

Integrated analysis of iron metabolic-related genes in hepatocellular carcinoma

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Research article

Keywords: Iron metabolic, Prognostic model, HCC, TCGA database, ICGC database.

Posted Date: January 18th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-147085/v1>

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Abstract

Background

Iron is a nutrient essential for hemoglobin synthesis, DNA synthesis, and energy metabolism in all mammals. Iron metabolic involved in numerous types of cancers including hepatocellular cancer. In this study, we aim to identify prognostic model that based on iron metabolic-related genes that could effectively predict the prognosis for HCC patients.

Methods

The RNA microarray and clinical data of HCC patients that obtained from The Cancer Genome Atlas (TCGA) database. We identify the clusters of HCC patients with different clinical outcome performed by consensus clustering analysis. Four iron metabolic-related genes (FLVCR1, FTL, HIF1A, HMOX1) were screen for prognostic model by performed the Cox regression analysis. The efficacy of prognostic model was validated by the International Cancer Genome Consortium (ICGC) database. Meantime, the expressions value of FLVCR1, FTL, HIF1A, HMOX1 was performed using Oncomine database, the Human Protein Atlas and Kaplan Meier-plotter.

Result

The patients with low-risk score have better prognosis than high risk score both in TCGA cohort and ICGC cohort. The prognostic model showed well performance for predicting the prognosis of HCC patients than other clinicopathological parameters by OS-related ROC curves.

Conclusion

Our survival models that based on Iron metabolic can be independent risk factors for hepatocellular carcinoma patients.

Background

Hepatocellular carcinoma (HCC) is a common type of cause of cancer-related death among human cancers [1]. Compare with other malignancies, the incidence of HCC is fifth highest in men and ninth highest in women, and it accounts for the second most cancer deaths worldwide[2]. And it's dismal prognosis with overall 1- and 3- year survival rates of only 36% and 17%[3]. Although the great progress has been obtained in chemotherapy, surgical resection, radiofrequency ablation, systemic therapy, and liver transplantation, the prognosis of Hepatocellular carcinoma still poor due to most of patients at an advanced stage[4]. Clearly, there exists an unmet need to explore innovative approaches to predictive the prognosis of HCC.

Iron is a nutrient essential for hemoglobin synthesis, DNA synthesis, and energy metabolism in all mammals. Iron metabolism is also an essential biological process including mitochondrial respiration, metabolism and detoxification, DNA synthesis, antioxidant defense, oxygen sensing, and immune defense[5]. Specially, a new type of cell death has been found in tumors called ferroptosis which is dependent on iron metabolism[6, 7]. Some studies have implicated that iron metabolism involved in numerous types of cancers including hepatocellular cancer, bladder cancer, breast cancer, colorectal cancer, gastric cancer, lung cancer, melanoma, and pancreatic cancer[8, 9]. Iron metabolism in the cell mainly including intake, utilization, and efflux processes that are regulated by numerous genes and proteins[10–12]. Iron metabolism-related genes mainly consist of iron intake genes (TFRC and DMT1), utilization genes (FTH1, FTL, HIF1A, HMOX1, SLC25A37, and SLC25A38), and efflux genes (FLVCR1 and SLC40A1)[13].

In order to explore the relationship between iron metabolism and the prognosis of HCC and whether iron metabolism-related genes can be used as a potential biomarker to prognosis of HCC and guiding clinical medication. we used the RNA microarray and clinical data of HCC patients to develop a prognostic model as an independent biomarker to predicting the survival of HCC patients.

Materials And Methods

Data download and processing

According to existing literature, the potential iron metabolism-related genes mainly including TFRC, DMT1, FTH1, FTL, HIF1A, HMOX1, SLC25A37, SLC25A38, FLVCR1, and SLC40A1 were obtained. RNA microarray and clinical data that consisting of 374 HCC and 50 adjacent normal tissues was downloaded from the TCGA database. The differential expression of iron metabolism-related genes comprising 374 HCC and 50 adjacent normal tissue were processed by the mean function, and normalized the mean expression level by log₂ transformation. We analysis the differential expression of iron metabolism-related genes between tumor tissue and normal tissues by “limma” package, and the thresholds was false discovery rate (FDR) < 0.05 and |log₂ fold change (FC)|> 1.

Consensus clustering analysis of iron metabolism-related genes

All of hepatocellular carcinoma patients were clustered into different groups by consensus clustering analysis with “ConsensusClusterPlus” in R[14]. Then, calculated the overall survival (OS) difference between different clusters by the Kaplan-Meier method. Compare the distribution of age, gender, grade and stage between different clusters by Chi-square test.

Prognostic model base on iron metabolism-related genes

The expression of the iron metabolism-related genes was analyses by using univariate Cox regression analysis to obtain the significantly related to survival genes. Then, we employed the multivariate Cox regression analysis to remove highly correlated survival-related iron metabolism-related genes. We

calculated the risk score of each HCC patients according to the prognostic model. The calculation of the risk score was conducted as follows: Risk score = (the v_j is the means expression level of gene, c_j means the regression coefficient of gene).

The Kaplan–Meier survival curve was plotted to evaluate the high- and low-risk groups by the log-rank test according to prognostic model. We determine the accuracy of the prognostic model by generated OS-related ROC curves. Independent prognostic analysis was we predict whether prognostic model can be as an independent prognostic factor for HCC patients.

Tumor-infiltrating immune cells of prognostic model

CIBERSORT is an analytical tool that estimates infiltrating immune composition of a tumor biopsy using gene expression data. In this study, we quantify the fractions of immune cells in the hepatocellular carcinoma samples by CIBERSORT method. The results of the inferred fractions of immune cell populations produced by CIBERSORT were considered accurate with a threshold of $P < 0.05$ [15].

Iron metabolism-related genes validation by Oncomine database, The Human Protein Atlas and Kaplan-Meier plotter

The Oncomine database, a cancer microarray database and web-based data-mining platform, for the purpose of mining cancer gene information [16]. The Oncomine database was applied for differential expression classification of common cancer types, and their respective normal tissues, as well as clinical and pathological analyses. The Human Pathology Atlas allowed for generation of personalized genome-scale metabolic models for cancer patients to identify key genes involved in tumor growth. In this study, we utilized Oncomine database and The Human Protein Atlas to explore the protein expression of the iron metabolism-related genes in HCC tissues and liver tissues. The prognostic value of the RBPs in HCC was verified by the Kaplan-Meier plotter online tool.

Validation of prognostic model

Our prognostic model external validation by the International Cancer Genome Consortium (ICGC) database. The risk score of each HCC patients was calculated by the same formula. ICGC database mainly contain 116 Japanese HCC patient and adjacent normal tissues.

Statistical analysis

All statistical analyses were performed by Perl language and R language. The Kaplan–Meier curve was plotted to showed the survival differences between the high- and low-risk groups and the log-rank test was performed to determine the significance of the differences. And all comparisons statistical significance was defined as a $P < 0.05$.

Results

Differentially expressed of iron metabolism-related genes

Heatmap was generated to visualize the expression level of m⁶A methylation-related genes between tumor tissue and normal tissue (**Figure 1**). The expression levels of SLC11A2, FLVCR1, FTH1, TFRC, SLC25A38, and FTL were significantly overexpressed in tumor samples than normal tissue. The expression levels of HMOX1 were significantly low expressed in tumor samples.

