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Decreased expression of HOXB9 is related to poor overall survival in patients with gastric carcinoma

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ABSTRACT

Background: Studies have demonstrated the implication of HOXB9 in tumorigenesis, but its role in gastric carcinoma remains unknown.
Aims: To investigate the expression and prognostic value of HOXB9 in patients with gastric carcinoma.
Methods: The localization and expression of HOXB9 in gastric cancer cells lines were detected by immunofluorescence and western blot. The mRNA and protein expression level of HOXB9 was detected in subjects with gastric carcinoma and paired non-cancerous tissues. Correlation between HOXB9 expression and clinicopathological parameters, the association of HOXB9 expression with the patients' survival rate was also assessed.
Results: HOXB9 was predominantly localized in the cell nucleus. A significant decrease in HOXB9 intensity in poorly differentiated gastric cancer cells is evident ($P<0.01$). A lower mRNA and protein expression level of HOXB9 was detected in gastric carcinoma ($P<0.01$). Decreased expression of HOXB9, poorly differentiation status and the presence of lymph node metastasis predict shorter overall survival ($P<0.05$). Patients without HOXB9 expression had a lower overall survival rate ($P<0.01$). Multivariate Cox regression analysis showed HOXB9 was an independent prognostic factor in gastric carcinoma ($P<0.01$).
Conclusions: HOXB9 is down-regulation in gastric carcinoma and may be a novel prognostic marker for poorer clinical outcome for patients with gastric carcinoma.

1. Introduction

The incidence of gastric carcinoma has been continuously decreasing during the past four decades, but it remains a major health problem worldwide [1]. It has become the second most common cause of cancer-related death, with around 700,000 deaths a year [2,3]. Although great progress has been made in earlier diagnosis and target therapy recent years, survival of patients with gastric carcinoma is poor. Even in the case of curative intent, the prognosis is poor and the 5-year survival is only 20–30% [4]. Gastric carcinoma patients with the same stage of the disease present different clinical courses and have different prognosis. This heterogeneity of gastric carcinoma is present at the molecular level and has a genetic predisposition to it [5]. Evidence indicates that there are many genetic alterations and unique chromosomal rearrangements that occur in the pathogenesis of gastric carcinoma, including the involvement of mTOR, Ras/Raf kinase/ERK, and/or NF-kappaB pathways [6,7]. Other canonical oncogenic pathways, such as E2F, K-RAS, p53, and Wnt/β-catenin signalling, are also known to be deregulated with varying frequencies in gastric carcinoma [8]. Recently, some molecular-based markers [9–11] were reported to be significant prognostic factors. However, few of them have been confirmed as independent predictive factor in large cohort. Therefore, identification of a preoperative useful indicator to better understand the biological basis for gastric carcinoma progression may provide important clinically relevant insights into disease management.

The HOX genes are a family of homeodomain-containing transcription factors that determine cellular identity during development. In mammals, 39 HOX genes have been identified and organized into four paralogous clusters (A, B, C and D) located on four different chromosomes [12]. They can play key roles in embryonic development, especially in the patterning of the anterior to posterior axis, from the level of the hindbrain to the end of the spine [13]. During the last decades, several homeobox genes were

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identified in normal tissue, in different diseases and metabolic alterations [14]. Recently, numerous studies have demonstrated deregulated HOX gene expression in cancer including lung, prostate, breast, colon, bladder and thyroid cancer, and also in ovarian cancer [15–20].

HOXB9, which is a direct transcriptional target of WNT/TCF4, is part of a HOX family gene cluster [21]. It is involved in cell proliferation and differentiation, also critical for embryonic segmentation and limb patterning [22]. HOXB9 can promote breast cancer progression by leading to epithelial-to-mesenchymal transition (EMT), increasing angiogenesis and distal metastasis [23]. Recent studies have demonstrated the implication of HOXB9 in tumorigenesis. Several studies have suggested that HOXB9 protein is up-regulated in breast tumours [24,25], by altering the microenvironment, induces several tumorigenic phenotypes and promotes disease progression [24]. HOXB9 expression has also been reported in lung cancer cell lines [15], but its function is unknown. In fact only limited insight is available into the functional and molecular consequences of HOX gene alterations in cancer.

Since the expression of HOXB9 in gastric carcinoma is unclear, we performed not only immunofluorescence and Western blotting analysis to detect the expression of HOXB9 in gastric cancer cell lines, but also used real time polymerase chain reaction (PCR) and immunohistochemical staining in a large number of gastric carcinoma and the corresponding non-tumour mucosa tissues in the present study for the first time. Furthermore, we analysed the relationship between HOXB9 expression and the clinical features, as well as the survival of patients.

2. Materials and methods

2.1. Cell lines and culture conditions

Human gastric cancer cells lines AGS, MKN-28, SGC-7901 and MKN-45 were preserved by our institute. These cell lines were maintained in RPMI 1640 medium (Gibco) supplemented with 10% foetal bovine serum (Hyclone), penicillin (100 units/mL) and streptomycin (100 units/mL) at 37 °C and 5% CO_2 in a humidified incubator.

