

A Novel Gene Signature Associated with Inflammatory Responses and Immune Status Assists in Prognosis and Intervention for Patients with HCC

Guofang Lu^{1,2,*}, Rui Du^{3,*}, Bin Feng^{1,4}, Jianlin Wang³, Fengrui Zhang⁵, Jianming Pei², Yuanyong Wang^{6,*}, Yulong Shang¹

¹State Key Laboratory of Cancer Biology and National Clinical Research Center for Digestive Diseases, Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi'an, People's Republic of China; ²Department of Physiology and Pathophysiology, National Key Discipline of Cell Biology, Fourth Military Medical University, Xi'an, People's Republic of China; ³Institute for Biomedical Sciences of Pain, Tangdu Hospital, Fourth Military Medical University, Xi'an, People's Republic of China; ⁴Department of Radiation Oncology, Xijing Hospital, Fourth Military Medical University, Xi'an, People's Republic of China; ⁵CAS Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing, People's Republic of China; ⁶Department of Thoracic Surgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yulong Shang; Jianming Pei, Tel +86 29 84771506, Fax +86 29 82539041, Email shangyul870222@163.com; jmpei8@fmmu.edu.cn

Background: Tumor growth depends on tumor cells and the tumor microenvironment, which are regulated by inflammation and immune responses. However, the roles of inflammation and immune status in hepatocellular carcinoma (HCC) remain unclear. The aim of this study was to evaluate the prognostic value of an inflammatory response-related gene signature associated with immune status, which may provide insight into new treatment options for HCC patients.

Materials and Methods: Differentially expressed genes associated with inflammation were obtained from The Cancer Genome Atlas (TCGA), the Gene Expression Omnibus, and the Molecular Signatures Database. An inflammation-associated prognostic gene signature was constructed and validated using TCGA and the International Cancer Genome Consortium datasets, respectively, using LASSO Cox regression analysis. Log-rank was performed to compare the overall survival of low- and high-risk score cohorts. Immune cell infiltration and immune-related functions were analyzed using single-sample gene enrichment analysis. The structures of the drugs identified by the prognostic model were predicted using PubChem. The drugs sensitivity of bleomycin, simvastatin and zoledronate detected by CCK8 colorimetric assay. The mRNA levels of 7 genes in HCC after drug treatment analyzed via qRT-PCR.

Results: Inflammation-associated genes, including ITGA5, MEPIA, P2RX4, RIPK2, SLC7A1 and SRI, were identified and found to be associated with the prognosis of HCC. We further found that the high-risk patients experienced poor prognosis, which was observed to be an independent and significant risk factor for prognosis. Moreover, we observed elevated expression levels in multiple immune cell types and immune function. Lastly, we validated that bleomycin, simvastatin and zoledronate could regulate these genes in HCC.

Conclusion: The inflammatory-response-associated gene signature could predict the prognosis and the immunological status of HCC patients. Additionally, bleomycin, simvastatin and zoledronate may represent potential drug candidates that could inhibit these genes. This may constitute a new approach for the treatment of HCC.

Keywords: inflammation-associated gene, prognostic gene signature, immune infiltration, immune checkpoint, hepatocellular carcinoma, drug structure

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer types in humans, killing millions of people worldwide each year; in some African and Asian countries, HCC has become a major cause of cancer death.¹ The incidence of HCC in China accounts for about one-half of the world's total, and most patients are already in the late stage

of the disease when they are clinically diagnosed, resulting in a 5-year overall survival (OS) rate of only about 14.1%.² Although a small number of patients can receive radical surgery, the recurrence rate is as high as 70% five years after intervention. Despite the great strides in diagnosis and treatment, HCC remains a highly lethal tumor due to metastasis and recurrence.

In recent years, several studies have examined the link between inflammation and cancer. In 1863, the German pathologist Rudolph Virchow proposed that tumors originated from sites of chronic inflammation, and that tissue damage and subsequent inflammation were important causes of tumor formation.³ Current research shows that, under normal conditions, inflammation is usually self-limited, but abnormalities in any of the intermediate links can lead to damage to cellular DNA and impaired cell proliferation, ultimately leading to tumorigenesis.^{4,5} Previous studies in our laboratory have demonstrated how pro-inflammatory signaling pathways promote gastric cancer cell proliferation and metastasis.^{6,7} Bu et al also found a relationship between chronic intestinal inflammation and colon cancer.⁸ Moreover, chronic liver injury can lead to chronic hepatitis and liver cell death, further promoting hepatic injury, cirrhosis and HCC, suggesting that a chronic inflammatory state may be required for the development and progression of HCC.⁹ Tumor growth depends not only on the genetic changes of malignant tumor cells, but also on alterations of the tumor microenvironment (TME) including stroma, blood vessels, and tumor-infiltrating cells. Immunity and inflammation constitute the two core elements of the TME.^{10,11} Innate and adaptive immunity play important roles in tumorigenesis, progression and metastasis.¹² Inflammation not only promotes an immune response, but can also lead to immunosuppression.¹³ However, the key inflammatory mechanisms leading to the development of HCC are still poorly understood. Therefore, characterizing the inflammatory mechanisms involved in HCC and targeting inflammation may become important for the treatment of HCC.

The combination of Atezolizumab with Bevacizumab (Atezo-Bev) is currently the first-choice first-line treatment, as it confers a superior survival benefit compared to Sorafenib.^{14,15} Whereas the clinical success of chemotherapy with HCC patients is limited. Previous studies have also described the development of various immune checkpoint inhibitors, such as anti-programmed cell death 1 (PD-1) and programmed cell death-ligand 1 (PD-L1), which have now entered clinical practice. Immunotherapy targets immune cells to enhance the anti-tumor immune response and improve the objective response rate to the treatment.¹⁶ Anti-PD-1 therapy in particular has proven successful for the treatment of HCC.¹⁷ Indeed, the effective rate of anti-PD-1 alone in the treatment of HCC is 15%.¹⁸ These study reminder us that immune checkpoint blockade immunotherapy and related combination therapies are the trends in the treatment of advanced hepatocellular carcinoma.

In the current study, we identified inflammation-associated differentially expressed genes using The Cancer Genome Atlas (TCGA), the Gene Expression Omnibus (GEO), and the Molecular Signatures Database. We then constructed and validated an inflammation-associated prognostic gene signature in TCGA and International Cancer Genome Consortium (ICGC) datasets. Furthermore, we analyzed the relationship between the inflammation-associated prognostic gene signature and the tumor immune microenvironment, including immune cell infiltration, immune-related functions, immune infiltration subtypes and immune checkpoint molecules. We also predicted three drugs using the prognostic gene signature and verified the expression levels of seven genes using reverse transcription-PCR. The results highlighted the combined role of inflammatory responses and immune status in prognosis and identified a new approach for the clinical management of HCC.

