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HOXB7 Expression is a Novel Biomarker for Long-term Prognosis After Resection of Hepatocellular Carcinoma

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Abstract. *Background/Aim: Homeobox B7 (HOXB7) gene is involved in various cellular functions. We investigated the clinical significance of HOXB7 expression in hepatocellular carcinoma (HCC). Materials and Methods: HOXB7 mRNA expression in 103 HCC samples and 58 matched non-cancerous liver tissues were examined by quantitative real-time polymerase chain reaction (qRT-PCR). HOXB7 protein expression was also examined by immunohistochemistry. Gene set enrichment analysis (GSEA) was performed using a public dataset. Results: HOXB7 expression was significantly higher in HCC tissues than in liver parenchyma. Ten-year overall survival (OS) and 5-year recurrence-free survival (RFS) of cases with higher HOXB7 expression were significantly poorer than those with lower HOXB7 expression. HOXB7 expression was significantly associated with larger tumor size and higher rate of biliary invasion and constituted an independent prognostic factor for OS by multivariate analysis. These results were supported by GSEA. Conclusion: HOXB7 expression in HCC could be a novel biomarker for long-term prognosis after tumor resection.*

Liver cancer is one of the most common malignancies and the second and sixth most frequent cause of cancer-related death in men and women, respectively (1). Among primary liver cancers, hepatocellular carcinoma (HCC) represents the major histological subtype, accounting for 70%-85% of the total liver cancer burden worldwide (2). Because of the relatively low efficacy of chemotherapy and radiotherapy, radical therapeutic strategies for HCC are currently restricted to surgical techniques (3, 4). Furthermore, even after curative resection, a high frequency of tumor recurrence is observed (5). Therefore, identification of novel clinical biomarkers to predict prognosis and recurrence, as well as further investigation of therapeutic targets, are critically required to improve outcomes in patients with HCC.

Here, homeotic (*Hox*) genes are a group of genes that control embryo development along the anterior-posterior (head-tail) axis, causing morphological diversity at the organismal and evolutionary levels (6). Additionally, functional disorders in *Hox* genes cause various diseases (7). *Hox* genes also play key roles in cancer biology (8) and are involved in the regulation of common cellular functions, such as cell proliferation and differentiation (9). The HOX family consists of 39 members; these members can be divided into four clusters, namely, A, B, C and D, which are located on four different chromosomes (10). Homeobox B7 (*HOXB7*) is a gene coding HOXB7 protein, which is categorized in the homeobox B cluster located on chromosome 17. Recent studies have reported the biological roles of HOXB7 in several cancer types (11-14). However, the clinical significance of HOXB7 in solid cancers, including HCC, is unclear. Accordingly, in this study, we aimed to evaluate the clinical significance of HOXB7 expression in HCC using surgically resected specimens.

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Key Words: HOXB7, hepatocellular carcinoma, prognostic biomarker.

Materials and Methods

All protocols used in this study were approved by the local ethical review board of Kyushu University.

Patients and collection of clinical samples. One hundred and three patients with HCC who underwent liver resection at Kyushu University Beppu Hospital and affiliated hospitals (Oita Red Cross

Hospital (Oita, Japan), Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital (Hiroshima, Japan) and Iizuka Hospital (Fukuoka, Japan)) between 2001 and 2004 were enrolled in this study. Tissues from resected tumors were immediately frozen by immersion in liquid nitrogen and then kept at –80˚C until RNA extraction. Corresponding normal liver tissues (available in 58 of 103 cases) were also collected. Intermittent follow-up was conducted after the operation, with a median period for the 103 patients of 59.4 months (range=3.0-120.0). In addition to survival and recurrence information, the clinicopathological data of the patients and corresponding specimens was extracted. Written informed consent was obtained from all patients.

