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ORIGINAL RESEARCH

HSPB11 is a Prognostic Biomarker Associated with Immune Infiltrates in Hepatocellular Carcinoma

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Purpose: Heat shock proteins (HSPs) play important roles in oncogenesis and malignant progression. HSPB11 is highly expressed in many malignant tumors, but research on its role in hepatocellular carcinoma (HCC) is insufficient.

Patients and Methods: A comprehensive analysis of HSPB11 in HCC was performed based on data of patients with HCC and those from online public databases.

Results: HSPB11 was overexpressed in HCC, with a high discrimination ability between tumor and normal tissues (area under the curve =0.923). HSPB11 overexpression correlated with advanced tumor stage, poorer tumor differentiation, and worse prognosis and was an independent risk factor for HCC prognosis. The nomogram and calibration models composed of HSPB11, T stage, and M stage had good abilities to predict the 1-, 3-, and 5-year survival rates of patients. HSPB11 was determined to be involved in multiple oncogenic processes, including cell cycle checkpoints, the G2M checkpoint, E2F targets, Rho GTPases, and KRAS signaling. HSPB11 expression was related to immune cell infiltration, especially that of Th2 cells and dendritic cells.

Conclusion: HSPB11 is involved in oncogenesis and immune regulation in HCC and is a potential prognostic biomarker and therapeutic target.

Keywords: heat shock protein B11, biomarker, immune cell infiltration, Th2 cell, dendritic cell

Introduction

Hepatocellular carcinoma (HCC) is characterized by high morbidity and mortality, making it the sixth most common cancer and one of the most common causes of cancer-related death.[1,2] Patients with early-stage HCC can be effectively treated with surgical resection, radiofrequency ablation, or liver transplantation, but most patients are not diagnosed until advanced disease stages, resulting in a poor prognosis.[3,4] Systemic drug therapies, such as sorafenib, lenvatinib, regorafenib, and cabozantinib, are recommended for advanced-stage HCC.[5–8] However, the mortality of patients with advanced HCC remains very high.[9] Therefore, researchers have been exploring valuable prognostic biomarkers and more effective treatment methods.[10]

Heat shock proteins (HSPs) are a family of highly conserved proteins that are expressed at low basal levels under normal physiological conditions, but their expression is upregulated upon exposure to heat stress and tumorigenic environments.[11–14] Several members of the HSP family, such as HSP27, HSP70, and HSP90, play important roles in the oncogenesis, therapeutic resistance, invasion, and metastasis of HCC.[15–20] Various inhibitors of HSPs have been proposed as possible treatment strategies for HCC, which could be used alone or in combination with other treatment approaches.[21] HSPB11 has been investigated in many animal models, such as fish and birds.[22,23] Its expression is closely related to several diseases, such as tumors and degenerative lesions of the nervous system.[15,24–29] For example, HSPB11 is overexpressed in high-grade gliomas, and its expression level correlates with the degree of malignancy and prognosis.[27,29,30] In esophageal cancer, the expression level of

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HSPB11 is closely related to the survival and prognosis of patients. Combined with other biomarkers, it can be used to predict the response to neoadjuvant radiochemotherapy in patients with esophageal cancer.[28,31] Norouzinia et al found that the expression of HSPB11 in gastric cancer tissues of patients infected with *Helicobacter pylori* is lower than that in tissues of individuals not infected with *H. pylori*, suggesting that it plays a role in the mechanism of *H. pylori* infection.[32] Yang et al reported that HSPB11 is highly expressed in HCC, but its roles in oncogenesis and immune regulation are still not clear.[15] Thus, we comprehensively analyzed the role of HSPB11 in HCC, including its diagnostic and prognostic values, biological functions, and association with the immune microenvironment.

Materials and Methods

Comparison of HSPB11 Expression Using Data from Online Public Databases

The expression level of HSPB11 was investigated in 17 common tumors using data from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. Additionally, 225 liver tumor tissues and 220 non-tumor tissues from GSE14520, as well as 81 tumor tissues and 10 non-tumor tissues from GSE62232 were retrieved to evaluate the expression level of HSPB11. The receiver operating characteristic (ROC) curve was created to explore the diagnostic value of HSPB11 in HCC.

Validation of HSPB11 Expression in Clinical Samples

Thirty tumor tissues and their adjacent normal tissues were collected from HCC patients in the Second Hospital of Dalian Medical University. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was performed using the ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA) and StepOnePlus apparatus (Applied Biosystems, Foster City, CA, USA). The amplification primers used were as follows: HSPB11 forward, TGATGGCTCCGCTACTTACTT and reverse, GCAGAAACGCTATGCACAGAT; glyceraldehyde-3-phosphate dehydrogenase (*GADPH*, the internal reference gene) forward, CAGCCTCAAGATCATCAGCAAT and reverse, ATGAGTCCTTCCACGATACCAAA. Three technical replicates were performed for each sample. The Ethics Committee of Second Hospital of Dalian Medical University approved this research (no. 0202159). Written informed consent was obtained from all patients before specimen collection.

