



Screening potential prognostic biomarkers for portal vein emboli in patients with hepatocellular carcinoma

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Background: The formation of portal vein tumor thrombus (PVTT) is closely related to the prognosis of patients with hepatocellular carcinoma (HCC). However, the mechanisms by which PVTTs form and the biomarkers involved are still little understood.

Methods: The Genome Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases were used to obtain transcriptome data from normal tissue, HCC tissue, primary tumors (PTs) of HCC, and paired PVTT tissue. Differentially expressed genes (DEGs) in PTs and PVTTs were analyzed. The differentially expressed immune genes were further investigated in terms of their prognostic significance, immune infiltration, function. Finally, we explored the relationship between risk scores and drug sensitivity based on the R package.

Results: In the two datasets, there were 458 DEGs identified in the PT and PVTT tissues, of which, 58 were immune-related genes. The differentially expressed immune genes may promote the progression of PVTT by participating in the regulation of non-cellular components such as the extracellular matrix, inflammatory factors, and chemokines. Furthermore, the immune genes *KDR*, *AKT3*, *FCGR2B*, *KLAA1429*, and *TPT1* were correlated with the prognosis of HCC in patients with PVTT. Using this data, a model was constructed to predict the prognosis of patients, thus allowing for the identification of high- and low-risk patients.

Conclusions: This study demonstrated that immune-related genes may be involved in the regulation of the extracellular matrix and acellular components, and subsequently, in the formation of PVTT. These five genes *KDR*, *AKT3*, *FCGR2B*, *KLAA1429*, and *TPT1* may be potential prognostic biomarkers and treatment targets for HCC patients with PVTT.

Keywords: Hepatocellular carcinoma (HCC); portal vein tumor thrombus (PVTT); immune; biomarker

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer and the third most deadly cancer worldwide (1). Due to a lack of typical clinical symptoms or a complete absence of any symptoms in the early stages, most HCC patients are

already in the middle and late stages of the disease at the time of diagnosis, and may even present with metastasis (2). The liver has a dual blood supply facilitated by the hepatic artery and portal vein. The anatomical structures of the arteries, veins, and bile ducts in the liver are complex. HCC

is highly aggressive and heterogeneous, and is prone to intrahepatic metastasis and invasion of blood vessels. Portal vein tumor thrombus (PVTT) is the main form of hepatic vascular invasion and metastasis. Studies have shown that the incidence of PVTT in patients at initial diagnosis of HCC ranges from 10% to 62.2% (3-5). The formation of PVTT accelerates the progression of the patient's disease course and induces portal hypertension, liver function deterioration, and other complications (6). The median overall survival (OS) of HCC patients with PVTT without any intervention is only 2.7 months (3). Indeed, PVTT is associated with the clinical stage of HCC and is an important factor for poor patient prognosis (6,7).

At present, there is no global unified guideline for the treatment of HCC patients with PVTT. Chinese guidelines recommend surgical resection, transcatheter chemoembolization, and systematic and symptomatic treatment. Multidisciplinary therapeutic assistance depends on liver function, tumor condition, and PVTT stage. The European Association for the Study of the Liver (EASL) and the National Comprehensive Cancer Network (NCCN) guidelines recommend targeted therapy and immunotherapy as first-line treatment for advanced HCC (7,8). As an inflammation-related tumor, HCC forms a complex immune tolerance microenvironment in the liver. Immunotherapy is therefore expected to be a promising treatment method for HCC (9).

Studies have shown that immune cells in the immune microenvironment contribute to the development of PVTT (10), but the specific mechanisms of action remain unclear. Thus, it is important to identify effective and sensitive immune-related molecular biomarkers for the diagnosis and treatment of patients with HCC complicated with PVTT. Wang *et al.* compared primary tumor (PT) and PVTT samples from HCC patients with matched adjacent normal tissue and identified 20 differentially expressed genes (DEGs) whose expression was significantly associated with OS and vascular invasion in HCC patients. Furthermore, these DEGs were found to modulate the aggressive phenotype of HCC cells (11). Other studies have shown that tumor-associated macrophages (TAM) are closely related to the expression of osteopontin, which is a key regulatory gene of HCC prognosis, angiogenesis, invasion, and metastasis (12-14). These reports confirm that immune cells in the immune microenvironment are closely related to the formation of PVTT and the prognosis of HCC patients.

