Int J Clin Exp Pathol 2014;7(6):2925-2933
www.ijcep.com /ISSN:1936-2625/IJCEP0000396

Original Article

High-level expression of HOXB13 is closely associated with tumor angiogenesis and poor prognosis of hepatocellular carcinoma

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Received April 1, 2014; Accepted May 25, 2014; Epub May 15, 2014; Published June 1, 2014

Abstract: Homeobox B13 (HOXB13) is generally considered as a crucial regulator of terminal cellular differentiation. More recently, the absent or aberrant expression of HOXB13 has been increasingly implicated in cancer development and metastasis. However, the expression of HOXB13 in hepatocellular carcinoma (HCC) and its correlation with tumor angiogenesis and prognosis still remain unclear. The aim of the study was to evaluate the expression of HOXB13 in patients with HCC and explore the relationship of HOXB13 expression with clinicopathologic factors, tumor angiogenesis and prognosis. Immunohistochemistry was performed to determine the expression of HOXB13 in HCC and corresponding paracarcinomatous tissues from 72 patients. Vascular endothelial growth factor (VEGF) and CD31 were only examined in tissues of HCC patients mentioned above. The results showed that HOXB13 expression was significantly ($P < 0.001$) higher in HCC (69.4%) than that in surrounding non-tumor tissues (26.4%), positively correlated with tumor VEGF ($P < 0.001$) and microvessel density (MVD) ($P = 0.013$). Besides, it was associated with tumor capsula ($P < 0.001$), vascular invasion ($P < 0.001$), Edmondson grade ($P < 0.001$), AFP ($P = 0.007$) and TNM stage ($P < 0.001$). Univariate analysis showed poorer overall survival (OS) rate and disease free survival (DFS) rate in patients expressing higher levels of HOXB13. HOXB13 was also found to be an independent poor prognostic factor of OS and DFS in multivariate analysis. Taken together, our results suggest that increased HOXB13 expression is associated with tumor angiogenesis and progression in HCC and may function as a promising biomarker for unfavorable prognosis of HCC.

Keywords: HOXB13, hepatocellular carcinoma, angiogenesis, expression, prognosis, biomarker

Introduction

Hepatocellular carcinoma (HCC) is the fifth most commonly diagnosed with malignant tumor worldwide and the second most often cause of cancer-related mortality in men. Among females, it is the seventh most common cancer and the sixth in lethal [1]. Its prognosis is gloomy with a 5-year survival of 11%. Hepatectomy or orthotopic liver transplantation remains the only potentially effective therapy [2]. Nevertheless, the vast majority of HCC patients are diagnosed at advanced stage, when surgical treatments are ineffective or unfeasible. Quest for distinguishing biomarkers for the early detection of HCC is of primary importance.

Homeobox B13 (HOXB13) locates in chromosomal region 17q21.3 which belongs to Homeobox gene family, approximately 70 kb upstream of the HOXB gene cluster [3]. HOXB13 gene functions as a regulator to promote cellular epidermal differentiation and it has been linked to cancer metastasis [4, 5]. Preceding studies have indicated that HOXB13 is overexpressed in several tumors such as prostate cancer [6, 7], endometrial cancer [8], ovarian cancer [9, 10], cervical cancer [11, 12] and breast cancer [13, 14], suggesting its contribution to carcinogenesis and tumor progression. Meanwhile, it may function as a tumor suppressor like colorectal cancer [15], renal cancer [16], melanoma cancers [17], etc. Its inactivation may play a critical role in tumorigenesis,

