Tohoku J. Exp. Med., 2014, **234**, 209-219

# Overexpression of HOXA13 as a Potential Marker for Diagnosis and Poor Prognosis of Hepatocellular Carcinoma

**Ting-Ting Pan,[1] Wei-Dong Jia,[2,3] Qi-Yang Yao,[2] Qi-Kai Sun,[2] Wei-Hua Ren,[2] Mei Huang,[2] Jie Ma,[2] Jian-Sheng Li,[3] Jin-Liang Ma,[3] Ji-Hai Yu,[3] Yong-Sheng Ge,[3] Wen-Bin Liu,[3] Chuan-Hai Zhang[3] and Ge-Liang Xu[2,3]**

[1]Graduate school, Tianjin Medical University, Tianjin, P.R. China
[2]Anhui Province Key Laboratory of Hepatopancreatobiliary Surgery, Hefei, Anhui Province, P.R. China
[3]Department of Hepatic Surgery, Affiliated Provincial Hospital of Anhui Medical University, Hefei, Anhui Province, P.R. China

HOXA13 is a member of homeobox genes that encode transcription factors regulating embryonic development and cell fate. Abnormal HOXA13 expression was reported in hepatocellular carcinoma (HCC), but its correlation with tumor angiogenesis and prognosis still remain unclear. This study was aimed to uncover the expression, diagnostic and prognostic significance of HOXA13 in HCC. Immunohistochemistry was performed to detect HOXA13 expression in HCC and corresponding paracarcinomatous tissues from 90 patients. Enzyme-linked immunosorbent assay was used to detect serum HOXA13 in 90 HCC patients and 20 healthy volunteers. Receiver operating characteristics was analyzed to calculate diagnostic accuracy of serum HOXA13, alpha-fetoprotein (AFP) and their combination. Immunoreactivity of HOXA13 was detected in 72.2% of HCC, and 12.2% of adjacent non-cancerous samples. HOXA13 expression was significantly associated with tumor size, microvascular invasion, pathological grade, tumor capsula status, AFP level, tumor-node-metastasis stage and positively correlated with VEGF ($p < 0.001$) and microvessel density ($p < 0.001$). The combination of serum HOXA13 and AFP had a markedly higher area under the curve than HOXA13 alone. HOXA13 expression was associated with unfavorable overall survival (OS) ($p < 0.001$) and disease-free survival (DFS) ($p < 0.001$). Multivariate analysis indicated that patients with HOXA13-expressing tumors had a significantly shorter OS ($p = 0.030$) and DFS ($p = 0.005$) than those with HOXA13-negative tumors. Thus, HOXA13 expression possibly plays an important role in tumor angiogenesis, progression and prognosis of HCC. Moreover, we demonstrate that serum HOXA13 may serve as a biomarker for early HCC diagnosing and predicting outcome.

**Keywords:** hepatocellular carcinoma; homeobox gene; HOXA13; prognosis; tumor angiogenesis
Tohoku J. Exp. Med., 2014 November, **234** (3), 209-219. 

## Introduction

Hepatocellular carcinoma (HCC) has become the fifth most common malignant tumor and the third leading cause of cancer-related death worldwide (Ferlay et al. 2010; Jemal et al. 2011). It is one of the most common malignant tumors in China. Despite improvements of surgical techniques and early diagnostic methods, HCC remains a major carcinoma with high mortality because of the high postoperative recurrence and metastatic rate (Siegel et al. 2013; Altekruse et al. 2014). The only chance for long-term, disease-free survival (DFS) is early diagnosis before symptoms develop. It needs to strengthen HCC surveillance in high-risk patients. Surveillance of HCC should be conducted based on abdominal ultrasonography, computed tomography, and tumor biomarkers. The accuracy of abdominal ultrasound is highly dependent on the operator's experience, while computed tomography is expensive and also its feasibility dependents on patients' compliance. Traditional biomarkers, such as alpha-fetoprotein (AFP), have limited clinical value in predicting prognosis and metastasis (Behne and Copur 2012; Forner and Bruix 2012). Thus, it is essential to identify new specific tumor biomarkers, especially for early stage tumors.

The HOX genes encode a family of transcription factors that play key roles in regulating embryonic development, cell fate, adult tissue homeostasis and organ functioning maintenance (Gehring and Hiromi 1986). The genes have a common sequence element of 183 bp, which encodes a conserved homeodomain region consisting of a 61-amino

Received July 28, 2014; revised and accepted October 9, 2014. Published online October 24, 2014; doi: 10.1620/tjem.234.209.
Correspondence: Ge-Liang Xu, Anhui Province Key Laboratory of Hepatopancreatobiliary Surgery, and Department of Hepatic Surgery, Affiliated Provincial Hospital of Anhui Medical University, NO. 17, Lujiang Road, Hefei, Anhui Province 230001, P.R. China.
e-mail: xugeliang2013@163.com

acid motif (Lewis 1978). Human HOX genes are 39 genes organized into four different chromosomal loci termed HOXA, HOXB, HOXC and HOXD, and each contains 9 to 11 genes aligned in 13 paralogue groups based on sequence similarity and position in the locus (Acampora et al. 1989; Scott 1992). Furthermore, increasing evidence indicates that expression of particular HOX genes is deregulated in a variety of solid tumors including lung, prostate, breast, colon, thyroid, ovarian, and liver cancer (De Vita et al. 1993; Cillo 1994; Hamada et al. 2001; Jung et al. 2004; Makiyama et al. 2005; Kanai et al. 2010; Cillo et al. 2011; Cantile et al. 2013). HOXA13, a member of paralogue 13 located on chromosome 7, is a determinant of gut primordia and posterior body structures and controls the lumbo-sacral region including analia and genitalia (Graham et al. 1989). HOXA13 thus plays a crucial role in extraembryonic vascularization (Shaut et al. 2008). In normal adult tissue, HOXA13 has a tendency for high expression in the hindgut region, while its expression is absent or extremely low in anterior areas of the body including liver (Graham et al. 1989; Takahashi et al. 2004). Latest evidence indicates that HOXA13 is upregulated in HCC and its expression may be associated with clinical progression of HCC and may be predictive of disease outcome (Cillo et al. 2011; Quagliata et al. 2014). However, its correlation with tumor angiogenesis, diagnostic value and prognostic significance still remains unclear. In this study, we further detected the expression of HOXA13 in tumor tissues from 90 patients with HCC to evaluate its clinical significance and explore the relation with clinicopathological parameters and tumor angiogenesis. In addition, we assessed the potential of serum HOXA13 in differentiating HCC patients from healthy individuals.