To date, there have been few reports examining

immunity and HCC complicated with PVTT. This current study used data from online bioinformatics databases to identify the DEGs, with a particular focus on differentially expressed immune-related genes, in PT and PVTT tissues. Bioinformatics analyses were performed to analyze the mRNA expression, biological function, immune invasion, and prognostic significance of the immune-related DEGs. The identification of novel immune biomarkers paves the way for improved diagnosis and the development of potential therapies for HCC patients complicated with PVTT.

We present the study in accordance with the REMARK reporting checklist (available at <https://dx.doi.org/10.21037/jgo-21-433>).

Methods

Data sets and preprocessing

The GSE69164 and GSE77509 datasets were obtained from the Genome Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>). There was a total of 22 samples in the GSE69164 dataset, including 11 cases of carcinoma and 11 paired cases of blood clots. The GSE77509 dataset had a total of 40 samples, including 20 cancers and 20 paired thrombus cases. The RNA-seq data for the HCC and normal samples (a total of 62 samples) were obtained from The Cancer Genome Atlas (TCGA; <https://cancer.genomes.nih.gov/>) and normalized using the R package software.

Identification of the DEGs

Differential analysis was performed on all RNA-seq data, and the DEGs in the PT and paired PVTT samples were identified. The intersection of the DEGs and immune genes was used to obtain the differentially expressed immune-related genes.

Establishment of the prognostic model

To evaluate the relationship between the differentially expressed immune genes and patient prognosis, univariate Cox, Lasso, and multivariate Cox analyses were performed on the downloaded TCGA data. After identifying independent prognostic genes and determining their coefficients, the HCC patients were divided into a high-risk group and a low-risk group based on the median risk score coefficient. Receiver operating characteristic (ROC) curves

were used to test the predictive power of the survival model.

Survival analysis

Univariate Cox regression analysis and the Kaplan-Meier method were used to evaluate the prognostic effect of the differentially expressed immune genes. When the Kaplan-Meier method was used to plot survival curves, a log-rank $P < 0.05$ was considered statistically significant.

Tumor-infiltrating immune cell (TIC) analysis

The single-sample Gene Set Enrichment Analysis (ssGSEA) method was used to calculate the content of 28 types of HCC immune cells in the TCGA cohort, and the correlation between gene expression and immune cell expression was analyzed.

Enrichment function analysis and protein-protein interaction (PPI) network construction

Using the clusterProfiler, enrichplot, and ggplot2 packages, the potential biological functions and pathways of the DEGs in HCC were explored using R language. The clusterProfiler and enrichplot packages use hypergeometric distributions to calculate each item of the P and Q values for Gene Ontology (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG). AP and Q values less than 0.05 were considered significant enrichment functions. The PPI network of different genes was constructed through the string website, and the top 10 genes were calculated by Cytoscape plug-in using Cytoscape software.

Drug sensitivity analysis

The HCC gene expression matrix of TCGA was used to calculate the sensitivity of the samples to the drug based on the amount of gene expression in each sample. The difference between the high-risk group and the low-risk group was compared using the rank-sum test to determine the sensitivity of the drug.

Statistical analysis

Wilcoxon rank-sum test was used to compare the differences in gene expression between tumor tissues and carcinoma tissues. The Kaplan-Meier method was used to draw survival curves, and the log-rank test was performed

to test its significance. The ssGSEA algorithm was used to evaluate TICs. All statistical data were completed using the R language software package (<https://www.r-project.org/>), and $P < 0.05$ was considered statistically significant.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Results

Identification of DEGs and differentially expressed immune genes in PT and PVT

The GSE69164 and GSE77509 datasets were used to screen the DEGs in the PT and PVT samples. A total of 458 DEGs were associated with tumor thrombi. When these DEGs were intersected with 1735 immune genes, a total of 58 immune-related DEGs were identified (Figure 1).

Construction and validation of a prognostic model

To further understand the prognostic value of the 58 differentially expressed immune genes, univariate and multivariate analyses were performed. A total of five predictive genes, *KDR*, *AKT3*, *FCGR2B*, *KLAA1429*, and *TPT1*, were identified ($P < 0.05$). The risk score formula for OS prediction was as follows: risk score = $(0.088 \times AKT3) + (0.042 \times FCGR2B) + (0.170 \times KLAA1429) + (0.002 \times TPT1) + (0.094 \times KDR)$. Patients were divided into a high-risk group ($n=185$) and a low-risk group ($n=185$) based on the median risk score. Survival analysis showed that patients with a high-risk score generally had poorer prognosis compared to patient with a low-risk score ($P < 0.001$; Figure 2A-2D). In addition, time-dependent ROCs were used to evaluate the prognostic value of the model. The results showed that the area under the ROC curve (AUC) of 1-, 3-, and 5-year OS was 0.704, 0.661, and 0.659, respectively, suggesting that the model has a good predictive ability for 1-year OS (Figure 2E). Subsequently, the expression of these five genes and patient prognosis was examined in HCC tumor tissues and normal tissues. The results demonstrated that there was no significant difference in *AKT3* and *KDR* expression between tumor tissues and normal tissues. However, patients with high *KDR* expression had a better prognosis ($P=0.026$). The expression of *FCGR2B* was significantly lower in tumor tissues compared to normal tissues, but its expression was not