Association Between HSPB11 and Cancer Stage and Prognosis

Correlation analyses were performed using the R *ggplot2* package based on data derived from TCGA to investigate the association between HSPB11 expression and tumor size (T stage), pathological stage, tumor differentiation (histological grade), and adjacent hepatic tissue inflammation. Kaplan–Meier plots and univariate and multivariate Cox proportional hazards models were created using the *survival* package to evaluate the effect of HSPB11 on the prognosis of HCC and to identify risk factors for HCC based on data available in TCGA database. Another online database (Kaplan–Meier Plotter, https://kmplot.com/analysis/) was also used to evaluate the effect of HSPB11 on the prognosis of patients with HCC.

Establishment of Prognostic Models for HCC

The time-dependent survival ROC curve was created using the R *timeROC* package to evaluate the predictive ability of HSPB11 for the prognosis of patients with HCC. By integrating risk factors identified in the aforementioned Cox regression model, the nomogram and calibration models were built using the R *rms* and *survival* packages to predict the survival probabilities.

Enrichment of HSPB11-Associated Genes in HCC

Patients with HCC from TCGA were divided into HSPB11-high and HSPB11-low groups, and the differentially expressed genes and their corresponding logFC values were screened using R with the *DESeq2* package. The gene set enrichment analysis (GSEA) was performed using the *clusterProfiler* package based on MSigDB Collections

(https://www.gsea-msigdb.org/). A false discovery rate <0.25 and adjusted P-value <0.05 were used as criteria for statistical significance. *h.all.v7.2.symbols.gmt Hallmarks* and *c2.cp.v7.2.symbols.gmt Curated* were used as the reference gene sets.

Correlation Analyses of HSPB11 and Immune Cell Infiltration

The ssGSEA analysis was performed using the R *GSVA* package to demonstrate the association between HSPB11 and the level of infiltration of 24 types of immune cells. Spearman correlation and Wilcoxon rank-sum tests were applied to investigate the relationship between HSPB11 expression and the immune cell infiltration level.

Statistical Analyses

R (v.3.6.3) and RStudio software were used for the statistical analyses, and $P < 0.05$ was set as the criterion for statistical significance.

