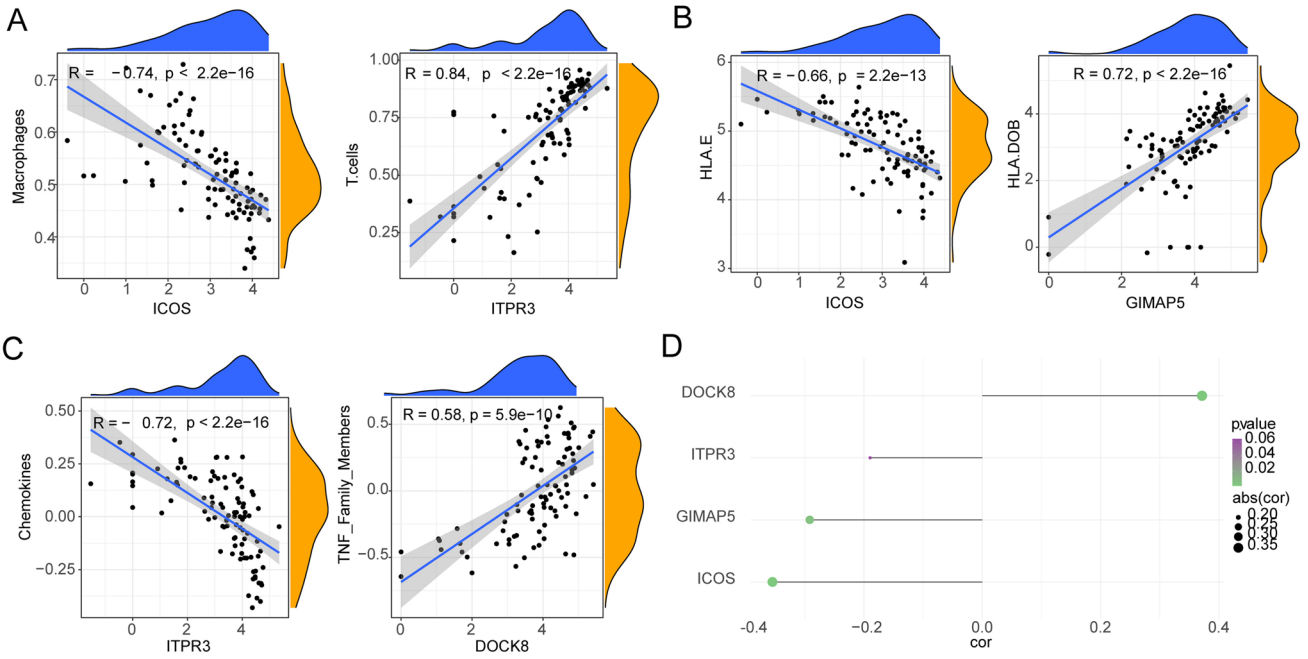

Fig. 4 Immune-related analyses of key genes. The TOP2 correlation scatterplots of key genes and significantly differential immune cells (**A**), significantly differential HLAs (**B**), and significantly differential immune reactions (**C**). **D** Lollipop chart for the correlations between key genes and inflammation index