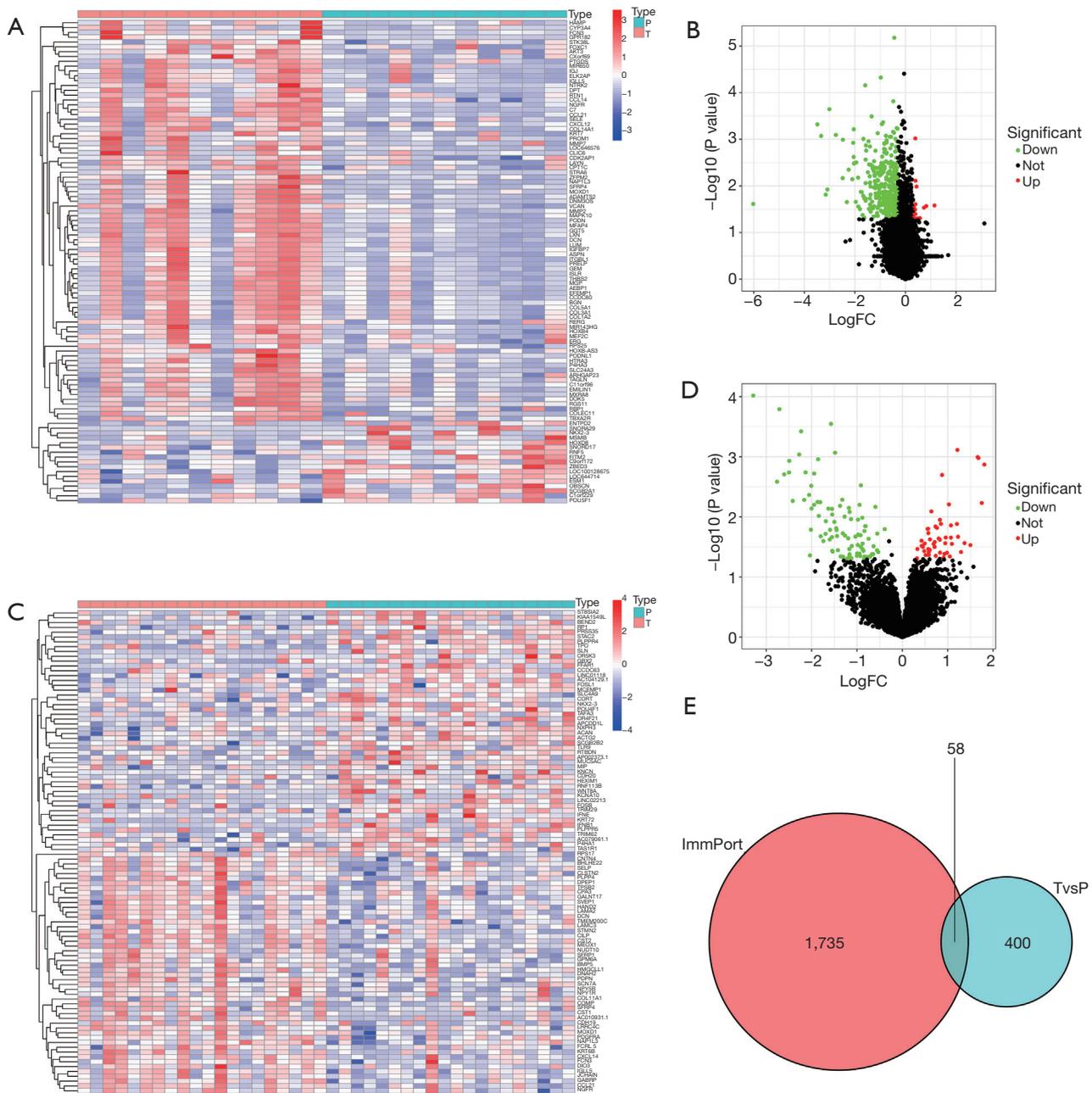


Figure 1 Identification of the DEGs in PTs and PVTs from patients with HCCs. (A) Heat map showing the DEGs in the GSE69164 dataset. (B) Volcanic maps showing the DEGs in the GSE69164 dataset. (C) Heat map showing the DEGs in the GSE77509 dataset. (D) Volcanic map showing the DEGs in the GSE77509 dataset. Compared with PVTT tissue, red or green dots represent up-regulated or down-regulated DEGs in PT, and gray dots represent genes with no significant difference in expression. (E) The Venn diagram of DEGs and immune genes. A total of 58 genes were overlapping between the DEGs and immune genes. DEGs, differentially expressed genes; PT, primary tumor; PVTT, portal vein tumor thrombus; HCC, hepatocellular carcinoma.