RNA preparation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA from frozen tissue specimens and HCC cell lines was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. Quality assessment of extracted RNA was performed by measuring absorbance. cDNA was synthesized from 8 μg total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using a LightCycler 480 Probe Master kit (Roche Applied Science, Basel, Switzerland). Gene-specific oligonucleotide primers were designed for qRT-PCR. The following primers were used: *HOXB7*, 5'-CTGGATGCGAAGCTCAGG-3' (sense) and 5'-CAGGTAGCG ATTGTAGTGAAATTCT-3' (antisense); and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 5'-TTGGTATCGTGGAA GGACTCA-3' (sense) and 5'-TGTCATCATATTTGGCAGGTT-3' (antisense). PCR amplification was performed in a LightCycler 480 instrument (Roche Applied Science) using a LightCycler 480 Probes Master kit (Roche Applied Science). Amplification conditions for the *HOXB7* mRNA consisted of initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 s, annealing at 62˚C for 10 s and elongation at 67˚C for 10 s. Melt curve analysis was performed to distinguish specific products from non-specific products and primer dimers. The relative expression levels of these genes were obtained by normalizing the amount of mRNA to that of *GAPDH* mRNA as an endogenous control in each sample.

Histology and immunohistochemical analysis. HCC tissues were surgically removed, embedded in paraffin and sectioned (5 μm thickness). Immunohistochemical analysis was applied to determine the expression level and localization of HOXB7 protein. A polyclonal rabbit anti-HOXB7 antibody (ab196007; Abcam, Cambridge, UK) was used as the primary antibody diluted 100:1. The tissues were counterstained with hematoxylin.

Acquisition of mRNA expression profiles from public datasets and application of gene set enrichment analysis (GSEA). We obtained mRNA expression profiles of 242 HCC tissues from the National Cancer for Biotechnology Information Gene Expression Omnibus database (accession code: GSE14520 (15, 16)). The correlations between *HOXB7* mRNA expression profiles and known gene signatures in public datasets listed above by GSEA (17) were investigated. The names of gene sets extracted from the Molecular Signatures Database and their Uniform Resource Locator were as follows. LEE_LIVER_CANCER_SURVIVAL_UP: http://software.broad institute.org/ gsea/msigdb/cards/LEE_LIVER_CANCER_SURVIVAL_ UP.html, WOO_LIVER_CANCER_RECURRENCE_DN: http:// software.broadinstitute.org/gsea/msigdb/cards/WOO_LIVER_CANC ER_RECURRENCE_DN.html, CHIANG_LIVER_CANCER_ SUBCLASS_PROLIFERATION_DN: http://software.broadinstitute. org/gsea/msigdb/cards/CHIANG_LIVER_CANCER_SUBCLASS_P ROLIFERATION_DN.html, COULOUARN_TEMPORAL_TGFB1_ SIGNATURE_DN: http://software.broadinstitute.org/gsea/msigdb/ cards/COULOUARN_TEMPORAL_TGFB1_SIGNATURE_DN.html. Results with p values of less than 0.05 were considered significant.

Statistical analysis. For continuous variables, data were expressed as means±standard deviations and statistical analyses were performed using Welch's *t*-tests. Categorical variables were compared using the Chi-square test or Fisher's exact test. Overall survival (OS) and recurrence-free survival (RFS) was estimated using the Kaplan-Meier method, whereas survival curves were compared using the log-rank test. Univariate and multivariate analyses were performed using the proportional hazard model to identify independent variables predictive of OS. Differences with *p*-values of less than 0.05 were considered statistically significant. Data analyses were performed using R version 3.1.1 (R Core Team (2014). R: A language and environment for statistical computing. The R Foundation for Statistical Computing, Vienna, Austria. URL: http://www.R-project.org/).

Results

First, we conducted qRT-PCR to examine *HOXB7* mRNA expression in 103 HCC tissues and 58 non-cancerous liver tissues. *HOXB7* expression was significantly lower in HCC samples than in non-cancerous tissues (Figure 1A, p=0.003). We also performed immunohistochemical analysis of representative samples and found that the staining was strong in tumor specimens but weak in adjacent liver parenchyma. In higher-magnification images, staining in tumors was enhanced in both the nuclei and cytoplasm. In contrast, staining was patchy and faint in the liver parenchyma (Figure 1B).