## Materials and Methods

*Patients and tissue samples*

This study was approved by the Human Research Ethics Committee of Anhui Medical University (Hefei, China), and consent was obtained from each patient. The tumor and paracarcinomatous specimens were obtained from 90 patients (75 males, 15 females) with a definitive diagnosis of HCC and underwent curative surgical resection surgery at the department of Hepatic Surgery, Affiliated Provincial Hospital of Anhui Medical University between January 2008 and December 2011. HCC diagnosis was verified by pathological examination, and none of those patients had received preoperative adjuvant therapy or suffered from severe postoperative complications, such as hemorrhage, anastomotic leakage, or hepatic failure. The clinicopathological data of the patients were retrieved from clinical and pathology reports including age, gender, number of tumor nodule, tumor size, tumor capsula, portal invasion, Edmondson grade, status of hepatitis B surface antigen (HBsAg), liver cirrhosis, Child-Pugh grade, levels of preoperative serum AFP and tumor stage. The age of patients ranges from 21 to 74 years (57 ± 11 years). Tumor differentiation was defined according to the Edmondson grading system, and the pathological tumor stage was defined according to the sixth edition of the tumor-node-metastasis (TNM) classification of Union for International Cancer Control (UICC). Hepatic function was assessed by using Child-Pugh classification. The follow up data were available from all patients. Follow-up was terminated on March 12, 2014. The mean follow-up was 35 months ranging from 6 to 66 months. The calculation of DFS time began on the date of surgery and ended when any of the following events happened: recurrence, metastasis, or oncological death. The overall survival (OS) was defined as the interval between the date of surgery and the date of death or the last observation taken. The data were censored at the last follow-up period for living patients. For the measurement of serum HOXA13, we collected peripheral blood samples from 90 HCC patients before surgery and from 20 age-matched, healthy volunteers as a control.

*Immunohistochemical staining for HOXA13, CD34, and vascular endothelial growth factor (VEGF)*

We used immunohistochemistry to detect the expression of HOXA13 in 90 HCC tissues and matched adjacent non-cancerous tissues while VEGF and CD34 were just examined in HCC tissues. Formalin-fixed and paraffin-embedded tissues were cut into serial sections with a thickness of 4 $\mu$m. Sections were stained with hematoxylin and eosin (HE) for histological examination. Sections were gradually deparaffinized and rehydrated with xylene and ethanol and subjected to microwave antigen retrieval in citrate buffer (10 mM, pH 6.0) for 20 min, and then cooled at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution for 10 minutes. Then the sections were separately incubated with rabbit anti-HOXA13 antibody (bs-12244R, Beijing Biosynthesis Biotechnology, Beijing, China), mouse anti-VEGF antibody (ZM-0265, ZSGB-BIO) or mouse anti-CD34 antibody (ZM-0046, ZSGB-BIO) at 4°C overnight. Following incubation in horse-radish peroxidase (HRP)-conjugated secondary antibody (PV-6000, ZSGB-BIO) for 20 min, sections were stained in 3,3′-diaminobenzidine tetrahydrochloride (DAB) (ZLI-9017, ZSGB-BIO) solution under microscopic observation and then counterstained with hematoxylin, dehydrated and mounted. Negative controls were processed with PBS instead of primary antibody.

The expression levels of HOXA13 and VEGF were assessed by a semi-quantitative scoring system including the intensity of staining and the percentage of positive tumor cells. No staining or staining < 10% of the tumor cells showed focal or weak immunopositivity was clarified as negative, moderate or patchy immunopositivity in 10-30% of tumor cells as "+", and strong or diffuse immunopositivity in > 30% of tumor cells as "++". Ten fields were selected, and expression in 1,000 tumor cells was evaluated with high-power (400×) microscope.

CD34 is an antigen present in vascular endothelial cells; thus, microvessels were detected by using anti-CD34 antibody. The field of maximal CD34 expression was found in tumor cells, and five areas of maximal angiogenesis (hotspot) of each tumor tissue section were selected on a low-power (100×) microscope, and then the maximum number of microvessels was counted for each area under high-power magnification (200×). The average of the number of microvessels in the five hotspots was recorded as the microvessel density (MVD) level of the tumor. The immunohistochemical results were evaluated by two pathologists who were blinded to clinical data.

*Measurement of serum HOXA13 levels by enzyme-linked immunosorbent assay (ELISA)*

A 5 ml venous blood sample was withdrawn from each subject and centrifuged for 10 min at 4,000 r/min and 4°C. Serum was sub-

sequently collected and stored at −80°C until testing. Serum HOXA13 levels were measured using a commercially available ELISA kit according to the manufacturer's instruction (Shanghai Yuan Ye Biological Technology Co., LTD, 1402589H). Briefly, standards (50 $\mu$l) were added to a standard well, and each serum sample (10 $\mu$l) and sample diluents (40 $\mu$l) were added to a testing well. After adding HRP-conjugate reagent (100 $\mu$l) to each well, the plate was covered with an adhesive strip and incubated for 60 minutes at 37°C. After incubation, each well was aspirated and washed five times. Then, chromogen solution A (50 $\mu$l) and chromogen solution B (50 $\mu$l) were added to each well, and the plate was gently mixed and incubated for 15 minutes at 37°C. The Optical Density (O.D.) at 450 nm was measured with a microtiter plate reader (Thermo Scientific, Waltham, MA, USA) after addition of stop solution (100 $\mu$l) to each well. Each assay was performed in triplicate and repeated three times.

*Statistical analysis*

All statistical analyses were performed using the statistical package SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Continuous data were expressed as mean ± standard deviation (S.D.). Significant differences in the means were determined using the Student's *t* test. Spearman's rank correlation test was used to analyze the relation between HOXA13 and VEGF. The chi-square test was used to ana-