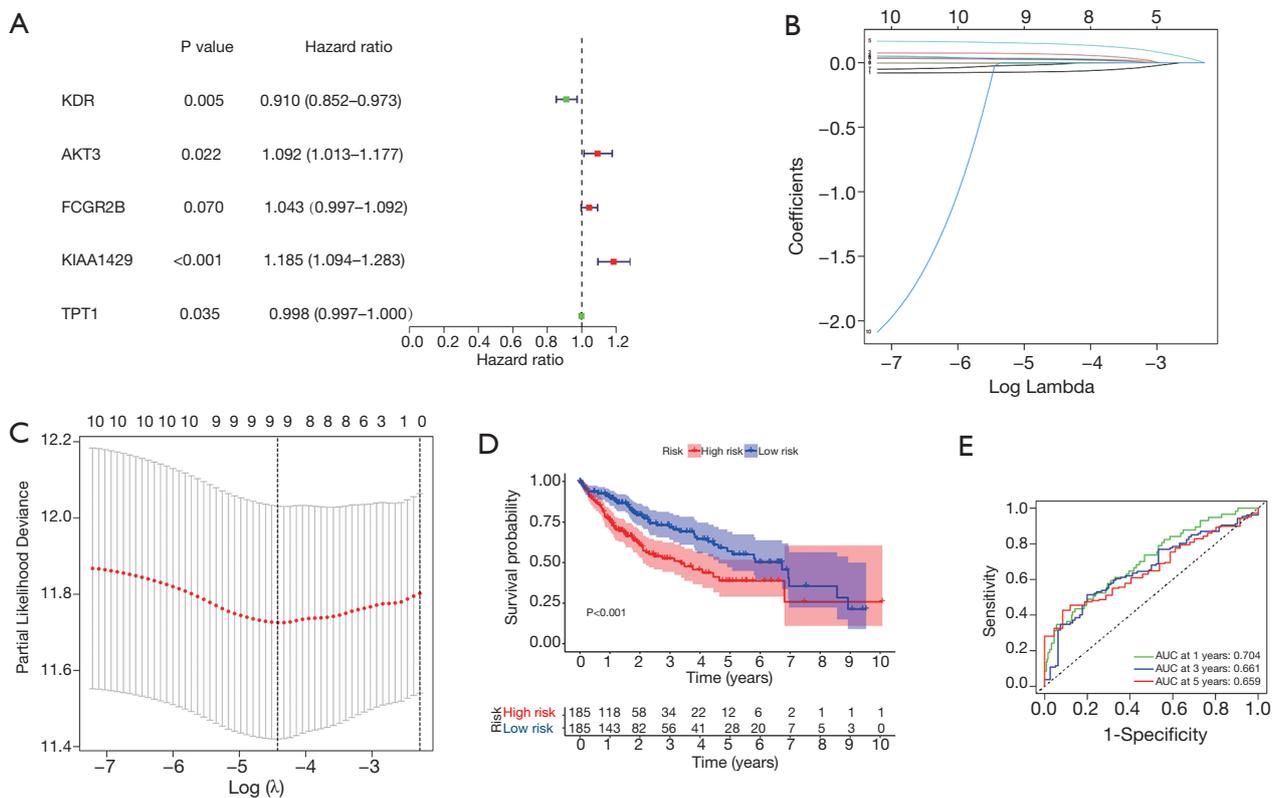


Figure 2 The effects of differentially expressed immune-related genes on the prognosis of HCC. (A) Multivariate Cox analysis of the differentially expressed immune-related genes. (B) and (C) Coefficients calculated using Lasso's multivariate Cox regression. (D) Kaplan-Meier survival curves of patients in the TCGA dataset in the high-risk and low-risk groups constructed according to risk scores. (E) The ROC curve shows the predictive efficiency of the immune-related genes. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; ROC, receiver operating characteristic; AUC, area under the ROC curve.

correlated with patient prognosis ($P=0.82$). Both *KIAA1429* and *TPT1* were significantly overexpressed in tumor tissues. Patients with low *KIAA1429* expression and high *TPT1* expression had better prognosis ($P=0.017$; *Figure 3*). HCC is highly heterogeneous, and this may be related to the evolution and heterogeneity of the tumor.

