

HOXB13 expression and promoter methylation as a candidate biomarker in gastric cancer

BAI-QIANG SUI, CHUN-DONG ZHANG, JI-CHAO LIU, LEI WANG and DONG-QIU DAI

Department of Gastrointestinal Surgery, The Fourth Affiliated Hospital of China Medical University,
Shenyang, Liaoning 110032, P.R. China

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Abstract. Homeobox b13 (HOXB13) is considered to be a tumor suppressor gene in multiple types of human cancer. The present study aimed to identify the difference in expression of HOXB13 mRNA between gastric cancer (GC) tissues and corresponding non-malignant gastric tissues. The clinical significance of HOXB13 mRNA expression was also assessed in GC and a potential association between HOXB13 mRNA expression and DNA promoter methylation was observed. The expression of HOXB13 mRNA was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and HOXB13 methylation status was assessed by methylation-specific PCR (MSP) in 5 GC cell lines and 85 paired GC and normal gastric tissues. Kaplan-Meier survival curves were used to assess the survival of patients with GC. HOXB13 mRNA expression was significantly lower in primary GC tissues than in corresponding nonmalignant gastric tissues, and decreased HOXB13 expression was associated with poorer differentiation, lymph node metastasis, invasion depth and Tumor-Node-Metastasis (TNM) stage. Kaplan-Meier survival analysis demonstrated that HOXB13 mRNA expression was a significant prognostic indicator of GC patient survival. Furthermore, MSP revealed that the proportion of GC samples with hypermethylated HOXB13 (60.0%, 51/85) was increased compared with the corresponding nonmalignant gastric tissues (11.8%, 10/85). Decreased HOXB13 mRNA expression was due to DNA hypermethylation as following treatment with the DNA methyltransferase inhibitor 5-Aza-dC, HOXB13 expression in the GC MKN-45 cell line was upregulated. The results of the present study indicate that decreased expression of HOXB13 mRNA was associated with tumor differentiation, depth of invasion, lymph node metastases and TNM stage in

GC, and it was a significant poor prognostic factor for patients with GC. Aberrant DNA promoter methylation was a crucial reason for the downregulation of HOXB13 mRNA expression.

Introduction

Gastric cancer (GC) is the fourth most common malignant disease globally and is the second highest mortality rate due to cancer globally (1). Owing to the poor response to treatment observed in patients with advanced-stage GC, the 5-year overall survival (OS) rate is in the range of 25-30% worldwide (2). Therefore, it is necessary to diagnose GC at an early stage. Understanding the molecular mechanisms underlying GC development is also essential for determining methods to inhibit tumor progression.

The homeobox B13 (HOXB13) gene belongs to the HOX family, which are known to function in encoding nuclear transcription factors involved in establishing and maintaining patterns of differentiation during development (3). The HOXB13 gene, which is located in chromosomal region 17q21.2, encodes a 31-kDa protein (4). The 5' end of HOXB13 contains two CpG islands, one in the promoter/exon1 and the other 4.5 kb upstream of the transcription start site, suggesting that HOXB13 gene expression may be controlled by DNA methylation (5). Mice with loss-of-function mutations in HOXB13 exhibited an overgrown-tail phenotype, with increased cell proliferation and decreased apoptosis in the tail; this evidence indicated that HOXB13 inhibited proliferation and activated programmed cell death (6). Overexpression of HOXB13 inhibited prostate cell proliferation, inducing G1-phase cell cycle arrest as mediated by downregulation of T-cell factor-4 (TCF-4) expression (7). HOXB13 also downregulated the expression of TCF-4 at the protein level, suppressing the growth of colorectal cancer cells (8). HOXB13 also serves diverse biological functions in embryonic development and terminally differentiated tissue (9,10).