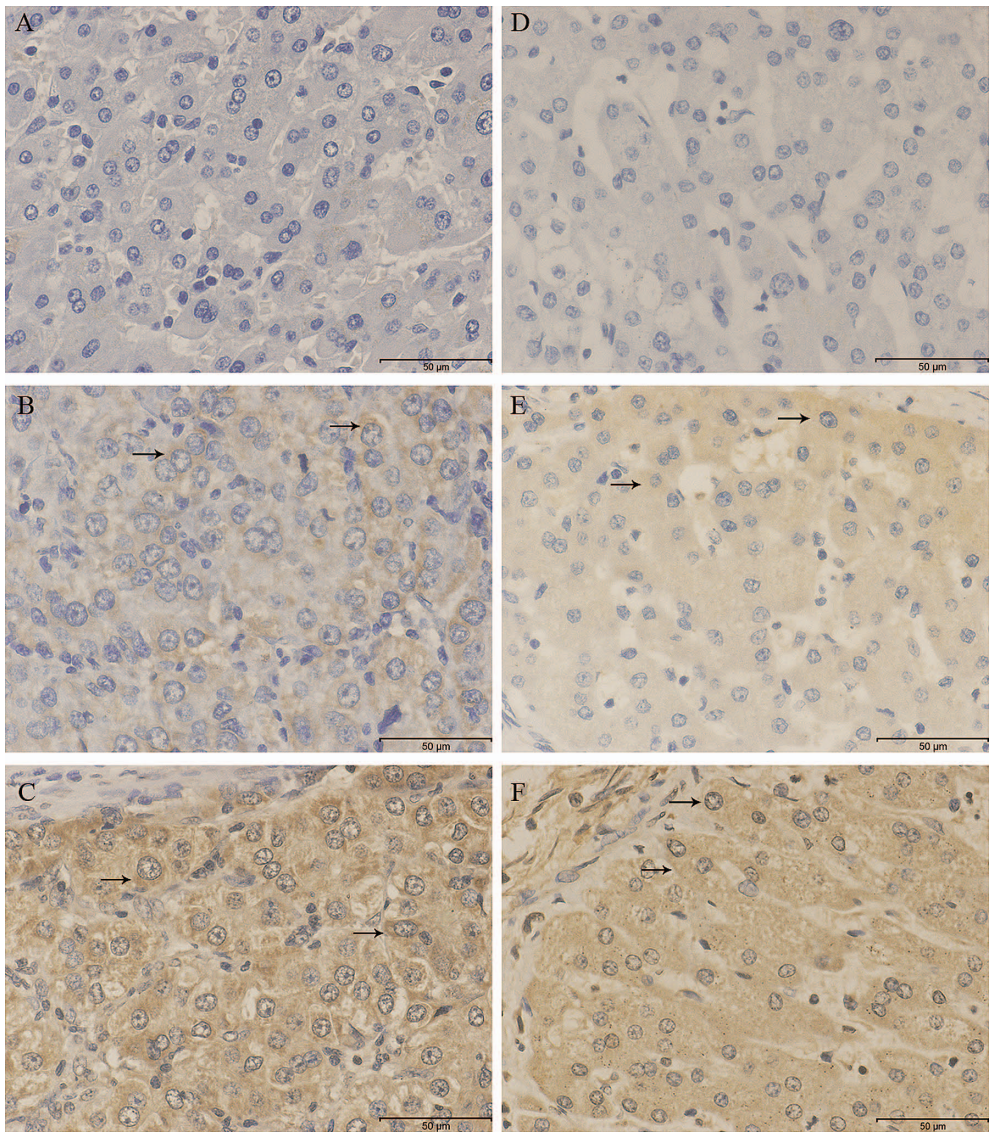


Fig. 1. Representative immunohistochemical staining of HOXA13 in hepatocellular carcinoma (HCC) and paracarcinomatous liver tissues.
HOXA13 expression showed mainly diffuse cytoplasmic staining in tumor and paracarcinomatous liver tissues (arrow). Hepatocytes showed positive HOXA13 expression in paracarcinomatous tissues present different levels of atypical hyperplasia. Sections were collected from 6 different patients. (A) Negative HOXA13 expression in HCC (×400). (B) Low HOXA13 expression in HCC (×400). (C) High HOXA13 expression in HCC (×400). (D) Negative HOXA13 expression in paracarcinomatous live tissue (×400). Paracarcinomatous liver tissue retained normal morphology. (E) Low HOXA13 expression in paracarcinomatous liver tissue (×400). (F) High HOXA13 expression in paracarcinomatous liver tissue (×400). Bar = 50 $\mu$m.

lyze the associations between HOXA13 expression and clinicopathological parameters. The Kaplan-Meier method was used for survival analysis, and the survival differences were assessed by the log-rank test. Receiver operating characteristics (ROC) curves were generated to determine the diagnostic performance of serum HOXA13, serum AFP, and their combination. The Cox regression model was used to analyze all parameters that were significant in the univariate analysis. $P < 0.05$ was considered statistically significant.

## Results

*Immunohistochemical staining for HOXA13 in HCC and paracarcinomatous liver tissues*

Immunohistochemistry showed that positive HOXA13 expression was mainly localized in the cytoplasm of tumor cells with varying staining intensity (Fig. 1). The positive rate of HOXA13 was significantly ($p < 0.05$) higher in

Table 1. HOXA13 expression status in relation to clinicopathological features in 90 HCC patients.

| Clinicopathologic data | HOXA13 immunohistochemical staining | | | Serum HOXA13 level | |
|---|---|---|---|---|---|
| | − | + ~ ++ | *p* | Mean ± S.D. | *p* |
| Age (years) | | | 0.208 | | 0.739 |
| < 60 | 16 | 32 | | 6.20 ± 3.65 | |
| ≥ 60 | 9 | 33 | | 5.96 ± 2.77 | |
| Gender | | | 0.916 | | 0.665 |
| Male | 21 | 54 | | 6.17 ± 3.37 | |
| Female | 4 | 11 | | 5.78 ± 2.96 | |
| Tumor size (cm) | | | 0.011 | | 0.011 |
| > 5 | 10 | 45 | | 6.74 ± 2.98 | |
| ≤ 5 | 15 | 20 | | 4.92 ± 3.52 | |
| Tumor nodule number | | | 0.072 | | 0.782 |
| Single | 19 | 36 | | 6.03 ± 3.45 | |
| Multiple | 6 | 29 | | 6.24 ± 2.94 | |
| Vascular invasion | | | 0.010 | | 0.003 |
| Present | 1 | 19 | | 7.19 ± 2.99 | |
| Absent | 24 | 46 | | 5.18 ± 3.26 | |
| Tumor capsula | | | 0.001 | | 0.014 |
| Present | 10 | 50 | | 7.78 ± 3.64 | |
| Absent | 15 | 15 | | 5.67 ± 3.07 | |
| HBsAg status | | | 0.695 | | 0.945 |
| Positive | 23 | 58 | | 6.09 ± 3.37 | |
| Negative | 2 | 7 | | 6.15 ± 2.92 | |
| TNM stage | | | 0.015 | | 0.018 |
| I-II | 22 | 40 | | 5.49 ± 3.40 | |
| III-IV | 3 | 25 | | 7.19 ± 2.79 | |
| AFP (ng/ml) | | | 0.039 | | 0.052 |
| > 20 | 11 | 44 | | 6.54 ± 3.26 | |
| ≤ 20 | 14 | 21 | | 5.06 ± 3.16 | |
| Edmondson grade | | | 0.003 | | 0.864 |
| I-II | 19 | 27 | | 6.06 ± 3.19 | |
| III-IV | 6 | 38 | | 6.18 ± 3.53 | |
| Cirrhosis | | | 0.910 | | 0.749 |
| Present | 17 | 45 | | 6.29 ± 2.46 | |
| Absent | 8 | 20 | | 6.03 ± 3.54 | |
| Child-Pugh grade | | | 0.310 | | 0.878 |
| A | 23 | 63 | | 5.87 ± 3.31 | |
| B | 2 | 2 | | 6.11 ± 3.30 | |

TNM (tumor-node-metastasis) status is based on primary liver cancer TNM standard 2003 of Union for International Cancer Control (UICC). Edmondson grade was short for Edmondson-Steiner grade which was used to evaluate the histologic grade of the tumor. Hepatic function was assessed by using Child-Pugh classification. HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein.

HCC tissues (65/90, 72.2%) than in corresponding paracarcinomatous non-cancerous liver tissues (11/90, 12.2%). All paracarcinomatous non-cancerous tissues were obtained from the liver at least 3 cm away from the tumor edge. HOXA13 was expressed in paracarcinomatous tissues of 11 patients, while it was also expressed in HCC tissues. In addition, HOXA13-positive paracarcinomatous liver tissues showed different levels of atypical hyperplasia, suggesting that up-regulation of HOXA13 may play an important role in tumorigenesis.

*Correlation of tissue HOXA13 expression with clinicopathological parameters*

In order to evaluate biological significance of HOXA13, we analyzed the associations between tissue HOXA13 expression and clinicopathological parameters including age, gender, number of tumor nodule, tumor size, tumor capsula, portal invasion, Edmondson grade, HBsAg status, liver cirrhosis, Child-Pugh grade, levels of preoperative serum AFP and tumor TNM stage in HCC. As shown in Table 1, the expression level of HOXA13 was significantly associated with tumor size ($p$ = 0.011), vascular invasion ($p$ = 0.010), tumor capsula ($p$ = 0.001), TNM stage ($p$ = 0.015), serum AFP ($p$ = 0.039), and Edmondson grade ($p$ = 0.003). There was no significant correlation with age, gender, HBsAg status, cirrhosis, Child-Pugh grade, and tumor nodule number.

