Original Article

Comprehensive bioinformatics analyses identified Homeobox B9 as a potential prognostic biomarker and therapeutic target for gastric cancer

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Background: The Homeobox B (*HOXB*) family promotes tumor progression, but the mechanism of its action in gastric cancer (GC) is unclear. We sought to identify the *HOXB* family members that are critical to the prognosis of GC patients.

Methods: The Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA), cBioPortal, UALCAN, Kaplan-Meier plotter, and the GeneMANIA databases were used to analyze the messenger RNA (mRNA) expression levels, prognostic value, and gene-gene interaction network of the *HOXB9* family members in GC. The expression of *HOXB9* in GC and its relationship with various clinicopathological parameters and the prognosis of patients were verified by immunohistochemistry.

Results: The expression of *HOXB3*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13* mRNA was significantly upregulated in GC. There was a significant correlation between the upregulation of *HOXB3*, *HOXB5*, and *HOXB9* mRNA and a low overall survival (OS) rate. The high expression of *HOXB7*, *HOXB9*, and *HOXB13* mRNA was closely correlated to tumor grade and stage. *HOXB9* was the *HOXB* family member most closely related to the occurrence and development of GC. A further analysis showed that *HOXB9* might be involved in deoxyribonucleic acid repair and division regulation. A validation study showed that the advanced cancer group had a higher level of *HOXB9* expression than the early cancer group. The high expression of *HOXB9* in gastric tissue plays an important role in the survival and prognosis of GC patients.

Conclusions: HOXB family members have different degrees of abnormal expression in GC. High *HOXB9* expression in GC tissues was significantly correlated with a worse prognosis. Thus, *HOXB9* is a potential novel biomarker and therapeutic target for GC.

Keywords: Gastric cancer (GC); Homeobox B9 (*HOXB9*); Homeobox B family (*HOXB* family); prognosis biomarker; therapeutic target

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Introduction

Gastric cancer (GC) is characterized by high morbidity and mortality, and is the 5th most common cancer type and the 3rd most common cause of cancer-related death worldwide (1). Great progress has been made in the surgical treatment and chemotherapy of GC; however, due to the recurrence and metastasis of GC, the 5-year survival rate of GC patients is still less than 20% (2,3). Additionally, the early diagnosis of GC remains a clinical challenge, as many patients are asymptomatic, the early lesions can be non-specific, and there is a lack of effective biomarkers. Previous research has focused on the potential pathogenesis of GC and identifying novel biomarkers and therapeutic targets. However, the mortality rate of GC patients remains very high (4). Thus, research urgently needs to be conducted to examine the mechanisms underlying the occurrence and development of GC and identify highly sensitive and specific tumor biomarkers.

The Homeobox (HOX) gene family encodes a variety of homeodomain transcription factors that play an important role in embryonic development by regulating cell proliferation and differentiation (5). It has been confirmed that there are 39 Hox genes in mammals that are composed of 4 homologous clusters on the autosomal chromosomes (6,7). The Homeobox B (*HOXB*) family is critical for cell morphogenesis and differentiation (8,9). The expressions of HOXB family members, including *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXB13*, have been confirmed to be associated with the progression of a variety of tumors. Specifically, HOXB1 has been shown to be downregulated in gliomas and lung cancers, and its expression inhibits the apoptosis and promotes the proliferation of cancer cells (10,11). Additionally, *HOXB2* has been shown to be upregulated in cervical cancer and pancreatic cancer (12,13), and was found to be the targets of multiple small RNAs in colorectal cancer (CRC), glioblastoma, and acute myeloid leukemia (14-16). *HOXB3* has been shown to be a target of microRNA-10a (miR-10a) and regulated by retinoic acid receptor signaling pathway in pancreatic cancer metastasis (17). *HOXB4* is an important transcription factor involved in the progression of many cancer types, including lung, breast, prostate, and bladder cancer, and promotes cancer cell proliferation through the STAT3 pathway (18,19). As an important factor in the development of the enteric nervous system, *HOXB5* has been shown to play a role in breast cancer biology by modifying intussusceptive angiogenesis (20). *HOXB6* is highly expressed in hematopoietic stem/progenitor cells, and is downregulated during terminal differentiation, which suggests that its aberrant expression is implicated in hematological malignancies (21). As an important transcription factor, *HOXB7* regulates many cancer cells' functions, including proliferation, invasion, migration, angiogenesis, and epithelial-mesenchymal transition (EMT) (22). The upregulated expression of *HOXB8* has been observed in all stages of CRC (23), while a LINC01006/miR-2682-5p/*HOXB8* feedback loop has been shown to promote cell growth and metastasis in prostate cancer (PCa) (24). *HOXB9* has been shown to be highly expressed in 43% of breast cancer tissues and positively correlated with tumor grade (25). The abnormal expression of *HOXB9* has also been shown to indicate poor prognosis and promote tumor progression in patients with many other cancer types (26,27). Many studies have identified *HOXB13* as a candidate tumor suppressor gene in several types of cancer, including CRC (28), melanoma (29), and breast cancer (30).