Next, to estimate the clinical significance of *HOXB7* expression in HCC, cases were divided into two groups by the median value according to the level of *HOXB7* expression in tumor tissues. In survival analyses, the 10-year OS and 5-year RFS rates of the high HOXB7 expression group were significantly lower than those of the low *HOXB7* expression group (p=0.011 and 0.032, respectively; Figure 2A, B). Analyses of clinicopathological factors revealed that tumors with high *HOXB7* expression were associated with a more malignant phenotype, *i.e.* larger tumor sizes, increased biliary invasion and poorer tumor differentiation (Table I). Moreover, multivariate analysis revealed that *HOXB7* expression was an independent prognostic factor for 10-year OS (hazard ratio (HR)=2.040, p=0.027; Table II).

Finally, to examine the validity of these results, we performed GSEA by using a public dataset. The results showed that *HOXB7* expression was negatively correlated with the expression of genes associated with good survival (Figure 3A). Moreover, *HOXB7* expression was also negatively associated with genes down-regulated in recurrent

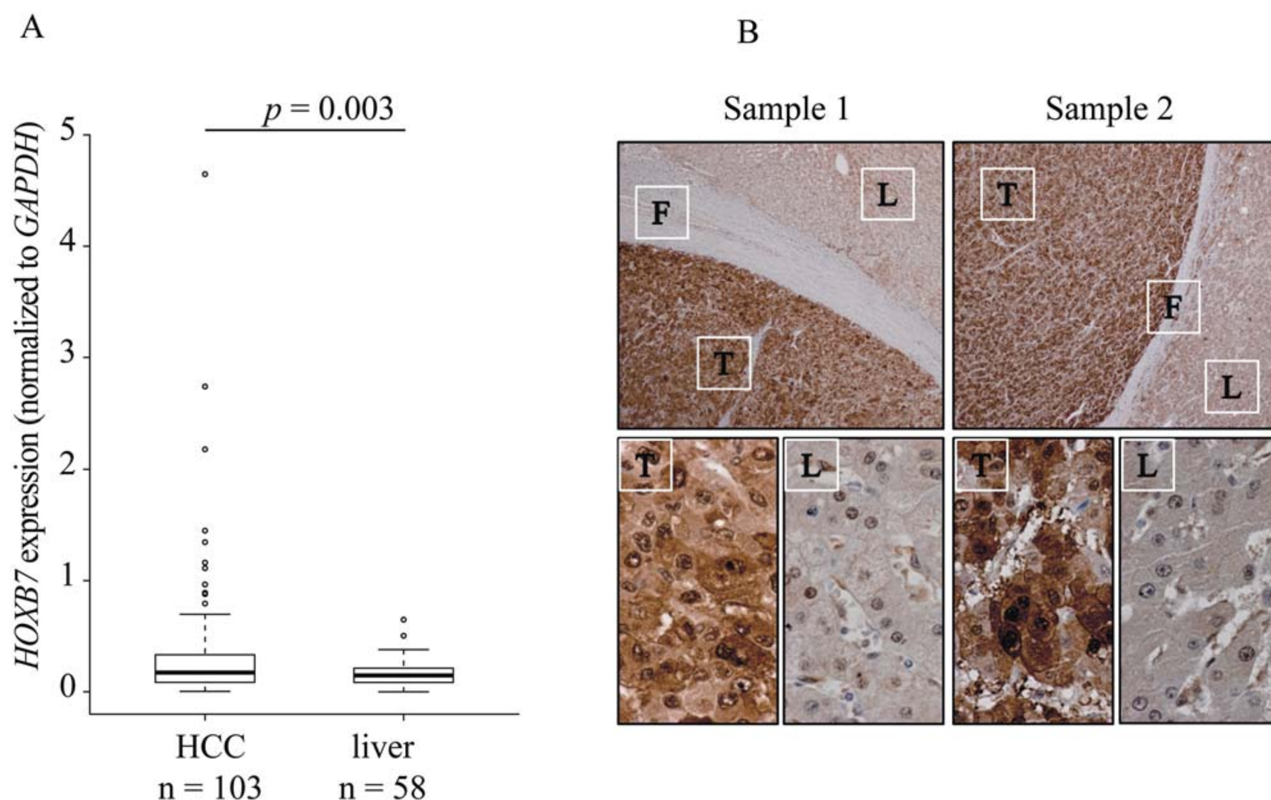

Figure 1. *Comparison of HOXB7 expression in HCC tissues and corresponding liver parenchyma. (A) HOXB7 expression levels as measured by qRT-PCR in 103 HCC tissues and 58 corresponding liver tissues. HOXB7 mRNA expression was up-regulated in tumor tissues compared to adjacent liver parenchyma (p=0.003, Welch's t-test). (B) Immunohistochemical staining of HOXB7 protein in two representative human clinical HCC samples. HOXB7 protein was strongly stained in tumor tissue compared with liver parenchyma (upper: magnification 40×; lower: magnification 400×, T, tumor; F, fibrous capsule; L, liver parenchyma).*

or proliferative tumors and with gene signatures related to a less invasive phenotype (Figure 3B-D).

Discussion