*Immunohistochemical expression of VEGF in HCC tissues and its correlation with HOXA13*

Neovascularization is a common phenomenon in solid tumors including HCC (Zhu et al. 2011). VEGF is one of the most important proangiogenic factors in angiogenetic process (Folkman 2002). It can stimulate endothelial cell proliferation and induce new blood vessels formation. In the present study, we found that among 90 patients with HCC, 74.4% (67/90) of them showed high VEGF expression (Fig. 2). Spearman's rank correlation test was used to analyze the relation between HOXA13 and VEGF. Finally, a significant positive correlation was found between tissue expression of HOXA13 and VEGF in HCC ($r$ = 0.474, $p$ < 0.001; Table 2). On this result, we can make an assumption that HOXA13 plays an important role in tumor angiogenesis in HCC via VEGF. To confirm this finding, further investigations will still be required to explore its possible molecular mechanisms.

*Correlation between HOXA13 expression and MVD*

MVD in HCC tumor tissues ranged from 0 to 190/200 per field (median, 79/200 × field) (Fig. 3). Tumors with positive HOXA13 expression had significantly greater MVD than tumors with negative HOXA13 expression (93.4

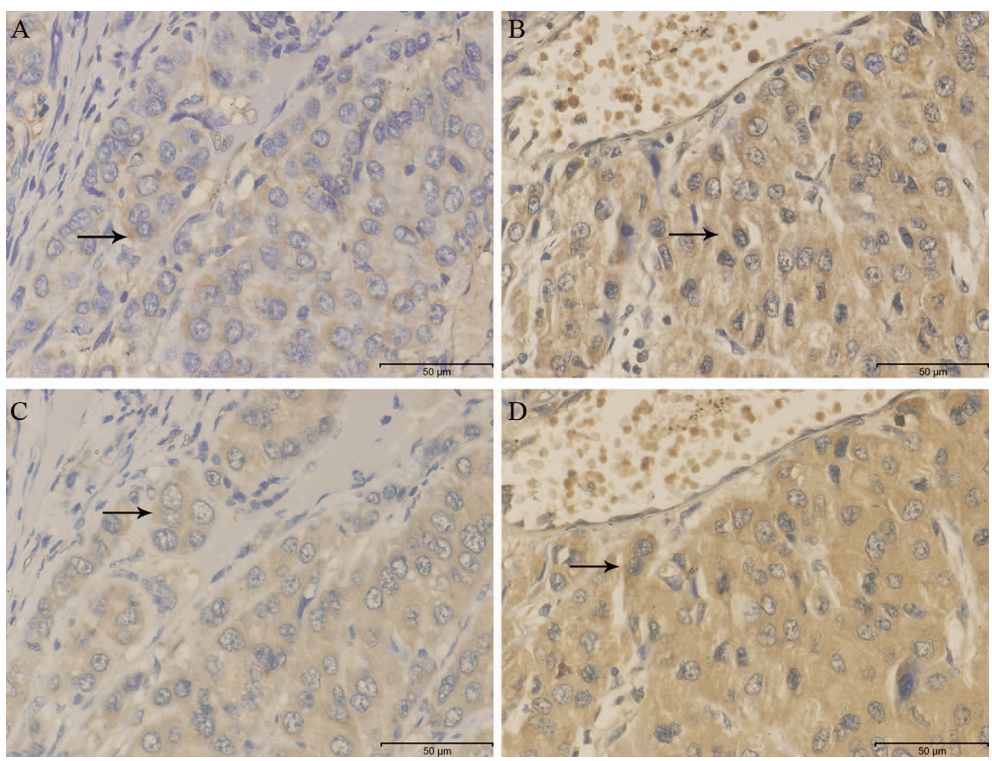


Fig. 2. Representative sections showing high and low cytoplasmic expression of HOXA13 and VEGF in HCC tissues. The expression of HOXA13 and VEGF in HCC had a significant positive correlation. (A) Low HOXA13 expression (×400). (B) High HOXA13 expression (×400). (C) Low VEGF expression (×400). (D) High VEGF expression (×400). Arrows indicate cytoplasmic HOXA13 or VEGF expression in HCC cancer cells. Bar = 50 $\mu$m. Panels A and C were representative serial sections from one patient and panels B and D were representative serial sections from another patient.

Table 2. Correlation between the expression of HOXA13 and VEGF in 90 HCC cases.

| Immunoreactivity | HOXA13 | | | |
|---|---|---|---|---|
| | − | + ~ ++ | *r* | *p* value |
| VEGF | | | 0.474 | < 0.001 |
| − | 15 | 8 | | |
| + ~ ++ | 10 | 57 | | |

VEGF, vascular endothelial growth factor.
*r*: Pearson contingency coefficient.

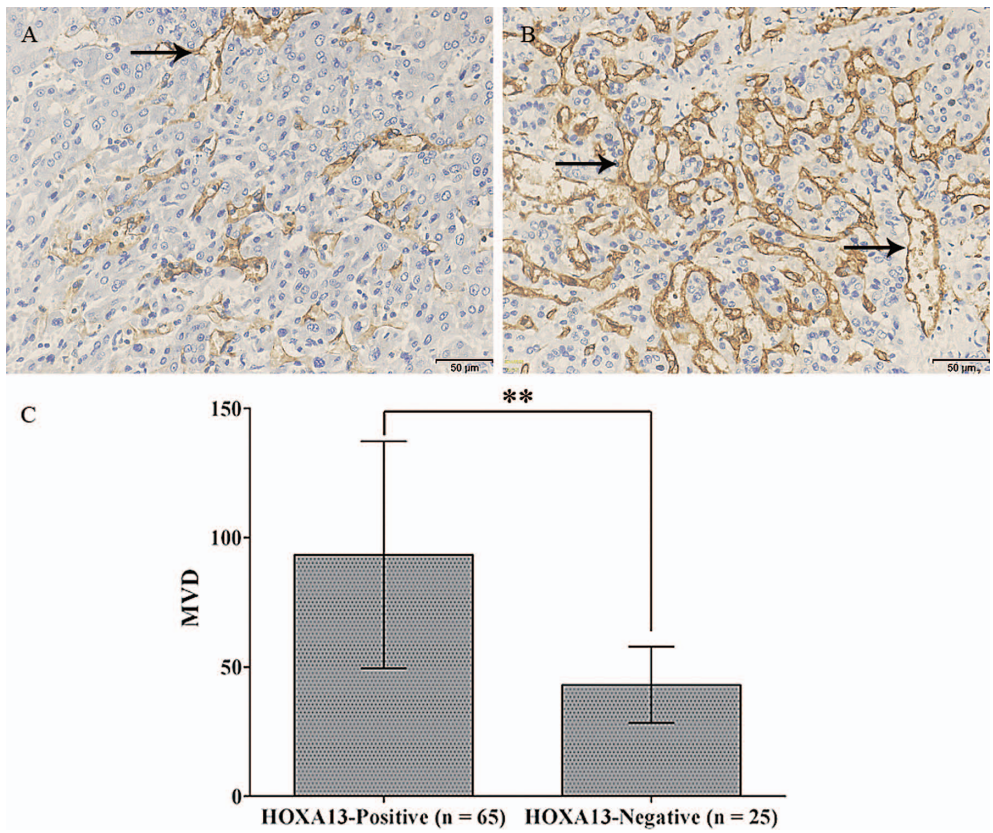


Fig. 3. Immunohistochemical staining of CD34 for microvessel density (MVD) in HCC tissues.
(A) Low expression of CD34 in HCC (×200). (B) Representative high expression of CD34 in HCC (×200). Arrows showed CD34-positive microvessels. Bar = 50 $\mu$m. (C) Tumors with HOXA13 expression had a significantly higher MVD compared to tumors without HOXA13 expression. Data are expressed as the number of CD34-positive microvessels counted under a high-power (×200) microscope (**$p < 0.001$). The data are shown as mean with standard deviation (S.D.).