Immunoinfiltration analysis

The HCC cohort data from TCGA were used to analyze the level of immune infiltration. Except for central memory CD4 T cells and memory B cells, the other 26 types of immune cells showed different degrees of immune infiltration in HCC (*Figure 4A*), and HCC was closely related to immune cells. Further analysis showed that *FCGR2B*, *KDR*, and *AKT3* were positively correlated with the expression of most immune cells, while *KIAA1429* was

negatively correlated with the expression of most immune cells. In addition, *TPT1* was mainly positively correlated with the high expression of immune cells. However, it was negatively correlated with CD56dim natural killer cells and central memory CD8 T cell (*Figure 4B*).

Rich set function analysis and PPI network construction

The potential function of the DEGs identified in the Venn diagram was further investigated using GO and KEGG pathway enrichment analysis. The enrichment function analysis revealed that the top three biological processes (BP) were cell chemotaxis, cellular calcium homeostasis, and positive regulation of epithelial cell proliferation. The top three cellular components (CC) were external side of the plasma membrane, collagen-containing extracellular matrix, and basal part of the cell. The identified molecular

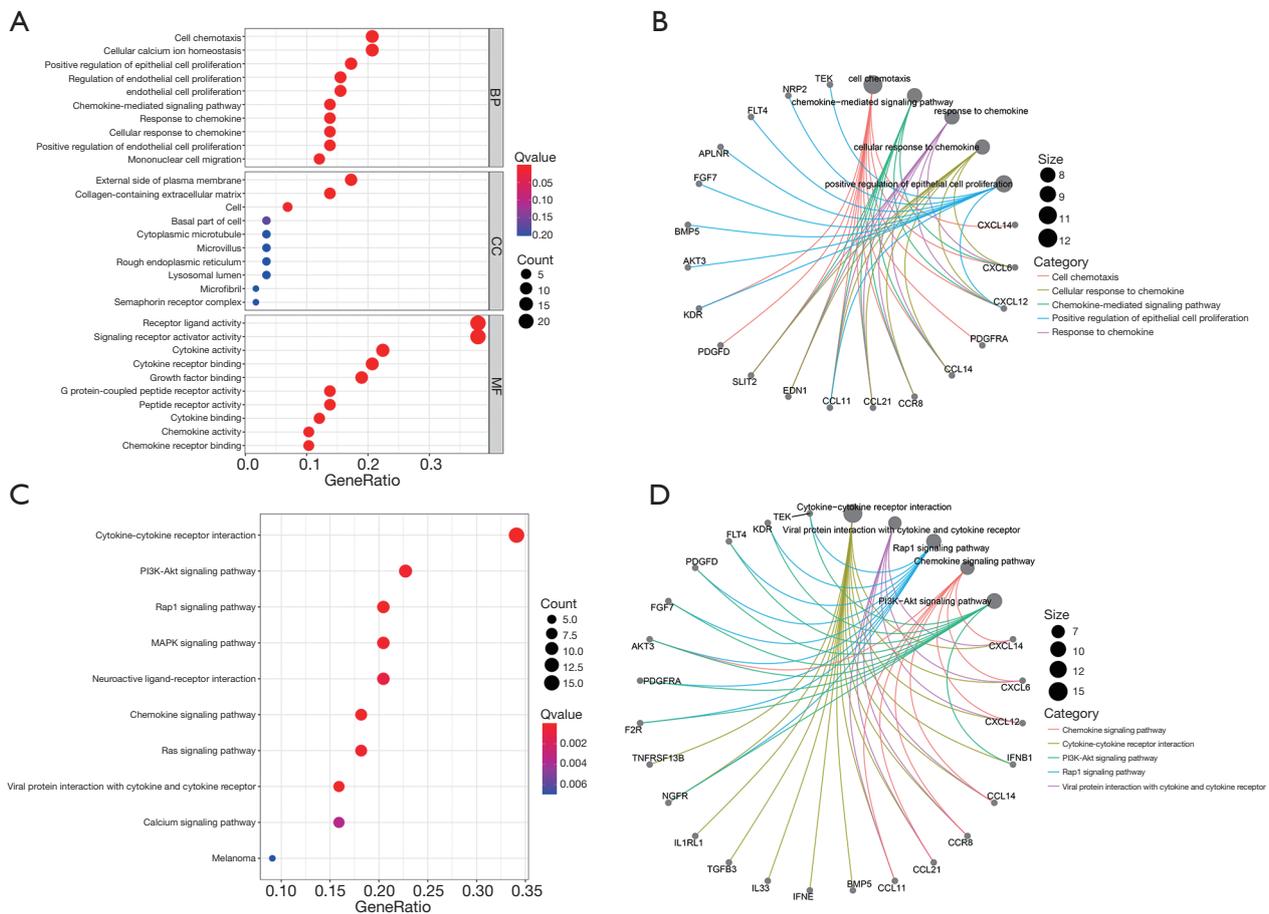


Figure 5 Analysis of the enrichment function of the DEGs. (A) A bubble diagram showing the enrichment function results. (B) A circus diagram of the enrichment function Results. The red and blue dots represent the Q value, and the radius of the dots represents the gene count. (C) Bubble diagram of KEGG pathway analysis results. (D) Circus diagram of KEGG pathway analysis results. The red and blue dots represent the Q value, and the radius of the dots represents the gene count. DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes.

functions (MF) included receptor-ligand activity, signaling receptor activator activity, and cytokine activity. KEGG signaling pathway analysis showed that the DEGs were mainly associated with the MAPK signaling pathway, the RAP1 signaling pathway, the PI3K-Akt signaling pathway, and cytokine-cytokine receptor interaction (Figure 5). The relationship between DEGs was screened and the PPI network was constructed (Figure 6). These results indicated that the screened DEGs have a certain role in the immune system.