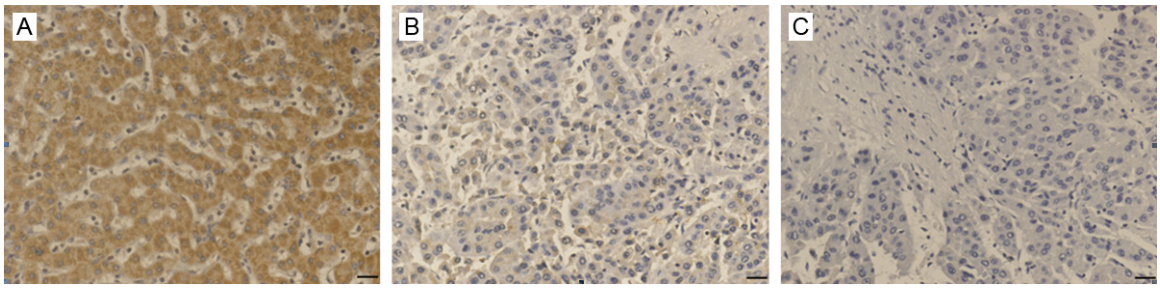

Figure 1. Representative immunohistochemical staining of HOXB13 in hepatocellular carcinoma (HCC) tissues. HOXB13 expression showed diffuse cytoplasmic staining in tumor cells. A: ++ HOXB13 staining (×100); B: + HOXB13 staining (×100); C: Negative expression of HOXB13 (×100). (All Bars = 50 μm).

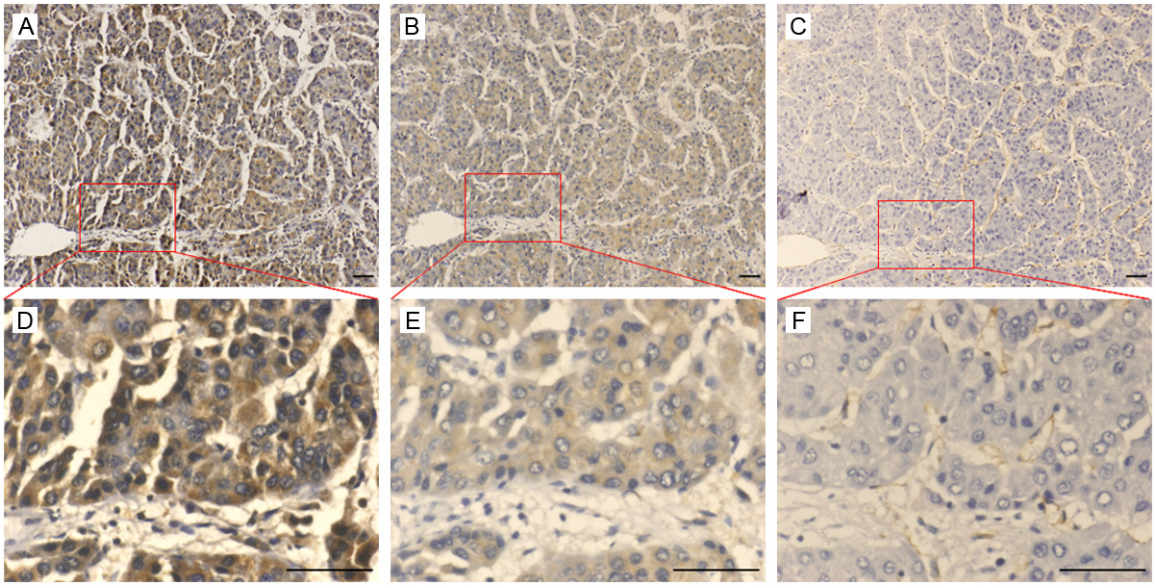

Figure 2. Immunohistochemical staining of HOXB13, VEGF and CD31 in HCC tumors. A: Positive for HOXB13 staining (×100); B: Positive for VEGF staining (×100); C: Positive for CD31 staining (×100); D: Partial enlargement of HOXB13 staining with the magnifying power of 400x; E: Partial enlargement of VEGF staining with the magnifying power of 400x; F: Partial enlargement of CD31 staining with the magnifying power of 400x. (All Bars = 50 μm; examined by using serial sections).

aggression and metastasis. Although studies have demonstrated HOXB13 was expressed as a promising molecular tumor marker for tumor invasiveness in different types of human cancers, few were reported about the expression of HOXB13 in HCC and its potential clinical significance still remains unclear.