Consensus clustering analysis of iron metabolism-related genes

The consensus clustering analysis showed that the most appropriated selection was divided HCC patient into two clusters (**Figure 2A-D**). The Kaplan–Meier curve showed that 5 year-OS of cluster 1 was significant longer than cluster 2 (P = 0.006) (**Figure 3A**). Furthermore, the relationship between the clustering and clinicopathological features were evaluated. Tumor T status, and stage were significant difference between the cluster 1 and cluster 2 (**Figure 3B**).

Prognostic model base on iron metabolism-related genes

Total of 4 iron metabolic-related genes (FLVCR1, FTL, HIF1A, HMOX1) were identified to construct the prognostic model. Our prognostic model that based on the 4 iron metabolic-related genes was formed using the following formula: risk score = (the means expression of FLVCR1 * 0.16713) + (the means expression of FTL * 2.52E-05) + (the means expression of HIF1A * 0.014361) + (the means expression of HMOX1 * 0.002315).

According the median level of the risk score, the HCC patients divided into low-risk and high-risk groups. Kaplan–Meier survival curves showed the low-risk group had a better survival rate than high-risk group (HR = 1.329, 95% CI = 1.210– 1.461, P < 0.001) (**Figure 4A**). In addition, we evaluated the accuracy of the OS-related prognostic model by constructed the ROC curve, and the AUC of risk score was significant than other clinicopathological parameters (**Figure 4B**). Finally, we ranked HCC patients by prognostic model to analyses the survival distribution. We could determine the mortality of HCC patients base on their risk scores. Moreover, with the increase of risk score, the patient's mortality rose (**Figure 4C, D**).

The relationships between prognostic model, clinicopathological parameters and tumor-infiltrating immune cells

The univariate and multivariate Cox regression analysis showed that the prognostic model can be as an independent prognostic factor for HCC patients (**Figure 5A, B**). Moreover, the prognostic model was significantly related to clinicopathological features such as survival state (p=0.001), stage(p=0.002), grade(p=0.001), and tumor T status(p=0.002) (**Figure 6A-D**).

We next evaluated the association of prognostic model and tumor-infiltrating immune cells in HCC microenvironment using CIBERSORT algorithm. The CIBERSORT analysis showed that the composition of 22 immune cell types in High-risk group and low-risk group of HCC varied significantly (**Figure 7A, B**).

Macrophages M0 were more enriched in High-risk group, while T cells CD8, NK cells activated were more concentrated in low-risk group (**Figure 7C**).

The expression of the iron metabolism-related genes in Oncomine database, The Human Protein Atlas and Kaplan Meier plotter

We analyze the expression level of FLVCR1, FTL, HIF1A, HMOX1 in liver cancer by Oncomine database. The expression level of FLVCR1, FTL, HIF1A in different hepatocellular carcinoma was higher than the normal group in the Wurmbach Liver (222906_at), Chen Liver (IMAGE: 1575419), Roessler Liver (200989_at) (**Figure 8A-C**). However, the expression level HMOX1 was no value in Oncomine. In addition, we verify the histological level of FLVCR1, FTL, HIF1A, HMOX1 by the Human Protein Atlas database, and the results showed that FLVCR1, FTL, HIF1A, HMOX1 is upregulated in HCC tissues and downregulated in normal tissue (**Figure 8D-G**). The prognostic significance of FLVCR1, FTL, HIF1A, HMOX1 were identified by Kaplan Meier-plotter server. The results showed that the FLVCR1, FTL, HIF1A, HMOX1 were closely related to the OS of HCC patients (**Figure 9 A-D**).

Validation of the prognostic model

We calculated the risk score of each HCC patient in the ICGC data portal project Liver Cancer - RIKEN, JP (LIRI-JP) as an independent external validation by the same formula. The HCC patients divided into high- and low-risk groups based on the median level of risk score. The Kaplan–Meier survival curves showed the prognostic value of our prognostic model ($P < 0.001$) (**Figure 10A**). In addition, the ROC curve also showed a good ability of the OS-related prognostic model to predict prognosis of HCC patients (**Figure 10B**). And with the increase of risk score, the mortality rate of patients rose (**Figure 10C, D**). Therefore, these validation results confirmed the predict prognosis ability of our prognostic model.

Discussion

The occurrence, development and treatment of hepatocellular carcinoma is a complex regulatory network. Compared to using a single clinicopathological parameter, gathering diverse biomarkers and establishing a prognostic model is a more effective way to predict tumor prognosis. Nowadays, iron metabolism has significant roles in many types of cancer, including hepatocellular carcinoma. Iron metabolism was ubiquitous in the occurrence and development of cancer. The prognostic model that base on iron metabolism-related genes may more precise than single clinicopathological parameter.

In this study, we aimed to analyze the relationship between iron metabolism-related genes and the prognosis of HCC patients. we obtained the RNA expression profiles of iron metabolism-related genes from the TCGA database. Then, we determine 4 iron metabolism-related genes (FLVCR1, FTL, HIF1A, HMOX1) and construct the prognostic model by Cox regression analysis. Finally, the prognostic model was verified by ICGC cohort with same formula. The prognostic model could be an independent prognostic biomarker for HCC patients.

The CIBERSORT analysis showed that T cells CD8, NK cells activated were more concentrated in low-risk group Macrophages M0 were more enriched in High-risk group, while Macrophages M0 were more enriched in High-risk group. CD8 + T cells are master effectors of antitumor immunity, and their presence at tumor sites correlates with favorable outcomes[17, 18]. Some studies suggested that the relative abundance of CD8 + T cells infiltrating human metastatic melanoma tumors strongly correlates with clinical response to immunotherapy[19, 20]. The quantity and quality of CD8 + T cells in the tumor microenvironment play a significant role in determining whether a robust immune response will be generated against the tumor upon initiating immunotherapy[18]. Meantime, NK cells are key components of the innate immune system and have potent antitumor and antimetastatic activity [21]. However, the relationship between Macrophages M0 and hepatocellular carcinoma still not clear. These results indicated that low-risk patients in prognostic model may be sensitive to immunotherapy.

FLVCR1 (FLVCR heme transporter 1) is a transmembrane protein involved in the trafficking of intracellular heme[22]. A study showed that Long non-coding RNA FLVCR1-AS1 contributes to the proliferation and invasion of lung cancer by sponging miR-573 to upregulate the expression of E2F transcription factor 3[23]. In addition, some studied showed LncRNA FLVCR1-AS1 promotes proliferation, migration and activates Wnt/ β -catenin pathway through miR-381-3p/CTNNB1 axis in breast cancer[24]. Moreover, LncRNA FLVCR1-AS1 mediates miR-513/YAP1 signaling to promote cell progression, migration, invasion and EMT process in ovarian cancer[25]. FTL (ferritin light chain), a key protein in iron metabolism, is associated with the survival of cancer patients. Some studies showed that FTL may play an important biomarker in various cancers including prostate cancer, lung cancer, renal cancer and ovarian cancer [26–28]. HIF1A (hypoxia inducible factor 1 subunit alpha), an important component of angiogenesis, is activated as a response to tumor hypoxia and facilitates tumor survival[29]. Previous study showed that hsa-circ-0000211 promoted lung adenocarcinoma cell migration and invasion by modulating the miR-622/HIF1-A network[30]. HMOX1 (heme oxygenase 1), an enzyme that catalyzes the reaction, that degrades the heme group contained in several important proteins, such as hemoglobin, myoglobin, and cytochrome p450[31]. Previous study showed that HMOX1 has an antitumor role in breast cancer[32]. Another study reported that HMOX1 inhibits tumor metastasis mediated by Notch1 pathway in murine mammary carcinoma[33]. In addition, a study showed that HMOX1 facilitates cell proliferation by the B-Raf-ERK signaling pathway in melanoma[34].