Materials and Methods

Data Preparation

RNA sequencing data and clinical characteristics from HCC patients were downloaded from TCGA (<http://www.cancer.gov/tcga>), GEO (<http://www.ncbi.nlm.nih.gov/geo/>) and ICGC (<https://icgc.org/>). The inflammation-associated genes were found in the Molecular Signatures Database. All data used in this study are freely available to the public. The “limma” R package was used in TCGA and ICGC datasets to identify differentially expressed genes (DEGs) in HCC tissue and adjacent non-tumor tissue using a false discovery rate <0.05 and a fold change (FC) >2. In the GSE87630 database,

DEGs in HCC and adjacent non-tumor tissue were identified using GEO2R with $\log_2|FC| \geq 0.5$ and adjusted P-value ≤ 0.05 . All DEGs were analyzed GraphPad Prism 8 software.

Identification and Validation of the Inflammation-Associated Prognostic Gene Signature

The prognostic significance of inflammatory response-associated genes was analyzed using Univariate Cox regression analysis. The prognostic gene signature was built using LASSO-penalized Cox analysis.¹⁹ A tenfold cross-validation was used to evaluate the penalty parameter (λ) of the prognostic gene signature. To determine the risk scores, we analyzed the levels of seven inflammation-associated genes and the matching regression coefficient. The score was defined as: esum (expression of each gene \times corresponding coefficient). According to the median risk score of the cohort, the patients were divided into low- and high-risk groups. To examine gene expression levels in these two groups, t-Distributed Stochastic Neighbor Embedding (t-SNE) and Principal Component Analysis (PCA) were carried out using the “Rtsne” and “ggplot2” R packages. The OS analysis of the low- and high-risk cohorts were performed using “survminer”. The predictive performance of the signature was assessed using the time-dependent ROC curve analysis. Additionally, multivariate and univariate Cox regression analyses were carried out to assess the prognostic value of the gene signature for HCC patients. All data were made using R software (Version 3.6.1).

Tumor Immune Status Analysis

The levels of immune cell infiltration and stromal cells were measured using immune and stromal scores by cell-type identification by estimating relative subsets of RNA transcript (CIBERSORT) web portal (<https://cibersort.stanford.edu/>). Spearman correlation was used to analyze the relationship between the different risk scores and the stromal or immune scores. The relationship between the different risk scores and the subtype of immune infiltration was analyzed using a two-way ANOVA.

Predicted of Drug Structure

We predicted 121 potential molecular drugs targeting the ITGA5, MEP1A, P2RX4, RIPK2, SERPINE1, SLC7A1 and SRI genes from TCGA. We identified three potential target drugs through the correlation analysis and published article review. The two-dimensional chemical structure images of bleomycin, simvastatin and zoledronate were obtained from PubChem (pubchem.ncbi.nlm.nih.gov). The 121 molecular drugs are listed in [Supplementary Table 1](#).

Cell Line

Human HL7701 cell, HCC cell Huh-1 and Hep3b were obtained from China Infrastructure of Cell Line Resources and Stem Cell Bank, Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) at 37°C in a 5% CO₂ incubator. The medium was supplemented with 10% FBS (South America origin; IC-1905; BioCytoSci, TX, USA), and 1% penicillin- streptomycin.

RNA Extraction and Quantitative Reverse Transcription PCR (qRT-PCR)

Total RNA from Huh-1 and Hep3b cells and normal liver cells were extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions as described previously.²⁰ Reverse transcription was performed using the Advantage RT-for-PCR Kit (Takara Bio). The RT-PCR was conducted using SYBR Premix Pro Taq HS qPCR kit (Accurate Biotechnology, Hunan, China) on a CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, USA). The primer sequences are listed in [Supplementary Table 2](#). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression levels.

Statistical Analysis

The Wilcoxon test was used to analyze the DEGs in tumor and adjacent tissues. Categorical data were analyzed using the χ^2 test. Mann-Whitney's *U*-test was used to compare the ssGSEA scores of immune cells or immunological pathways in

different cohorts. The Log rank test was to analyze the differences in OS. Multivariate and univariate analysis was carried out to comprehensive analyze the prognostic risk factors of HCC based on the clinical baseline factors. The R software (version 3.6.1) and other tools, including “survminer”, “corrplot”, “venn”, “ggplot2”, “pheatmap”, “igraph”, and “ggpubr” were used to generate the graphs.

Results

Identification and Construction a Prognostic Gene Signature Using TCGA Cohorts

To evaluate the role of inflammation-associated genes in HCC, we first identified DEGs from TCGA and GSE87630 in HCC (the clinicopathological characteristics of the patients were presented in Table 1), and 200 inflammatory response-related genes were identified from the Molecular Signatures Database (Supplementary Table 3). Next, we obtained 49 inflammation-associated DEGs that were shared between the three databases (Figure 1A). We then evaluated functional enrichment in these 49 genes using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation. As shown in Figure S1A and B, the datasets were enriched with four functional categories: GO biological process (BP), GO molecular function (MF), GO cellular component (CC), and KEGG pathway (KEGG).

Using univariate Cox analysis, 12 of the aforementioned genes were associated with OS in patients with HCC (Figure 1B). The hazard ratio for the OS of the patients with high 12 inflammation-related DEGs is shown in Figure 1C. The analysis demonstrated that high gene expression of the following genes was an independent risk factor for HCC: HRH1 [1.623 (1.089–2.419), p-value = 0.017], ADORA2B [1.517 (1.146–2.008), p-value = 0.004] and P2RX4 [1.455 (1.142–1.855, p-value = 0.002]. Furthermore, we analyzed the interaction between these 12 inflammation-related genes using STRING and found that 11 genes exhibited strong positive significant correlation each other (Figure 1D).

The prognostic gene signature of 12 genes was assessed in TCGA (training) cohort using the penalized LASSO Cox PH regression (GLMnet R Package) in order to investigate the linear relationship among different genes. The graph for all combinations of λ values is shown in Figure S2A and B. The results demonstrated that the λ values at seven-gene

Table 1 Clinicopathological Characteristics in HCC from TCGA and ICGC Database

	TCGA Cohort	ICGC Cohort
All patients, n	377	260
Age, n(%)		
≤65	235 (62.33%)	98 (37.69%)
>65	142 (37.67%)	162 (62.31%)
Gender, n(%)		
Female	122 (32.36%)	68 (26.15%)
Male	255 (67.64%)	192 (73.85%)
Pathologic stage, n(%)		
I–II	262 (69.5%)	157 (60.38%)
III–IV	91 (23.44%)	103 (39.62%)
Unknown	24 (6.36%)	
T stage, n(%)		
I	185 (49.07%)	
II	95 (25.2%)	
III	84 (22.28%)	
IV	13 (3.45%)	
Grade, n(%)		
I+II	237 (62.86%)	
III+IV	140 (37.14%)	
Survival status, n(%)		
Alive	244 (64.89%)	
Death	132 (35.11%)	