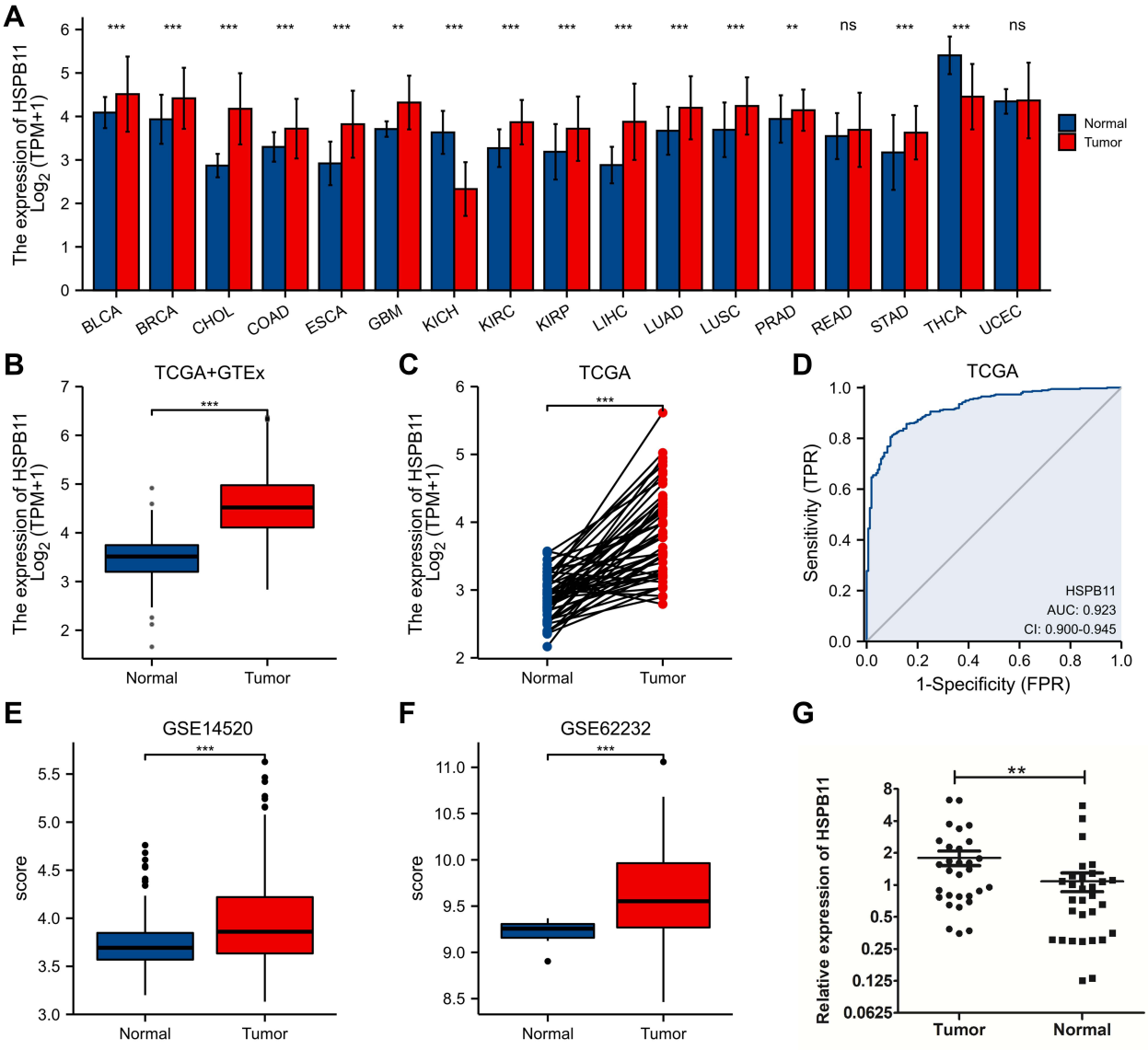

Figure 1 Expression level of HSPB11 in pan-cancers and hepatocellular carcinoma (HCC). (**A**) HSPB11 expression in pan-cancers compared with that in normal tissues in The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) databases. (**B**–**E**) Expression levels of HSPB11 in HCC in several online public databases. (**F**) Receiver operating characteristic curve to test the value of HSPB11 to identify HCC tissues. (**G**) The quantitative polymerase chain reaction of clinical HCC samples confirmed that the mRNA level of *HSPB11* in tumor tissues was higher than that in the adjacent normal liver tissue. **p < 0.01, ***p < 0.001, ns, no significance.

Results

Expression Level of HSPB11 in Pan-Cancers and HCC

HSPB11 was more highly expressed in 13 types of tumors (breast infiltrating carcinoma, bladder urothelial carcinoma, cholangiocarcinoma, colon adenocarcinoma, esophageal carcinoma, pleomorphic glioma, renal clear cell carcinoma, renal papillary cell carcinoma, liver hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, prostate cancer, and gastric cancer) than in their adjacent normal tissues (Figure 1A). The hyperexpression of HSPB11 was observed in HCC tissues in both unmatched (Figure 1B) and paired (Figure 1C) comparative studies based on TCGA database. Furthermore, in the ROC curve, the area under the curve (AUC) was 0.923, indicating a high ability of HSPB11 to differentiate tumor and normal tissues (Figure 1D). The comparative analysis of public databases of GSE14520 (Figure 1E) and GSE62232 (Figure 1F), accompanied by the qRT-PCR analysis of clinical samples from our hospital (Figure 1G), all confirmed higher *HSPB11* mRNA expression in HCC than in the adjacent normal liver tissues.

HSPB11 Correlates with HCC Stage and Prognosis

The clinical data used to identify the relationship between HSPB11 and the cancer stage and prognosis of HCC were retrieved from TCGA database, and the baseline characteristics of the 374 HCC patients are presented in Table S1. The expression level of HSPB11 was positively correlated with tumor size, pathological stage, and histological grade, indicating that patients with advanced stage and poorer tumor differentiation tend to express higher level of HSPB11.