In our study, our prognostic model that based on iron metabolism-related genes for hepatocellular carcinoma patient are validation by ICGC cohort. Moreover, we also showed the relationships between prognostic model, clinicopathological parameters and tumor-infiltrating immune cells. In addition, the expressions level of 4 iron metabolism-related genes was performed using Oncomine database, the Human Protein Atlas. Moreover, CIBERSORT results indicated that low-risk patients in prognostic model may be sensitive to immunotherapy. Iron metabolism is a dynamic and continuous process. It is still not clear that if analyzing changes in iron metabolism-related genes are sufficient to reflect iron metabolism activity. Therefore, there are some limitations in our work. First, there is no further experiment to explore the relationship between iron metabolism-related genes and HCC. Second, lack of numerous clinical researches to confirm our results.

In conclusion, our study constructed a prognostic model based on iron metabolism-related genes for hepatocellular carcinoma patients. Our prognostic model showed great potential for application in clinical. However, these iron metabolism-related genes need further research explore whether they may be helpful for selecting targets for immunotherapies and individualize treatment.

Declarations

Authors' contributions

W.L and Z.X.C contributed to the conception and design of the study; All authors participated in data download, analysis and statistical analysis, writing and editing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Declaration of Competing Interest

The authors report no conflicts of interest in this work.

Funding

This work was supported by the Taishan Scholar foundation of Shandong Province (No tshw201502061).

Acknowledgments

Thanks for the support of my family

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Figures

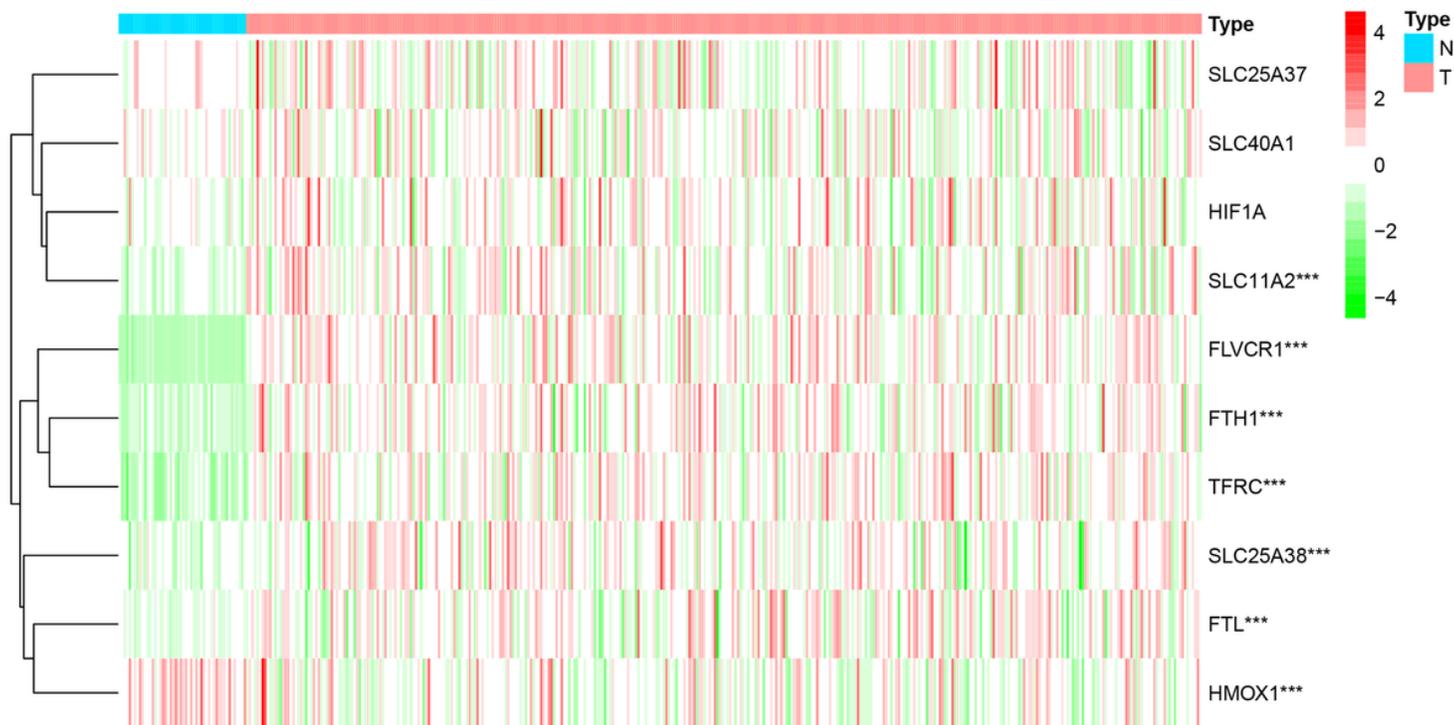


Figure 1

Heatmap of differentially expressed iron metabolism-related genes between HCC and normal tissues.