2.2. Confocal microscopy and Western blot analysis

In confocal microscopy experiments, AGS, MKN-28, SGC-7901 and MKN-45 cells were grown in Lab-Tek II four-well chamber slides (Thermo Fisher Scientific, USA). After overnight culture, cells were fixed, washed and permeabilized with 0.3% Triton X-100 in PBS for 10 min. Then cells were incubated with primary antibody HOXB9 (dilution 1:100, Santa Cruz Biotechnology, USA) overnight at 4 °C. The cells were incubated with Cy3-conjugated anti-Rabbit IgG (dilution 1:500 (Jackson Immuno Research, West grove, PA, USA) for 2 h at room temperature in the dark. Cell nucleus was counterstained using DAPI. Fluorescence was monitored and photographed on a confocal microscope (Thermo Fisher Scientific, USA).

As for the Western blot analysis, 35 micrograms of protein were separated on a 12% SDS-PAGE and transferred onto nitrocellulose (NC) membrane (0.45 mm, Millipore, USA). And the NC membrane was incubated with anti-HOXB9 (1:200 dilution, Santa Cruz Biotechnology, USA) and anti-β-actin (1:2000 dilution, Sigma, USA) at 4 °C overnight. The NC membrane was then incubated with horseradish peroxidase (HRP)-coupled with goat anti-Rabbit (1:2000 dilution, BIOS) at room temperature for 90 min, respectively. Then reaction products were visualized by enhanced chemiluminescence (ECL-kit, Thermo Fisher Scientific, USA).

2.3. Real time polymerase chain reaction (PCR)

Total RNA of 10 carcinomas and 10 normal gastric tissues were extracted by Trizol (Invitrogen, Carlsbad, CA). Quantification was performed with the LightCycler System II (Roche, Mannheim, Germany). The PCR reaction conditions for HOXB9 included an initial denaturation step at 95 °C for 30 s followed by 45 cycles of amplification at 95 °C for 10 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 20 s. The housekeeping gene GAPDH was quantified as internal control, and the reaction mix only without the template DNA was used as the negative control. The result of fluorescence curves represented the number of copies. Primer sequences of HOXB9 were as follows: 5′-GCTGTCTAATCAAAGACCCGGCTA-3′ as the forward primer and 5′-CTCCAGCGTCTGGTATTTGGTG-3′ as the reverse primer.

2.4. Tissue microarray and immunohistochemistry

This study was approved by the Ethics Committee of the Fourth Military Medical University, and informed consent was acquired in accordance with the Declaration of Helsinki. The gastric carcinoma microarray (Shanghai Biochip Co., Ltd.) was constructed from patient's samples acquired from the Department of Gastrointestinal Surgery of Xijing Hospital at the Fourth Military Medical University (Xi'an, China) between October 2004 and June 2006. All patients did not receive chemotherapy prior to surgery and were followed up. The microarray contains 380 gastric tissue samples, including 190 carcinomas and 190 normal gastric tissues. Histomorphology of all tumour specimens and regional lymph nodes was confirmed with haematoxylin–eosin staining according to the International Union against Cancer TNM classification. Each tissue sample comes with detailed information regarding the patient, including gender, age and tumour histotype. Table 1 summarizes the data regarding the samples.

The tissue microarray paraffin blocks were cut into 4 μm sections. The sections were heated at 70 °C for 1 h, dewaxed in xylene and dehydrated through a gradient concentration of alcohol. After retrieving and blocking the endogenous peroxidase and non-specific staining with 3% (v/v) H_2O_2 and normal goat serum, the sections were incubated with anti-HOXB9 antibody (1:100 dilution, Santa Cruz Biotechnology, USA) overnight at 4 °C. The slides were then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody for 10 min at 37 °C. Finally, the sections were visualized by DAB solution (Zhongshan Goldenbridge Biotechnology Co., Ltd.), and counterstained with haematoxylin (Zhongshan Goldenbridge Biotechnology Co., Ltd.). A slide incubated with anti-actin primary antibodies served as the positive control, and a slide maintained in 0.01 mol/L PBS (pH 7.4), instead of the primary antibody solution, served as a negative control.

2.5. Immunohistochemistry scoring criteria

The histology of the samples was examined by two histopathologists independently without knowing the clinicopathologic information. We used the scoring method of Sinicrope et al. [26] to examine the immunohistochemistry. Each case was evaluated to estimate the intensity of the cell staining and the percentage of positive tumour cells. Sections with no labelling or with fewer than 5% labelled cells were scored as 0. Sections were scored as a 1 with labelling of 5–25% of cells, as a 2 with 25–50% of cells and as a 3 with 50–75% of cells. Finally, labelling of $\geq$75% of the cells was scored as a 4. The staining intensity was scored similarly, with 0 used for negative staining, 1 for weakly positive, 2 for moderately positive and 3 for strongly positive. The final score of each sample was multiplied by the results of the intensity and extent of staining. The product of the quantity and intensity scores was calculated

Table 1
Correlation of HOXB9 expression with clinicopathologic features in gastric carcinoma.