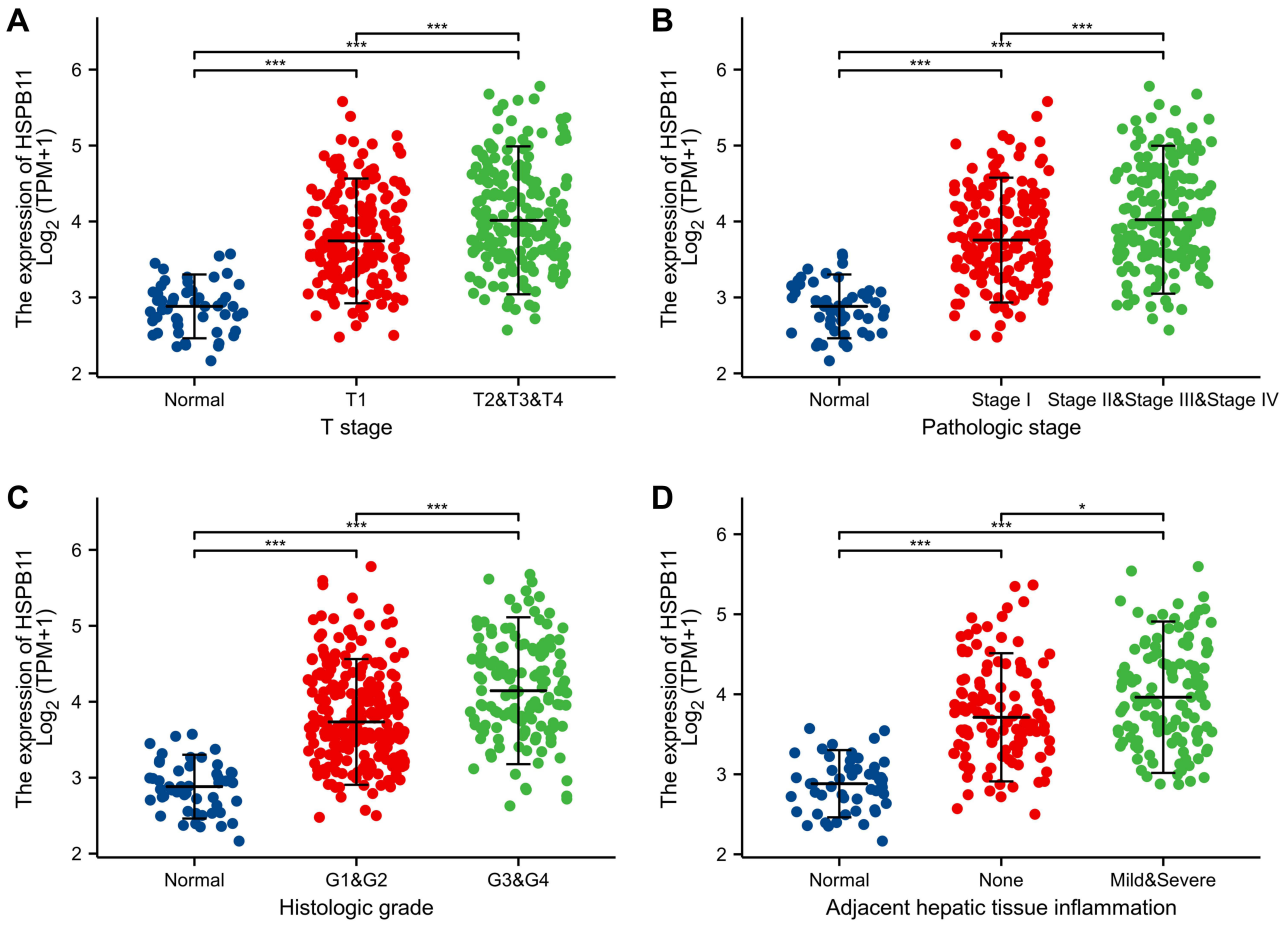

Figure 2 Relationship between the expression level of HSPB11 and stage of hepatocellular carcinoma. The expression level of HSPB11 was highly correlated with (**A**) T stage, (**B**) pathological stage, (**C**) histological grade, and (**D**) adjacent hepatic tissue inflammation. *p < 0.05, ***p < 0.001.

(Figure 2A–C). Higher HSPB11 expression was also observed in HCC patients with adjacent hepatic tissue inflammation (Figure 2D).

The association between the HSPB11 level and the prognosis of HCC was evaluated. According to the Kaplan–Meier survival curves based on TCGA, HCC patients with higher HSPB11 expression showed a lower overall survival (OS) (hazard ratio (HR) = 2.09, 95% confidence interval (CI) = 1.45–2.99, $p < 0.001$), poorer disease-specific survival (DSS; HR = 2.19, CI = 1.38–3.48, p = 0.001), and a worse progression-free interval (PFI; HR = 1.70, CI = 1.25–2.32, p = 0.001) (Figure 3A–C). An analysis of the Kaplan–Meier Plotter database confirmed the poorer prognosis of HCC patients with high HSPB11 expression (Figure 3D–F). The multivariate Cox models showed that tumor size, distant metastasis, and the expression level of HSPB11 were all independent risk factors for the prognosis of HCC (Table 1).

Establishment of Prognostic Models for HCC

The AUC values of the time-dependent survival ROC curve for 1-, 3-, and 5-year survival were all greater than 0.6 (Figure 4A). By integrating risk factors for prognosis identified in the Cox regression models, including tumor size, distant metastasis, and the expression level of HSPB11, nomogram and calibration models were built to predict the survival probabilities at 1, 3, and 5 years. The prediction models showed good prediction ability; the dotted line predicted by the model was close to the ideal line (Figure 4B and C).