Currently, hepatectomy is the only curative treatment available in most patients with HCC. However, its long-term prognosis is not satisfactory and the probability of survival decreases further, even at more than 5 years after the first operation (18). Therefore, there is a great need to identify biomarkers that can be used to predict long-term clinical outcomes in patients with HCC, although few previous studies have mentioned them. To the best of our knowledge, there is no preceding work reporting clinical significance of *HOXB7* in HCC. In this study, we showed that the 10-year OS rate of patients with high *HOXB7* expression was significantly lower than that in patients with low *HOXB7* expression. Moreover, *HOXB7* expression was an independent prognostic factor for 10-year OS, suggesting its usefulness as a promising predictor of long-term prognosis. HCC is also characterized by its high probability of recurrence (up to 70%) within 5 years after curative resection (19). Thus, it is clinically important to search for useful biomarkers that can predict the possibility of recurrence. Our results showed that patients with high *HOXB7* expression exhibited higher rates of recurrence than those with low *HOXB7* expression with 5 years after curative resection. Besides, GSEA indicated significant associations between *HOXB7* expression and prognostic or recurrent gene signatures, which supported our findings.

We also showed that the *HOXB7* mRNA expression was up-regulated in HCC tissues compared to that in the liver parenchyma. In immunohistochemistry, HOXB7 protein expression in tumor tissues was enhanced in both the cytoplasm and nuclei. HOXB7 is reported to function as a transcription factor for various oncogenes by binding chromatin in nuclei (20); therefore, our results provided us insight into the role of aberrant *HOXB7* expression in tumor progression in HCC. Indeed, HOXB7 has been shown to act as a tumor-promoting factor in several types of cancers. In