Many studies have identified HOXB13 as a candidate tumor suppressor gene in several types of cancer, including colorectal cancer (8), renal cancer (11), melanoma (12) and breast cancer (13). Additionally, hypermethylation of the HOXB13 gene promoter was a potential mechanism for decrease expression (8,13-15). Other studies, however, revealed that HOXB13 was overexpressed in numerous types of tumor, potentially contributing to carcinogenesis and

Correspondence to: Professor Dong-Qiu Dai, Department of Gastrointestinal Surgery, The Fourth Affiliated Hospital of China Medical University, 4 Chongshan East Road, Shenyang, Liaoning 110032, P.R. China
E-mail: daidq63@126.com; daidq63@163.com

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tumor progression in prostate (16), breast (17), ovarian (18,19), cervical (20) and oral cancer (21). The HOXB13 germline variant G84E mutation may be involved in promoting tumor progression, with the function of HOXB13 depending on the tissue in which expressed (22,23). Although previous studies have demonstrated that HOXB13 is a contrasting biomarker for tumor development in numerous types of cancer, the function of HOXB13 in gastric cancer is unclear (8,11-21).

To understand the function of the HOXB13 gene in GC, the present study detected the expression levels of HOXB13 mRNA in gastric cancer tissues and corresponding non-malignant gastric tissues and then evaluated the association between HOXB13 mRNA expression and survival time. The methylation status of HOXB13 in GC cell lines and GC tissues was also detected, and then the association between its expression and DNA methylation was assessed.

Materials and methods

Patients and tissue samples. The present study included 85 patients in total, which included 45 males and 40 females. The average age was 61 years old and the age range was 35-75. All patients underwent gastrectomy between September 2007 and August 2012 at the Fourth Affiliated Hospital of China Medical University (Shenyang, China). Gastric tissue specimens and corresponding nonmalignant gastric tissues were collected following tumor excision during gastrectomy and were diagnosed by at least two pathologists. Tumor classification was performed according to the Tumor-Node-Metastasis (TNM) grading system, seventh edition (24). Following this, patients were monitored periodically and their tumor marker levels, including carcinoembryonic antigen and carbohydrate antigen 19-9 assessed, their blood tested, chest and abdominal computed tomography images captured and gastroscopy performed. OS rates were defined as the time from surgery to mortality or the last follow-up. The final follow-up date was February 2016. The median duration of follow-up was 26 months (range 5-90 months). The present study was approved by the Research Ethics Committee of China Medical University (Shenyang, China). All patients provided written informed consent to participate in this research.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from tissues or cultured cells. cDNA was synthesized from RNA by using an Expanding Reverse Transcriptase kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. qPCR was used to detect the expression of HOXB13 mRNA in a reaction volume of 25 μ l, including 12.5 μ l SYBR Green (Takara Bio, Inc.), 2 μ l cDNA, 1 μ l of each primer and 8.5 μ l diethyl pyrocarbonate water. The mixture was incubated by the following program: 95°C for 30 sec, 40 cycles of 95°C for 5 sec, 60°C for 32 sec. The primers used were: HOXB13 forward, 5'-TGTTGCCAGGGAGACAACAAC-3' and reverse, 5'-CGTGTGGAGTCTGCAAATGCT-3' (25); and β -actin (ACTB) forward, 5'-TGGCACCCAGCACAATGA A-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGC A-3'. For each PCR, diethyl pyrocarbonate water was used as a negative control. The expression level of HOXB13 mRNA was

standardized to the ACTB mRNA expression level, and data was quantified using the $2^{-\Delta\Delta C_q}$ method (26).

Gastric cells and culture. The SGC-7901, BGC-823, MGC-803, MKN-45 and AGS GC cell lines, and the immortalized normal gastric GES-1 cell line (as the control), were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 (Invitrogen; Thermo Fisher Scientific, Inc.). Cell lines were cultured with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator with 5% CO₂.