Characteristics	No. of patients	HOXB9 staining		*P* value
		Weak	Strong	
Normal tissue	190	8(4.2)	182(95.8)	<0.001
Gastric carcinoma	190	104(54.7)	86(45.3)	
Gender				
M	102	60(58.8)	42(41.2)	0.389
F	88	44(50.0)	44(50.0)	
Age				
≥60	116	66(56.9)	50(43.1)	0.596
<60	74	38(51.4)	36(48.6)	
Histologic grade				
Well differentiated	32	2(6.3)	30(93.7)	<0.001
Moderately differentiated	72	36(50.0)	36(50.0)	
Poorly differentiated	86	66(76.7)	20(23.3)	
TNM stage				
I	46	20(43.5)	26(56.5)	0.006
II	54	18(33.3)	36(66.7)	
III	70	52(74.3)	18(25.7)	
IV	20	14(70.0)	6(30.0)	
LN metastasis				
Absence	88	36(40.9)	52(59.1)	0.012
Presence	102	68(66.7)	34(33.3)	

Histomorphology of all tumour specimens and regional lymph nodes was confirmed with haematoxylin–eosin staining according to the International Union against Cancer TNM classification; LN, lymph nodes; M, male; F, female.

such that a final score of 0–4 indicated weak expression (− to +), 4–12 indicated strong expression (++ to +++).

2.6. Statistical analysis

Statistical analysis was performed using the SPSS version 17.0 software. χ^2 test was used to determine differences in expression of HOXB9. Associations between HOXB9 expression and clinicopathological variables were analysed using the Kruskal–Wallis *H* test and Mann–Whitney *U* test. Survival curves were analysed by Kaplan–Meier method, and differences in survival distributions were evaluated by the log-rank test. Univariate Cox regression and multivariate Cox regression analysis were used to identify factors that might affect the survival status. Statistical significance was set at $p < 0.05$.

3. Results

3.1. HOXB9 expression is predominantly localized in the cell nucleus with also cytoplasmic distribution and at a lower level

We initially examined the expression and subcellular localization of HOXB9 in gastric cancer cell lines. Immunofluorescence staining showed HOXB9 was predominantly localized in the cell nucleus with also cytoplasmic distribution. When fluorescence was quantified in these cells, a significant decrease in HOXB9 intensity in SGC-7901 and MKN-45 cells is evident ($P < 0.01$) compared with MKN-28 cells (Fig. 1G–L). But while there is a trend towards decreased HOXB9 fluorescence in the AGS cells, this effect is not statistically significant (Fig. 1A–C, $P > 0.05$). To identify the expression of HOXB9 in gastric carcinoma cell lines, the protein levels of HOXB9 were determined in four gastric carcinoma cell lines by western blot. As shown in Fig. 1M, a lower expression level of HOXB9 protein was detected in AGS compared with MKN-28, lower in SGC-7901 and the lowest in MKN-45.

3.2. HOXB9 expression is decreased in gastric carcinoma

The mRNA expression levels of HOXB9 were examined by real-time PCR in primary gastric carcinoma and matched adjacent nontumorous tissues taken from other 10 patients in our hospital. We found HOXB9 was decreased in cancerous tissues (Fig. 2A). To further confirm the different HOXB9 expression levels between tumour and normal tissues, we examined HOXB9 expression in gastric carcinoma and the non-cancerous tissues by immunohistochemistry analysis. A total of 380 men and women, age distribution 50–64 years were involved in our study. Based on immunohistochemistry analysis and the hierarchical scores of the staining, we observed that HOXB9 was expressed in the cytoplasm and nucleus of gastric carcinoma cells (Fig. 2B–I). Among the 190 gastric carcinoma specimens, weak positive staining (− to +) and strong positive staining (++ to +++) were detected in 104 (54.7%) and 86 (45.3%) samples, respectively. However, the weak expression (− to +) and strong expression (++ to +++) of HOXB9 were detected in 8 (4.2%) and 182 (98.5%) cases of the paired adjacent non-cancerous tissues specimens, respectively. Compared with the paired adjacent non-cancerous tissues, HOXB9 expression in gastric carcinoma was statistically significantly ($P < 0.001$) decreased.

3.3. HOXB9 expression was associated with clinicopathological variables in gastric carcinoma

Associations between HOXB9 expression level and clinicopathological variables are summarized in Table 1. For statistical analysis, the most salient finding was the close correlation between HOXB9 expression and degrees of differentiation of gastric carcinoma. The expression of HOXB9 level decreased from well-differentiated gastric carcinoma patients to moderately differentiated and poorly differentiated patients ($P < 0.001$). With regard to TNM stage, HOXB9 expression tended to decrease from I to II stages ($P = 0.006$). In the case of metastatic status, decreased HOXB9 expression was observed in gastric carcinoma patients with lymph node metastasis ($P = 0.012$). In contrast, HOXB9 expression was not significantly affected by the gender ($P = 0.389$) or age ($P = 0.596$) of the patients.