Drug sensitivity analysis

To examine the relationship between the risk scores and

sensitivity, the TCGA data was analyzed. The genes identified were highly sensitive to certain drugs including all-trans retinoic acid (P=0.0019), axitinib (P=0.00095), gefitinib (P=0.00017), imatinib (P=0.0093), and lapatinib (P=0.018) (Figure 7). These results suggested that the model can not only predict the prognosis of patients but also assist in the clinical treatment decision-making process.

Discussion

The prognosis of HCC patients complicated with PVT is extremely poor, and to date, there is no specific sensitive marker that can predict the formation of PVT and the prognosis of patients. Studies have shown that serum S100p

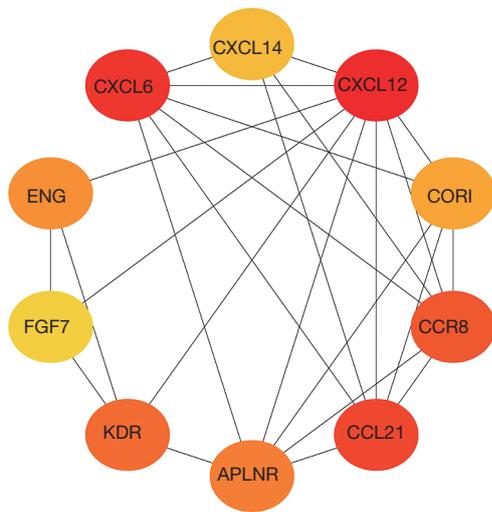


Figure 6 The PPI network of the top 10 genes with the number of nodes. The more intense the red color, the more linked nodes present. PPI, protein-protein interaction.

is overexpressed in HCC and is highly correlated with the formation of PVTT and microvascular invasion (MVI). Thus, S100p may represent a novel differential diagnostic marker for HCC and may serve as a potential predictor of preoperative MVI status (15). Xu *et al.* demonstrated that the levels of serum *SCAND3* methylation is an independent risk factor for predicting PVTT in HCC patients (16). Liu *et al.* showed that microRNA-135a promoted the formation of PVTT in nude mice by inhibiting metastasis suppressor 1, suggesting that microRNA-135a may be a potential therapeutic target for the treatment of PVTT at the molecular level (17). However, to date, there have been few studies examining the DEGs in PT and PVTT tissues. Compared with adjacent normal liver tissue, HCC with PVTT show significant molecular changes. Differential expression of some genes can regulate the invasion of HCC cells. Gene analysis between HCC and PVTT also suggested that extracellular matrix receptor interactions are associated with venous invasion of HCC (11,18). Studies have shown that the circular RNA circ0003998 is highly expressed in PVTT and PT tissues and is associated with invasive characteristics. Further *in vitro* and *in vivo* experiments confirmed that circ0003998 promotes the epithelial-mesenchymal transformation of HCC (19). This current study identified a large number of DEGs in PT and PVTT samples, suggesting that these genes may be potential prognostic markers in HCC patients with PVTT.