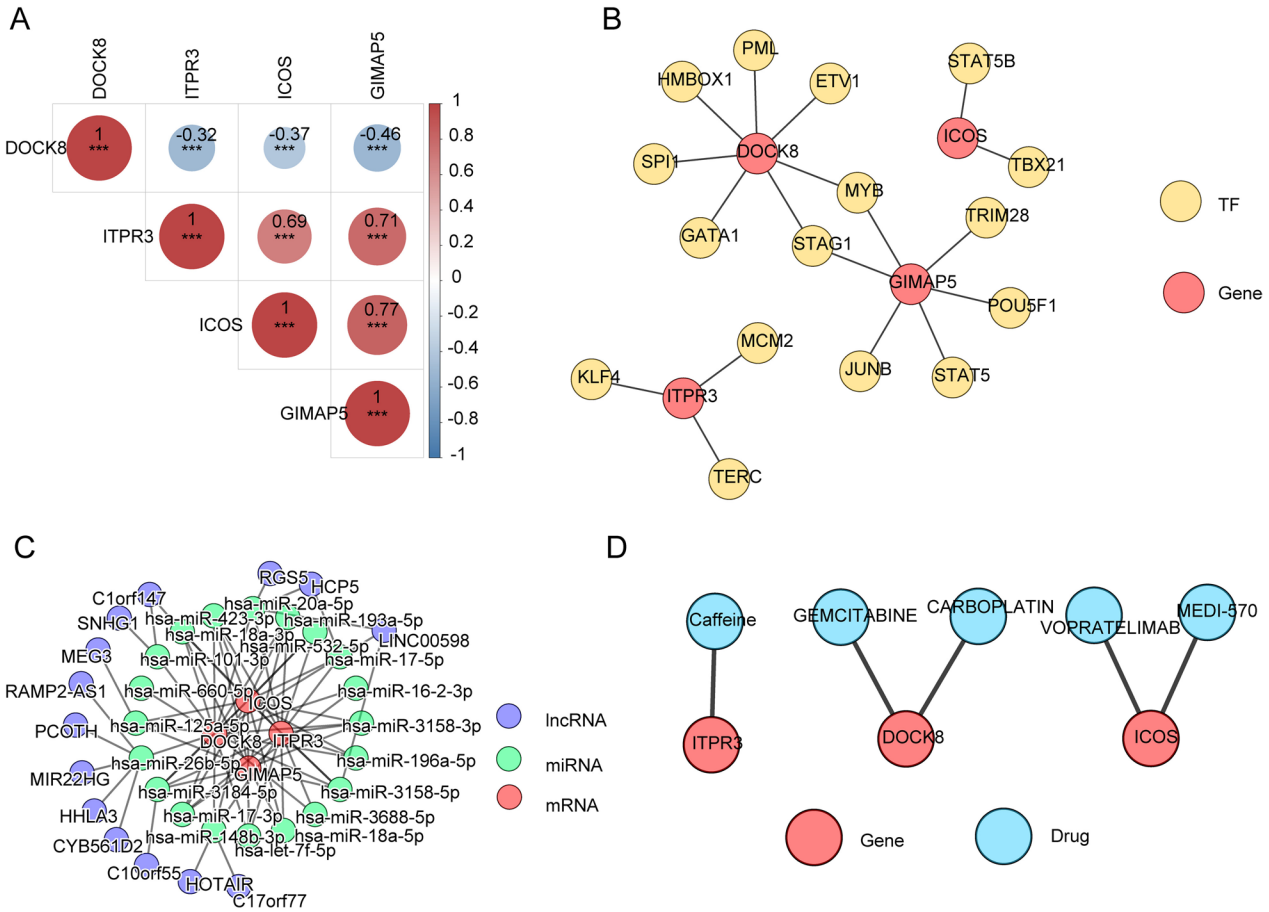

Fig. 5 Analysis of the potential regulatory network of key genes and drug prediction. **A** Correlation heatmap among four key genes. **B** The transcription factors (TFs)-mRNA network targeting key genes. **C** The competitive endogenous RNAs (ceRNA) network targeting key genes. **D** The gene–drug network through DGIdb database

injury (Huang et al. 2021). Jin confirmed that HOTAIR binds to UPF1, which exacerbates nerve injury by binding to acyl coenzyme a ligase 4 (ACSL4) binding promotes ACSL4 degradation and mediates the progression of cerebral hemorrhage (Jin et al. 2021). Li et al. found that HIF-1α mediated the inhibition of ACSL4 expression in the early post-ischemic period, which could attenuate cerebral ischemic injury (Cui et al. 2021). Notably, Wang et al. demonstrated that the HOTAIR/EZH2 axis may be involved in OGD/R-mediated endothelial dysfunction based on the fact that miR-130a-3p mediated the effect of HOTAIR on IR injury (Wang et al. 2022). Thus, the mechanism of HOTAIR involvement in stroke injury is unclear and how it involves pathways such as C17orf77 impact hsa-miR-148b-3p, and the connection of these with ceRNA networks deserves further exploration. All these findings confirm that the interaction between ceRNA networks and hub genes will be an exciting new area of exploration and will provide new ideas for ischemic cerebrovascular disease.

At last, five targeted drugs of key genes (DOCK8, ICOS and ITPR3) were predicted, which contains *Caffeine*, *Carboplatin*, *Gemcitabine*, *MEDI-570* and *Vopratelimab*. The benefits of *coffee* consumption for stroke prevention have been revealed in protective pathway might involve ITPR3,expressing activation markers ICOS, HLA-DR, CTLA-4 and PD-1 was significantly increased in patients after treatment (Yi et al. 2017; Brunekreeft et al. 2020). It can be inferred that treatment with *carboplatin* involves immune-related mechanisms of ICOS. Pharmacological targeting of MDSCs (*Gemcitabine*) may be an effective and promising therapeutic strategy for IS treatment (Yan et al. 2022). *MEDI-570* is a monoclonal antibody against ICOS that eliminates ICOS+ cells in preclinical models. *MEDI-570* is well tolerated and has good clinical activity in refractory T-cell non-Hodgkin's lymphoma (T-NHL). The mechanism may be that *MEDI-570* leads to a sustained reduction of ICOS+ T lymphocytes (Chavez et al. 2023). The presence and persistence of this pharmacodynamic biomarker was found to confirm the agonistic effect