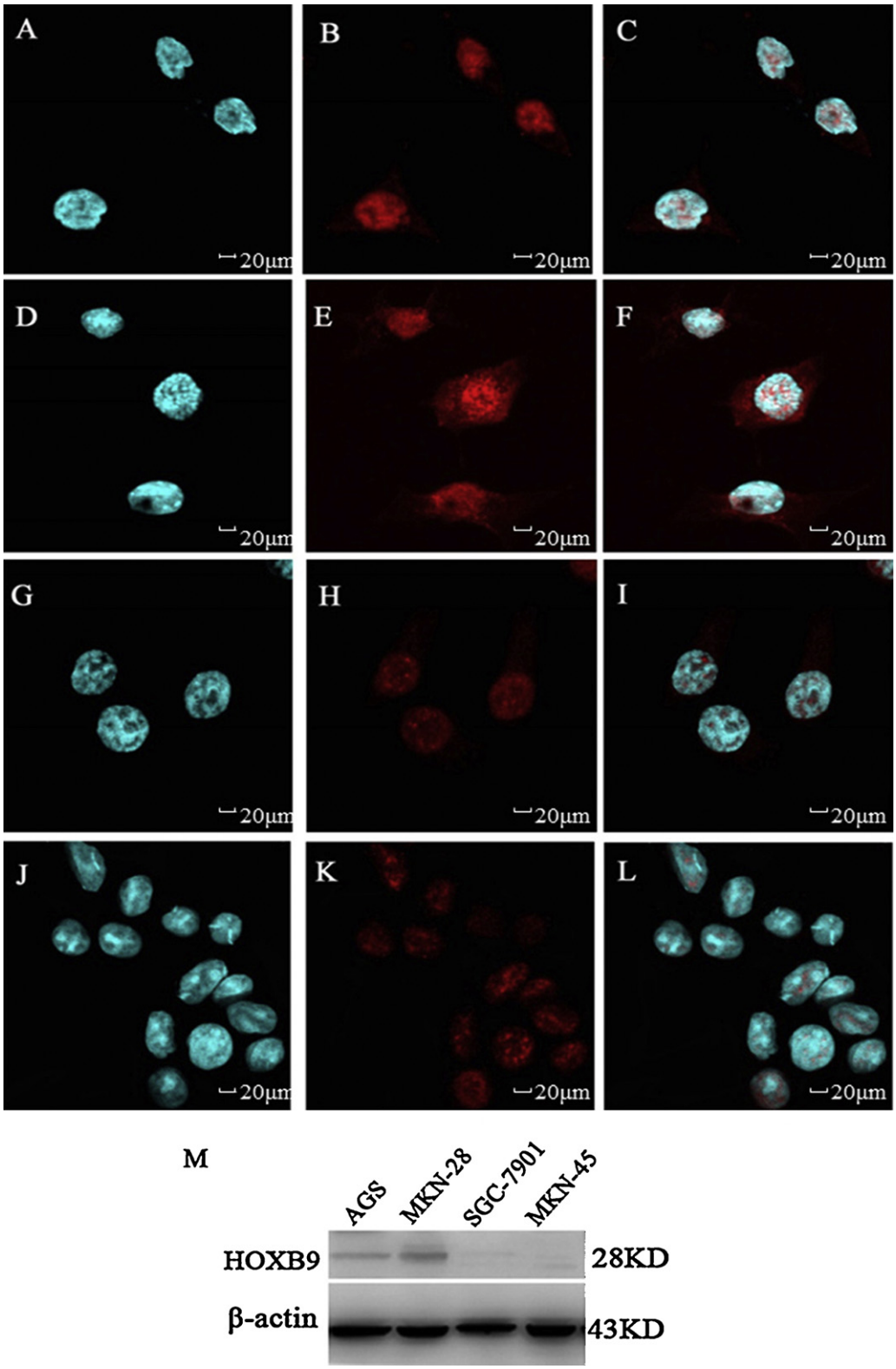

Fig. 1. Immunofluorescence staining of HOXB9 (red) was performed in four gastric cancer cell lines (AGS, MKN-28, SGC-7901 and MKN-45). Nuclei were counterstained with DAPI (blue). Merged images are showed on the right. A–C, AGS (A, DAPI; B, anti-HOXB9; C, merge); D–F, MKN-28 (D, DAPI; E, anti-HOXB9; F, merge); G–I, SGC-7901 (G, DAPI; H, anti-HOXB9; I, merge); J–L, MKN-45 (J, DAPI; K, anti-HOXB9; L, merge). HOXB9 protein is localized mainly in the cell nucleus with also cytoplasmic distribution (A–L); HOXB9 immunostaining intensity is higher in well differentiated MKN-28 (A–C). Scale bars = 20 μm. Consistent with immunofluorescence staining, protein of HOXB9 (M) was expressed at a lower level in AGS compared with MKN-28, lower in SGC-7901 and the lowest in MKN-45. β-Actin was used as internal control.