± 43.9 vs. 43.1 ± 14.8, $p < 0.001$; Fig. 3). These findings further verify the conclusion that HOXA13 may play an essential role in tumor angiogenesis in HCC.

*Relationship between HOXA13 expression and prognosis*

Kaplan-Meier survival analysis was used to assess the relationship between HOXA13 expression and patients' OS and DFS (Fig. 4). Patients with HOXA13-positive expression [28.6 months; 95% confidence interval (CI): 25.5-31.8] had a shorter OS than HOXA13-negative patients (51.8 months; 95% CI: 45.2-58.3; $p < 0.001$). Similarly, the DFS was significantly lower in patients with HOXA13-positive expression (16.5 months; 95% CI: 14.3-18.7) than in those with HOXA13-negative expression (39.2 months; 95% CI: 32.9-45.7; $p < 0.001$). Univariate analysis indicated that tumor expression of HOXA13, tumor size, vascular invasion, Edmondson grade, tumor capsula status, serum AFP and TNM stage had significant prognostic influence on OS and DFS (Table 3). Multivariate survival analysis (Table 4) further revealed intra-tumoral HOXA13 staining as an independent poor prognostic marker for OS [hazard ratio (HR) = 2.249; 95% CI: 1.079-4.685; $p = 0.030$] and DFS (HR = 2.773; 95% CI: 1.358-5.663; $p = 0.005$). In addition, tumor size, vascular invasion, tumor capsula status, Edmondson

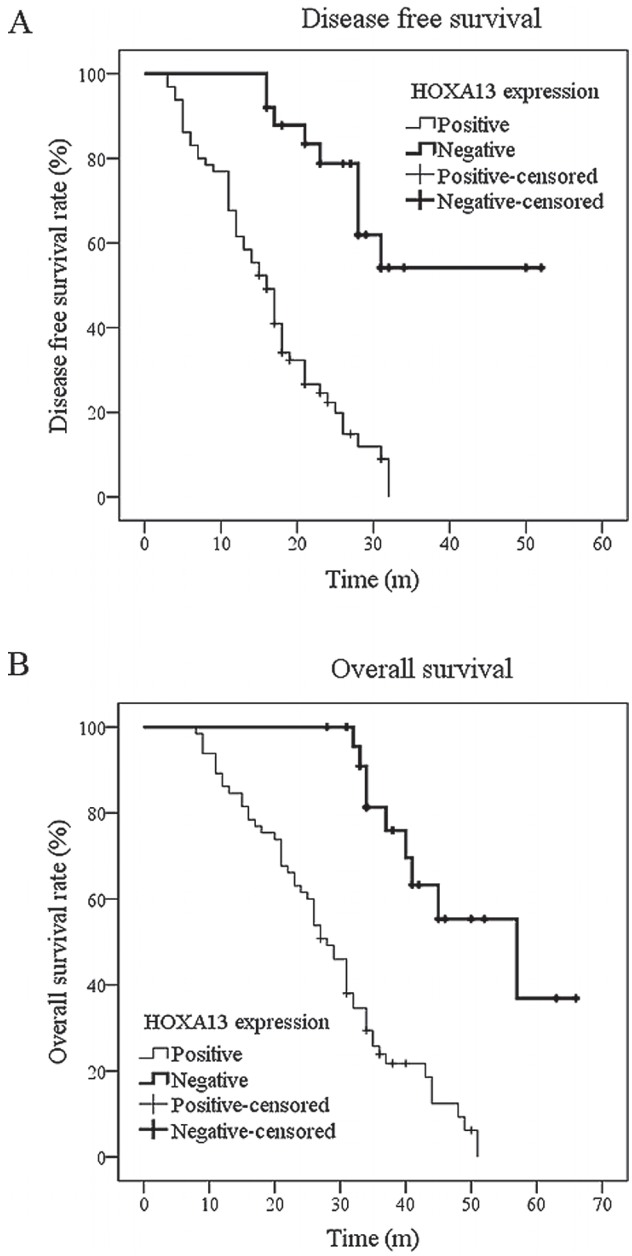


Fig. 4. Kaplan-Meier analysis of overall survival (OS) and disease-free survival (DFS) of patients with HCC. Kaplan-Meier analysis of OS and DFS of patients with HCC ($n$ = 90) was based on HOXA13 expression as positive ($n$ = 65) or negative ($n$ = 25). (A) DFS curve of patients with HCC based on HOXA13 expression. (B) OS curve of patients with HCC based on HOXA13 expression. The HCC patients with positive HOXA13 expression showed notably poorer DFS and OS rate than those with negative HOXA13 expression.

grade and serum AFP were also independent prognostic factors for OS and DFS. These findings further indicate that HOXA13 had a tumor-promoting role in HCC and it was a negative independent predictor of OS and DFS of patients with HCC.

We also assessed the correlation between HOXA13 expression in paracarcinomatous tissues and prognosis. Patients with HOXA13 expression in paracarcinomatous tissues had OS of 26.6 months (95% CI: 21.1-32.1) and DFS of 16.5 months (95% CI: 11.86-21.2), while patients without HOXA13 expression had OS of 35.8 months (95% CI: 21.1-31.1) and DFS of 23.5 months (95% CI: 19.7-27.2). There was no significant difference in OS ($p$ = 0.070) and DFS ($p$ = 0.227) between the two groups.

*Clinical significance of serum HOXA13 in HCC*

The results of ELISA showed that HCC patients had a significantly higher level of serum HOXA13 than healthy controls (6.17 ± 2.78 ng/ml vs. 1.44 ± 2.10 ng/ml; $p$ < 0.001, Fig. 5A). That indicates HOXA13 may be a potential diagnostic marker in HCC. Furthermore, as shown in Table 1, there were significantly higher serum levels of HOX13 in patients with tumor size ($p$ = 0.011), vascular invasion ($p$ = 0.003), incomplete capsule ($p$ = 0.014), and more advanced TNM ($p$ = 0.018). There were no significant correlation with age, gender, tumor nodule number, HBsAg status, serum AFP level, cirrhosis, Child-Pugh grade, and Edmondson grade. ROC curves were established to compare the diagnostic potential of serum HOXA13 and AFP level in distinguishing HCC patients from healthy subjects (Fig. 5B). The area under the curve (AUC) was 0.775 (95% CI: 0.655-0.895) for HOXA13 and 0.822 (95% CI: 0.727-0.917) for AFP. Moreover, the addition of serum AFP increased the ability of serum HOXA13 to detect HCC with an AUC of 0.878 and 95% CI was 0.790-0.965. These findings indicate that serum HOXA13 had a similar diagnostic accuracy to serum AFP in differentiating HCC from healthy individuals, moreover, the combination of HOXA13 and AFP had a significant higher AUC than each of them alone. When used in combination with other biomarkers, serum HOXA13 may provide additional diagnostic power in HCC.