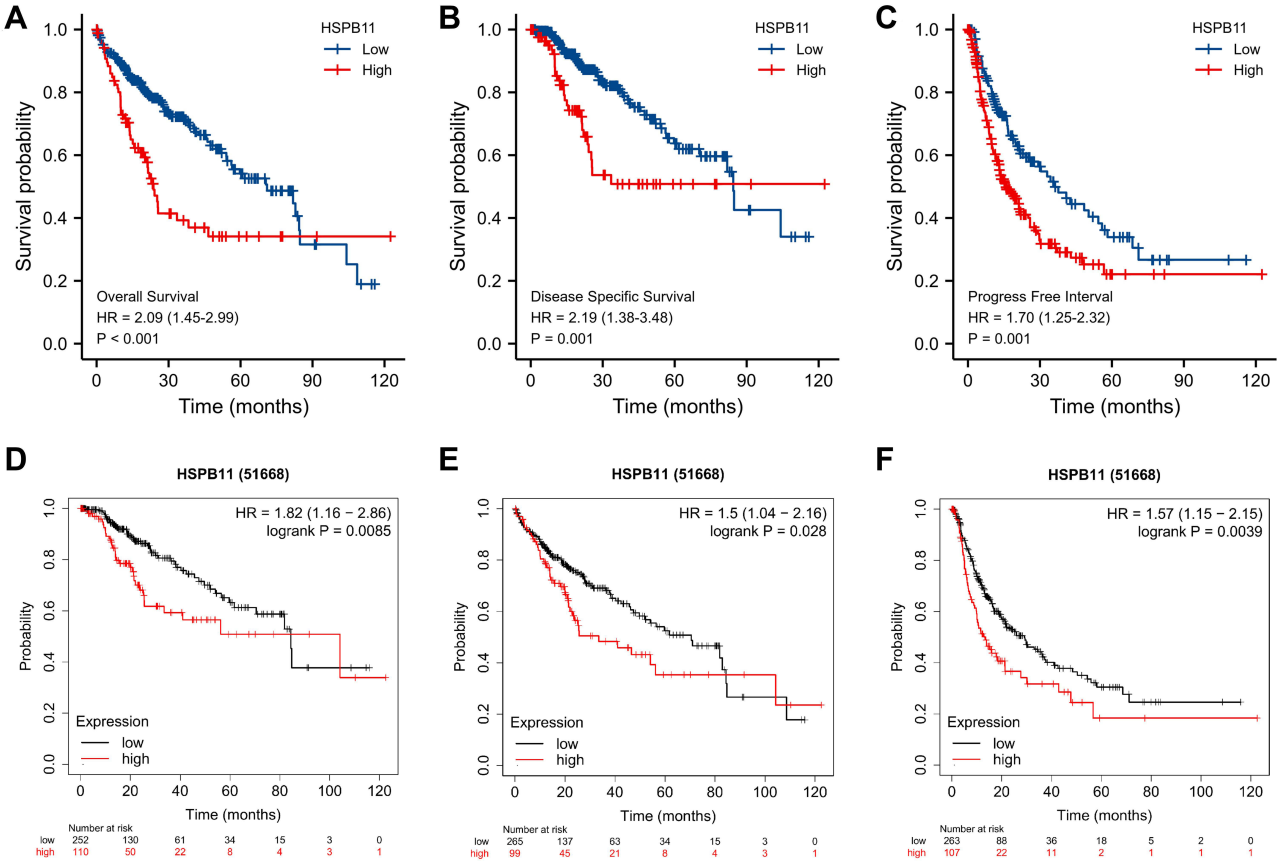

Figure 3 Prognostic value of the mRNA level of *HSPB11* in patients with hepatocellular carcinoma (HCC). (**A**) Overall survival (OS), (**B**) disease-specific survival (DSS), and (**C**) progression-free interval (PFI) survival curves comparing patients with high (red) and low (blue) HSPB11 expression based on TCGA database. Analysis of the Kaplan–Meier Plotter database confirmed the poorer prognosis of HCC patients with a high HSPB11 expression level (**D**–**F**). HSPB11 expression in HCC was plotted at the threshold of $p < 0.05$.

Table 1 Univariate and Multivariate Survival Analysis (Overall Survival) of Prognostic Covariates in Patients with Hepatocellular Carcinoma

Characteristic	Total (N)	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	P	HR (95% CI)	P
Age (>60 vs ≤60)	373	1.205 (0.850–1.708)	0.295		
Sex (Male vs Female)	373	1.261 (0.885–1.796)	0.200		
T stage (T2& T3&T4 vs T1)	370	2.126 (1.481–3.052)	<0.001	2.255 (1.419–3.583)	<0.001
N stage (N1 vs N0)	258	2.029 (0.497–8.281)	0.324		
M stage (M1vs.M0)	272	4.077 (1.281–12.973)	0.017	4.105 (1.229–13.707)	0.022
Histological grade (G4&G3 vs G1&G2)	368	1.091 (0.761–1.564)	0.636		
Vascular invasion (Yes vs No)	317	1.344 (0.887–2.035)	0.163		
Albumin (g/dl) (≥3.5 vs.<3.5)	299	0.897 (0.549–1.464)	0.662		
AFP (ng/mL) (>400 vs ≤400)	279	1.075 (0.658–1.759)	0.772		
Child-Pugh grade (B&C vs A)	240	1.643 (0.811–3.330)	0.168		
Adjacent hepatic tissue inflammation (Yes vs No)	236	1.194 (0.734–1.942)	0.475		
Prothrombin time (>4 vs ≤4)	296	1.335 (0.881–2.023)	0.174		
HSPB11 (High vs Low)	373	1.799 (1.269–2.550)	<0.001	1.848 (1.179–2.897)	0.007

Enrichment Analysis of HSPB11 and Associated Genes in HCC

The GSEA between the HSPB11-high and HSPB11-low patients was performed to explore HSPB11-associated pathways. The GSEA, based on *h.all.v7.2.symbols.gmt*, revealed that the G2M checkpoint, E2F targets, mitotic spindle, and KRAS signaling were significantly enriched. Cell cycle checkpoints, the G2M checkpoint, mitotic prometaphase, and signaling via Rho GTPases were significantly enriched based on the *c2.cp.v7.2.symbols.gmt* database (Figure 5).

Association Between HSPB11 Expression and Immune Cell Infiltration

As shown in Figure 6A, the expression level of HSPB11 was correlated with several immune cells including NK cells, neutrophils, mast cells, T cells, B cells, and macrophages. Significantly, the expression of HSPB11 was positively correlated with the abundance of Th2 cells ($R = 0.426$, $P < 0.001$) and negatively associated with the abundance of DCs ($R = -0.222$, $P < 0.001$). HCC with high HSPB11 expression presented higher infiltration levels of Th2 cells and lower infiltration levels of DCs (Figure 6B–E).