Therefore, in the present study, immunohistochemistry was used to examine the expression of HOXB13, vascular endothelial growth factor (VEGF) and microvessel density (MVD) in HCC or paracarcinomatous tissues. Furthermore, the survival analysis was observed by Kaplan-Meier method. A multivariate survival analysis was performed for all parameters that were significant in the univariate analysis using the Cox regression model. All the above mentioned aimed to elucidate the expression of HOXB13 in HCC and its association with clinicopathological characteristics, angiogenesis and prognosis.

Materials and methods

Patients and specimens

The tumor tissues and paracarcinomatous specimens were obtained from 72 patients with HCC undergone curative hepatectomy from 2006 to 2010 at the Affiliated Provincial Hospital of Anhui Medical University. Patients

Table 1. Differential expression of HOXB13 between HCC and corresponding paracarcinomatous tissues (cases)

Tissues	Case number	HOXB13 expression		Positive rate (%)
		+~++	-	
HCC tissues	72	50	22	69.4
Paracarcinomatous tissues	72	19	53	26.4

who had undergone preoperative radiotherapy or chemotherapy were excluded from this study. The clinical data and pathological materials gathered by retrospective medical records were consisted of age, gender, tumor size, tumor nodule, tumor capsula, vascular invasion, Edmondson grade, cirrhosis, HBeAg status, Child-Pugh grade, alpha-fetoprotein (AFP) and TNM stage. The 72 patients included 59 males and 13 females with a mean age of 55.5 years (range 22-74 years). Tumor differentiation was defined according to the Edmondson grading system [18]. The tumor stage was performed according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer (UICC). Tumor size was measured as the largest dimension of the tumor by gross examination. Follow-up data were obtained from all 72 patients. The mean follow-up period was 24 months (range 2-72 months). This study was carried out in accordance with the Helsinki Declaration. Prior approval of the project was granted by the Research Ethics Committee of Anhui Medical University, Hefei, China. All patients provided written informed consent.

Immunohistochemistry analysis

Immunohistochemistry was implemented using a two-step protocol according to the manufacturer's instructions. In a nutshell, the tumor tissues were fixed in 10% formalin, then embedded in paraffin wax and cut into 4-μm sections. Slides were deparaffinized in xylene, rehydrated in graded alcohol and washed with phosphate-buffered saline (PBS). After pretreated with citrate buffer (pH 6.0) for 20 min in a microwave oven, slides were cooled at room temperature. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide. Then sections were respectively incubated with primary polyclonal rabbit anti-HOXB13 antibody or anti-VEGF antibody or anti-CD31 antibody (titer 1:100, Zhongshan Jinqiao Co., Beijing, China) at 4°C overnight in a moist chamber. Following incubation, slides were rinsed with PBS 3 times for 5 min each. After incubating with biotin-labeled secondary antibody (mouse antirabbit IgG; Zhongshan Jinqiao Co., Beijing, China) for 30 min at 37°C, color was developed by incubating sections with 3, 3-diaminobenzidine (DAB; Zhongshan Jinqiao Co., Beijing, China). Finally, nuclei were counterstained with hematoxylin followed by dehydration and mounting. Under the same conditions, PBS was used in place of the primary antibodies for negative control and known immunopositive slide was used as positive control.

Immunohistochemical evaluation

Semiquantitative estimation was made to interpretate the results of immunohistochemistry according to the percentage of staining cells per 100 cells in 10 microscopic fields with high-power (400×) microscope, as follows: 0-10%, negative (-); 10-30%, weak positive (+); >30%, strong positive (++). CD31 antibody was used to stain vascular endothelial cells and then calculate MVD [19]. Evaluation acted in accordance with following principles. It was quantified in 5 fields which high expression using high-power lens (400×) and values were expressed by averages measurements. All sections were assessed by two pathologists blindly without knowing clinical characteristics.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL, USA). The corrections between HOXB13 expression and clinicopathological features were assessed by χ^2 test or Fisher's exact test. The Kaplan-Meier method and the log-rank test were used for survival analysis. Cox proportional hazards regression model was used for multivariate survival analysis in order to assess prognostic factors that were significant in the univariate analysis. P <0.05 was considered statistically significant.