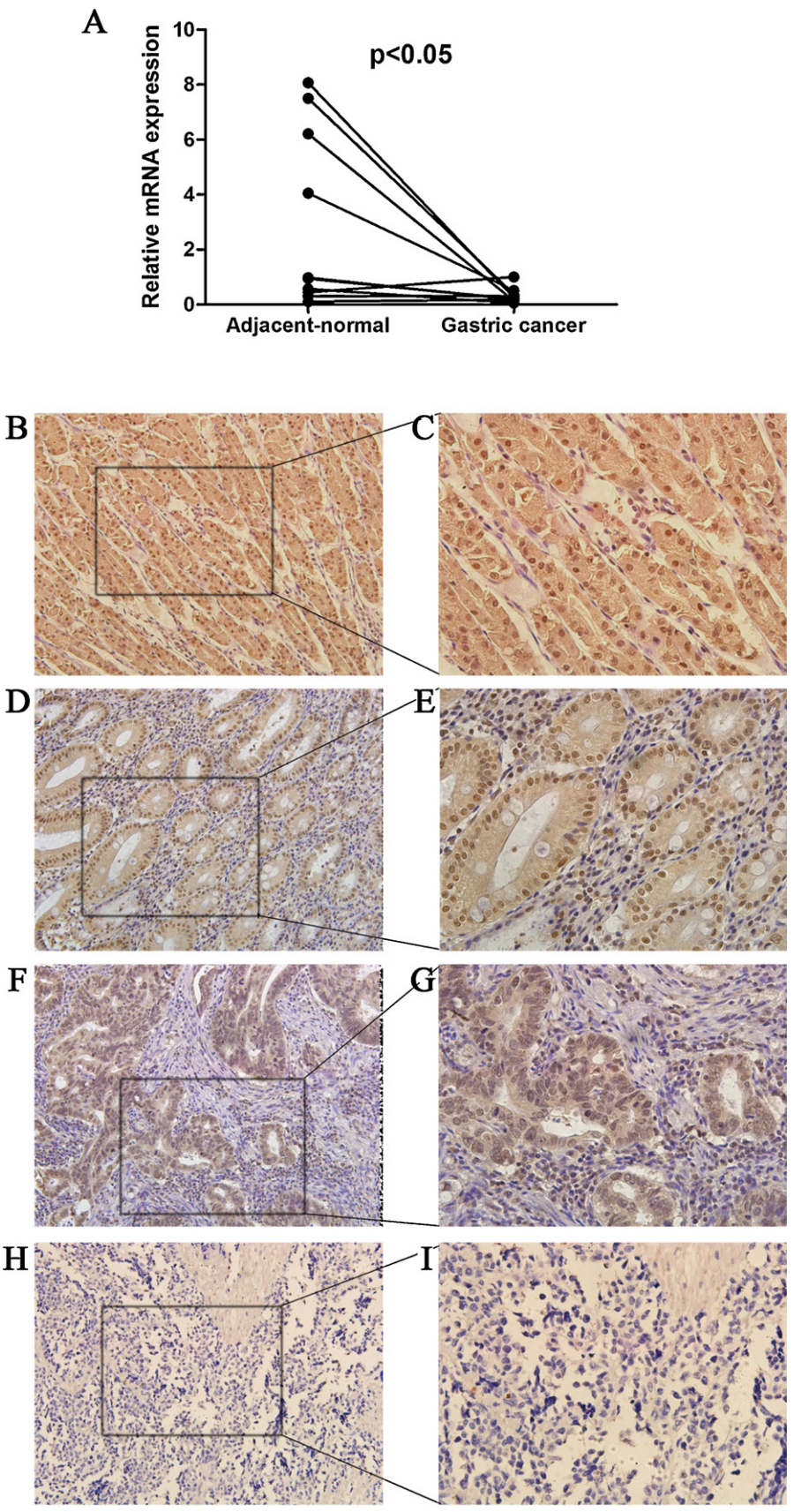

Fig. 2. Expression of HOXB9 in gastric carcinoma. Relative HOXB9 mRNA expression level in 10 subjects with gastric carcinoma and adjacent non-cancer tissues was detected by real-time PCR analysis (A). GAPDH was used as internal control. Immunohistochemical (IHC) staining was performed to examine the expression of HOXB9 in gastric carcinoma and adjacent non-cancer tissues, and the representative pictures were shown (magnification, 100× and 200×). (B) Adjacent non-cancer tissues (100×), (C) adjacent non-cancer tissues (200×), (D) well differentiated gastric carcinoma(100×), (E) well differentiated gastric carcinoma (200×), (F) moderately differentiated gastric carcinoma (100×), (G) moderately differentiated gastric carcinoma (200×), (H) poorly differentiated gastric carcinoma (100×), (I) poorly differentiated gastric carcinoma (200×).

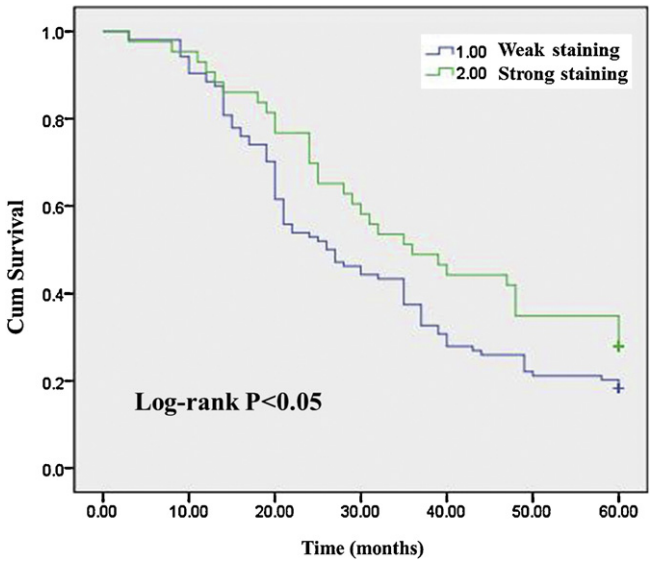

Fig. 3. Kaplan–Meier survival curves in gastric carcinoma according to HOXB9 staining. $P < 0.05$.

These observations indicated that decreased HOXB9 expression is associated with cancer malignancy.

3.4. HOXB9 expression was correlated with survival in gastric carcinoma

We reviewed clinical information of these gastric carcinoma patients. During the 5 years follow-up, 147 of the 190 patients (77.4%) had died. The postoperative median survival time of all patients was 31 months. Patients with weak positive and strong expression of HOXB9 had median survival times of 26 months and 36 months, respectively. The survival rate of patients with weak positive staining, as determined by the log-rank test, was lower than those showing strong positive staining ($P < 0.05$; Fig. 3). Univariate Cox regression analysis (Table 2) revealed that expression of HOXB9 was significantly associated with higher overall mortality. Compared with hazard ratio (HR) in the HOXB9 weak expression patients, the hazard ratio (HR) of death was 1.42-fold in patients with high expression of HOXB9. Moreover, poorly differentiated status ($P < 0.01$), TNM stage ($P < 0.001$) and the presence of lymph node metastasis also predicted shorter overall survival in gastric carcinoma ($P < 0.001$). However, no prognostic significance on overall survival was found in sex or age ($P > 0.05$).