Discussion

As molecular chaperones, HSPs can protect cells to ensure survival under stress conditions, and increased expression of HSPs is observed in pathophysiological processes of cancer.[33] Studies have found that HSPs can enhance the survival and aggressiveness of cancer cells under stress conditions,[34] and specific inhibitors of HSPs or combined treatment with chemotherapy drugs can improve the prognosis of HCC.[21] In recent years, few studies have reported that HSPB11 is overexpressed in a variety of malignant tumors.[15,27–29,35] However, research on HSPB11 in HCC is insufficient. Based on data from public databases and those of clinical samples, we confirmed that HSPB11 is highly expressed in HCC, which is consistent with the findings of a previous study,[15] and the expression level had a high diagnostic value. The expression level of HSPB11 was significantly correlated with a more advanced tumor stage and worse prognosis. In multivariate Cox survival analysis models, the expression level of HSPB11 was an independent risk factor for the prognosis of

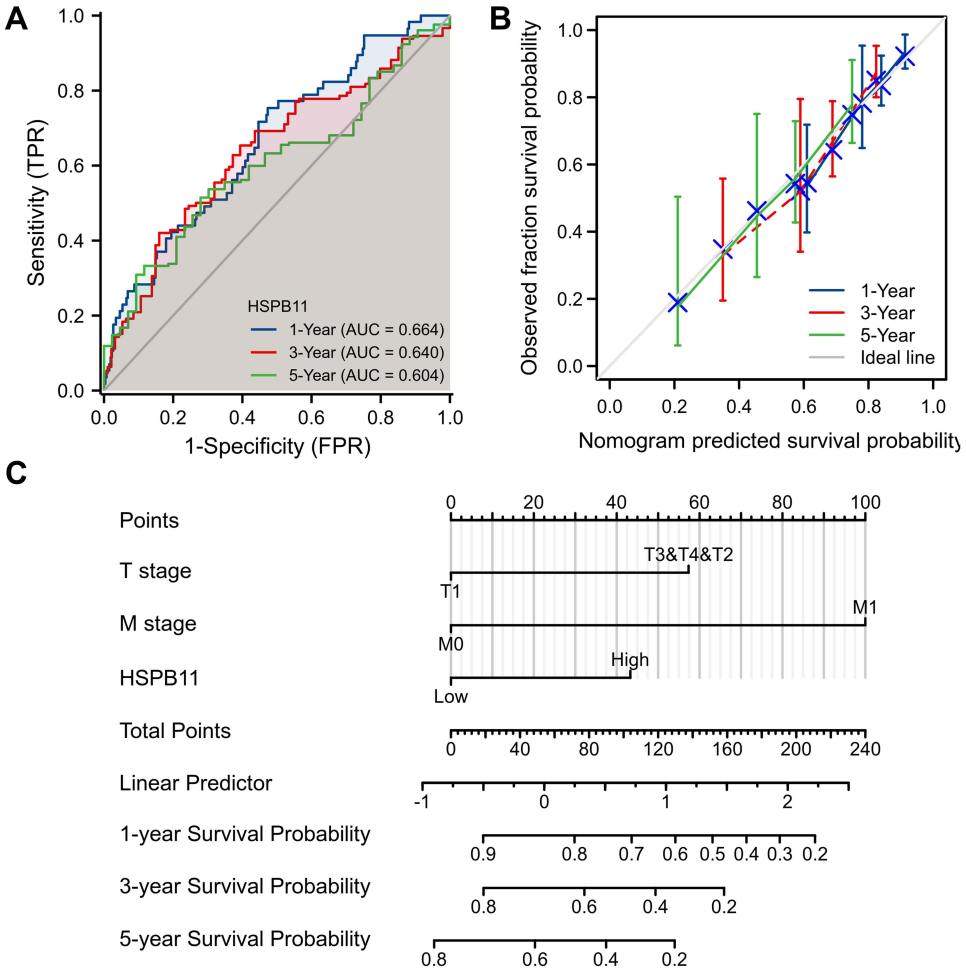

Figure 4 Establishment of prediction models to evaluate the probability of 1-, 3-, and 5-year overall survival (OS) of hepatocellular carcinoma patients. (**A**) Time-dependent survival receiver operating characteristic curve to evaluate the expression of HSPB11 for 1-, 3-, and 5-year OS. (**B**) Nomogram for estimating the probability of 1-, 3-, and 5-year OS. (**C**) Calibration plots of the nomogram for evaluating the probability of OS at 1, 3, and 5 years.