## Discussion

In this study, we evaluated the expression and clinical significance of HOXA13 in HCC. Available evidence indicates that HOXA13 plays a prominent role in tumor survival and progression (Gu et al. 2009; Cillo et al. 2011). In embryonic development, each HOX gene is expressed in a spatiotemporal pattern, and thus HOXA13 is expressed specifically in the cloacal mesoderm and hindgut, but not in liver (Burke et al. 1995; Roberts et al. 1995; Takahashi et al. 2004). In our study, we found that HOXA13 was expressed in 72.2% HCC tissues and 12.2% paracarcinomatous tissues, and all HOXA13-positive paracarcinomatous tissues had different levels of atypical hyperplasia. We thus suggest that the up-regulation of HOXA13 may play a crucial role in carcinogenesis of hepatocytes.

In this study, HOXA13 expression is significantly higher in HCC compared with corresponding paracarcinomatous tissues, and positive HOXA13 expression was mainly localized in the cytoplasm of tumor cells. As a transcription factor, immunohistochemical labeling for

Table 3. Univariate analysis of factors associated with OS and DFS.

| variable | OS | | DFS | |
|---|---|---|---|---|
| | Median survival time (m) | *p* | Median survival time (m) | *p* |
| HOXA13 | | < 0.001 | | < 0.001 |
| Negative | 51.7 | | 36.7 | |
| Positive | 28.6 | | 15.2 | |
| Age (years) | | 0.690 | | 0.992 |
| < 60 | 36.7 | | 22.3 | |
| ≥ 60 | 33.6 | | 23.2 | |
| Gender | | 0.583 | | 0.985 |
| Male | 35.4 | | 23.3 | |
| Female | 33.5 | | 20.2 | |
| Tumor size (cm) | | 0.001 | | 0.001 |
| > 5 | 28.7 | | 18.1 | |
| ≤ 5 | 44.5 | | 26.2 | |
| Tumor nodule number | | 0.178 | | 0.144 |
| Single | 32.8 | | 20.6 | |
| Multiple | 40.1 | | 28.1 | |
| Vascular invasion | | < 0.001 | | < 0.001 |
| Present | 17.6 | | 8.6 | |
| Absent | 40.1 | | 27.1 | |
| Tumor capsula | | < 0.001 | | < 0.001 |
| Present | 28.7 | | 16.6 | |
| Absent | 46.4 | | 33.4 | |
| HBsAg status | | 0.169 | | 0.147 |
| Positive | 35.7 | | 23.6 | |
| Negative | 27.7 | | 16.2 | |
| TNM stage | | 0.016 | | 0.020 |
| I-II | 38.0 | | 25.3 | |
| III-IV | 28.1 | | 16.1 | |
| AFP (ng/ml) | | 0.019 | | 0.023 |
| > 20 | 30.3 | | 18.2 | |
| ≤ 20 | 40.1 | | 27.7 | |
| Edmondson grade | | < 0.001 | | < 0.001 |
| I-II | 35.3 | | 29.5 | |
| III-IV | 25.6 | | 20.0 | |
| Cirrhosis | | 0.424 | | 0.387 |
| Present | 33.6 | | 19.8 | |
| Absent | 36.5 | | 25.3 | |
| Child-Pugh grade | | 0.625 | | 0.562 |
| A | 34.8 | | 22.7 | |
| B | 30.8 | | 18.5 | |

TNM (tumor-node-metastasis) status is based on primary liver cancer TNM standard 2003 of Union for International Cancer Control (UICC). Edmondson grade was short for Edmondson-Steiner grade which was used to evaluate the histologic grade of the tumor. Hepatic function was assessed by using Child-Pugh classification. HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; OS, overall survival; DFS, disease-free survival.

HOXA13 is expected to localize in nuclei of HCC cells, as previously reported (Knosp et al. 2004; Cillo et al. 2011). However, Gu et al. (2009) also found cytoplasmic localization of HOXA13 in esophageal squamous cell carcinoma and this phenomenon was also found in many other transcription factors (Sun et al. 2013). The regulation of transcription factor activity plays an important role in many biological processes. Mechanisms known to influence transcription factor activity include post-translational modification, expression levels, protein stability, and subcellular

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

| Variable | OS | | | DFS | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *p* | HR | 95% CI | *p* |
| HOXA13 (low vs. high) | 2.249 | 1.079-4.685 | 0.030 | 2.773 | 1.358-5.663 | 0.005 |
| Tumor size, cm (≤ 5 vs. > 5) | 2.087 | 1.066-4.088 | 0.032 | 2.233 | 1.055-4.725 | 0.036 |
| TNM (I-II vs. III-IV) | 3.505 | 1.895-6.482 | < 0.001 | 1.897 | 1.082-3.323 | 0.025 |
| Edmondson grade (I-II vs. III-IV) | 1.823 | 0.965-3.442 | 0.064 | 2.264 | 1.161-4.416 | 0.016 |
| Tumor capsula (complete vs. none) | 2.313 | 1.081-4.946 | 0.032 | 2.408 | 1.130-5.133 | 0.023 |
| Vascular invasion (present vs. absent) | 6.186 | 2.987-12.811 | < 0.001 | 5.140 | 2.527-10.454 | < 0.001 |
| AFP (≤ 20 vs. > 20 ng/ml) | 2.207 | 1.161-4.196 | 0.016 | 1.524 | 0.824-2.815 | 0.179 |

TNM (tumor-node-metastasis) status is based on primary liver cancer TNM standard 2003 of Union for International Cancer Control (UICC). Edmondson grade was short for Edmondson-Steiner grade which was used to evaluate the histologic grade of the tumor. HBsAg, hepatitis B surface antigen; AFP, alpha-fetoprotein; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; 95%CI, 95% confidence interval.