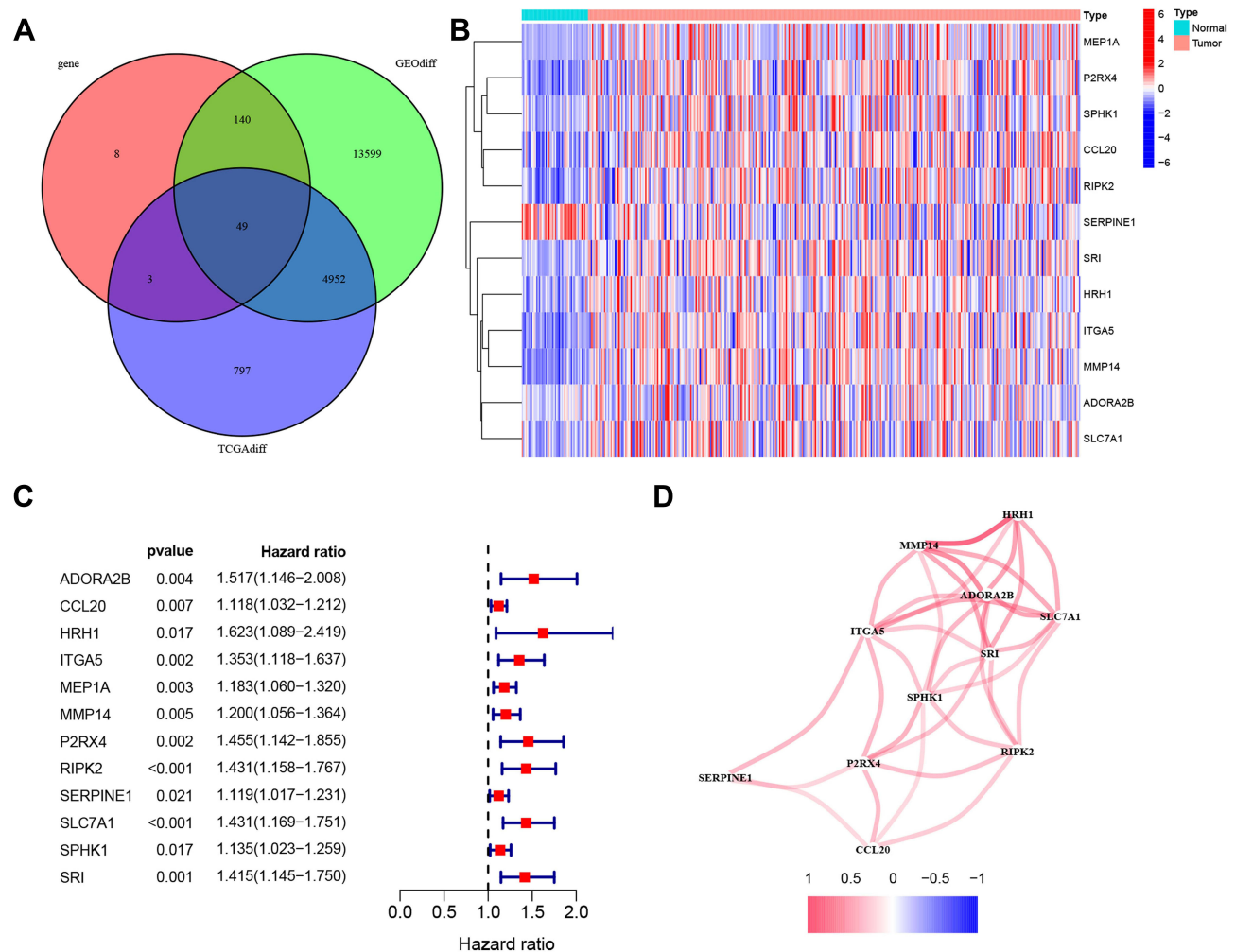


Figure 1 The 12 inflammation-associated differentially expressed genes identified in HCC. **(A)** A Venn diagram was used to identify 49 significant differentially expressed genes from TCGA, GSE87630, and inflammation-associated genes. **(B)** Heatmap of the expression levels of 12 candidate genes in HCC (red) and adjacent non-tumor tissue (blue). **(C)** Forest plot of the relationship between the expression of 12 genes and overall survival in HCC. All 12 genes were risk factors (hazard ratio >1). **(D)** Construction the protein-protein interaction network of 12 genes. Among these, 11 interacted with each other.

maker was the optimal value. The risk score was calculated as follows: expression level of ITGA5 * 0.067+expression level of MEP1A * 0.093+expression level of P2RX4 * 0.141+expression level of RIPK2 * 0.163+expression level of SERPINE1 * 0.040+expression level of SLC7A1 * 0.121+expression level of SRI * 0.164. The patients were divided into a high- and a low-risk group according to the median value of the groups. The correlation between gene expression and clinicopathological characteristics of HCC in the TCGA and ICGC cohorts were presented in Table 1. To compare the prognostic gene signature between the high- and low-risk groups, a scatter plot was generated. The results revealed that high-risk patients with HCC had an increased likelihood of dying earlier compared to those in the low-risk groups (Figure 2A). Moreover, we analyzed the two risk groups using PCA and t-SNE, and the data points were found to be clearly separated in the PCA and t-SNE analysis (Figure 2C). To evaluate the effect of these genes on HCC prognosis, we then examined the relationship between their expression levels and OS in TCGA cohort (Figure S3). In addition, Kaplan-Meier survival analysis indicated that high expression of these 7 genes (ITGA5, MEP1A, P2RX4, RIPK2, SERPINE1, SLC7A1 and SRI) was associated with poor prognosis in HCC patients (Figure 2E). Moreover, we used a time-dependent ROC curve and AUC to assess the prognostic performance of the gene signature. For the prognostic gene signature, the AUCs at 1, 2 and 3 years were 0.673, 0.627 or 0.607, respectively (Figure 2F).