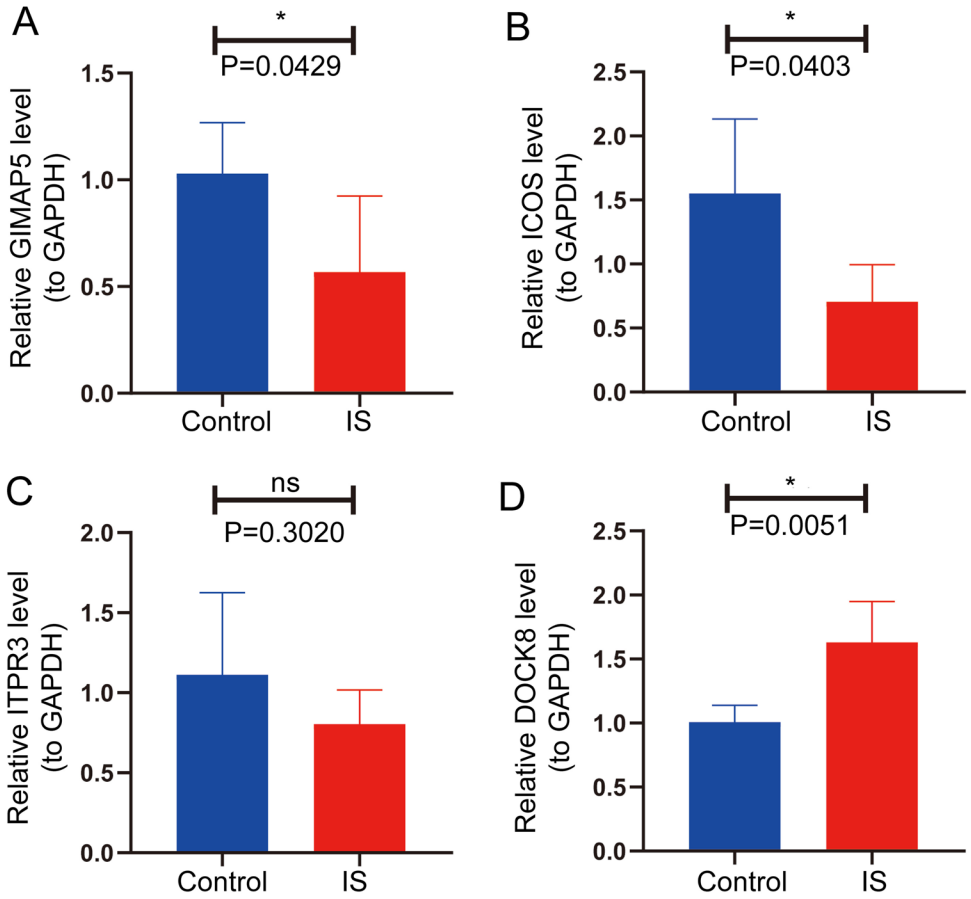

Fig. 6 Results of quantitative real-time PCR (qRT-PCR) for the expressions levels of four key genes in the clinical IS and control blood samples. **A** GIMAP5. **B** ICOS. **C** ITPR3. **D** DOCK8. *$p < 0.05$, **$p < 0.01$

of *Vopratelimab* on ICOS leading to proliferation and sustained activation of ICOS-hi CD4 T cells, including Th1, Tcm and Tfh subpopulations (Yap et al. 2022).

Overall, the nomogram model by converting the gene expression into a total score exhibited effective performance for risk prediction of IS and took all of the expression of four platelet-related key genes into consideration for clinical decision-making with the help of peripheral blood tests. Besides, given consideration to correlation of various immune cells, immune reactions as well as drugs and key genes, the treatment strategy targeting corresponding mechanisms to take anti-inflammatory response in IS are expected to develop in the future.

Limitations and Conclusions

In conclusion, our results suggested that DOCK8, GIMAP5, ICOS and ITPR3 were key PRGs of IS, where DOCK8 plays a key role in the TLR signaling pathway and further influences the progression of IS. Notably, we found that HOTAIR could affect hsa-miR-148b-3p, which could further affect the expression of DOCK8, ICOS and ITPR3, we will further explore this in our subsequent research based on a larger amount of IS cohorts. This provides additional evidence for potential biomarkers of immune, energy metabolism and therapeutic relevance in IS.

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Data Availability The datasets analysed during the current study are available in the GEO (https://www.ncbi.nlm.nih.gov/geo/) repositories (GSE16561, GSE37587, GSE58294, GSE158312, and GSE110993).

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval The study was conducted following Declaration of Helsinki and approved by the Chinese Clinical Trial Registry (Guizhou

Stroke Registration Study, No. ChiCTR1900022140) Ethical Review Committee.

Informed Consent The patients provided their written informed consent to participate in this study.

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