Results

HOXB13 and VEGF expression in HCC tissues

In the present study, we examined the expression of HOXB13 protein in 72 HCC samples and

Table 2. The expression correlation between HOXB13 and VEGF (cases)

Stain	HOXB13		*r*	*P* value
	+~++	-		
VEGF				
+~++	43	6	0.641	<0.001
-	7	16		

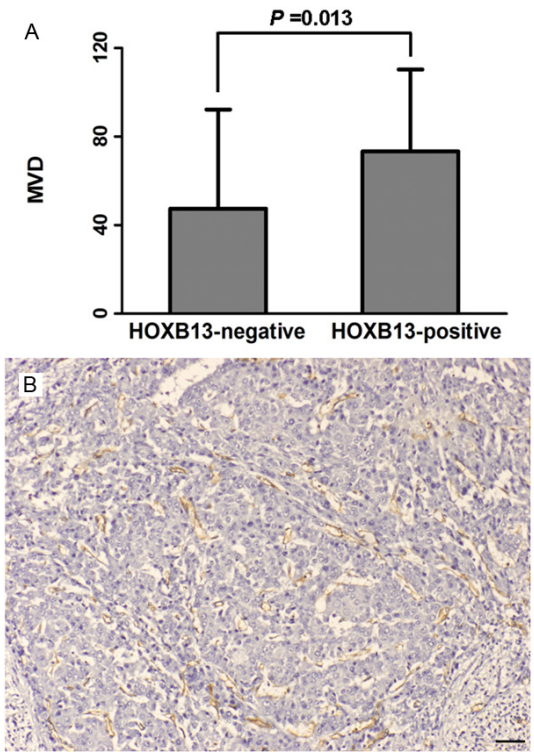

Figure 3. Immunohistochemical staining of CD31 for MVD in HCC tissues. A: Tumors with positive HOXB13 expression had a significantly higher MVD compared to tumors with negative HOXB13 expression (*P* = 0.013). Data are expressed as the number of CD31-positive microvessels per field; B: Representative section of HCC with immunohistochemical staining of CD31. Bar = 50 μm.

the matched corresponding paracarcinomatous specimens by immunohistochemical analysis, which showed that HOXB13 staining mainly located at cytoplasm with varied staining intensity (**Figures 1**, **2A** and **2D**). The positive rate of HOXB13 protein expression was 69.4% (50/72) and 26.4% (19/72) in adjacent non-cancerous samples (**Table 1**). The protein expression level of HOXB13 was significantly higher in HCC tissues than the level in adjacent non-cancerous liver tissues (*P* <0.001). VEGF expression was positive in 49 out of 72 HCC samples (68.1%; **Figure 2B** and **2E**). Spearman's rank correlation test proved a significant correlation between tissue expression of HOXB13 and VEGF in HCC tissues (*r* = 0.641, *P* <0.001; **Table 2**)

Relationship between HOXB13 expression and MVD in HCC tissues

To further evaluate the association between HOXB13 and angiogenesis, MVD was calculated by immunohistochemical staining of endothelial cells with CD31 (**Figure 2C** and **2F**). The results indicated that tumors with HOXB13-positive expression had significantly higher MVD (73.4 ± 34.0 vs. 47.5 ± 44.8; *P* = 0.013) compared to those in HOXB13-negative tissues (**Figure 3**).

Correlation of tissue HOXB13 expression with clinicopathological parameters

Clinicopathological parameters related to the HOXB13 expression status of the 72 patients were assessed in **Table 3**. Positive expression of HOXB13 in HCC tissues were significantly correlated with tumor capsula (*P* <0.001), vascular invasion (*P* <0.001), Edmondson grade (*P* <0.001), AFP (*P* = 0.007) and TNM stage (*P* <0.001). However, HOXB13 expression was not correlated with age, gender, tumor size, tumor nodule number, cirrhosis, HBeAg status and Child-Pugh grade.