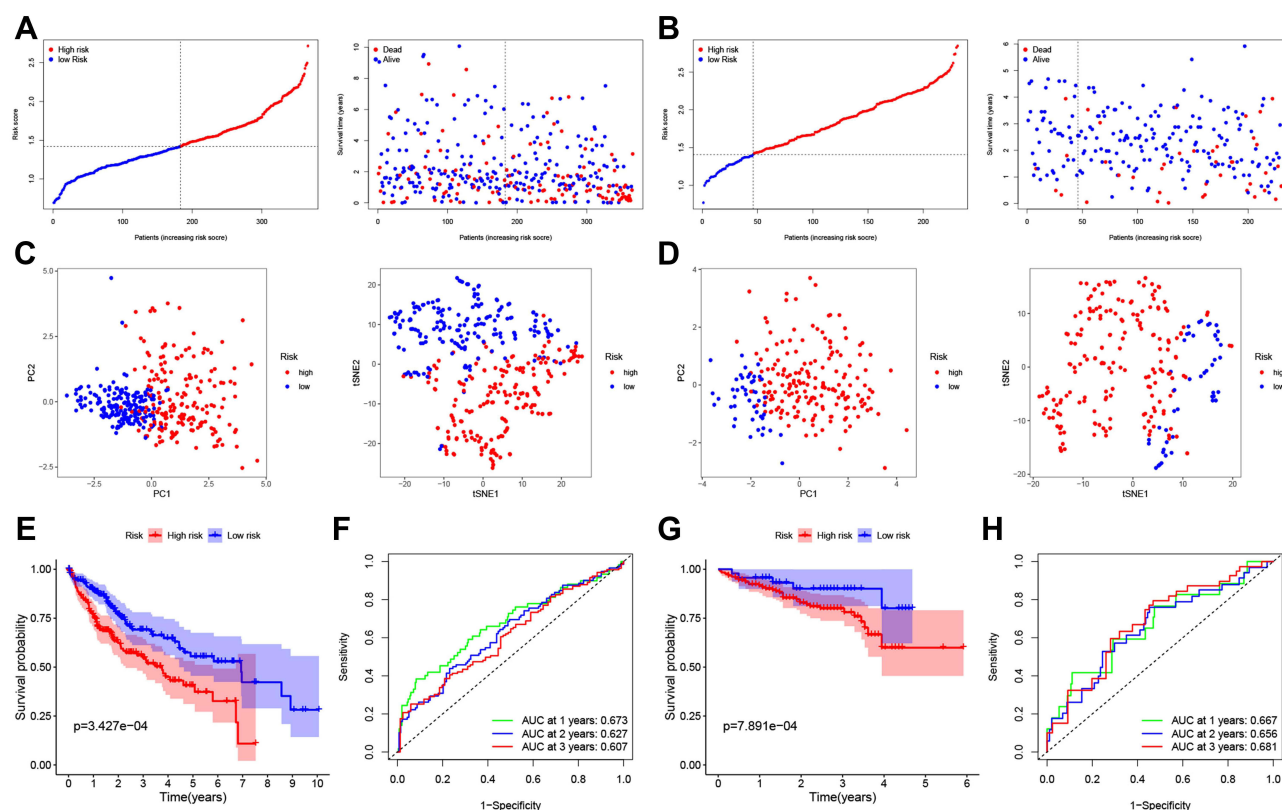


Figure 2 Construction and validation of the 7-gene prognostic gene signature in TCGA (A, C, E and F) and ICGC (B, D, G and H) datasets. (A and B). The expression of the 7 genes was used to divide the patients into high- and low-risk groups according to the median value of the cohort. Red: high risk; blue: low risk (left). Association between different risk groups and OS in HCC (right). (C and D). PCA (left) and t-SNE (right) analysis showing samples divided according to expression levels. (E and G). Kaplan-Meier analysis of the association between high/low-risk group expression and the OS of HCC patients. Red: high risk; blue: low risk, $P < 0.05$. (F and H). AUC of 1-, 2- and 3-year OS in patients with HCC.

Validation of the Seven-Gene Gene Signature in HCC Using the ICGC Database

The robustness of the association between the expression of the 7 genes and prognosis in HCC was further validated in another cohort (ICGC cohort) (Figure 2B, D, G–H). The ICGC datasets were also divided into a high- and a low-risk group based on the median of the dataset. The result of the scatter plot suggested that high-risk patients in HCC had an increased likelihood of dying earlier, compared with the low-risk group (Figure 2B). The results of PCA and t-SNE are shown in Figure 2D, in which unsupervised clustering accurately separated the samples according to the expression levels. There was a similar trend for OS in HCC from ICGC datasets, as high expression of all seven genes was associated with shorter OS (Figure 2G). For the seven-gene time-dependent ROC analysis, the AUCs at 1, 2 or 3 years were 0.667, 0.656 and 0.681, respectively (Figure 2H).

Prognostic Value of the Seven-Gene Gene Signature in HCC in TCGA and ICGC Cohorts

We performed univariate and multivariate Cox regression analysis of parameters potentially associated with OS in HCC in order to determine whether the risk score could be an independent predictive indicator. First, univariate Cox regression analysis was carried out on age, gender, grade (only TCGA cohort) and stage. The results demonstrated that these factors are positive prognostic factors informing HCC patient OS. In addition, the hazard ratio of the risk scores in TCGA cohort was 3.237 (95% CI = 2.035–5.183, $p < 0.001$) (Figure 3A). Subsequently, HCC stage was evaluated using multivariate Cox regression analysis, and the results suggested that this parameter could be an independent predictive factor for OS (HR = 2.721, 95% CI = 1.653–4.364, $p < 0.001$) (Figure 3B). Moreover, we also validated the role of relevant factors for OS in ICGC cohorts. The results also identified stage as an independent prognostic indicator using univariate (4.481, 95%