In GC, the *HOXB* factors have been shown to play critical roles in disease progression. The relationship between the abnormal expression of *HOXB* genes and clinicopathological features and prognosis of GC has been reported. For example, previous studies have shown that *HOXB3* and *HOXB9* are associated with the development and prognosis of GC (31). Recent studies have shown that *HOXB5* may be an important regulator of the Wnt/β-catenin signaling pathway and involved in the progression and metastasis of GC (32). *HOXB8* plays an important role in the occurrence and metastasis of GC (33). The low expression of *HOXB13* promotes the occurrence, invasion and progression of GC, and is a poor prognostic factor of GC (34). However, systematic bioinformatics analyses on the expressions of *HOXB* factors in tumors and their roles in GC prognosis are lacking.

In this study, using published databases, we comprehensively analyzed the expression patterns of different *HOXBs* in normal tissues and the tumor tissues of GC patients, and determined their potential functions and prognostic value in GC. We used several online analysis tools to analyze and evaluate the correlation between *HOXB9* expression, prognosis and clinicopathological factors in patients with GC, which was further verified by immunohistochemistry. In addition, using a new cohort of GC patients at our hospital, we confirmed that *HOXB9* is a potential biomarker that can be used to diagnose and

predict the prognosis of GC. However, this was different from previous studies. The results of Chang *et al.* (34) study suggested that *HOXB9* was a tumor suppressor in gastric carcinoma, and its activity was controlled by different regulatory mechanisms such as the hexapeptide motif as a "brake" in that case. The results of these regulatory effects could lead to either oncogenic or tumor suppressive roles of *HOXB9*, depending on the context of the particular type of cancer involved (35). We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-598).

Methods

Ethics statement

From 2010 to 2014, a total of 70 paraffin sections of GC tissues were selected from The First Affiliated Hospital of Jinzhou Medical University. Written informed consent was obtained from all the participants. This study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University (No. 1900034790). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). None of the patients in the analysis received radiotherapy or chemotherapy before surgery. Clinical data were collected for all patients.

Bioinformatics analyses of gene expressions

Using the Oncomine gene expression array data set (https://www.oncomine.org/resource/login.html), we analyzed the expression of *HOBX* transcriptome data among different cancers. There were 86,733 normal tissue specimens and tumor tissue specimens (36). The Student's *t*-test was used, and the P value and fold change value were set to 1E-4 and 2, respectively.

The Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) online database was used to analyze the expression of *HOXBs* in GC and normal tissues. Normal tissues include The Cancer Genome Atlas (TCGA) paired normal samples and Genotype-Tissue Expression (GTX) normal samples (37). The UALCAN online database (http://ualcan.path.uab.edu) was used to analyze the expression levels of *HOXBs* in different grades and stages of GC (38). A P value <0.05 was considered statistically significant.

Bioinformatics analyses of patient survival data

The Kaplan-Meier plotter GC database (http://kmplot.com/analysis/index.php?p=service&cancer=gastric) was used to analyze the quantity and expression of *HOXBs*, and overall survival (OS), progression-free survival (PFS), and post-progression survival (PPS) in patients with GC. Patients were allocated to high or low HOXB expression groups, and the survival analysis was performed using the Kaplan-Meier method. The hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated. The difference analysis was performed using the log-rank test.

Bioinformatics analyses of the gene-gene interaction network

The gene-gene interaction network of *HOXBs* was constructed with the GeneMANIA database (http://www.genemania.org). The GeneMANIA database is used to generate assumptions about gene function, analyze lists of genes, prioritize genes for functional analyses, and predict gene function (39). The GeneMANIA database uses extensive genomic and proteomic data to find genes with similar functions.