3.5. HOXB9 expression was an independent prognostic factor for survival outcome in gastric carcinoma

Prognostic value of HOXB9 was further evaluated by multivariate Cox regression analysis. Variables clinical pathological parameters, such as histologic type, TNM stage and lymph node metastasis, were considered in the analysis. As shown in Table 3, the downregulation of HOXB9 was a significant and independent prognostic indicator for patients with gastric carcinoma. Compared to the HR in the patients with HOXB9 weak expression, the HR of death was 0.63-fold great in patients with strong staining of HOXB9 ($P < 0.01$). The Cox proportional hazards model showed that higher HOXB9 expression was associated with decreased overall mortality. In addition, histo-differentiation, TNM stage and lymph node metastasis were associated with overall mortality, but sex and age were not (Table 3).

Table 2
The effect of clinicopathologic characteristics on overall survival by Univariate Cox regression analysis.

Characteristics	No. of patients	HR (95% CI)	*P* value
HOXB9 staining			
Weak	104	1	
Strong	86	1.42 (1.03–1.98)	<0.05
Age			
≥60	116	1	
<60	74	0.85 (0.61–1.18)	0.34
Gender			
M	102	1	
F	88	0.93 (0.67–1.29)	0.67
Histologic grade			
Well differentiated	32	1	
Moderately differentiated	72	1.73 (1.04–2.88)	<0.05
Poorly differentiated	86	2.18 (1.33–3.58)	<0.01
TNM stage			
I	46	1	
II	54	1.21 (0.75–1.94)	0.43
III	70	1.98 (1.28–3.07)	<0.01
IV	20	8.89 (4.87–16.23)	<0.001
LN metastasis			
Absence	88	1	
Presence	102	3.37 (2.35–4.83)	<0.001

Histomorphology of all tumour specimens and regional lymph nodes was confirmed with haematoxylin–eosin staining according to the International Union against Cancer TNM classification; LN, lymph nodes; M, male; F, female; HR, hazard ratio.

4. Discussion

Gastric carcinoma is one of the most common cancer and a major public health problem worldwide. Data for individual countries show that gastric carcinoma is the most common cancer in Japan and the second most common in China and Korea [27–29]. Unfortunately, due to the high rate of local recurrence and early lymph node and systemic metastases, the prognosis for patients remains poor, the overall 5-year survival rate for all patients ranges from 15% to 38% [30]. Recently, some studies have shown that some pathological factors, such as the number of positive lymph nodes, the presence of extracapsular lymph node involvement, and tumour size had additional prognostic value [31]. Meanwhile, many scientists were dedicated to searching for the new prognostic factors with molecular markers involved in oncogenes, tumour-suppressor genes, cell-cycle regulators and DNA repair genes, but the results

Table 3
Multivariate Cox regression analysis for overall survival.

Characteristics	No. of patients	HR (95% CI)	*P* value
HOXB9 staining			
Weak	104	1	
Strong	86	0.63 (0.41–0.97)	<0.05
Histologic grade			
Well differentiated	32	1	
Moderately differentiated	72	1.98 (1.13–3.47)	<0.05
Poorly differentiated	86	1.91 (1.05–3.47)	<0.05
TNM stage			
I	46	1	
II	54	0.96 (0.59–1.56)	0.86
III	70	2.10 (1.29–3.43)	<0.01
IV	20	6.65 (3.59–12.31)	<0.001
LN metastasis			
Absence	88	1	
Presence	102	0.28 (0.19–0.43)	<0.001

Histomorphology of all tumour specimens and regional lymph nodes was confirmed with haematoxylin–eosin staining according to the International Union against Cancer TNM classification; LN, lymph nodes; HR, hazard ratio.

were controversial [32]. Therefore, identification of reliable molecular prognostic markers is important in gastric carcinoma, and their measurement in serum or small biopsy samples should provide important prognostic information.

HOX genes regulate several cellular processes, including angiogenesis and maintenance of cell fate [33–35]. HOXB9 is included in a cluster of Homeobox genes and the encoded protein functions as a sequence-specific transcription factor. Previous results have shown that HOXB9 expressed differentially in normal and cancer tissues. Overexpression of HOXB9 has been associated with progression and metastasis in leukaemia, paediatric Acute Myeloid Leukaemia (AML) [36], lung cancer [15], Hodgkin lymphoma (HL) cell lines [37] and breast cancer. But the role of altered HOXB9 expression in the progression of malignancies remains elusive, and the role of HOXB9 in digestive cancer invasion and metastasis is unclear. Since the possible clinical significance of HOXB9 remains unknown in gastric carcinoma patients. We examined the subcellular localization and protein expression of HOXB9 in gastric cancer cell lines, then explored the relationships between HOXB9 expression and the clinicopathologic characteristics of patients with gastric carcinoma. We found that HOXB9 was predominantly localized in the cell nucleus with also cytoplasmic distribution. The current study has, for the first time, showed that HOXB9 expression level down-regulated in poorly differentiated gastric cancer cell lines compared with well differentiated gastric cancer cells. Similar findings were obtained in gastric carcinoma tissue. HOXB9 expressions were decreased both in the mRNA and protein levels. Furthermore, HOXB9 expression was significantly affected by the degrees of differentiation, TNM stage and lymph node metastatic status of the tumour. But no significant correlations were found when the cases were stratified by age and gender. This suggested that down-regulation of HOXB9 played some role in the malignant transformation of gastric epithelial cells as a late molecular event. The TNM staging system analysed in this research is according to clinical standards, which means that the higher staging value stands for poor outcome and higher malignancy of the tumour associated with lower HOXB9 expression levels. The present study provides the first evidence that HOXB9 expression is decreased in gastric carcinoma, indicating that HOXB9 may play an inhibitory role during the development of gastric carcinoma.