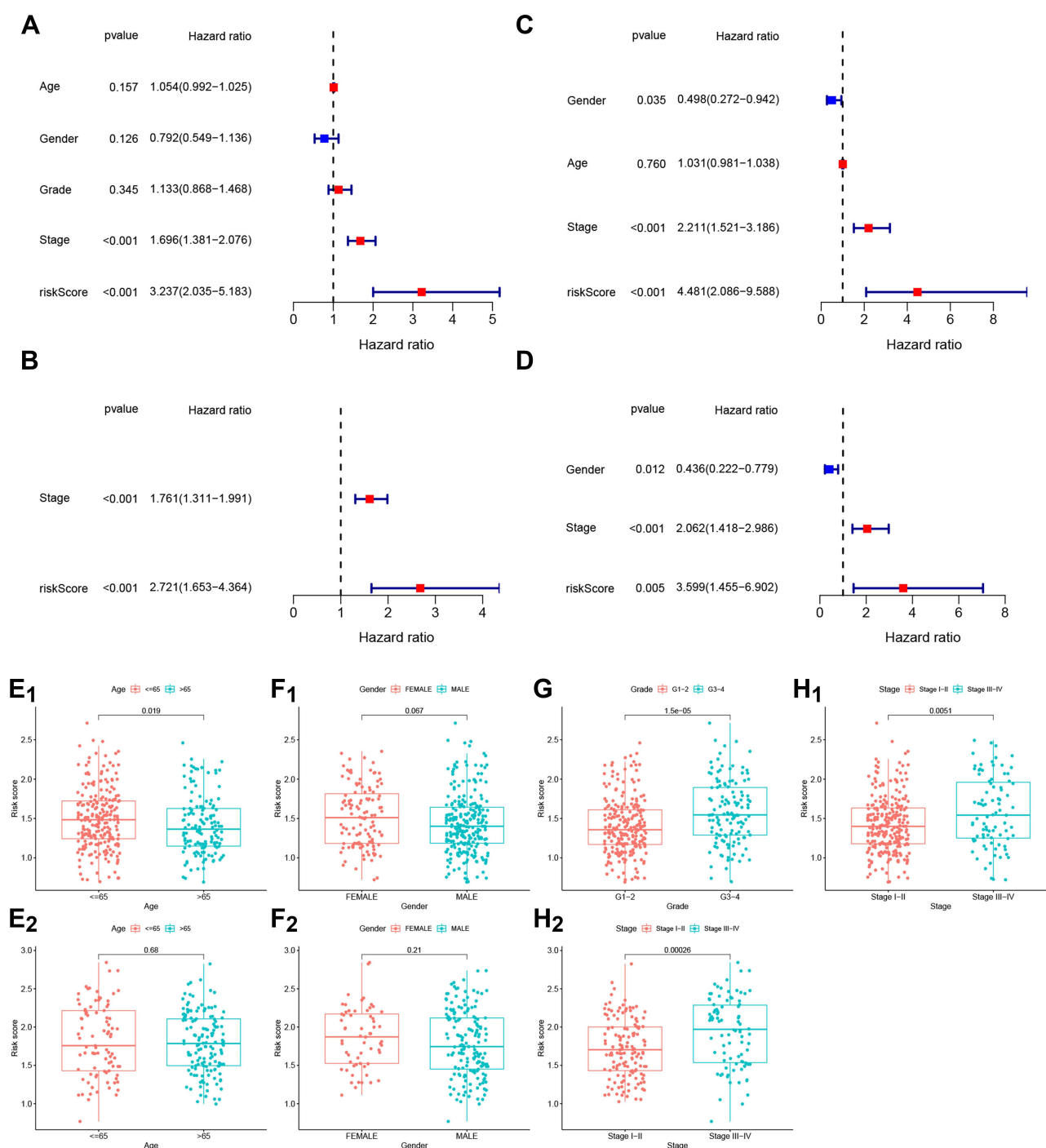


Figure 3 The relationship between prognostic value and clinicopathological characteristics of the 7-gene signature in TCGA (**A**, **B**, **E1**, **F1**, **G** and **H1**) and ICGC (**C**, **D**, **E2**, **F2** and **H2**) datasets. (**A** and **C**). Univariate Cox regression analysis of OS-associated factors (age, gender, grade and stage) in HCC patients. (**B** and **D**). OS-associated factors in HCC were validated using multivariate Cox regression analysis. (**E1–H2**). Association between risk score and clinicopathological features, including age (**E1** and **E2**, red: age ≤ 65 , blue: age > 65), sex (**F1** and **F2**, red: female, blue: male), grade (**G**, red: G1-2, blue: G3-4) and stage (**H1** and **H2**, red: stage I–II, blue: stage III–IV).

CI = 2.086–9.588, $p < 0.001$) and multivariate (3.599, 95% CI = 1.455–6.902) Cox regression analysis (Figure 3C–D). These results identified the prognostic value of tumor stage phase for HCC patient OS.

Next, we analyzed the association between age, sex, grade and stage and OS for HCC patients. Results demonstrated that patients aged ≤ 65 years had significantly higher risk scores than patients aged > 65 years ($p = 0.019$; Figure 3E1).

There was no difference in risk score between age groups (> 65 or ≤ 65 , $p = 0.68$) (Figure 3E₂) or sex (female or male) in TCGA ($p = 0.067$) and ICGC ($p = 0.21$) datasets, respectively (Figure 3F₁ and F₂). However, a significant difference in risk score was observed between patients with stage-I/II tumors and patients with stage-III/IV tumors, both in TCGA ($p = 0.0051$) and ICGC datasets ($p = 0.00026$) (Figure 3H₁ and H₂). Additionally, the difference in risk score in grade-1/2 vs grade-3/4 patients was significant in TCGA dataset ($p = 1.5e-05$) (Figure 3G). Altogether, this suggested that tumor stage and high risk scores in patients with stage-III/IV tumors may be a prognostic factor for HCC patients.

Immune Status and Tumor Immune Microenvironment of Different Risk Groups from TCGA Datasets

Enrichment scores from ssGSEA represent the infiltration levels of 16 immune cell types and 13 immunological functions from TCGA and ICGC cohorts, which was used to assess the relationship between 2 risk groups (Figure 2A) and immune status in HCC. The expression scores of classic dendritic cells (aDCs), CD8+ T-cells (ICGC only), inflammatory dendritic cell (iDCs), macrophages, mast cells (TCGA only), neutrophils, plasmacytoid dendritic cells (pDCs), T follicular helper cells (Tfh cells), Th1-cells, Th2-cells, Tumor infiltrating Lymphocytes (TILs) and Tregs in the high-risk group were significantly higher than those in the low-risk group, in both TCGA and ICGC datasets (Figure 4A and B). For immune-related functions, the enrichment scores for APC co-inhibition, APC co-stimulation, Chemokine receptor (CCR), Checkpoint, Human Leukocyte Antigen (HLA), Major Histocompatibility Complex (MHC) class-1, Proinflammation, T-cell co-inhibition, T-cell co-stimulation and Type II IFN response in the high-risk were greater than those in the low-risk group (Figure 4C and D). The data suggested immunological activation.

Subsequently, as immune infiltration level is associated with OS and prognosis in tumors, we investigated the link between immune infiltrates and risk scores. Immune cell infiltration is commonly seen in many types of tumors, including HCC, and its prognostic value has been reported.²¹ Here, we analyzed four types of immune infiltrate, including C1 (wound healing), C2 (IFN- γ dominant), C3 (inflammatory) and C4 (lymphocyte-depleted) in HCC from TCGA database. The findings revealed that a high-risk score was linked to C1, whereas a reduced risk score was linked to C4 (Figure 4E). Moreover, using TCGA data, we analyzed the correlation between the RNA stemness score (RNAss), which is premised on mRNA expression, the DNA stemness score (DNAss), which is premised on DNA methylation pattern, can both be used to test tumor stemness.²² We also analyzed the immune and stromal score, which could affect the status of tumor immune microenvironment and the risk score. The results suggested that the risk score negatively correlated with DNAss ($r = -0.23$, $p = 1.2e-05$), indicating that a high-risk score might be associated with reduced stemness. In addition, the risk score positively correlated with the immune score ($r = 0.25$, $p = 1.5e-06$), suggesting that a high-risk score might promote the stemness property in TAMs. However, there were no significant correlations between RNAss ($r = 0.06$, $p = 0.25$) and stromal score ($r = 0.082$, $p = 0.12$) with risk score (Figure 4F).

The PD-1/PD-L1 and PD-2/PD-L2 axis play a pivotal role in the progression of tumors by regulating immune surveillance. In the current study, we investigated the expression of the immune checkpoint molecules PD-L1/2, B7-1 (known as CD80) and B7-2 (known as CD86) in the high- and low-risk groups. The results showed that the expression levels of these four checkpoint molecules in the high-risk group were significantly increased compared with those in low-risk group ($p < 0.05$) (Figure 5A–D). And expression of these 4 check molecules had a positive correlation with risk scores, ($p < 0.01$) (Figure 5E–H). Thus, the risk factor may be associated with immune cell infiltration, immune-related functions and status of tumor immune microenvironment.