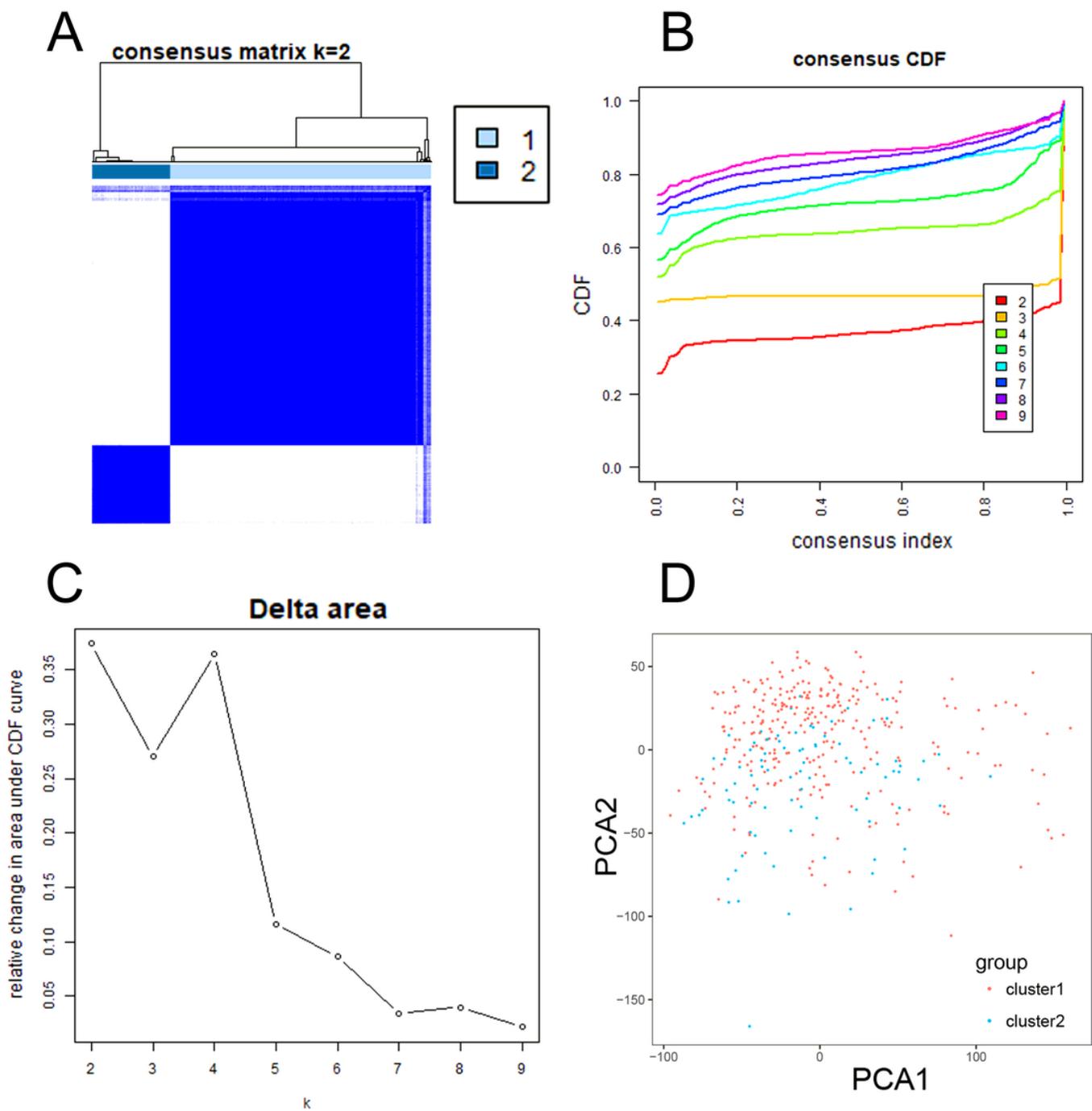


Figure 2

Differential overall survival and grade of HCC patients in the two different clusters. (A) The HCC patients divided into two distinct clusters, $k = 2$. (B) Consensus clustering cumulative distribution function for $k = 2 - 9$. (C) Relative change in area under cumulative distribution function curve for $k = 2 - 9$. (D) the principle components analysis of the iron metabolism-related genes.

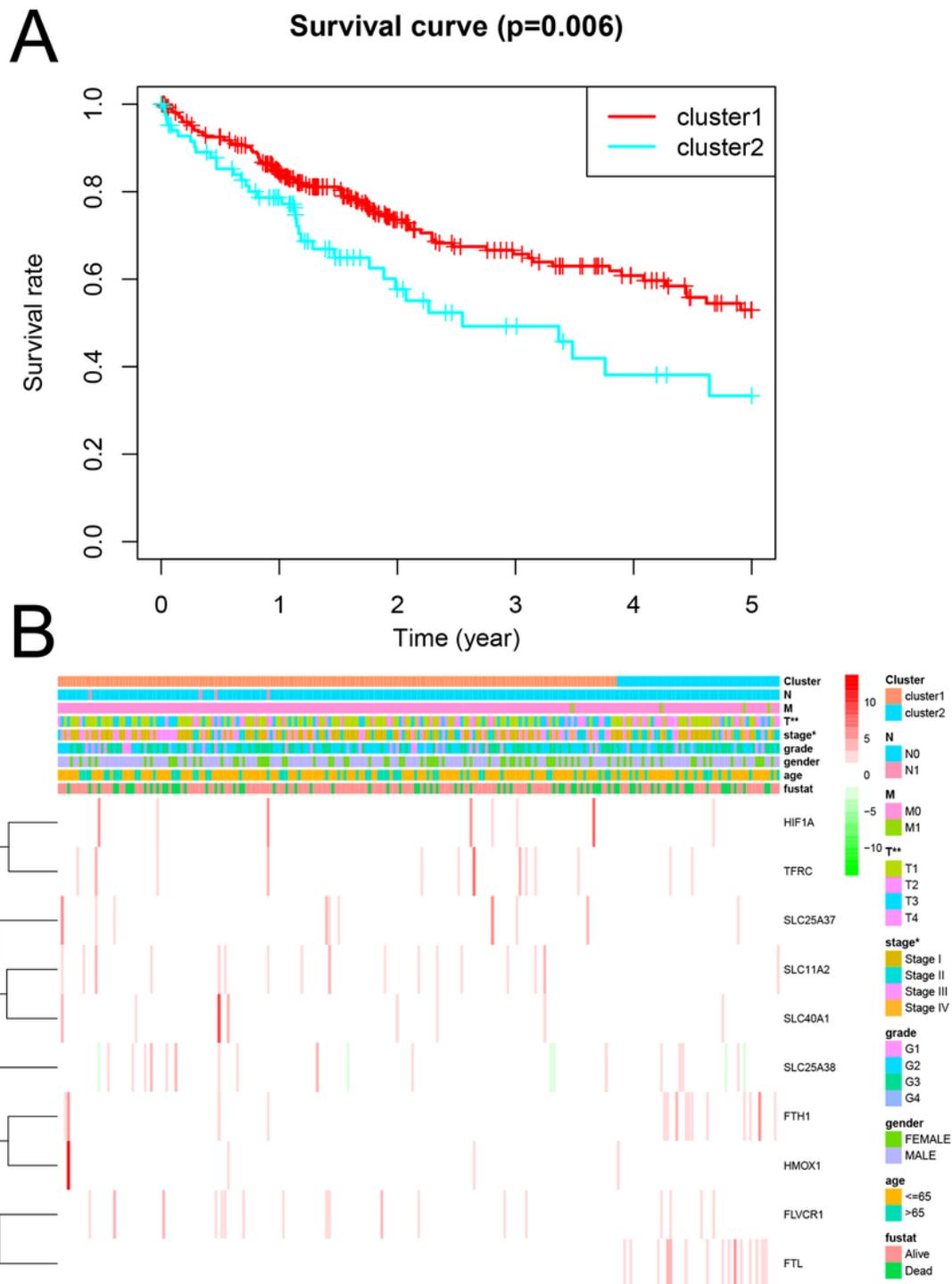


Figure 3

(A) Kaplan–Meier curve of cluster 1 and cluster 2 HCC patients. (B) Heatmap of expression of iron metabolism-related genes and clinicopathological features between cluster 1 and cluster 2.