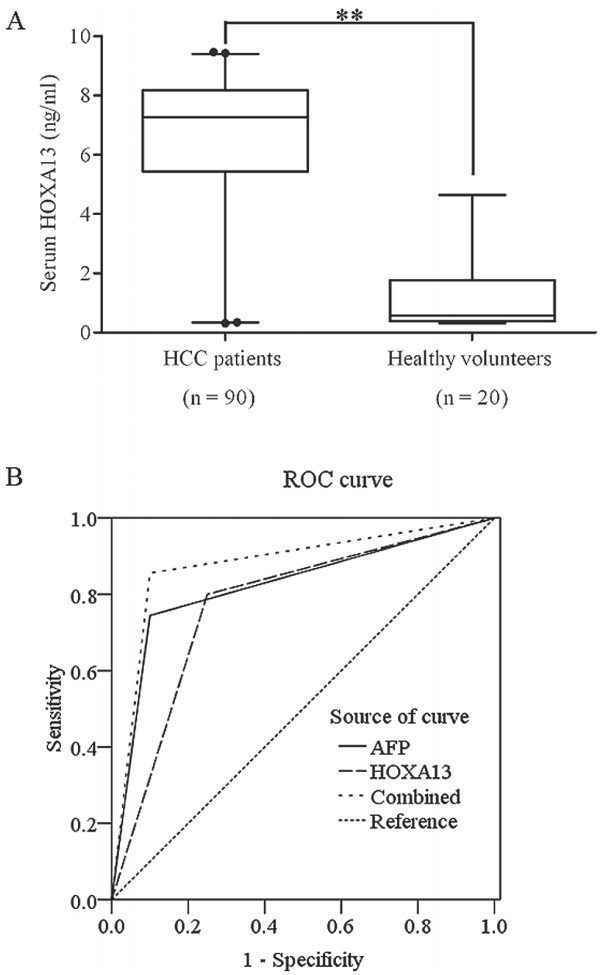


Fig. 5. Diagnostic potential of serum HOXA13 in HCC. (A) Measurement of serum HOXA13 levels in 90 HCC patients before surgery and 20 healthy individuals by enzyme-linked immunosorbent assay (ELISA). $^{**}p < 0.001$. (B) Receiver operating characteristic (ROC) curves for serum HOXA13, serum alpha-fetoprotein (AFP), and their combination in patients with HCC versus healthy controls. Combination of HOXA13 and AFP had a significant higher area under the curve (AUC) than HOXA13 and AFP alone. The addition of serum HOXA13 increased the ability of serum AFP to detect HCC.

localization. It is speculated that the cytoplasmic localization may be due to a modulation of nuclear localization signals with nuclear export and interaction with a cytoplasmic anchoring factor (Ziegelbauer et al. 2001; Haller et al. 2004; Stevens and Mann 2007; Gu et al. 2009).

By analyzing association between HOXA13 expression in HCC tissues and clinicopathological parameters, we found that the expression of HOXA13 was significantly associated with numerous parameters of HCC, including larger tumor size, portal vein invasion present, advanced Edmondson grade, incomplete tumor capsula, higher level of serum AFP and advanced TNM stage. These data indicate that HOXA13 plays an important role in HCC survival and metastasis.

In tumors, angiogenesis is considered to be one of the essential factors underlying tumor growth and metastasis. VEGF is one of the most important proangiogenic factors in angiogenetic process (Folkman 2002). In the present study, we found that tumors with positive HOXA13 expression group expressed higher VEGF and had higher MVD than those in negative HOXA13 expression group. A significant positive correlation was found between tissue expression of HOXA13 and VEGF in HCC. These findings suggest that HOXA13 plays an important role in tumor angiogenesis in HCC via VEGF. However, more research is needed to confirm and explore the findings.

Serum tumor biomarkers are attractive potential alternative tool for surveillance and diagnosis of HCC because of many advantages including noninvasive, relative objective, and repeatable. AFP is the most widely used biomarker, but it is not specific for HCC. Raised concentrations can also be tested in patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, pregnancy, and some reproductive system diseases. Other proposed tumor biomarkers, such as des-$\gamma$-carboxyprothrombin, the ratio of glycosylated AFP to total AFP, $\alpha$-fucosidase, osteopontin, and glypican3, have also shown similar shortcomings (Forner and Bruix 2012). According to these, it is essential to identify new biomarkers which can make up

such limitation. In the present study, we first demonstrated that serum HOXA13 levels are significantly raised in HCC patients and had a similar diagnostic accuracy to serum AFP in differentiating HCC from healthy individuals. Moreover, the combination of HOXA13 and AFP had a significant higher AUC than each of them alone. These finding indicates that serum HOXA13 may be a potential diagnostic biomarker for HCC. Nevertheless, there is still a long way to go before HOXA13 can be accepted as a diagnostic tool, or an instrument for screening. Further validation studies should be done in patients with HBV, HCV infection and alcohol-related HCC. Patients with other prevalent liver cancers, such as cholangiocarcinoma, should be investigated to rule out potential sources of false-positive results (Kim et al. 2011). Further multicenter research using larger sample size is needed.

Since the final purpose of these diagnosis and treatment strategies is to improve the survival of patients, it is urgent to clarify if the promotion of carcinogenesis and growth from HOXA13 proteins exerts any effect on the survival of patients. To investigate this, we confirmed that HOXA13 was overexpressed in HCC tumor tissues, and then we used the Kaplan-Meier analysis and log-rank test for further survival analysis. We found that HCC patients with HOXA13-expressing tumors had a significantly shorter OS and DFS than those with HOXA13-negative expressed tumors. Furthermore, using the COX proportional hazards regression model, we found that high HOXA13 expression was an independent predictor of poor prognosis for both OS and DFS in HCC. These findings further verify HOXA13 was involved in HCC tumorigenesis and was a negative independent predictor for OS and DFS of patients with HCC.

A major limitation of this study is that it is a single institute study and the sample size is small. Additionally, there is a potential selection bias inherent to any retrospective study. So to confirm these findings, a prospective study with a larger cohort of patients and further investigations will still be required to explore its possible molecular mechanisms.

In conclusion, we found that HOXA13 expression is significantly higher in HCC tissues compared with corresponding paracarcinomatous tissues. HOXA13 overexpression may be associated with tumor angiogenesis in HCC and progression of HCC. HCC patients have significantly higher levels of serum HOXA13 than normal controls. And serum HOXA13, in conjunction with serum AFP, is effective in differentiating HCC from healthy individuals. HOXA13-high expression can be used as an independent predictor of poor prognosis for both OS and DFS of HCC patients after curative surgery. Because HOXA13 is involved in the tumorigenesis of HCC, it may provide a new aspect for drug development.

## Acknowledgments

This project was supported by the Programs for Science and Technology Development of Anhui Province, China (Grant No. 1106c0805028 and No. 11010402163) and Natural Science Foundation of China (Grant No. 81101877). We acknowledge two pathologists Chen Ke and Wang Xiao-Qiu for their excellent professional support.

## Conflict of Interest

The authors declare no conflict of interest.

## References

Acampora, D., D'Esposito, M., Faiella, A., Pannese, M., Migliaccio, E., Morelli, F., Stornaiuolo, A., Nigro, V., Simeone, A. & Boncinelli, E. (1989) The human HOX gene family. *Nucleic Acids Res*., **17**, 10385-10402.

Altekruse, S.F., Henley, S.J., Cucinelli, J.E. & McGlynn, K.A. (2014) Changing hepatocellular carcinoma incidence and liver cancer mortality rates in the United States. *Am. J. Gastroenterol*., **109**, 542-553.

Behne, T. & Copur, M.S. (2012) Biomarkers for hepatocellular carcinoma. *Int. J. Hepatol*., **2012**, 859076.

Burke, A.C., Nelson, C.E., Morgan, B.A. & Tabin, C. (1995) Hox genes and the evolution of vertebrate axial morphology. *Development*, **121**, 333-346.

Cantile, M., Scognamiglio, G., La Sala, L., La Mantia, E., Scaramuzza, V., Valentino, E., Tatangelo, F., Losito, S., Pezzullo, L., Chiofalo, M.G., Fulciniti, F., Franco, R. & Botti, G. (2013) Aberrant expression of posterior HOX genes in well differentiated histotypes of thyroid cancers. *Int. J. Mol. Sci*., **14**, 21727-21740.