Western blot analysis. Total protein in cultured cells and tissue specimens was extracted using the Qproteome Mammalian Protein Prep kit (Qiagen GmbH, Hilden, German) and protein concentration was determined with a BCA Protein Assay kit (Bio-Rad, Milan, Italy). Protein samples were denatured by boiling for 5 min and 30 μ g protein samples were electrophoresed by 12% SDS-PAGE. The protein samples were then transferred to polyvinylidene fluoride membranes. Following blocking with blocking buffer (5% skim milk in 50 mM Tris-HCl, 200 mM NaCl and 0.05% Tween-20, pH 7.5) for 2 h at room temperature, membranes were incubated overnight at 4°C with the HOXB13 antibody (1:1,000; cat. no. ab28575) or ACTB antibody (cat. no. ab6276; both Abcam, Cambridge, MA, USA). The next day, those membranes were washed three times with PBS and incubated for 120 min at room temperature with a horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. A0545; 1:1,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following washing, the immunoreactive protein bands were visualized using an Electrochemiluminescence Detection kit (cat. no. P0018; Beyotime Institute of Biotechnology, Haimen, China). Each experiment was repeated at least three times.

DNA extraction and methylation-specific PCR (MSP). Total DNA was extracted from MKN-45 and tissue specimens using the Takara Universal Genomic DNA Extraction kit Ver.3.0 (Takara Bio, Inc.), according to the manufacturer's protocol. The EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) was used to perform bisulfite conversion for subsequent methylation analysis. The primers used for MSP were complementary to the promoter region of the HOXB13 gene. The primers were as follows: Methylated HOXB13 CpG islands forward, 5'-TATTTTGGATGGAGTTAAGGA TATC-3' and reverse, 5'-ATAATTAACAACAAACATCAA CGTA-3'; and unmethylated HOXB13 CpG islands forward, 5'-ATTTTGGATGGAGTTAAGGATATTG-3' and reverse, 5'-CATAATTAACAACAAACATCAACATA-3' (27). The reaction mixture was in a volume of 50 μ l containing 1 μ l DNA, 2X GC buffer I, 1 μ l of each primer, 2.5 mM dNTP Mix and 2.5 U LA Taq polymerase (Takara Bio, Inc.). The MSP conditions were as follows: 94°C for 5 min, 40 cycles of 94°C for 30 sec, 54°C for 30 sec, 72°C for 45 sec and 72°C for 10 min. The GES-1 cell line and peripheral blood cell-derived DNAs treated with the CpG methyltransferase SssI (New England Biolabs, Ipswich, MA, USA) were used as negative and positive controls respectively. All procedures were repeated at least three times. The PCR products were subjected to 2% agarose

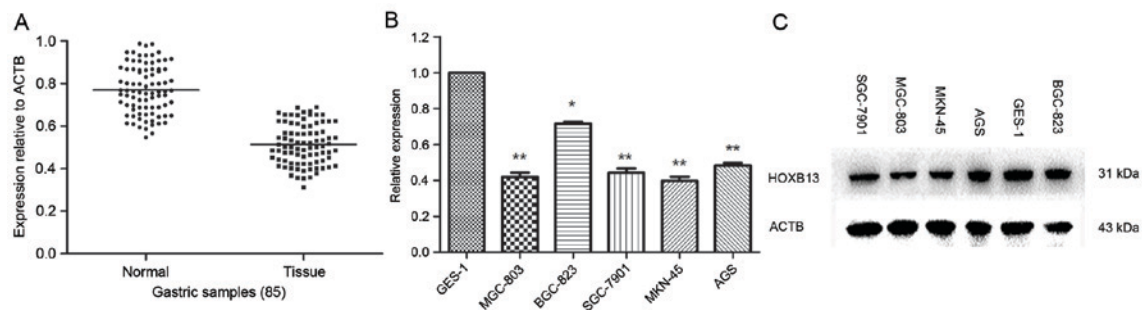


Figure 1. HOXB13 expression in GC tissues and GC cell lines. (A) HOXB13 mRNA expression in 85 gastric specimens and corresponding nonmalignant gastric tissues. (B) HOXB13 mRNA expression in five GC cell lines and GES-1. (C) HOXB13 protein expression in five GC cell lines and GES-1. * $P<0.05$, ** $P<0.01$ vs. GES-1. GC, gastric cancer; HOXB13, homeobox B13; ACTB, β -actin.

gel electrophoresis at 120 V for 40 min and quantified using the Fluor Chen 2.0 system.