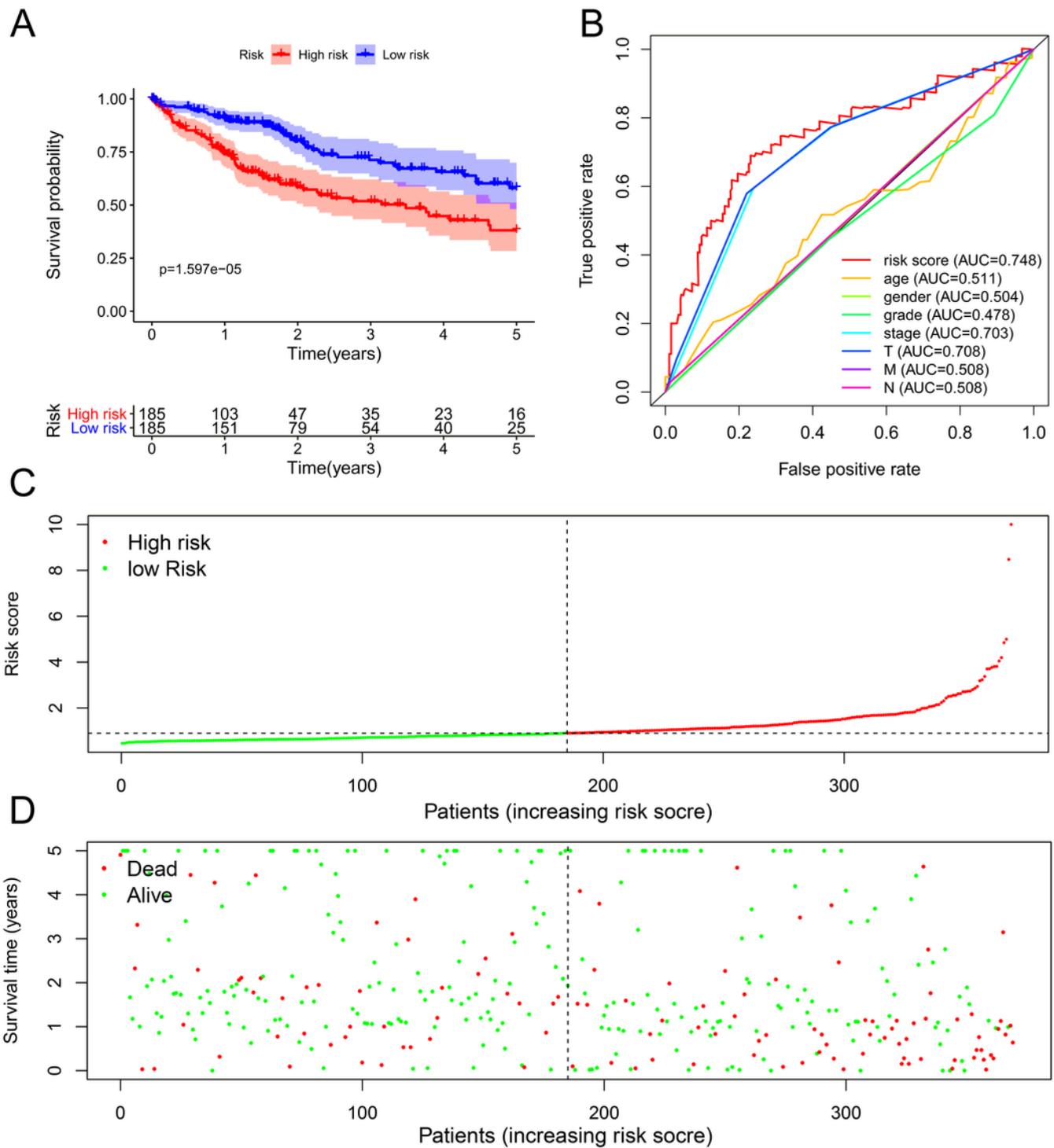


Figure 4

Prognostic model of hepatocellular carcinoma patients in TCGA cohort. (A) Kaplan–Meier curve of high-risk and low-risk HCC patients. (B) ROC curve of OS-related prognostic model. (C) Risk score distribution of HCC patients with different risks. (D) Scatterplots of HCC patients with different survival status.

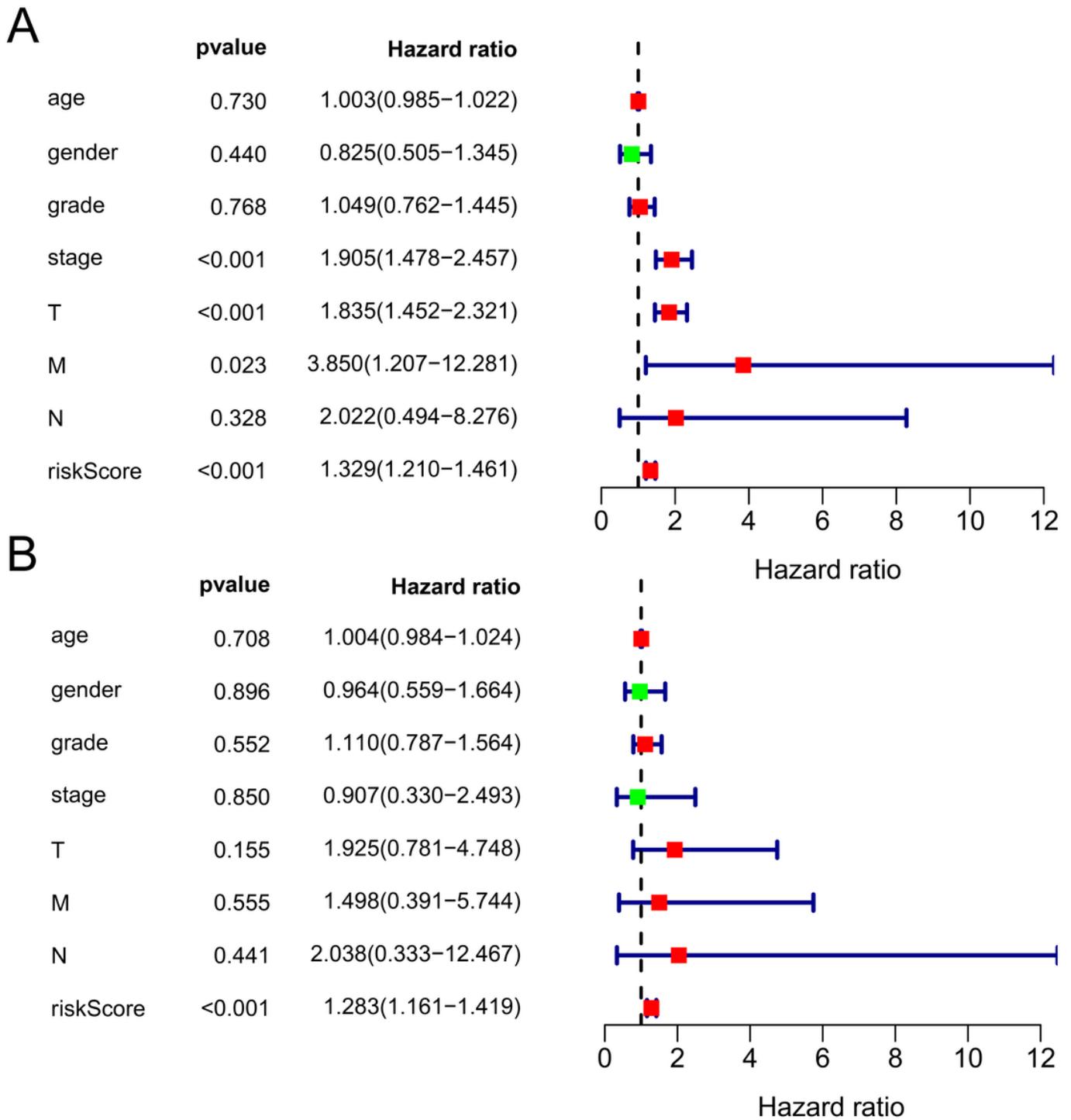


Figure 5

Independent prognostic analysis. (A) Univariate factor independent prognostic analysis. (B) Multivariate factor independent prognostic analysis.