To clarify the prognostic significant, we here analysed the relation of HOXB9 expression with survival of 190 patients with gastric carcinoma and revealed a link between loss and poor survival. The 5-year survival rate of patients with a high expression of HOXB9 was significantly higher than those in patients with low expression, especially in stage IV. HOXB9 expression may also be a significant and independent prognostic indicator for gastric carcinoma. Our study indicates that HOXB9 expression is reduced in gastric carcinoma, which disagrees with previous study in many other types of tumours. And the cause of the expression profiles is still not clear. Major differences in HOX gene expression are detectable in primary solid tumours (kidney, colon, breast, prostate and small cell lung cancer) compared with the corresponding normal adult organs [38]. To be summary, most cases of deregulated homeobox gene expression in cancer can be considered in three broad categories: first, homeobox genes that are normally expressed in undifferentiated cells are up-regulated in cancer; second, homeobox genes that are normally expressed in differentiated tissues are down-regulated in cancer; third, those that are not expressed in differentiated and undifferentiated tissues are expressed in cancer. HOXB9 showed tissue specificity with respect to its normal expression patterns and the consequences of its loss of function in carcinoma. Due to the tissue specificity and epigenetic loss of function, homeobox genes are best described as “tumour modulators” rather than oncogenes or tumour-suppressor genes. However, the current state of our knowledge is insufficient to establish a precise, causal relationship between HOXB9 and the specific cancer phenotypes to which they contribute, and to understand how HOXB9 genes contribute to the tissue-specific features of cancer. Therefore, there is a need for further investigating of mechanisms involved in the expression profile of the gene.

In conclusion, we tested the subcellular localization and protein expression of HOXB9 in four human gastric cancer cells lines (AGS, MKN-28, SGC-7901 and MKN-45) by immunofluorescence and western blot for the first time. We further examined HOXB9 protein expression in primary gastric carcinom and matched adjacent non-tumorous tissues by real time PCR and immunohistochemistry, and analysed their relationship with clinicopathological features. Our study has provided a basis for the development of a novel biomarker for the prognosis of gastric carcinoma. Our study suggests that decreased expression of HOXB9 in gastric carcinoma might play an important role in the progression and metastases of gastric carcinoma. We herein substantiate, in the largest series studied so far, that loss of HOXB9 expression is an independent marker of poor clinical outcome in gastric carcinoma patients.

Conflict of interest statement

There are no conflicts of interest to disclose in this paper.

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References

[1] Sano T, Sasako M, Yamamoto S, et al. Gastric cancer surgery: morbidity and mortality results from a prospective randomized controlled trial comparing D2 and extended para-aortic lymphadenectomy – Japan Clinical Oncology Group Study 9501. Journal of Clinical Oncology 2004;22:2767–73.
[2] Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. Methods in Molecular Biology 2009;472:467–77.
[3] Winer E, Gralow J, Diller L, et al. Clinical cancer advances 2008: major research advances in cancer treatment, prevention, and screening – a report from the American Society of Clinical Oncology. Journal of Clinical Oncology 2009;27:812–26.
[4] Dicken BJ, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: review and considerations for future directions. Annals of Surgery 2005;241:27–39.
[5] Cho JY, Lim JY, Cheong JH, et al. Gene expression signature-based prognostic risk score in gastric cancer. Clinical Cancer Research 2011;17:1850–7.
[6] Feng W, Brown RE, Trung CD, et al. Morphoproteomic profile of mTOR, Ras/Raf kinase/ERK, and NF-kappaB pathways in human gastric adenocarcinoma. Annals of Clinical and Laboratory Science 2008;38:195–209.
[7] Wang K, Liu R, Li J, et al. Quercetin induces protective autophagy in gastric cancer cells: involvement of Akt-mTOR- and hypoxia-induced factor 1alpha-mediated signaling. Autophagy 2011;7:966–78.
[8] Tan IB, Ivanova T, Lim KH, et al. Intrinsic subtypes of gastric cancer, based on gene expression pattern, predict survival and respond differently to chemotherapy. Gastroenterology 2011;141:476–85, 485e1–11.
[9] Wang W, Lv L, Pan K, et al. Reduced expression of transcription factor AP-2alpha is associated with gastric adenocarcinoma prognosis. PLoS One 2011;6:e24897.
[10] Wang S, Wu X, Chen Yet al. Prognostic and predictive role of JWA and XRCC1 expressions in gastric cancer. Clinical Cancer Research 2012;18:2987–96.
[11] Kim J, Min SY, Lee HE, Kim WH. Aberrant DNA methylation and tumor suppressive activity of the EBF3 gene in gastric carcinoma. International Journal of Cancer 2012;130:817–26.
[12] Garcia-Fernandez J. The genesis and evolution of homeobox gene clusters. Nature Reviews Genetics 2005;6:881–92.
[13] Kelly ZL, Michael A, Butler-Manuel S, Pandha HS, Morgan RG. HOX genes in ovarian cancer. Journal of Ovarian Research 2011;4:16.
[14] Nunes FD, de Almeida FC, Tucci R, de Sousa SC. Homeobox genes: a molecular link between development and cancer. Pesquisa Odontologica Brasileira 2003;17:94–8.
[15] Calvo R, West J, Franklin W, et al. Altered HOX and WNT7A expression in human lung cancer. Proceedings of the National Academy of Sciences of the United States of America 2000;97:12776–81.
[16] Miller GJ, Miller HL, van Bokhoven A, et al. Aberrant HOXC expression accompanies the malignant phenotype in human prostate. Cancer Research 2003;63:5879–88.
[17] Raman V, Martensen SA, Reisman D, et al. Compromised HOXA5 function can limit p53 expression in human breast tumours. Nature 2000;405:974–8.