5-Aza-cytidine treatment. MKN-45 cells were plated at a density of 5×10^5 cells per well into 6-well cell culture plates and incubated at 37°C in a humidified incubator with 5% CO_2 . Following culturing overnight, 0, 5 or 10 $\mu\text{mol/l}$ 5-Aza-dC (Sigma-Aldrich; Merck KGaA) was added and cells were incubated for 3 days, with 1.0 $\mu\text{mol/l}$ trichostatin A (TSA; Beyotime Institute of Biotechnology) added on the final day at room temperature. Next, total RNA and DNA was extracted for detecting HOXB13 mRNA and protein expression levels as aforementioned.

Statistical analysis. All statistical analysis was performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Paired Student's t-test was used to analyze the association between HOXB13 mRNA expression and clinicopathological features. Kaplan-Meier curves and log-rank tests were used to estimate the influence of HOXB13 mRNA expression on OS. Cox's proportional hazard model was used to assess hazard ratios and corresponding 95% confidence intervals. One-way analysis of variance (ANOVA) was used to assess the associations between HOXB13 expression and methylation status. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of HOXB13 mRNA expression in gastric cell lines and tissues. HOXB13 mRNA expression levels were analyzed in 85 GC tissues and corresponding non-malignant tissues. RT-qPCR revealed that the expression of HOXB13 mRNA in GC tissues was distinctly lower than those in corresponding non-malignant tissues (Fig. 1A). The expression of HOXB13 mRNA and protein levels were further examined by RT-qPCR and western blotting in six gastric cell lines (five cancer, one non-cancer). The results which used Student's t-test revealed that HOXB13 mRNA and protein expression levels were significantly lower in three of the five GC cells lines compared with GES-1 (the non-cancer control; Fig. 1B and C). It was worth noting that HOXB13 protein expression levels were lower in MGC-803 and MKN-45 cells compared with GES-1 cells in Fig. 1C. As there may exist translation level

adjustment, protein expression levels are not necessarily in accordance with mRNA expression levels.

Association between expression of HOXB13 mRNA and clinicopathological features. The associations between HOXB13 mRNA expression and the clinicopathological factors of 85 patients with GC were assessed. As presented in Table I, HOXB13 mRNA expression was significantly associated with tumor differentiation ($P=0.008$), tumor invasion depth ($P=0.027$), the presence of lymph node metastases ($P=0.016$) and TNM stage ($P=0.007$). However, HOXB13 mRNA expression was not associated with the clinicopathological features tested, including age, sex, tumor location, tumor size and Borrmann type (Table I) (28).

Expression of HOXB13 mRNA relative to prognosis. Kaplan-Meier survival curves were constructed to assess the prognostic significance of HOXB13 mRNA expression and various clinicopathological features (Table II). mRNA expression levels in GC tissues were distinguished by comparing with the mean expression quantity (0.771) of HOXB13 mRNA in 85 non-malignant gastric tissues. A total of 22 patients expressed higher quantities and 63 patients expressed lower quantities of HOXB13. HOXB13 mRNA expression was inversely associated with 5-year OS. In patients expressing low amounts of HOXB13 mRNA, OS rate was 42.9%, which was lower compared with the 5-year OS rate of 68.2% (Log-rank test, $P<0.05$) in patients expressing high levels of HOXB13. Similarly, tumor differentiation, invasion depth, Borrmann type and TNM stage were revealed to be associated with OS rates. Cox multivariate analysis was performed on expression of HOXB13 mRNA, invasion depth, differentiation, Borrmann type and TNM stage. This analysis revealed that HOXB13 mRNA expression, invasion depth, differentiation and Borrmann type all acted as independent prognostic factors for OS (Table III and Fig. 2).