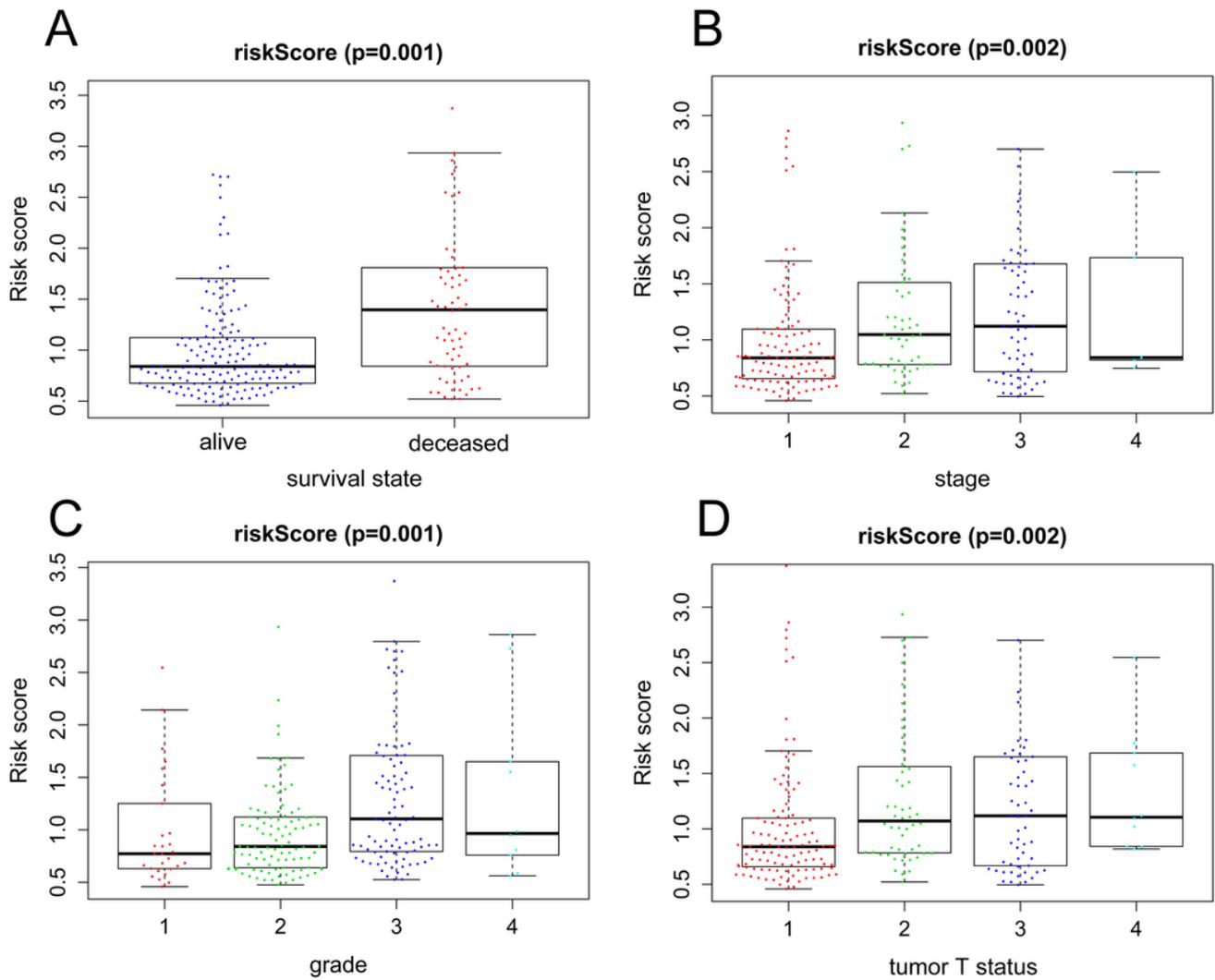


Figure 6

The relationships between prognostic model and Clinicopathological features. (A) survival state. (B) stage. (C) grade. (D) tumor T status.

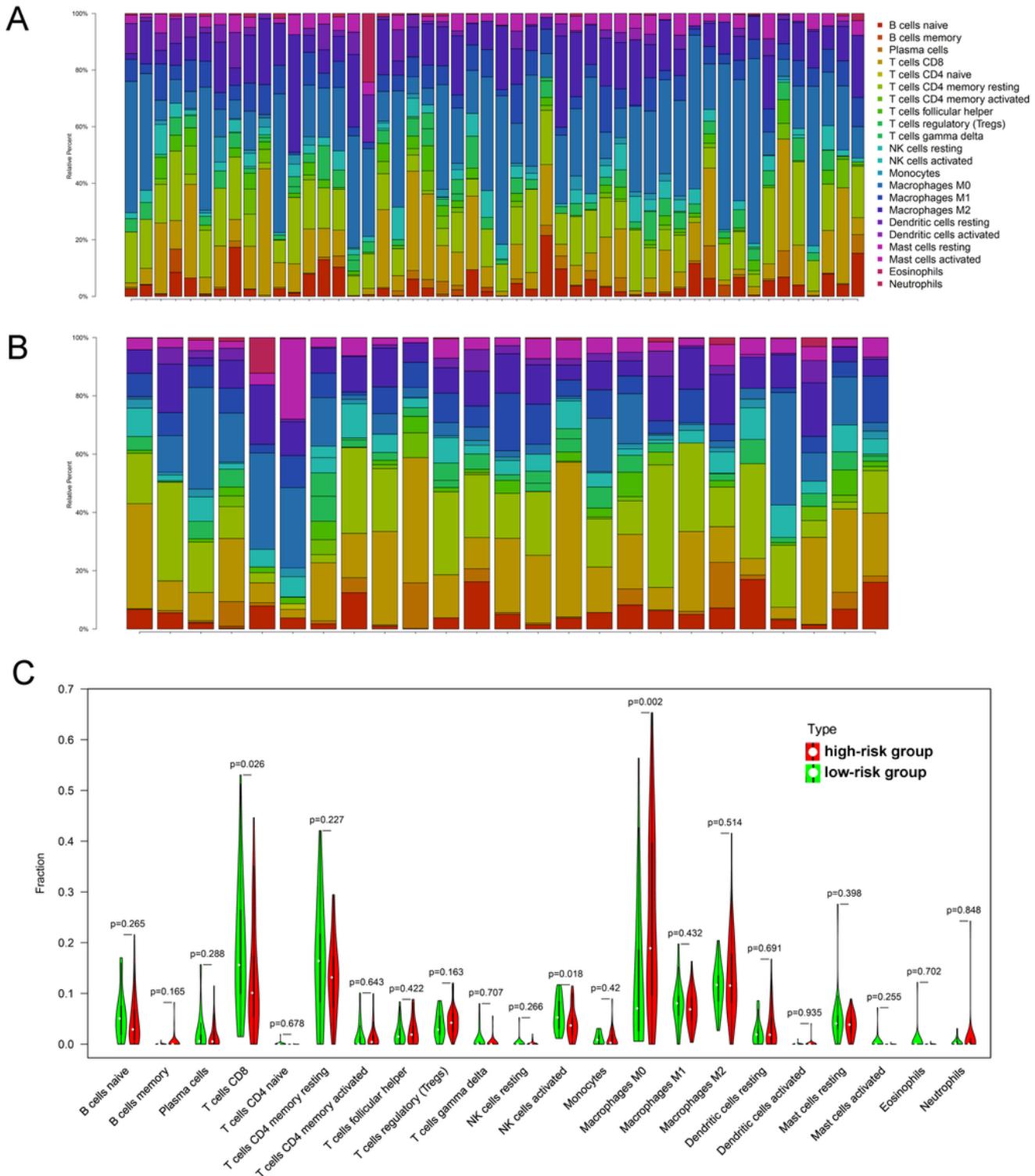


Figure 7

The relationships between prognostic model and tumor-infiltrating immune cells. (A) 22 immune cells in High-risk group. (B) 22 immune cells in low-risk group. (C) Violin plot displays the differentially infiltrated immune cells between High-risk group and low-risk group.