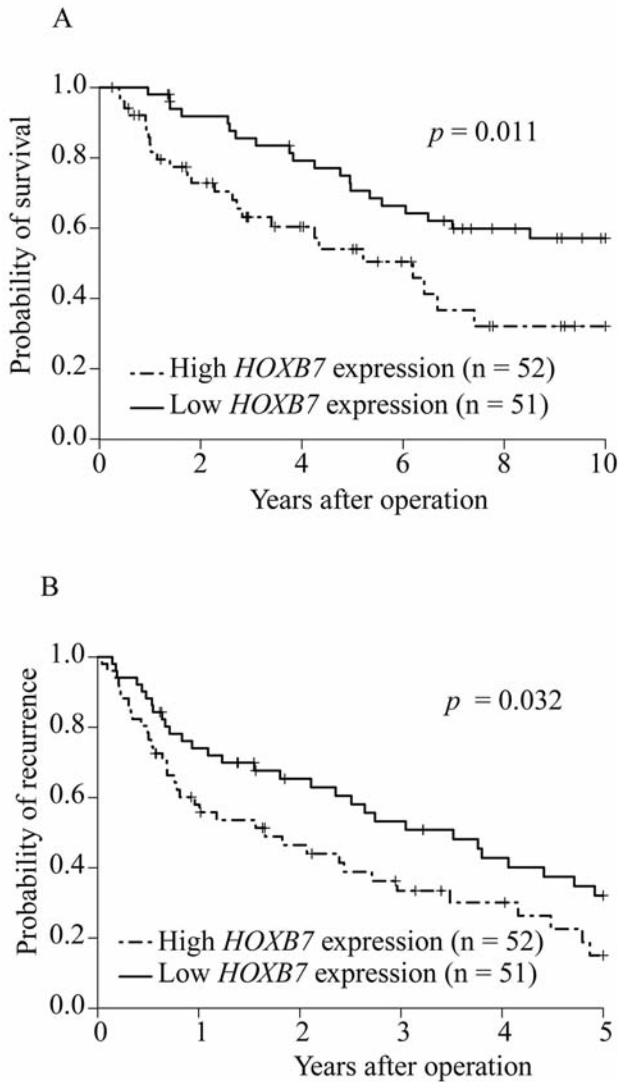

Figure 2. *Prognostic significance of HOXB7 expression in HCC cases. (A) Ten-year OS in patients with high HOXB7 (n=52) expression was significantly poorer than that in patients with low HOXB7 expression (n=51; p=0.011, log-rank test). (B) Five-year RFS in patients with high HOXB7 expression (n=52) was significantly shorter than that in patients with low HOXB7 expression (n=51; p=0.032, log-rank test).*

Table I. *HOXB7 expression and clinicopathological factors in patients with HCC (n=103).*

Factors	High *HOXB7* expression (n=52)	Low *HOXB7* expression (n=51)	*p*-Value
Age (year; mean±SD)	66.6±9.7	66.2±8.4	0.823
Gender			0.702
Male (%)	37 (71.2)	38 (74.5)	
Female (%)	15 (28.8)	13 (25.5)	
Etiology of HBV infection			0.287
Present (%)	16 (30.77)	11 (21.6)	
Absent (%)	36 (60.2)	40 (78.4)	
Etiology of HCV infection			0.780
Present (%)	34 (65.4)	32 (62.7)	
Absent (%)	18 (34.6)	19 (37.3)	
Child-Pugh classification			0.738
A (%)	46 (88.5)	44 (86.3)	
B or C (%)	8 (11.5)	7 (13.7)	
AFP			0.485
> 20 ng/ml (%)	24 (46.2)	21 (41.2)	
≤ 20 ng/ml (%)	25 (48.1)	29 (56.9)	
NA (%)	3 (5.8)	1 (2.0)	
Maximum tumor size			0.019*
> 2.5 cm (%)	13 (25.0)	24 (47.1)	
≤ 2.5 cm (%)	39 (75.0)	27 (52.9)	
Number of tumors			0.702
Single (%)	15 (28.8)	13 (25.5)	
Multiple (%)	37 (71.2)	38 (74.5)	
Fibrous capsule formation			0.954
Present (%)	30 (57.7)	28 (54.9)	
Absent (%)	3 (5.8)	8 (15.7)	
NA (%)	9 (17.3)	5 (9.8)	
Invasion to fibrous capsule			0.123
Present (%)	19 (36.5)	15 (29.4)	
Absent (%)	33 (63.5)	36 (70.6)	
Vascular invasion**			0.483
Present (%)	37 (71.2)	33 (64.7)	
Absent (%)	15 (28.8)	18 (35.3)	
Biliary invasion			0.018*
Present (%)	4 (7.69)	0 (0.0)	
Absent (%)	48 (92.3)	51 (100.0)	
Histological differentiation***			0.029*
Well or moderately (%)	39 (75)	46 (90.2)	
Poorly or undifferentiated (%)	12 (23.1)	4 (7.8)	
NA (%)	1 (1.9)	1 (2.0)	