Promoter methylation status of HOXB13 gene in gastric cell lines and tissues. The methylation status of HOXB13 gene promoter CpG islands was assessed by MSP. The data revealed that the HOXB13 gene promoter was hypermethylated in BGC823, AGS, MKN45 and SGC-7901 cells, partially methylated in MGC-803 cells, and unmethylated in GES1 cells (Fig. 3A). Hypermethylation of the HOXB13 gene promoter was observed in 60% (51/85) of gastric cancer tissue

Table I. Associations between the clinicopathological features and HOXB13 mRNA expression.

Variable	Patients (n)	HOXB13 mRNA level ^a	P-value
Non-malignant tissues	85	0.771±0.114	<0.001 ^b
Tumor tissues	85	0.514±0.095	
Age, years			0.200
≥65	42	0.535±0.088	
<65	43	0.508±0.102	
Sex			0.165
Male	45	0.508±0.095	
Female	40	0.537±0.095	
Location			0.738
Upper/middle	38	0.517±0.100	
Lower	47	0.524±0.092	
Size, cm			0.216
<5	41	0.508±0.930	
≥5	44	0.534±0.098	
Borrmann type			0.077
I+II	30	0.546±0.095	
III+IV	55	0.508±0.094	
Differentiation			0.008 ^b
Well/moderate	39	0.551±0.087	
Poor	46	0.496±0.097	
Invasion depth			0.027 ^b
T1+T2	36	0.548±0.091	
T3+T4	49	0.502±0.095	
TNM stage			0.007 ^b
I+II	34	0.556±0.095	
III+IV	51	0.499±0.090	
Lymph node metastasis			0.016 ^b
No	32	0.554±0.083	
Yes	53	0.502±0.099	

^aData presented as the mean ± standard deviation. ^bP<0.05. HOXB13, homeobox B13; TNM, Tumor-Node-Metastasis.

specimens (Fig. 3B), but in only 11.8% (10/85) of corresponding non-malignant gastric tissues. The 10 hypermethylated adjacent non-malignant gastric tissues had postoperative pathological diagnoses of intestinal metaplasia or hyperplasia.

5-Aza-dC treatment induces upregulated expression of HOXB13. To assess whether methylation of the HOXB13 gene promoter was responsible for downregulation of HOXB13 expression, the cell line with the lowest expression (MKN-45) was treated with the DNA methylation inhibitor 5-Aza-dC and the histone deacetylase inhibitor TSA. The results which were assessed by one-way ANOVA demonstrated that 5-Aza-dC upregulated HOXB13 expression, with the highest expression occurring at a concentration of 15 μ M 5-Aza-dC. HOXB13 expression was also restored with a combination treatment of 5-Aza-dC and TSA, but this was only slightly higher than that induced by 5-Aza-dC alone (P<0.05; Fig. 4A). HOXB13 protein expression was also increased in MKN-45 cells following 5-Aza-dC treatment (Fig. 4B).

Association between expression of HOXB13 mRNA and methylation status. One-way ANOVA was used to assess the association between HOXB13 mRNA expression and DNA methylation status in 85 GC tissues: mRNA expression of HOXB13 was significantly lower in tissues with methylated promoter regions than in tissues with partial or no methylation. However, there was no difference in expression of HOXB13 between tissues with partial HOXB13 promoter methylation and no HOXB13 promoter methylation. Therefore, low HOXB13 expression was associated with DNA hypermethylation.

Discussion

DNA methylation is a heritable epigenetic alteration that does not alter the DNA nucleotide sequence but is involved in transcriptional repression (29). Methylation of DNA at promoter CpG islands where transcription is initiated leads to the silencing of tumor suppressor genes, which contributes directly to cancer development (30,31). Previous studies have

Table II. Univariate analysis of survival in gastric cancer cases, assessed using the log rank test.