Relationship between HOXB13 expression and HCC patients' survival

Kaplan-Meier survival analysis was used to assess the relationship between HOXB13 expression and patients' survival. The log-rank test revealed that the overall survival time of HCC patients with HOXB13-positive expression (24.62 ± 2.74 months) was markedly shorter than that with HOXB13-negative expression (45.57 ± 6.25 months; *P* = 0.001; **Figure 4A**). Likewise, patients with HOXB13-positive expression (23.28 ± 3.01 months) had a shorter disease free survival time compared to HOXB13-negative patients (44.55 ± 6.50 months; *P* = 0.001; **Figure 4B**). To further analyze the relationship between the HOXB13 positive expression and prognosis, two different levels of positive expression were analyzed by using the Kaplan-Meier method (**Figure 5**).

Univariate analysis showed that variables including HOXB13 expression, tumor size,

Table 3. HOXB13 expression status in relation to clinicopathologic features in 72 HCC patients

Clinicopathologic data	Case number	HOXB13 tissue samples		
		-	+~++	*P* value
Age (years)				
<60	48	17	31	0.205
≥60	24	5	19	
Gender				
Male	59	20	39	0.327
Female	13	2	11	
Tumor size (cm)				
≤5	30	10	20	0.665
>5	42	12	30	
Tumor nodule number				
Single	60	21	39	0.137
Multiple	12	1	11	
Tumor capsula				
Absent	26	1	25	<0.001
Present	46	21	25	
Vascular invasion				
No	51	22	29	<0.001
Yes	21	0	21	
Edmondson grade				
I–II	35	18	17	<0.001
III–IV	37	4	33	
Cirrhosis				
Absent	5	2	3	1.000
Present	67	20	47	
HBeAg status				
Positive	61	19	42	1.000
Negative	11	3	8	
Child-Pugh grade				
A	69	22	47	0.548
B	3	0	3	
AFP (ng/ml)				
≤20	26	13	13	0.007
>20	46	9	37	
TNM stage				
I/II	41	21	20	<0.001
III/IV	31	1	30	

tumor capsula, vascular invasion, Edmondson grade and TNM stage had significantly prognostic influences on OS and DFS (**Table 4**). Moreover, multivariate survival analysis using Cox proportional hazard analyses of factors that were appeared significant in the univariate analyses revealed that HOXB13 expression as an independent prognostic factors for OS [hazard ratio (HR) 3.478; 95% CI 1.625-7.445; *P* = 0.001] and DFS [HR 3.123; 95% CI 1.514-6.442; *P* = 0.002], along with tumor capsula, vascular invasion, Edmondson grade and TNM stage (*P* <0.05, **Table 5**).

Discussion

Homeobox proteins are commonly regarded as modulators for growth and differentiation and their loss or gain of expression is cumulatively reported in human tumors. Increasing numbers of studies have demonstrated that HOXB13, as a member of homebox gene family, was unregulated in several kinds of cancers, including endometrial, ovarian, cervical, breast and prostate cancer [5-14]. Accumulated evidences suggested that HOXB13 played a key role in tumorigenesis, invasion, migration and apoptosis. Kim et al. [5] reported that HOXB13 promoted prostate cancer invasion and metastasis by decreasing intracellular zinc levels and stimulating nuclear factor kappa B (NF-κB) signals. Miao et al. [10] suggested that HOXB13 collaborated with activated ras to markedly promote ovarian cancer growth in vivo and then conferred resistance to tamoxifen-mediated apoptosis. However, it has also been reported that HOXB13 was downregulated in colorectal cancer and the underlying mechanism was mediated through the regulation of T-cell factor-4 (TCF4) protein stability [15]. These turmoil findings suggested that HOXB13 may act as a dual role due to different pathological types of cancer. However, the relationship between HOXB13 expression and tumor angiogenesis and/or clinicopathological parameters of HCC has not been clarified up till now.