SD, Standard deviation; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; NA, not available. *Statistically significant. **Invasion to portal vein or hepatic vein. ***WHO grade 1/2 or Edmondson-Steiner grade 1/2 were classified as well or moderately. WHO grade 3/4 or Edmondson-Steiner grade 3/4 were classified as poorly or undifferentiated.

colorectal cancer, forced overexpression of HOXB7 has been reported to significantly enhance cell growth, proliferation and tumorigenesis (11). In pancreatic adenocarcinoma, knockdown of *HOXB7* induces cell-cycle arrest and apoptosis (12). These results were consistent with our findings, showing that *HOXB7* expression was significantly associated with tumor size in HCC. Thus, aberrant expression of HOXB7 likely contributed to growth of HCC. On the other hand, in breast cancer, HOXB7 has been shown to exert its tumor-promoting role through activating the transforming growth factor (TGF) signaling pathway and inducing the epithelial mesenchymal transition (EMT), which results in increased invasion of breast cancer cells (13, 21). TGFβ signaling is reported to be up-regulated in HCC and involved in tumor progression *via* EMT (22, 23).

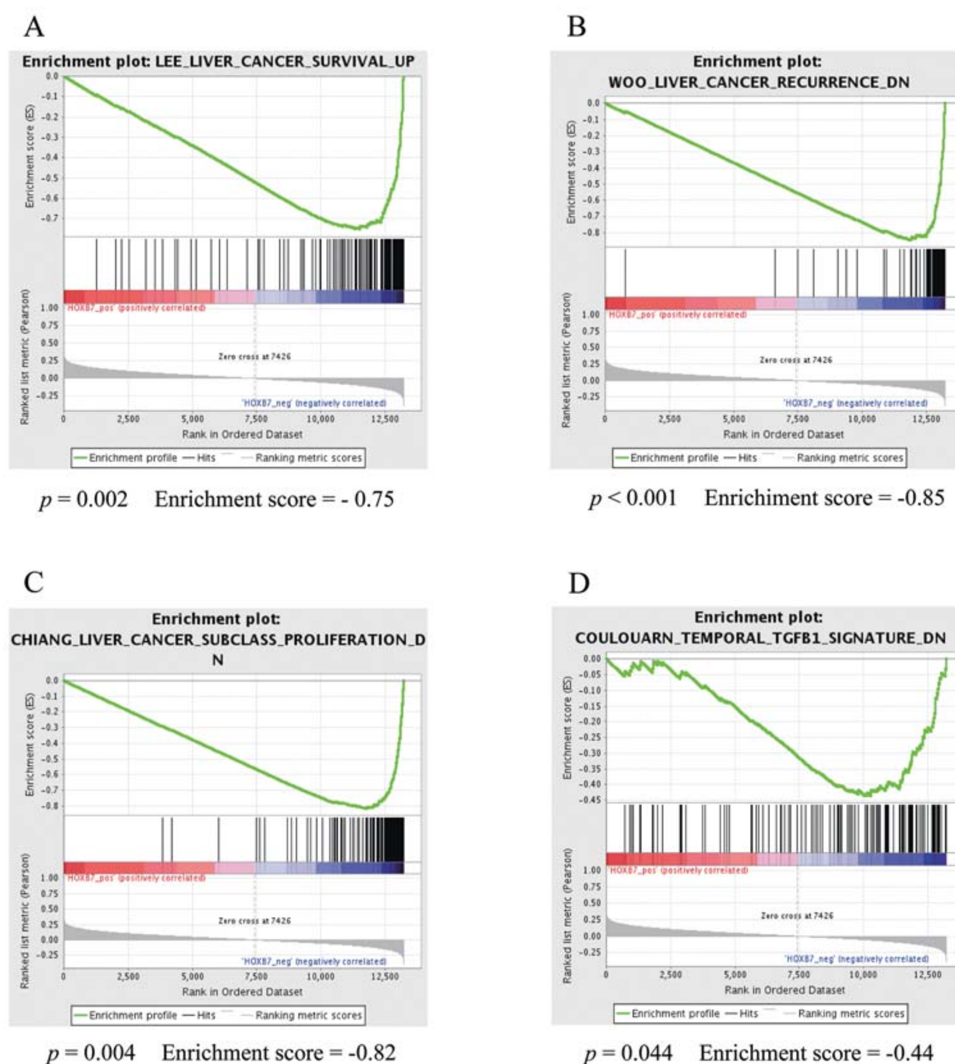

Figure 3. *Gene set enrichment analysis on HOXB7 expression in GSE14520 dataset. HOXB7 expression had significant negative correlations with expression of (A) genes highly expressed with good survival in patients with HCC, (B) genes down-regulated in high-risk group of early recurrence, (C) genes down-regulated in the proliferative phenotype and (D) genes associated with the less invasive phenotype.*

Therefore, these findings may explain the significant associations between *HOXB7* expression and biliary invasion in our analysis of HCC. In addition, we conducted GSEA using publically available datasets and found that *HOXB7* expression was significantly related to proliferative and invasive signatures, that could further support our findings.