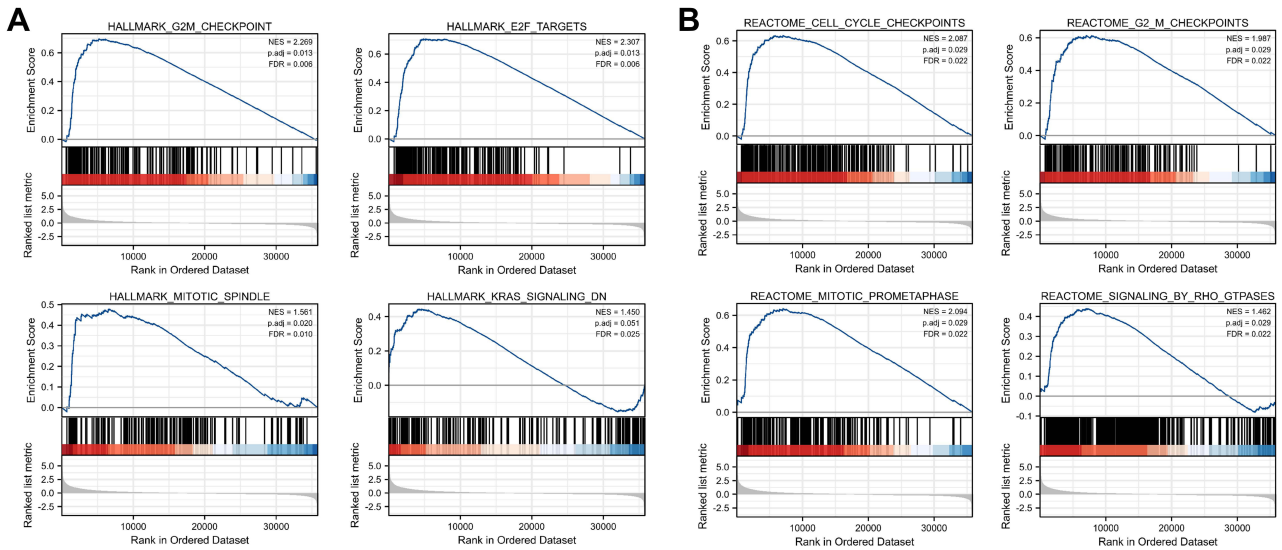

Figure 5 Plots from gene set enrichment analyses based on (**A**) *h.all.v7.2symbols.gmt* and (**B**) *cp. v7.2. symbols.gmt*. HSPB11 is involved in multiple oncogenic processes including the G2M checkpoint, mitotic spindle, E2F targets, KRAS signaling, cell cycle checkpoints, mitotic prometaphase, and signaling via Rho GTPase. NES, normalized enrichment score; p. adj, adjusted P value; FDR, false discovery rate.