Variable	5-year OS	P-value
Age, years		0.499
≥65	41.9	
<65	38.1	
Sex		0.409
Male	40.0	
Female	40.0	
Location		0.701
Upper/middle	44.7	
Lower	42.5	
Size, cm		0.763
<5	38.6	
≥5	41.5	
Borrmann type		<0.001 ^a
I+II	73.3	
III+IV	21.8	
Differentiation		<0.001 ^a
Well/moderate	66.7	
Poor	17.4	
Invasion depth		<0.001 ^a
T1+T2	66.7	
T3+T4	20.4	
Lymph node metastasis		0.072
No	53.1	
Yes	32.1	
TNM stage		0.021 ^a
I+II	50.0	
III+IV	33.3	
HOXB13 mRNA expression		0.010 ^a
High	68.2	
Low	42.9	

^aP<0.05. HOXB13, homeobox B13; TNM, Tumor-Node-Metastasis.

revealed that numerous tumor suppressor genes undergo aberrant DNA methylation in GC (32,33).

HOXB13 gene expression was observed during the late development of the tailbud and posterior of mouse embryos and HOXB13 expressed in the spinal, digestive and urogenital system in humans (34). A number of studies have revealed a decrease in HOXB13 expression in colorectal cancer (5), renal cancer (11), melanoma cancer (12) and breast cancer (13), making it a candidate tumor suppressor gene. Promoter hypermethylation of the HOXB13 gene is considered to be a potential mechanism for decreased expression (13-15,34).

In the present study, HOXB13 mRNA expression was revealed to be decreased in five GC cell lines, compared with the normal gastric cell line GES-1; HOXB13 mRNA expression was also significantly lower in GC tissues than in non-malignant gastric tissues. These data demonstrated

that HOXB13 may act as a candidate tumor suppressor in GC. In the 22 GC cases where HOXB13 mRNA expression was higher than the mean expression quantity in adjacent nonmalignant gastric tissues, the HOXB13 promoter was unmethylated in 12 cases and partially methylated in 10 cases (none were hypermethylated). This result indicated that the expression of HOXB13 mRNA in GC was markedly affected by DNA methylation. HOXB13 mRNA expression was associated with tumor differentiation, invasion depth, lymph node metastasis and TNM stage. Notably, the expression of HOXB13 mRNA in poorly differentiated GC tissues was lower than that in well/moderately differentiated GC tissues. Poorly differentiated GC cells possess higher malignancy than well/moderate differentiated cells, indicating that the decrease in HOXB13 mRNA expression occurs in more malignant cases of GC. HOXB13 mRNA expression is lower in GC tissues than adjacent non-malignant gastric tissues, which, along with later TNM stage and deeper invasion depth, means greater disease progression of GC. The expression of HOXB13 mRNA in GC with lymph node metastasis is significantly lower than GC without lymph node metastasis. Marra *et al* (35) reported that the loss of HOXB13 expression in non-muscle-invasive bladder transitional cancer is significantly associated with shorter disease-free survival. The results of a log-rank test revealed that there were significant differences in OS between patients of HOXB13 mRNA low expression and patients of HOXB13 mRNA high expression. The results of Cox proportional hazards model analysis demonstrated that expression of HOXB13 mRNA was an independent prognostic marker for OS. Decreased HOXB13 mRNA expression may therefore act as a potential predictor of the degree of GC malignancy and overall disease prognosis, results that are also supported by Okuda *et al* (14).