Cillo, C. (1994) HOX genes in human cancers. *Invasion Metastasis*, **14**, 38-49.

Cillo, C., Schiavo, G., Cantile, M., Bihl, M.P., Sorrentino, P., Carafa, V., D'Armiento, M., Roncalli, M., Sansano, S., Vecchione, R., Tornillo, L., Mori, L., De Libero, G., Zucman-Rossi, J. & Terracciano, L. (2011) The HOX gene network in hepatocellular carcinoma. *Int. J. Cancer*, **129**, 2577-2587.

De Vita, G., Barba, P., Odartchenko, N., Givel, J.C., Freschi, G., Bucciarelli, G., Magli, M.C., Boncinelli, E. & Cillo, C. (1993) Expression of homeobox-containing genes in primary and metastatic colorectal cancer. *Eur. J. Cancer*, **29A**, 887-893.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. & Parkin, D.M. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer*, **127**, 2893-2917.

Folkman, J. (2002) Role of angiogenesis in tumor growth and metastasis. *Semin. Oncol*., **29**, 15-18.

Forner, A. & Bruix, J. (2012) Biomarkers for early diagnosis of hepatocellular carcinoma. *Lancet Oncol*., **13**, 750-751.

Gehring, W.J. & Hiromi, Y. (1986) Homeotic genes and the homeobox. *Annu. Rev. Genet*., **20**, 147-173.

Graham, A., Papalopulu, N. & Krumlauf, R. (1989) The murine and Drosophila homeobox gene complexes have common features of organization and expression. *Cell*, **57**, 367-378.

Gu, Z.D., Shen, L.Y., Wang, H., Chen, X.M., Li, Y., Ning, T. & Chen, K.N. (2009) HOXA13 promotes cancer cell growth and predicts poor survival of patients with esophageal squamous cell carcinoma. *Cancer Res*., **69**, 4969-4973.

Haller, K., Rambaldi, I., Daniels, E. & Featherstone, M. (2004) Subcellular localization of multiple PREP2 isoforms is regulated by actin, tubulin, and nuclear export. *J. Biol. Chem*., **279**, 49384-49394.

Hamada, J., Omatsu, T., Okada, F., Furuuchi, K., Okubo, Y., Takahashi, Y., Tada, M., Miyazaki, Y.J., Taniguchi, Y., Shirato, H., Miyasaka, K. & Moriuchi, T. (2001) Overexpression of homeobox gene HOXD3 induces coordinate expression of

metastasis-related genes in human lung cancer cells. *Int. J. Cancer*, **93**, 516-525.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. & Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin*., **61**, 69-90.

Jung, C., Kim, R.S., Lee, S.J., Wang, C. & Jeng, M.H. (2004) HOXB13 homeodomain protein suppresses the growth of prostate cancer cells by the negative regulation of T-cell factor 4. *Cancer Res.*, **64**, 3046-3051.

Kanai, M., Hamada, J., Takada, M., Asano, T., Murakawa, K., Takahashi, Y., Murai, T., Tada, M., Miyamoto, M., Kondo, S. & Moriuchi, T. (2010) Aberrant expressions of HOX genes in colorectal and hepatocellular carcinomas. *Oncol. Rep.*, **23**, 843-851.

Kim, S.E., Lee, H.C., Shim, J.H., Park, H.J., Kim, K.M., Kim, P.N., Shin, Y.M., Yu, E.S., Chung, Y.H. & Suh, D.J. (2011) Noninvasive diagnostic criteria for hepatocellular carcinoma in hepatic masses >2 cm in a hepatitis B virus-endemic area. *Liver Int*., **31**, 1468-1476.

Knosp, W.M., Scott, V., Bachinger, H.P. & Stadler, H.S. (2004) HOXA13 regulates the expression of bone morphogenetic proteins 2 and 7 to control distal limb morphogenesis. *Development*, **131**, 4581-4592.

Lewis, E.B. (1978) A gene complex controlling segmentation in Drosophila. *Nature*, **276**, 565-570.

Makiyama, K., Hamada, J., Takada, M., Murakawa, K., Takahashi, Y., Tada, M., Tamoto, E., Shindo, G., Matsunaga, A., Teramoto, K., Komuro, K., Kondo, S., Katoh, H., Koike, T. & Moriuchi, T. (2005) Aberrant expression of HOX genes in human invasive breast carcinoma. *Oncol. Rep.*, **13**, 673-679.

Quagliata, L., Matter, M.S., Piscuoglio, S., Arabi, L., Ruiz, C., Procino, A., Kovac, M., Moretti, F., Makowska, Z., Boldanova, T., Andersen, J.B., Hämmerle, M., Tornillo, L., Heim, M.H., Diederichs, S., et al. (2014) Long noncoding RNA HOTTIP/HOXA13 expression is associated with disease progression and predicts outcome in hepatocellular carcinoma patients. *Hepatology*, **59**, 911-923.

Roberts, D.J., Johnson, R.L., Burke, A.C., Nelson, C.E., Morgan, B.A. & Tabin, C. (1995) Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. *Development*, **121**, 3163-3174.

Scott, M.P. (1992) Vertebrate homeobox gene nomenclature. *Cell*, **71**, 551-553.

Shaut, C.A., Keene, D.R., Sorensen, L.K., Li, D.Y. & Stadler, H.S. (2008) HOXA13 is essential for placental vascular patterning and labyrinth endothelial specification. *PLoS Genet*., **4**, e1000073.

Siegel, R., Naishadham, D. & Jemal, A. (2013) Cancer statistics, 2013. *CA Cancer J. Clin*., **63**, 11-30.

Stevens, K.E. & Mann, R.S. (2007) A balance between two nuclear localization sequences and a nuclear export sequence governs extradenticle subcellular localization. *Genetics*, **175**, 1625-1636.

Sun, H.X., Xu, Y., Yang, X.R., Wang, W.M., Bai, H., Shi, R.Y., Nayar, S.K., Devbhandari, R.P., He, Y.Z., Zhu, Q.F., Sun, Y.F., Hu, B., Khan, M., Anders, R.A. & Fan, J. (2013) Hypoxia inducible factor 2 alpha inhibits hepatocellular carcinoma growth through the transcription factor dimerization partner 3/E2F transcription factor 1-dependent apoptotic pathway. *Hepatology*, **57**,1088-1097.

Takahashi, Y., Hamada, J., Murakawa, K., Takada, M., Tada, M., Nogami, I., Hayashi, N., Nakamori, S., Monden, M., Miyamoto, M., Katoh, H. & Moriuchi, T. (2004) Expression profiles of 39 HOX genes in normal human adult organs and anaplastic thyroid cancer cell lines by quantitative real-time RT-PCR system. *Exp. Cell Res*., **293**, 144-153.

Zhu, A.X., Duda, D.G., Sahani, D.V. & Jain, R.K. (2011) HCC and angiogenesis: possible targets and future directions. *Nat. Rev. Clin. Oncol*., **8**, 292-301.

Ziegelbauer, J., Shan, B., Yager, D., Larabell, C., Hoffmann, B. & Tjian, R. (2001) Transcription factor MIZ-1 is regulated via microtubule association. *Mol. Cell*, **8**, 339-349.