Studies have shown that KDR is the main receptor that

promotes the pro-angiogenesis of vascular endothelial growth factor (VEGF), and KDR/FLK-1 is one of the main receptors of VEGF. Treatment targeting KDR/FLK-1 can inhibit tumor angiogenesis, increase tumor cell proliferation, and inhibit the development of HCC (20). In addition, studies have shown that *KDR* can serve as an independent prognostic biomarker in patients with unresectable HCC (21). *AKT3* is an adverse prognostic factor in patients with HCC (22), and activation of *AKT3* promotes HCC stem cell properties and reduces chemosensitivity (23). In addition, *KIAA1429* is significantly overexpressed in HCC tissues and is associated with poor prognosis. Inhibition of *KIAA1429* impedes tumor cell proliferation and metastasis *in vitro* and *in vivo* (24). Our current study identified the differentially expressed immune genes, *KDR*, *AKT3*, *FCGR2B*, *KIAA1429*, and *TPT1*, all of which are correlated with the prognosis of HCC patients with PVTT. Furthermore, the model constructed showed a certain predictive ability for patient prognosis.

HCC is a typical inflammatory-related malignancy, and its microenvironment contains a large number of macrophages as well as innate immune and adaptive cells, forming a complex immune tolerance microenvironment (25,26). In this study, a significant degree of immune cell infiltration was observed in HCC tissue. Furthermore, the differentially expressed immune genes *FCGR2B*, *KDR*, and *AKT3* were mainly positively correlated with the expression of immune cells, while *KIAA1429* was negatively correlated with the expression of immune cells. Interestingly, *TPT1* was positively correlated with certain types of immune cells, and negatively correlated with other immune cells. A growing number of studies have shown that TAM affect the angiogenesis, invasion, and metastasis of HCC, as well as patient prognosis and tumor progression (13,27-29). In addition, increased numbers of T regulatory (Treg) cells in HCC tissues is associated with PVTT formation, metastasis and recurrence, and poor prognosis (30). Immune cells can also interact with each other. Tumor-associated neutrophils can recruit TAM cells and Treg cells to promote HCC growth and angiogenesis (31). To further understand the function of the differentially expressed immune genes, GO and KEGG analyses were performed. The results demonstrated that the DEGs were mainly involved in cytokine-cytokine receptor interaction, the MAPK signaling pathway, and the PI3K-Akt signaling pathway. The PI3K and MAPK signaling pathways rely on cells that adhere to the external matrix via integrins, which are molecules that may amplify these signals (32). Further PPI analysis