Prediction of the Effects of Targeted Drugs on the Seven Genes in HCC

Molecular targeted drugs are the first choice for systemic treatment of HCC.²³ We identified that seven inflammation-related DEGs could serve critical role in inflammatory and immune microenvironment in HCC. Thus, we next sought to identify the potential key tumor targeted drugs that could inhibit the expression of these seven genes using public datasets. First, we predicted 121 potential molecular drugs targeting the ITGA5, MEP1A, P2RX4, SERPINE1, SLC7A1 and SRI genes in TCGA (Table S2), then identified the top three drugs as: Bleomycin, simvastatin and zoledronate ($p < 0.01$). We then carefully read and studied existing research, to verify the effectiveness of these drugs in HCC. Figure 6A

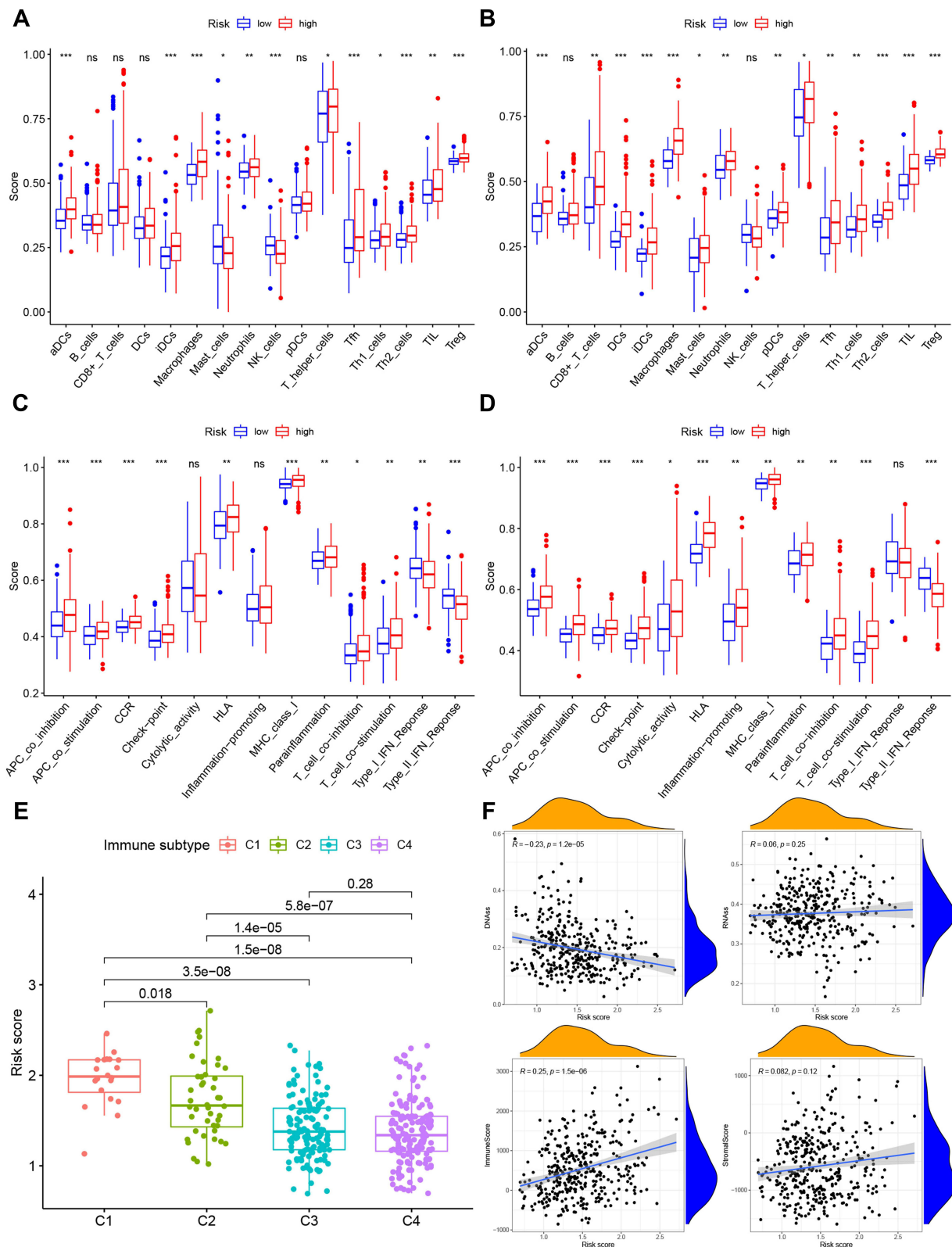


Figure 4 Immune status of high- and low-risk groups in TCGA (A and C) and ICGC (B and D) cohorts and association between tumor immune microenvironment and reference risk score in TCGA datasets (E and F). Boxplots of the score of 16 immune cell types (A and B) and 13 immunological functions (B and D) in the high- and low-risk groups. Red: high risk, blue: low risk, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant. (E). Comparison of the risk score in different tumor infiltration subtypes. C1, wound healing, C2, INF- γ dominant, C3, inflammatory, and C4, lymphocyte-depleted. (F). Correlation analysis between the risk score and DNAss (DNA stemness score), RNAss (RNA stemness score), immune score and stromal score in TCGA data.

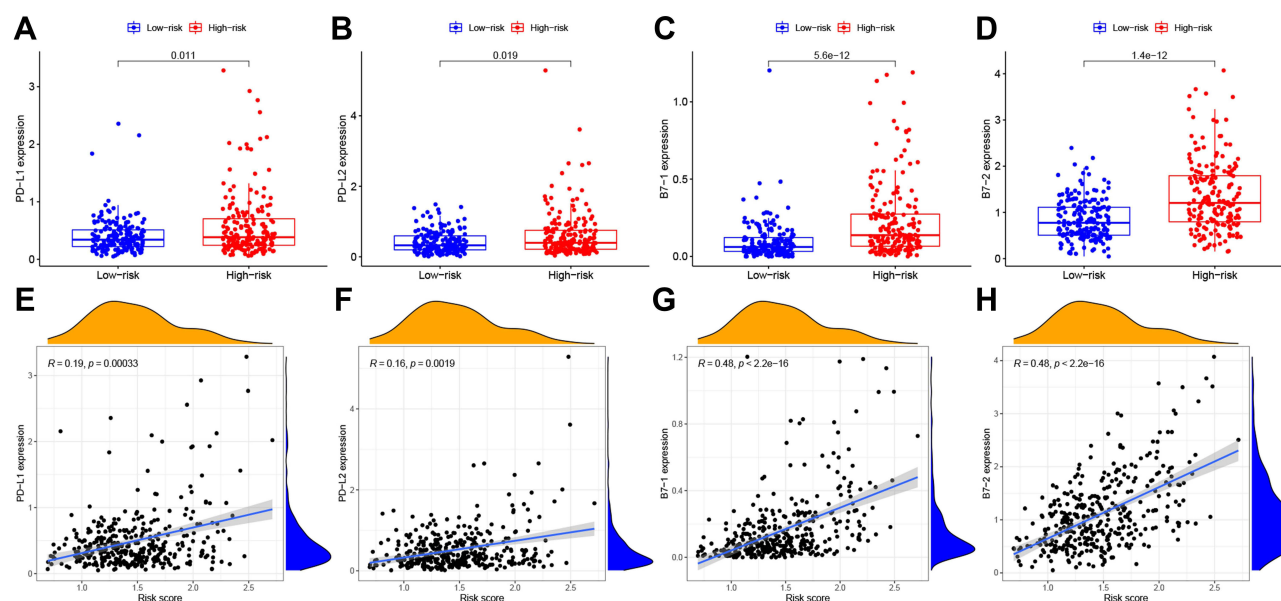


Figure 5 Comparison of the expression levels of immune checkpoint molecules in the high- and low risk groups in TCGA. (A–D). Expression of PD-L1, PD-L2, B7-1 (CD80) and B7-2 (CD86) in both groups. Red: high risk, blue: low risk, $P < 0.05$. (E–H). Correlation analysis between the risk score and the expression of four immune checkpoint molecules.