Although the biological functions of *HOXB7* in HCC have not been clearly elucidated and further studies are needed to determine the mechanisms through which *HOXB7* exerts its tumor-promoting role, the expression of *HOXB7* was a predictor of OS and RFS and correlated with various clinicopathological factors, possibly rendering *HOXB7* expression a prognostic biomarker and a useful indicator of tumor aggressiveness in patients with HCC.

In conclusion, we demonstrated that *HOXB7* could be a novel powerful prognostic biomarker in HCC. In particular, our results showed that long-term prognosis could be predicted on the basis of *HOXB7* gene expression levels in HCC tissues. Overall, our findings showed that evaluation of *HOXB7* expression in HCC tissues may help clinicians predict survival and recurrence in patients, suggesting the possibility of utilization of HOXB7 as a therapeutic target in HCC.

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Table II. *Univariate and multivariate analyses of clinicopathological factors for overall survival.*

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Age (year)	0.995	0.965-1.031	0.786			
Gender (male/female)	1.190	0.610-2.540	0.624			
Etiology of HBV infection (present/absent)	0.655	0.283-1.334	0.256			
Etiology of HCV infection (present/absent)	1.479	0.794-2.921	0.223			
Child-Pugh classification (A/B or C)	0.608	0.297-1.410	0.228			
AFP (20 ng/ml >/20 ng/ml ≤)	0.450	0.243-0.825	0.010*	0.581	0.291-1.155	0.121
Maximum tumor size (2.5 cm >/2.5 cm ≤)	0.502	0.254-0.936	0.030*	0.639	0.313-1.249	0.193
Number of tumors (single/multiple)	0.479	0.264-0.895	0.022*	0.621	0.317-1.263	0.184
Fibrous capsule formation (present/absent)	1.065	0.578-2.065	0.844			
Vascular invasion** (present/absent)	0.981	0.539-1.850	0.951			
Biliary invasion (present/absent)	4.894	0.767-17.545	0.084			
Tumor differentiation*** (well or moderately/poorly or undifferentiated)	0.364	0.181-0.814	0.016*	0.532	0.235–1.323	0.165
HOXB7 expression (high/low)	2.145	1.182-3.941	0.012*	2.040	1.084-3.097	0.027*

HR, Hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein. *Statistically significant.

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Disclosure

All Authors have no conflicts of interest to disclose.

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