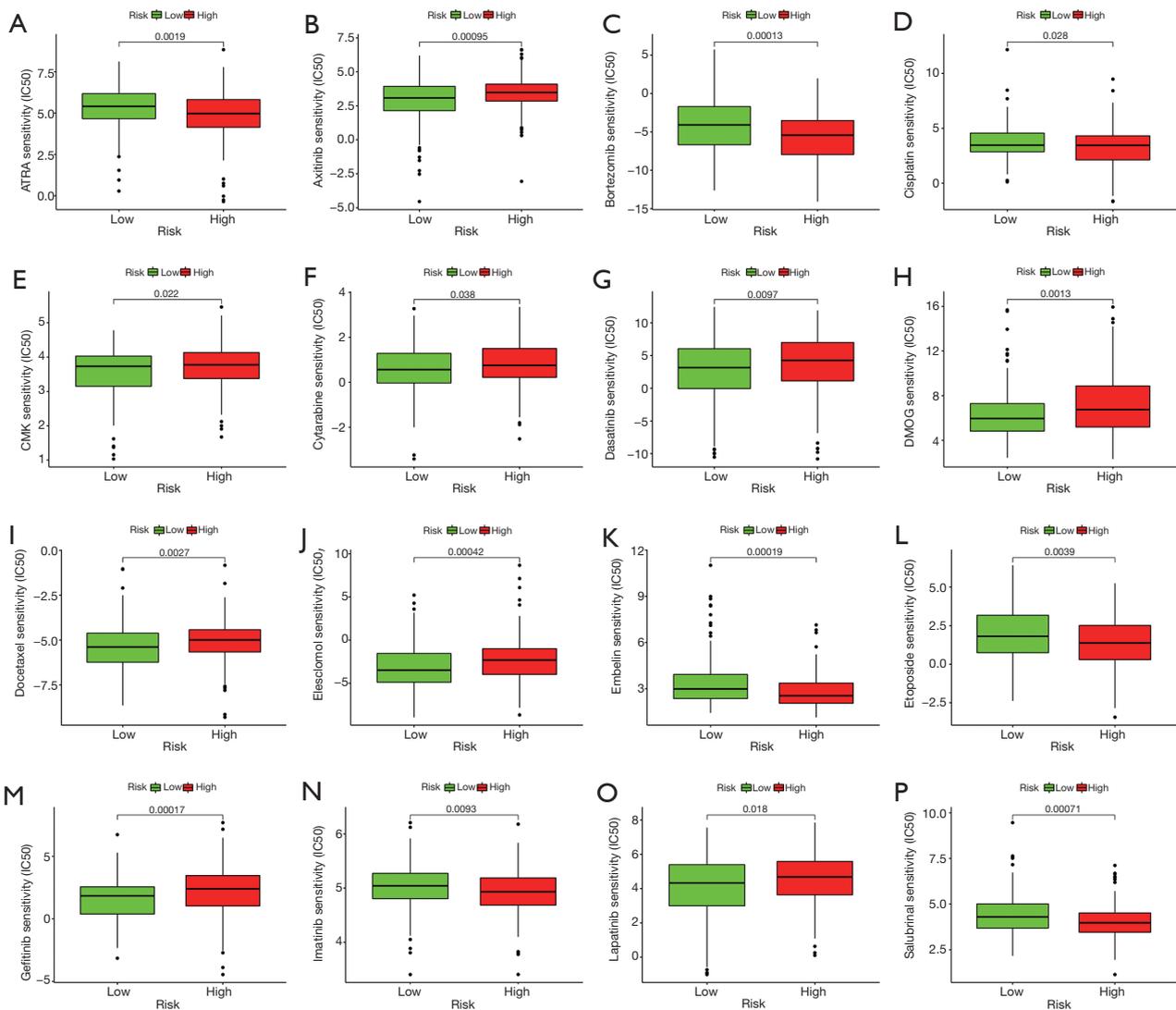


Figure 7 Drug sensitivity analysis. (A) ATRA; (B) axitinib; (C) bortezomib; (D) cisplatin; (E) CMK; (F) cytarabine; (G) dasatinib; (H) DMOG; (I) docetaxel; (J) elesclomol; (K) embelin; (L) etoposide; (M) gefitinib; (N) imatinib; (O) lapatinib; and (P) salubrinal. ATRA, all-trans retinoic acid; DMOG, dimethylallyl glycine.

suggested that the DEGs may be involved in the regulation of various non-cellular components such as inflammatory factors and chemokines. Recent studies have found that CXCL12 and its receptors, CXCR7 and CXCR4, promote invasion and metastasis of HCC, and are closely related to tumor progression, neovascularization, epithelial-mesenchymal transformation, and poor prognosis of HCC (33,34). Therefore, the DEGs identified in this study may be involved in the regulation of immune infiltration, and thereby regulate the progression of HCC.

In recent years, the emergence of immunotherapy

has provided a new option for the treatment of HCC. However, the efficacy of immunotherapy can be limited by low objective response rates, increased incidence of adverse reactions, and drug resistance. This current study demonstrated that the differentially expressed immune genes identified were highly sensitive to atrt, axitinib, gefitinib, imatinib, and lapatinib. A randomized phase II clinical study of second-line treatment for advanced HCC demonstrated that axitinib was safe and significantly improved the progression-free survival, the time to tumor progression, and the clinical benefit rates in a subset of the

Asian population (35). Lapatinib has also been shown to be well tolerated in a multi-institution phase II clinical study in patients with advanced HCC. However, the molecular and clinical features of the patients were not fully defined (36). Additional clinical trials are warranted to confirm the potential efficacy of axitinib and lapatinib in the treatment of patients with HCC complicated with PVTT.

There were some limitations to this present investigation. First, the small number of cases in the study may affect the accuracy and reliability of the results. Second, TCGA data mainly relates to American patients, and this may lead to selection bias. Expanded case numbers and basic studies in different populations are needed to verify our results.

Conclusions

In conclusion, this study identified a large number of DEGs in the PT and PVTT of HCC patients. The differentially expressed immune genes identified may promote the progression of PVTT by participating in the regulation of non-cellular components such as the extracellular matrix, inflammatory factors, and chemokines. Specifically, the differentially expressed immune genes *KDR*, *AKT3*, *FCGR2B*, *KIAA1429*, and *TPT1* may be potential predictors and therapeutic targets for HCC patients with PVTT. Further basic studies and functional experiments are warranted to confirm these results.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://dx.doi.org/10.21037/jgo-21-433>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/jgo-21-433>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was

conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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