A Potential drugs involved in HCC from TCGA in this paper

Drugs	Structure	Weigh	Effects	Reference	Correlation
Bleomycin	<chem>C55H84N17O21S3+</chem>	1415.6	efficacy for HCC	Y.litaka et al	0.496035
Simvastatin	<chem>C25H38O5</chem>	418.6	efficacy for HCC	J.Cejka et al	0.448673
Zoledronate	<chem>C5H10N2O7P2</chem>	272.09	efficacy for HCC	A.Sarkar et al	0.449141

B Bleomycin Simvastatin Zoledronic

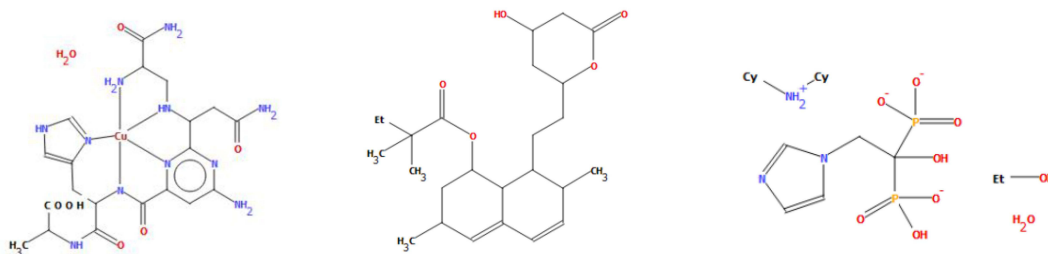


Figure 6 Prediction of the targeted drugs of 7 genes in HCC. (A). Characteristics of 3 potential targeted drugs involved in HCC identified in TCGA. (B). Two-dimensional chemical structure of bleomycin, simvastatin and zoledronate.

presents detailed information on these three drugs, including their structure, molecular weight, effects and references. In addition, two-dimensional chemical structure images of all three drugs were generated using PubChem (Figure 6B). Moreover, we verified the drug sensitivity of Bleomycin, Simvastatin and Zoledronate drugs in HCC Huh-1 and Hep3b cells in vitro analysis (Figure S4).

Lastly, the mRNA expression levels of all 7 genes in Huh-1 and Hep3b cells and in HL7702, a normal liver cell line, were verified using RT-PCR. The expression levels of ITGA5, MEP1A, P2RX4, RIPK2, SLC7A1 and SRI in the Huh-1 and Hep3b cells cell line were significantly higher than in the HL7702 cell line ($p < 0.05$) (Figure 7). Moreover, the

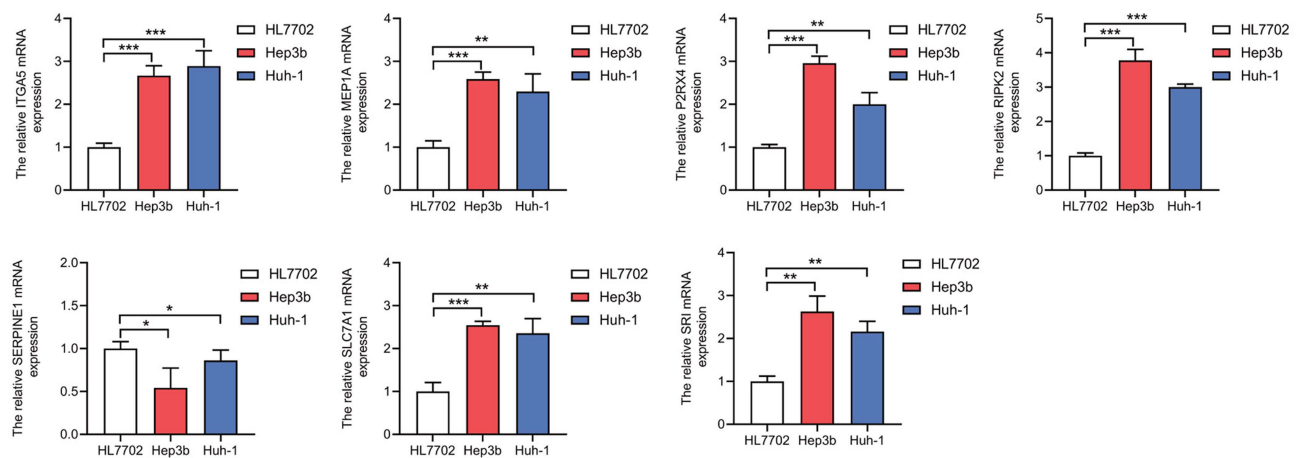


Figure 7 Validation of the mRNA expression levels of 7 genes in HCC cell lines. Real-time PCR was used to detect the expression of the ITGA5, MEPIA, P2RX4, RIPK2, SERPINE1, SLC7A1 and SRI genes. The expression of ITGA5, MEPIA, P2RX4, RIPK2, SLC7A1 and SRI was upregulated in the HCCLM3 cell line compared with normal liver cells, whereas that of SERPINE1 was downregulated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

downregulated expression of ITGA5, SERPINE1 and P2RX4 were observed after Bleomycin treatment, the down-regulated level of ITGA5, SERPINE1, SRI and SLC7A1 were determined by Simvastatin treatment, and the down-regulated expression of ITGA5, SERPINE1, RIPK2 and MEPIA were observed after Zoledronate (Figure S5).

Discussion

HCC is the fifth most prevalent cancer worldwide and the third most common cause of cancer mortality.²⁴ Most patients with HCC are in either sub-Saharan Africa or in Eastern Asia. In China, HCC accounts for more than 50% of global cases (age-standardized incidence rate: men, 35.2/100,000; women, 13.3/100,000).¹ Although the key mechanisms leading to the development of HCC are still unclear, one possible explanation for hepatocarcinogenesis is cirrhosis induced by inflammatory responses, as hepatitis causes alterations of the microenvironment including altered cytokine secretion from activated stellate cells and inflammatory signaling from infiltrating immune cells, which can influence the progression of HCC.^{1,25} Moreover, recent studies have shown that several types of tumors come from infected and chronically stimulated inflammatory sites, and the tumor microenvironment, which is composed of inflammatory cells, is an important factor leading to tumorigenesis. In other terms, long-term chronic inflammation increases the risk of developing tumors and promotes tumorigenesis.^{26,27} In addition, the combination of inflammatory responses and immune status plays an important role in HCC. Previous studies have reported that inflammation-associated genes risk signature can be used to predict patient prognosis and guide treatment for esophageal squamous cell carcinoma, lung adenocarcinoma and urothelial bladder cancer.^{28–31} Other hand, Sia et al determined that inflammation response-related gene signature could be correlated with the immune exhausted.³² Many cancers are characterized by simultaneous immunosuppression and inflammation, their regulation is integrally linked, which can induce a dysfunctional immune state unable to eliminate disease. One possibility limit excessive immunopathology while maintaining some level of immunological control.³³ In the current study, we first constructed and validated a prognostic gene signature consisting of inflammation-associated DEGs, namely ITGA5, MEPIA, P2RX4, RIPK2, SERPINE1, SLC7A1 and SRI, which could be used as an independent risk factor to predict the prognosis of HCC patients.