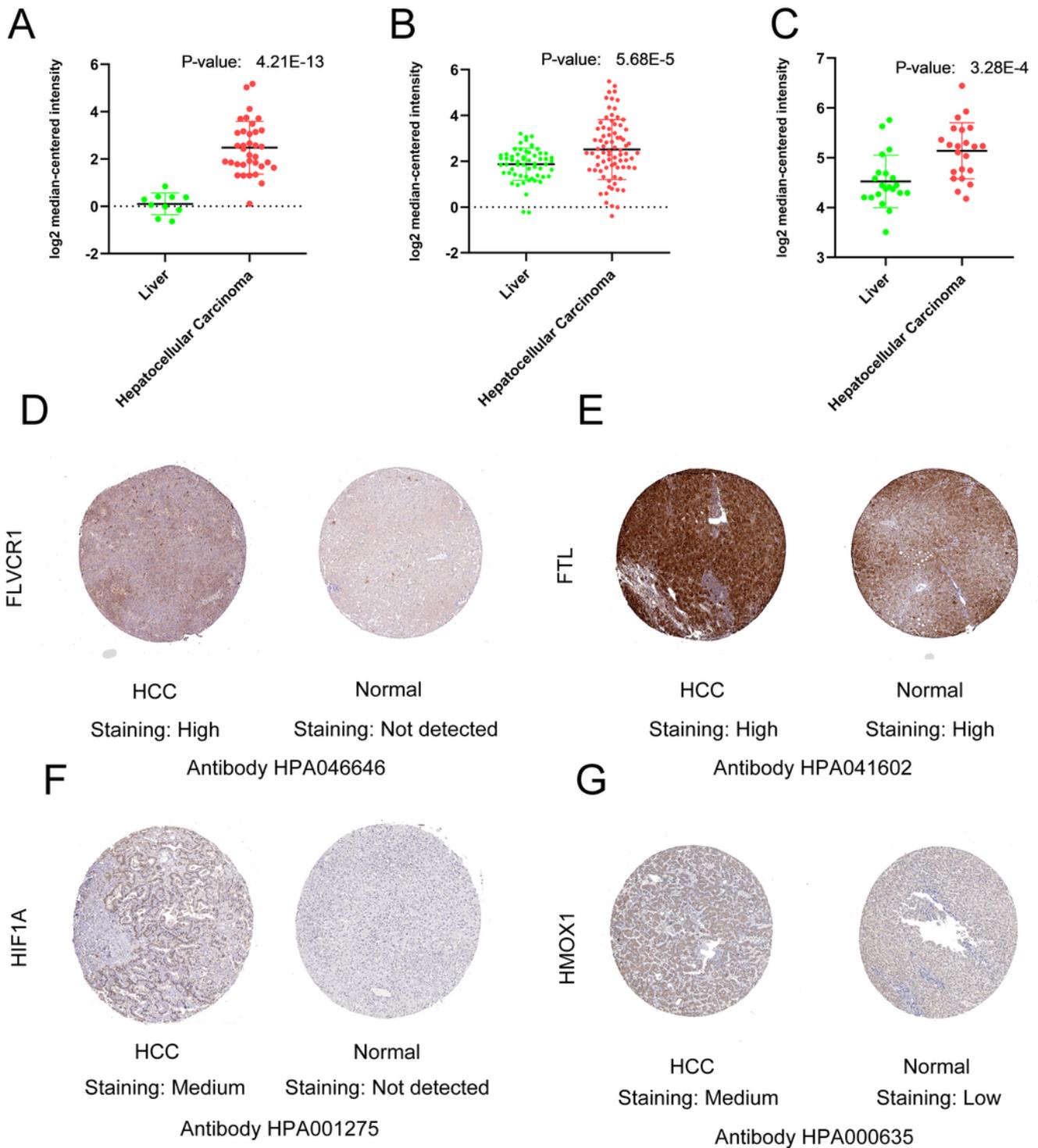


Figure 8

The expression level of FLVCR1, FTL, HIF1A, HMOX1 in the Oncomine database, and The Human Protein Atlas. (A) Expression level of FLVCR1 in HCC and Liver in Oncomine database. (B) Expression level of FTL in HCC and Liver in Oncomine database. (C) Expression level of HIF1A in HCC and Liver in Oncomine database. (D) Immunohistochemistry results of FLVCR1 in HCC (Staining: High; Intensity: Strong; Quantity: 75%-25%; Location: Cytoplasmic/ membra) and in normal tissue (Staining: Not detected;

Intensity: Negative; Quantity: None; Location: None). (E) Immunohistochemistry results of FTL in HCC (Staining: High; Intensity: Strong; Quantity: >75%; Location: Cytoplasmic/ membranous) and in normal tissue (Staining: High; Intensity: Strong; Quantity: >75%; Location: Cytoplasmic/ membra). (F) Immunohistochemistry results of HIF1A in HCC (Staining: Medium; Intensity: Moderate; Quantity: >75%; Location: Cytoplasmic/ membranous/ nuclear) and in normal tissue (Staining: Not detected; Intensity: Negative; Quantity: None; Location: None). (G) Immunohistochemistry results of HMOX1 in HCC (Staining: Medium; Intensity: Moderate; Quantity: 75%-25%; Location: Cytoplasmic/ membranous) and in normal tissue (Staining: Low; Intensity: Weak; Quantity: 75%-25%; Location: Cytoplasmic/ membranous).