In the present study, we first report the prognostic relevance of HOXB13 in patients with HCC and the correlation between HOXB13 expression and clinicopathological features. The levels of HOXB13 protein were significantly higher in HCC tissues when compared with that in paracarcinomatous tissues. Besides, we demonstrated that HCC tumors with HOXB13-

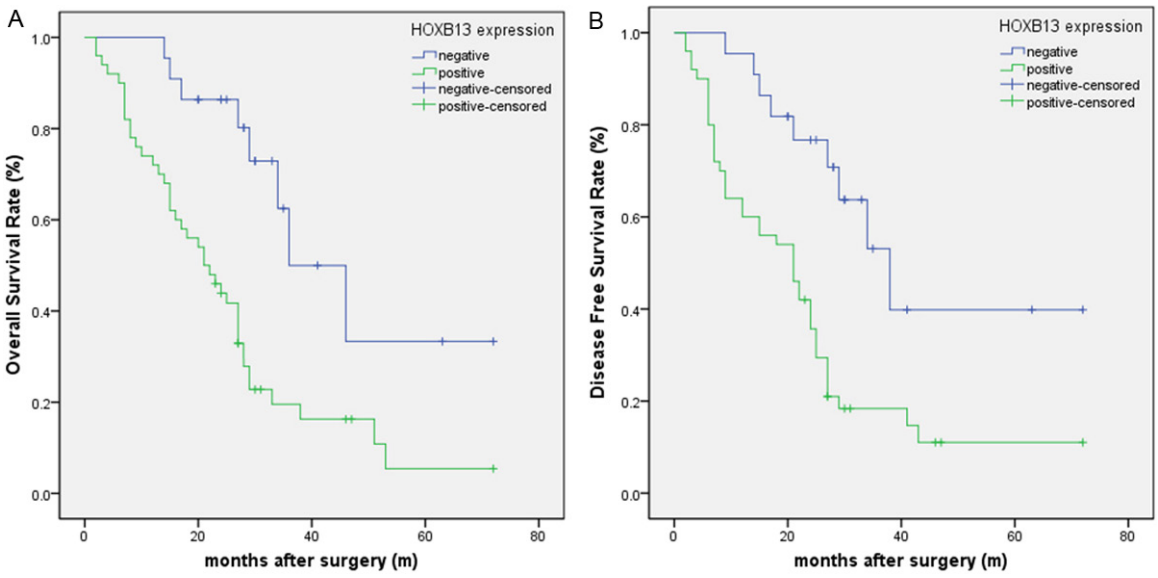

Figure 4. Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) curves of patients with HCC based on HOXB13 expression as positive or negative. A: OS curve of patients with HCC based on HOXB13 expression; B: DFS curve of patients with HCC based on HOXB13 expression. The HCC patients with HOXB13 positive showed notably worse OS and DFS rates than those with HOXB13 negative.