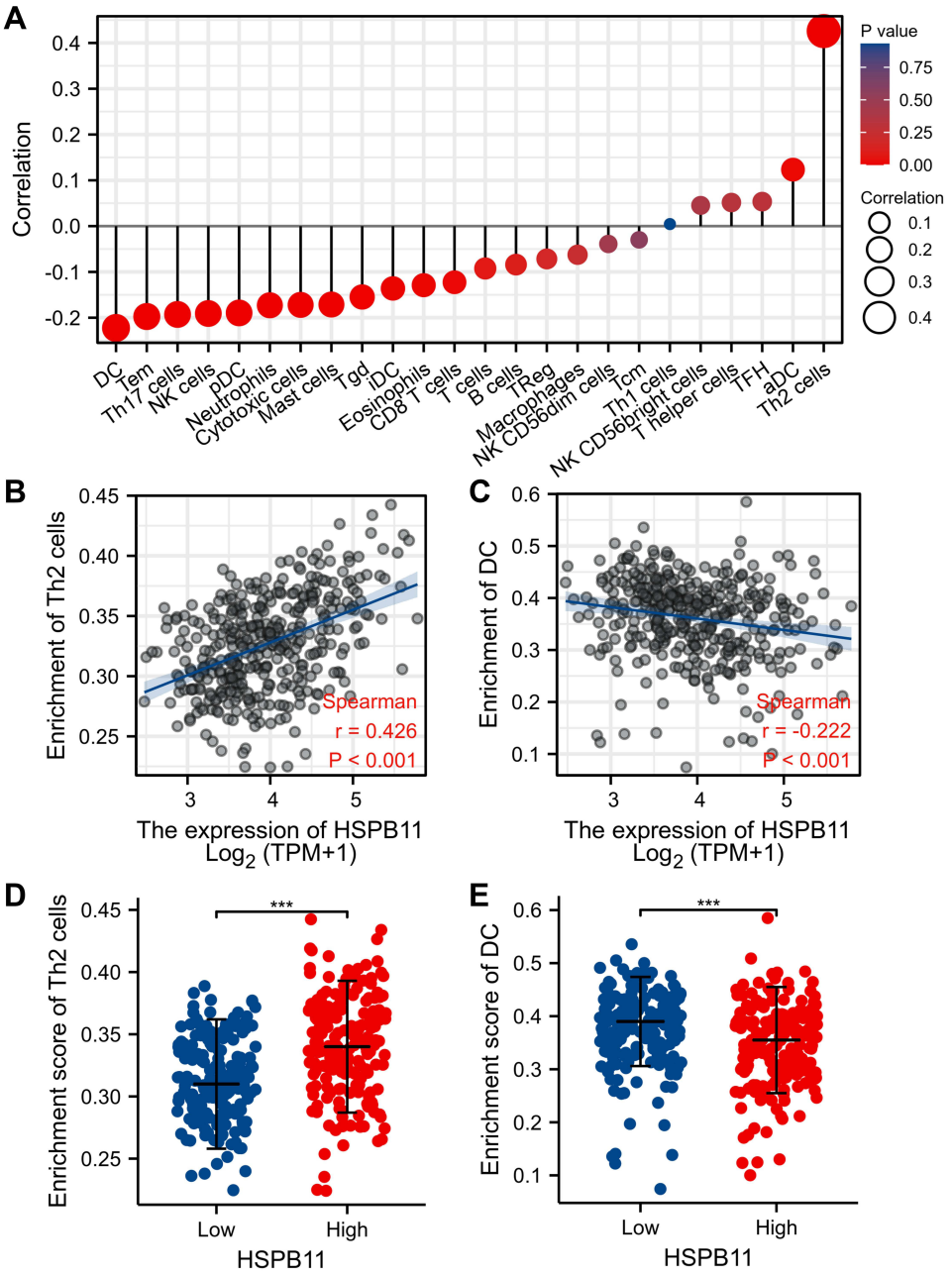

Figure 6 Expression level of HSPB11 is related to immune cell infiltration. (**A**) Association between the expression level of HSPB11 and relative abundance of 24 immune cell types. The size of dots reflects the absolute values of Spearman R. (**B**–**E**) Correlation diagrams and scatter plots indicate the differentiation of T helper (Th)2 and dendritic cell (DC) infiltration levels between HSPB11-high and HSPB11-low groups. ***p < 0.001.

patients with HCC. By combining the expression levels of HSPB11 with T stage and M stage, we established a nomogram and calibration models that could be used to predict the 1-, 3-, and 5-year survival probabilities of patients. The results showed that HSPB11 has good prediction ability, and the predicted dotted line was close to the ideal line. These results indicate that HSPB11 might be a useful prognostic and diagnostic marker for HCC.

A GSEA using data from patients with HSPB11-high and HSPB11-low tumors was performed to explore HSPB11-associated functions and pathways. The results revealed that HSPB11 is involved in multiple oncogenic processes, including cell cycle checkpoints, the G2M checkpoint, E2F targets, Rho GTPase signaling, and KRAS signaling. Inactivation of cell cycle checkpoints has been reported to correlate with the progression of multiple malignancies.[36,37]

The G2/M checkpoint can inhibit cells with genomic DNA damage from entering the M phase, thereby facilitating the DNA damage repair process, which is critical for protection against malignant transformation.[38,39] The E2F family is critical in regulating the cell cycle, proliferation, apoptosis, DNA damage signaling, and tumorigenesis. E2F1 is a proto-oncogenic gene that could induce both cell proliferation and tumor suppression by promoting apoptosis in the liver.[40] Rho GTPases are involved in regulating cell proliferation, differentiation, and apoptosis, and are closely related to the infiltration and distant metastasis of liver cancer.[41–46] KRAS participates in the RAS/RAF/MEK/ERK signaling pathway and is an important regulator of cell growth. Studies have confirmed KRAS mutations and activation of the Ras signaling pathway in HCC.[47–49] Our GSEA revealed that HSPB11 might be involved in these signaling pathways and functions in HCC, but further studies are needed to confirm the specific mechanism.

HCC is an inflammation-induced cancer, and the immune microenvironment plays a central role in regulation of the anti-tumor immune response.[50,51] In the current study, we found that the hyperexpression of HSPB11 is related to the infiltration level of immune cells in HCC. More specifically, HSPB11 was positively correlated with the abundance of Th2 cells and negatively associated with the abundance of DCs. Th2 cells secrete interleukin-4 and 10, promoting tumor growth and inducing metastasis via immunosuppression.[52–55] A Th1/Th2 imbalance has been observed in HCC patients with elevated Th2-released cytokines.[56] The dysfunction of DCs will result in the suppression of CD8+ T cell responses, leading to immune tolerance and cancer immunosurveillance failure.[57,58] DC-based vaccines have emerged as potential cancer immunotherapeutics.[59,60] Considering the significant correlations among HSPB11, Th2 cells, and DCs, we believe that HSPB11 could be involved in the immune regulation of HCC through its interaction with tumor immune cells.

The clinical data used in this study were mainly retrieved from public databases, and thus, some limitations are unavoidable. First, large clinical trials are needed to validate its diagnostic and prognostic values. Second, the mechanism through which HSPB11 affects oncogenesis and immune regulation in HCC should be confirmed through further basic molecular and animal experiments.

Conclusion

In this study, we comprehensively analyzed the role of HSPB11 in HCC. The value of HSPB11 in the diagnosis and prediction of HCC prognosis was confirmed. HSPB11 may not only be involved in the development but also in the immune regulation of HCC. However, cell line-based experiments and clinical studies are still needed to validate the potential diagnostic and prognostic value in HCC.

Abbreviations

HSPB11, heat shock protein B11; HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression (GTEx); OS, overall survival; DSS, disease-specific survival; PFI, progression-free interval; HR, hazard ratio; AUC, area under the curve; GSEA, Gene Set Enrichment Analysis; DCs, dendritic cells.

Research Ethics and Consent

The study complies with the Declaration of Helsinki, and the Ethics Committee of Second Hospital of Dalian Medical University approved this research (no. 0202159). Written informed consent was obtained from all patients before specimen collection.

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Disclosure

The authors report no conflicts of interest in this work.

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