Integrins are transmembrane proteins that regulate communication between cells and the extracellular matrix. Integrin $\alpha 5$ (ITGA5), a member of the integrin family, primarily interacts with the integrin $\beta 1$ subunit to form an $\alpha 5\beta 1$ heterodimer that recognizes its specific ligand fibronectin.³⁴ Increasing evidence demonstrates that ITGA5 is strongly associated with poor prognosis, and its expression level is significantly associated with tumor purity and infiltration levels of different immune cells in gastrointestinal and HCC tumors.^{35,36} MEPIA belongs to the metzincin superfamily and can cleave a wide variety of substrates, including membrane proteins, cytokines and protein kinases.³⁷ A previous study has suggested that MEPIA promotes HCC cell proliferation, migration, and invasion by regulating cytoskeletal events and

induces epithelial-to-mesenchymal transition in HCC cells.³⁸ P2RX2 is an ion channel purinoreceptor for adenosine triphosphate associated with cellular stress and inflammation and has been reported as a potential diagnostic and prognostic marker.³⁹ The amino acid transporter SLC7A1 is significantly associated with the prognosis of HCC patients⁴⁰ and impaired T cell differentiation through the modulation of mTORC1 signaling.⁴¹ SRI is a soluble resistance-related calcium-binding protein. A previous study has demonstrated that the interaction between ANXA7 and SRI regulates epithelial-to-mesenchymal transition, migration, invasion, and proliferation in HCC cells.⁴²

Our results determined that these genes, alone or in combination, may be independent risk factors for the progression of HCC. Tumors are complex environments, composed of transformed cells as well as stromal and immune infiltrates. In this study, we found that the scores of aDCs, iDCs, macrophages, neutrophils, pDCs, Tfh cells, Th1-cells, Th2-cells, TILs and Treg in the high-risk group was significantly higher than those of the low-risk group. In addition, APCs, T cells, HLA and type-II IFN responses were activated. The results suggested that immune cell infiltration and activated immunological functions in the high-risk group could result in poor prognosis.

Immunotherapy targets immune cells in order to enhance the anti-tumor immune response. The rapid development of immunotherapy has opened up new avenues for tumor treatment and has achieved outstanding results in the treatment of HCC. The successive approval of single-drug and combination therapy has also brought more options for patients with advanced HCC.^{15,43} Checkpoint inhibitors, such as, durvalumab (MEDI4736) and atezolizumab (MPDL3280A) directed at the PD-1/PD-L1 axis are now approved for the treatment of multiple cancers, including HCC.^{44–46} Previous study has determined that Anti-PD-L1, Anti- B7-1 (CD80) and B7-2 (CD86) checkpoint has been an innovative immunotherapeutic strategy.^{47,48} In our study, we also found that expression levels of the PD-L1/2, B7-1 (CD80) and B7-2 (CD86) checkpoint molecules in the high-risk group were significantly increased compared with those in the low-risk group. This suggests that inhibition of these four immune checkpoint molecules can suppress the expression level of 7 gene signature, further inhibition of HCC progression.

Previous study by Lee et al reported longer progression-free survival following combination treatment with atezolizumab and bevacizumab, compared with atezolizumab alone, in patients with unresectable HCC not previously treated with systemic therapy.⁴⁹ Another study also determined that lenvatinib plus pembrolizumab treatment resulted in effective antitumor activity in HCC.⁴³ Thus, combined immunotherapy may exert a therapeutic effect in HCC. Although immunotherapy has limited value for patients with late-stage HCC, patients with early and mid-stage HCC can benefit from combined immunotherapy. Using publicly available datasets, we identified that bleomycin,⁵⁰ simvastatin⁵¹ and zoledronate⁵² were potential key tumor-targeting drugs that can suppress the expression of seven genes in HCC. Bleomycins are a family of compounds produced by *Streptomyces verticillus* that have potent tumor-killing properties and thus occupy an important place in cancer chemotherapy.⁵³ Bleomycin-induced upregulation of BCLXL/L1 and MDM2 suggests that the ratio of pro-apoptotic to anti-apoptotic proteins regulates the responsiveness of HCC cells to chemotherapy; that is, treatment sensitivity or drug resistance.⁵⁴ Simvastatin is a cholesterol-reducing drug that has been reported to inhibit the activity of hydroxymethylglutaryl coenzyme A reductase and the HIF-1 α /PPAR- γ /PKM2 axis by suppressing PKM2-mediated glycolysis, resulting in decreased proliferation and increased apoptosis in HCC cells and re-sensitizing these cells to sorafenib. Additionally, sorafenib and simvastatin co-treatment can improve sorafenib resistance in HCC.^{55,56} Zoledronate is a bisphosphonate reported to be effective for the treatment of bone metastasis in patients with various cancers, including HCC. One possible mechanism is that zoledronate delays both the development and severity of pain associated with bone metastasis in patients with HCC and has a direct effect on HCC cells.⁵⁷ Our findings suggest that bleomycin, simvastatin and zoledronate can inhibit the expression levels of the genes identified using the inflammation-associated prognostic gene signature and may represent therapeutic options for HCC patients.

Conclusion

In summary, the results of this study indicate that the inflammation-related gene signature could predict OS for HCC patients. The analysis of the relationship between the inflammation-associated prognostic gene signature and the tumor immune microenvironment, including immune cell infiltration, immune-related functions, immune infiltration subtypes and immune checkpoint molecules, provides new insight for HCC immunotherapy. Moreover, bleomycin, simvastatin

and zoledronate could regulate the expression levels of these genes. These findings may help identify a new strategy for the clinical treatment of HCC.

Study Approval

All datasets in the present study were downloaded from public databases, including TCGA, GEO and ICGC. These public databases allowed researchers to download and analyze public datasets for scientific purposes. The current research follows the TCGA, GEO and ICGC data access policies and publication guidelines. Users can download relevant data for free, our study is based on open-source data, there are no ethical issues and other conflicts of interest. Inclusion of the Human cell lines in the study were approved by Xijing Hospital review board.

Author Contributions

Guofang Lu, Yuanyong Wang, Rui Du and Yulong Shang conceived, designed and carried out the study and the relevant data analysis and interpretation. Bin Feng, Jianglin Wang and Fengrui Zhang carried out the study and the relevant data analysis and interpretation. Guofang Lu, Yuanyong Wang and Rui Du wrote the manuscript. Jianming Pei and Yulong Shang reviewed and revised the article. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to Journal of Inflammation Research the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors have declared no competing interest in this work.

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