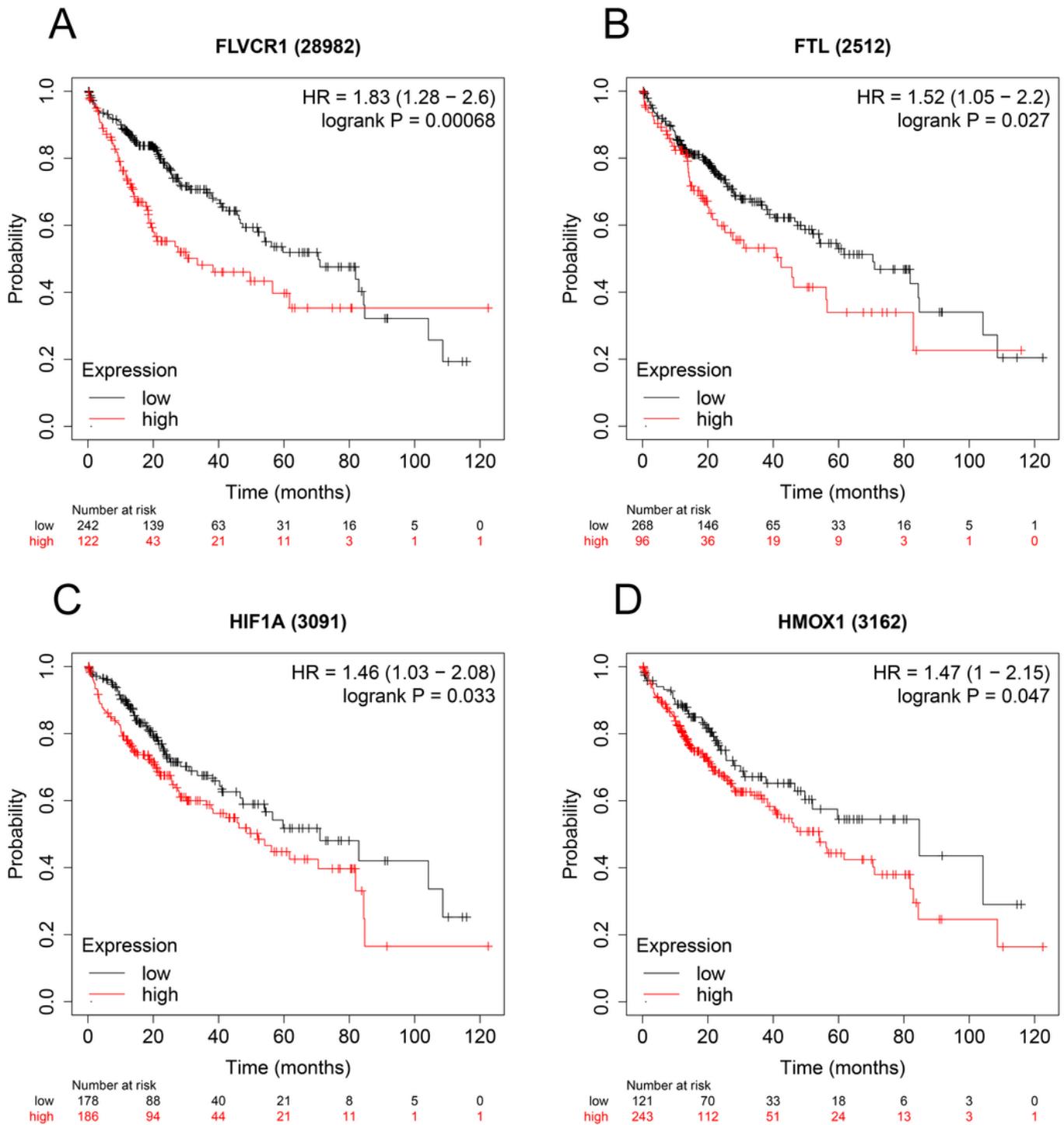


Figure 9

Validation the prognostic value of FLVCR1, FTL, HIF1A, HMOX1 in HCC by Kaplan Meier-plotter. (A) FLVCR1. (B) FTL. (C) HIF1A. (D) HMOX1.

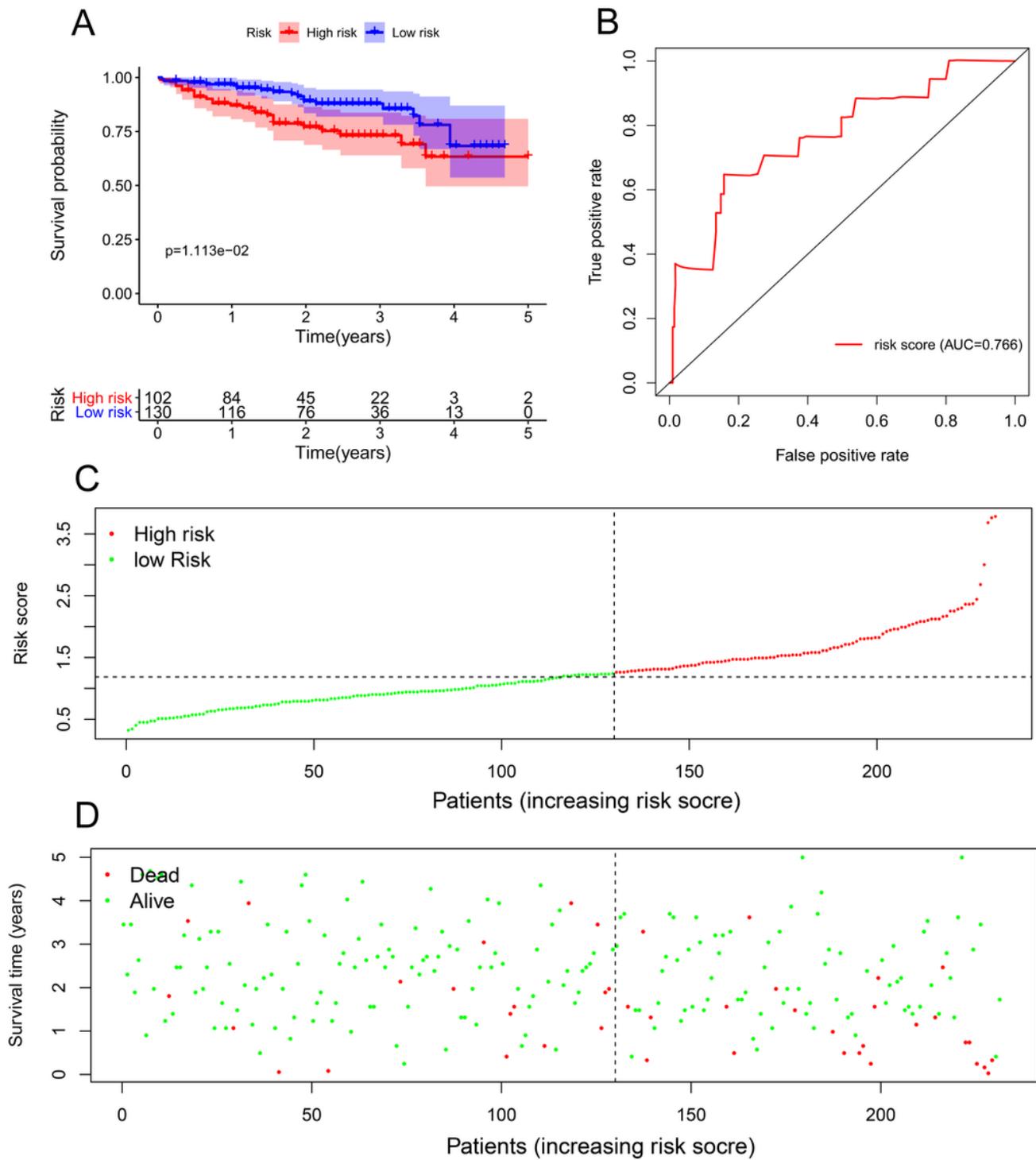


Figure 10

Prognostic model of hepatocellular carcinoma patients in ICGC cohort. (A) Kaplan–Meier curve of high-risk and low-risk HCC patients. (B) ROC curve of OS-related prognostic model. (C) Risk score distribution of HCC patients with different risks. (D) Scatterplots of HCC patients with different survival status.