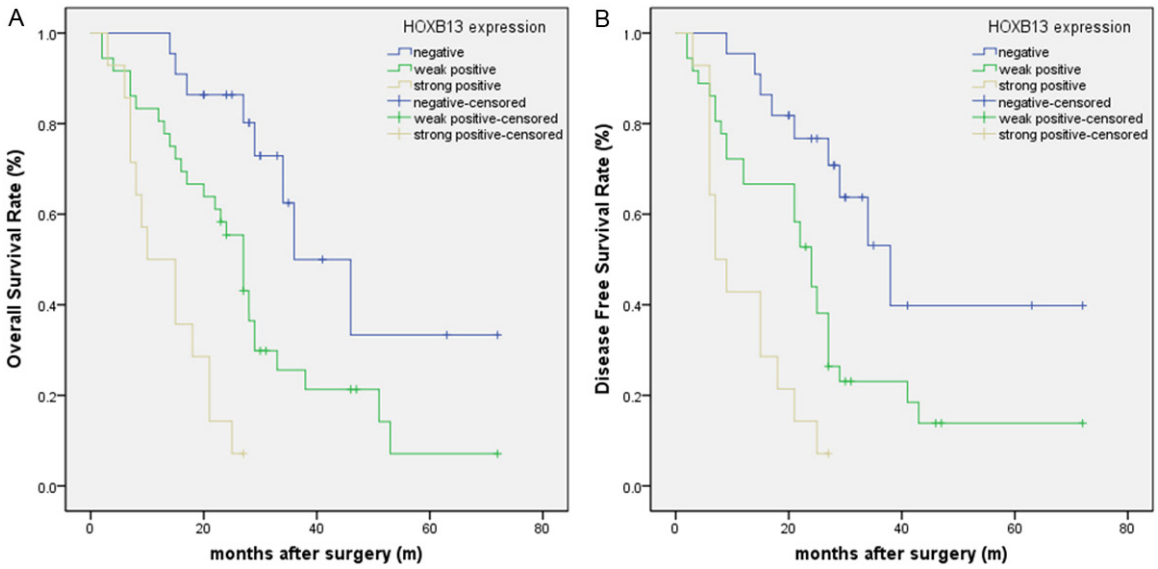

Figure 5. Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) curves of patients with HCC based on HOXB13 expression as strongly positive, weakly positive or negative. A: OS curve of patients with HCC based on HOXB13 expression; B: DFS curve of patients with HCC based on HOXB13 expression. The HCC patients with HOXB13 positive had a much worse OS and DFS rates than those with HOXB13 negative. The survival of patients with strongly positive HOXB13 expression was poorest.

positive expression were more significant related to incomplete tumor capsula, vascular invasion, advanced Edmondson grade, higher serum AFP and advanced TNM stage than HCC tumors with HOXB13-negative expression. Furthermore, the results of Kaplan-Meier survival analysis indicated that patients with high HOXB13 expression showed poor OS and DFS compared with patients with low HOXB13 expression. By Cox regression analysis, overexpression of HOXB13 was an independent predictor of poor prognosis for both OS and DFS in patients with HCC. Therefore, these results suggested that high HOXB13 expression had

Table 4. Univariate analysis of factors associated with OS and DFS

Variable	OS		DFS	
	95% CI	*P* value	95% CI	*P* value
HOXB13				
Negative	33.319-57.826	0.001	31.808-57.296	0.001
Positive	19.238-29.994		17.377-29.186	
Age (years)				
<60	24.992-39.526	0.564	23.440-38.343	0.517
≥60	21.123-34.177		16.777-28.448	
Gender				
Male	26.225-38.683	0.100	24.280-37.855	0.172
Female	10.945-34.132		10.016-32.805	
Tumor size (cm)				
≤5	30.123-49.817	0.013	27.584-48.314	0.015
>5	19.142-30.896		16.759-28.540	
Tumor nodule number				
Single	25.365-38.583	0.373	24.066-38.003	0.274
Multiple	15.665-36.668		12.399-33.768	
Tumor capsula				
Absent	14.550-25.629	0.000	12.104-22.204	0.001
Present	29.593-45.260		27.907-44.270	
Vascular invasion				
No	28.511-42.737	0.003	25.797-40.789	0.003
Yes	14.120-24.785		12.266-22.781	
Edmondson grade				
I–II	32.950-50.085	0.000	29.212-47.114	0.000
III–IV	16.016-23.947		14.001-22.435	
Cirrhosis				
Absent	12.472-37.528	0.818	11.759-27.841	0.525
Present	25.349-37.217		23.338-35.735	
HBeAg status				
Positive	25.683-38.078	0.579	23.700-36.804	0.282
Negative	15.186-31.686		13.078-25.286	
Child-Pugh grade				
A	25.866-37.468	0.085	23.960-36.232	0.022
B	1.338-29.995		0.000-21.540	
AFP (ng/ml)				
≤20	30.146-49.252	0.013	24.618-45.251	0.083
>20	19.259-27.531		17.881-26.399	
TNM stage				
I/II	31.753-48.873	0.000	29.804-47.474	0.000
III/IV	14.860-24.753		12.501-21.176	

an adverse outcome in HCC. HOXB13 may be used as a prognostic marker.