[18] Vider BZ, Zimber A, Hirsch D, et al. Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. Biochemical and Biophysical Research Communications 1997;232:742–8.
[19] Cantile M, Cindolo L, Napodano G, Altieri V, Cillo C. Hyperexpression of locus C genes in the HOX network is strongly associated in vivo with human bladder transitional cell carcinomas. Oncogene 2003;22:6462–8.
[20] Cheng W, Liu J, Yoshida H, Rosen D, Naora H. Lineage infidelity of epithelial ovarian cancers is controlled by HOX genes that specify regional identity in the reproductive tract. Nature Medicine 2005;11:531–7.
[21] Hatzis P, van der Flier LG, van Driel MA, et al. Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Molecular and Cellular Biology 2008;28:2732–44.
[22] Abate-Shen C. Deregulated homeobox gene expression in cancer: cause or consequence? Nature Reviews Cancer 2002;2:777–85.
[23] Chiba N, Comaills V, Shiotani B, et al. Homeobox B9 induces epithelial-to-mesenchymal transition-associated radioresistance by accelerating DNA damage responses. Proceedings of the National Academy of Sciences of the United States of America 2012;109:2760–5.
[24] Hayashida T, Takahashi F, Chiba N, et al. HOXB9, a gene overexpressed in breast cancer, promotes tumorigenicity and lung metastasis. Proceedings of the National Academy of Sciences of the United States of America 2010;107:1100–5.
[25] Seki H, Hayashida T, Jinno H, et al. HOXB9 expression promoting tumor cell proliferation and angiogenesis is associated with clinical outcomes in breast cancer patients. Annals of Surgical Oncology 2012;19: 1831–40.
[26] Sinicrope FA, Ruan SB, Cleary KR, Stephens LC, Lee JJ, Levin B. bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. Cancer Research 1995;55:237–41.
[27] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA: A Cancer Journal for Clinicians 2005;55:74–108.
[28] Crew KD, Neugut AI. Epidemiology of gastric cancer. World Journal of Gastroenterology 2006;12:354–62.
[29] Leung WK, Wu MS, Kakugawa Y, et al. Screening for gastric cancer in Asia: current evidence and practice. Lancet Oncology 2008;9:279–87.
[30] Hundahl SA, Phillips JL, Menck HR. The National Cancer Data Base Report on poor survival of U.S. gastric carcinoma patients treated with gastrectomy: Fifth Edition American Joint Committee on Cancer staging, proximal disease, and the "different disease" hypothesis. Cancer 2000;88:921–32.
[31] Liu X, Xu Y, Long Z, Zhu H, Wang Y. Prognostic significance of tumor size in T3 gastric cancer. Annals of Surgical Oncology 2009;16:1875–82.
[32] Liu X, Cai H, Huang H, Long Z, Shi Y, Wang Y. The prognostic significance of apoptosis-related biological markers in Chinese gastric cancer patients. PLoS One 2011;6:e29670.
[33] Kapur RP, Gershon MD, Milla PJ, Pachnis V. The influence of Hox genes and three intercellular signalling pathways on enteric neuromuscular development. Neurogastroenterology and Motility 2004;16(Suppl. 1):8–13.
[34] Cantile M, Schiavo G, Terracciano L, Cillo C. Homeobox genes in normal and abnormal vasculogenesis. Nutrition, Metabolism and Cardiovascular Diseases 2008;18:651–8.
[35] Duboule D. The rise and fall of Hox gene clusters. Development 2007; 134:2549–60.
[36] Danen-van Oorschot AA, Kuipers JE, Arentsen-Peters S, et al. Differentially expressed miRNAs in cytogenetic and molecular subtypes of pediatric acute myeloid leukemia. Pediatric Blood and Cancer 2012;58:715–21.
[37] Nagel S, Burek C, Venturini L, et al. Comprehensive analysis of homeobox genes in Hodgkin lymphoma cell lines identifies dysregulated expression of HOXB9 mediated via ERK5 signaling and BMI1. Blood 2007;109:3015–23.
[38] Takahashi Y, Hamada J, Murakawa K, et al. Expression profiles of 39 HOX genes in normal human adult organs and anaplastic thyroid cancer cell lines by quantitative real-time RT-PCR system. Experimental Cell Research 2004;293:144–53.