A previous study revealed that epigenetic mechanisms, including DNA methylation, were associated with tumor development and progression (36). Promoter hypermethylation was the mechanism underlying the decreased expression of HOXB13. In the present study, the HOXB13 gene was hypermethylated in all but one GC cell lines, and partially methylated in MGC-803. Aberrant methylation of the HOXB13 promoter was also observed in GC tissue samples. Hypermethylation of the HOXB13 gene promoter was identified in 51 (60.0%) of the 85 gastric tissues, while methylation in was only detected in 10 non-malignant adjacent gastric tissues (11.8%). Additionally, adjacent nonmalignant gastric tissues in hypermethylation status may be associated with tissue accompanied with precancerous lesions. To examine the association between the methylation status of the HOXB13 promoter and HOXB13 expression, a one-way ANOVA was used. The results of this analysis demonstrated that expression of HOXB13 in GC tissues with a hypermethylated HOXB13 promoter was markedly lower compared with the expression of HOXB13 in GC tissues with partially methylated or unmethylated HOXB13 promoters. However, there was no marked difference between the expression of HOXB13 in GC tissues with partially methylated promoter and the expression of HOXB13 in GC tissues with unmethylated promoters. These data revealed that low HOXB13 expression was caused by DNA hypermethylation and indicated that the methylation of HOXB13 may enhance the degree of malignancy of GC.

Table III. Multivariate analysis of survival in gastric cancer cases, assessed using the log rank test.

Variable	B	SE	P-value	HR (95% CI)
Borrmann type	1.068	0.267	<0.001 ^a	2.901 (1.723-4.914)
Differentiation	0.918	0.259	<0.001 ^a	2.504 (1.506-4.163)
Invasion depth	0.930	0.274	0.001 ^a	2.535 (1.480-4.341)
TNM stage	0.062	0.147	0.675	1.063 (0.798-1.418)
HOXB13 mRNA expression	-0.699	0.312	0.025 ^a	0.497 (0.270-0.915)

^aP<0.05. HOXB13, homeobox B13; B, regression coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval; TNM, Tumor-Node-Metastasis.

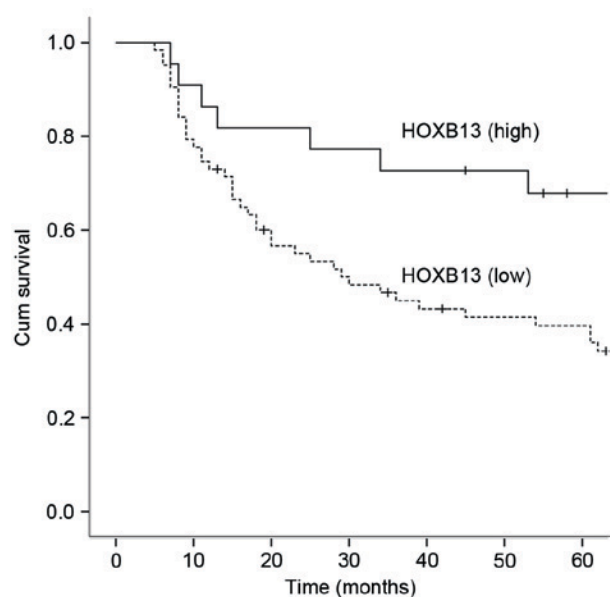


Figure 2. Univariate survival analysis of HOXB13 mRNA expression in gastric cancer. Kaplan-Meier curves for cumulative survival rate stratified by HOXB13 mRNA expression. A direct association between low expression of HOXB13 mRNA and a poor survival is presented. HOXB13, homeobox B13.

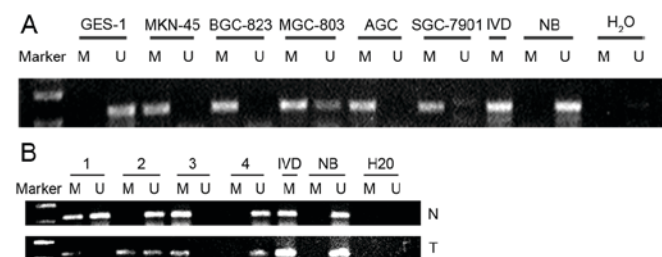


Figure 3. Methylation status of the HOXB13 gene in GC cell lines and GC tissues. (A) HOXB13 gene promoter was hypermethylated in BGC-823, AGS, MKN-45 and SGC-7901 cells, partially methylated in MGC-803 cells and unmethylated in GES1 cell. (B) HOXB13 gene promoter methylation status in GC tissues and corresponding nonmalignant gastric tissues. IVD was used as a positive control for methylation status, and NB samples were used as non-methylation positive control. GC, gastric cancer; HOXB13, homeobox B13; IVD, *in vitro* methylation DNA; NB, normal blood; M, methylated; U, unmethylated.