HCC is a hypervascular tumor characterized by neoangiogenesis and it is widely recognized that tumor angiogenesis are intrinsically connected with the development of metastases [20]. There is a plentiful of evidence to support significance of angiogenesis in the initiation, development and aggressiveness of HCC. VEGF, a primary driving force for both physiological and pathological angiogenesis, regulates the tumor angiogenesis. Zhang et al. [21] have found that VEGF played a crucial role in tumor angiogenesis and its functions in HCC proliferation and migration were mediated by P65, Protein kinase C alpha (PKCα) and/or p53. Mukozu et al. [22] have demonstrated that the serum levels of VEGF might be a useful predictor of HCC in patients with hepatitis C virus (HCV)-related liver cirrhosis, while serum levels of VEGF could predict vascular invasion in HCC. Additionally, Yao et al. [23] showed that high expression of VEGF and MVD were useful predictors for vascular invasion and metastasis of HCC.

Thus, in order to verify whether there is a relationship between HOXB13 protein and tumor angiogenesis, we quantified the levels of VEGF and MVD in this study, which were the most widely accepted markers of tumor angiogenesis. Our results showed that tumors with HOXB13-positive group expressed higher VEGF and had higher MVD than those in HOXB13-negative group. We analyzed the relation between VEGF and HOXB13 by spearman's rank correlation test and found out there were a significant positive correlation between HOXB13 and VEGF. Coincidentally, overexpression of HOXB13 in the epidermis is associated with an upregulation of VEGF in the skin of K14-directed HOXB13 transgenic mice, which indicated HOXB13 as a potential clinical target in wound healing and other pathologies

Table 5. Multivariate analysis of prognostic parameters associated with OS and DFS

Characteristics	OS			DFS		
	Hazard ratio	95% CI	*P*	Hazard ratio	95% CI	*P*
HOXB13 (Negative/positive)	3.478	1.625-7.445	0.001	3.123	1.514-6.442	0.002
Tumor capsula (Absent/present)	2.659	1.510-4.681	0.001	2.379	1.368-4.137	0.002
Vascular invasion (No/Yes)	2.354	1.308-4.236	0.004	2.276	1.277-4.057	0.005
Edmondson grade (I-II/III-IV)	1.772	1.301-2.415	0.000	1.625	1.215-2.173	0.001
Child-Pugh grade (A/B)	2.671	0.819-8.709	0.103	3.589	1.090-11.816	0.036
AFP (ng/ml) (≤20/>20)	0.454	0.236-0.871	0.018	0.601	0.330-1.094	0.096
TNM stage (I-II/III-IV)	1.759	1.319-2.346	0.000	1.755	1.325-2.326	0.000

characterized by abnormal or excessive inflammation, angiogenesis, or epidermal proliferation [24]. Taken together, these findings suggested that HOXB13 plays crucial role in HCC tumorigenesis by inducing and/or promoting tumor angiogenesis.

However, this study is not without its limitations, most notably its small sample size and the possible selection bias of the cohort. A prospective study with a larger, randomized and multicenter is thus needed to corroborate the present findings.

In conclusion, our study found that HOXB13 is overexpressed in HCC tissues compared with their adjacent tissues. It is possible that high-level expression of HOXB13 has a close correlation with tumor angiogenesis and metastasis, and its overexpression is of predictive value on HCC development and progression. In future, if this activity could be blocked by some specific inhibitors, we may provide a new target for the anti-angiogenic therapy of HCC.

Acknowledgements

We sincerely appreciate Dr. Hang-Cheng Zhou and Dr. Wen Zhong (two pathologists, Department of Pathology, Anhui Provincial Hospital, Hefei, China) for their kind assistance in pathology. This research was partly supported by the National Natural Science Foundation of China (No. 81201906, No. 81172364).

Disclosure of conflict of interest

None.

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