To verify the association between methylation of HOXB13 and HOXB13 expression further, 5-Aza-dC and TSA were used to treat the MKN-45 GC cell line, which had the lowest HOXB13

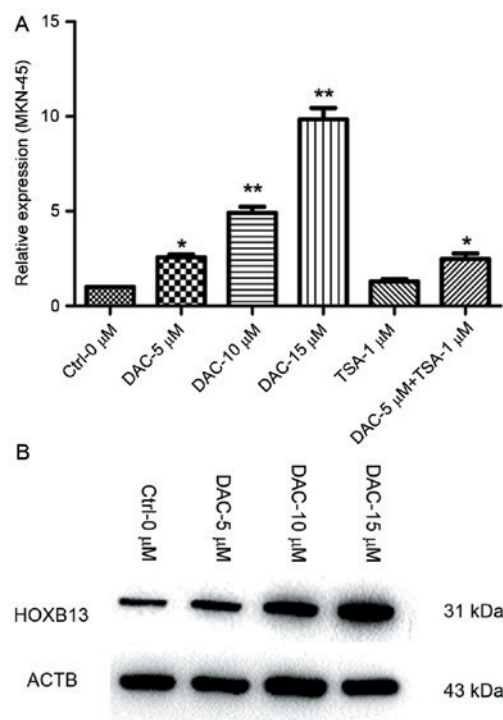


Figure 4. Effect of HOXB13 expression in the MKN-45 gastric cancer cell line following treatment with 5-Aza-dC. HOXB13 (A) mRNA and (B) protein expression in MKN45 cells following treatment with 5-Aza-dC (5, 10 or 15 μ M), TSA (1.0 μ M), or 5-aza-dC (5 μ M) combined with TSA (1 μ M). *P<0.05 and **P<0.01 vs. Ctrl. HOXB13, homeobox B13; TSA, trichostatin A; Ctrl, control.

of all tested cell lines. The results demonstrated that 5-Aza-dC enhanced HOXB13 RNA and protein expression, but TSA had a weaker effect on HOXB13 expression. Additionally, the expression of HOXB13 was positively associated with 5-Aza-dC concentration. This result further demonstrated that expression of HOXB13 was regulated by DNA hypermethylation.

In conclusion, the present study indicated that HOXB13 was downregulated in GC, which was caused by DNA hypermethylation. HOXB13 mRNA expression was associated with tumor differentiation, invasion depth, lymph node metastases and TNM stage, and may be a potential prognostic factor for GC. The present study demonstrated that HOXB13 is a tumor suppressor gene in GC and has potential as a prognostic biomarker and as a target for pharmacological intervention. Further studies *in vivo* are necessary to determine the involvement of HOXB13 in GC progression.

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Availability of data and materials

The data and materials used analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DQD was responsible for the design of the experiment and analysis and interpretation of data. BQS, CDZ, LW and JCL carried out the acquisition of data. BQS and CDZ were involved in drafting the manuscript and revising it critically for important intellectual content. All authors provided final approval of the version to be published.

Ethics approval and consent to participate

Research involving human subjects, human material or human data had been performed in accordance with the Declaration of Helsinki and had been approved by Ethics committee of the Fourth Affiliated Hospital of China Medical University. All participants consented to participate in this study.

Consent for publication

The patient, or parent, guardian or next of kin (in case of deceased patients) had provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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