Bioinformatics analyses of gene function and pathway

The genes co-expressed with *HOXB9* were identified using the cBioPortal database (https://www.cbioportal.org/) (40). The data set for stomach adenocarcinoma from TCGA, which included 478 cases with pathology reports, was used to further analyze gene expression using the cBioPortal database. In addition, the cBioPortal database was used to obtain the correlation coefficients among *HOXB* members and the gene sets co-expressed with *HOXB9*. We set the Spearman correlation coefficient >0.4 to explore genes that were significantly related to *HOBX*. The Database for Annotation Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov) was used to analyze the function and pathway of the genes co-expressed with *HOXB9*. A difference was considered statistically significant when the P value was <0.05 Gene Ontology (GO; http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/

kegg/pathway.html) pathway enrichment analyses were subsequently conducted to identify genes and pathways at the biologically functional level.

Sample sources and clinical data

A total of 70 effective GC tissue samples were collected from GC patients who underwent surgery at The First Affiliated Hospital of Jinzhou Medical University from 2010 to 2014. All the samples were embedded in paraffin. No patients received radiotherapy, chemotherapy, or hormone therapy before surgery. All the pathological sections were evaluated by pathologists and the diagnoses were clear. We identified 21 cases of tubular adenocarcinoma, 15 cases of papillary adenocarcinoma, 19 cases of mucinous adenocarcinoma, 9 cases of signet ring cell carcinoma, and 6 cases of undifferentiated carcinoma.

Immunohistochemical analysis

Paraffin sections of the GC tissues were made every 5 μm. The expression of *HOXB9* was detected using the streptavidin-peroxidase method. The gastric tissue sections with *HOXB9* expression were positive, and phosphate buffer was used as the negative control. We used *HOXB9* polyclonal antibody (Abcam, Cambridge, UK; 1:75) to explore the patient's tissue expression. The following scores were assigned according to the dyeing intensity: no pigment (0 points), light yellow (1 point), brown yellow (2 points), and dark brown (3 points). The following scores were assigned according to the percentage of stained cells: <5% (0 points), 5–25% (1 point), 26–50% (2 points), 51–75% (3 points), and >75% (4 points). The staining intensity was multiplied by the proportion of the stained cells. A score of 3–5 points indicated low expression, and a score of 6–12 points indicated high expression. Two professionals read the film independently.

Statistical analysis

The relationship between the expression of *HOXB9* protein and the clinicopathological features was analyzed by a non-parametric test. The Mann-Whitney U test was used to compare two groups, and the Kruskal-Wallis test was used to compare multiple groups. A Cox proportional hazards model was used for the multivariate analysis. A P value <0.05 indicated a statistically significant difference.

Results

Transcriptional levels of HOXBs in patients with stomach cancer

First, we used the Oncomine database to examine the transcriptional levels of *HOXBs* in GC and normal tissues (see *Figure 1*). Most cancer types had significantly increased expressions of 1 or more *HOXB* family members (see *Figure 1*). Multiple *HOXBs*, including *HOXB3*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13*, were more upregulated in GC samples than normal tissue samples in different publications (41-44). For example, D'Errico *et al.* (41) and Cho *et al.* (42) reported that the expression of *HOXB3*, *HOXB5*, *HOXB6*, *HOXB7*, and *HOXB9* was more than 2-fold higher in gastric intestinal-type adenocarcinoma tissue samples than normal tissue samples (see *Table 1*). Additionally, in the gastric statistics data of Wang *et al.* (43), Cho *et al.* (42), and D'Errico *et al.* (41), the expression of *HOXB7* was elevated (2- to 5-fold) in GC samples in 6 paired comparisons. According to the Cui gastric statistics data, the expression of *HOXB9* was 39-fold higher in GC samples than normal tissue samples (see *Table 1*).

Relationship between the messenger RNA (mRNA) levels of HOXBs and the clinicopathological parameters of patients with GC

Using the GEPIA data set, we compared the mRNA expression of HOXB factors between GC and adjacent normal tissues. The results indicated that the expression levels of *HOXB5*, *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13* were significantly higher in GC tissues than normal tissues (see *Figure 2A*,*2B*). We then used the UALCAN online analysis tool to examine the relationship between the mRNA expression level of *HOXB* family members and cancer stage and tumor grade in GC patients. As *Figure 2C* shows, the mRNA levels of *HOXB* family members were highly correlated with patients' individual cancer stages, and the tumors of patients with more advanced cancer stages tended to have higher *HOXB* mRNA levels. Compared to normal tissues, tumor tissues from patients in stage 4 had the highest mRNA expressions of *HOXB1* and *HOXB9* (see *Figure 2C*), while tumor tissues from patients in stages 2 and 3 had the highest mRNA expressions of *HOXB3*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, and *HOXB9* (see *Figure 2C*).

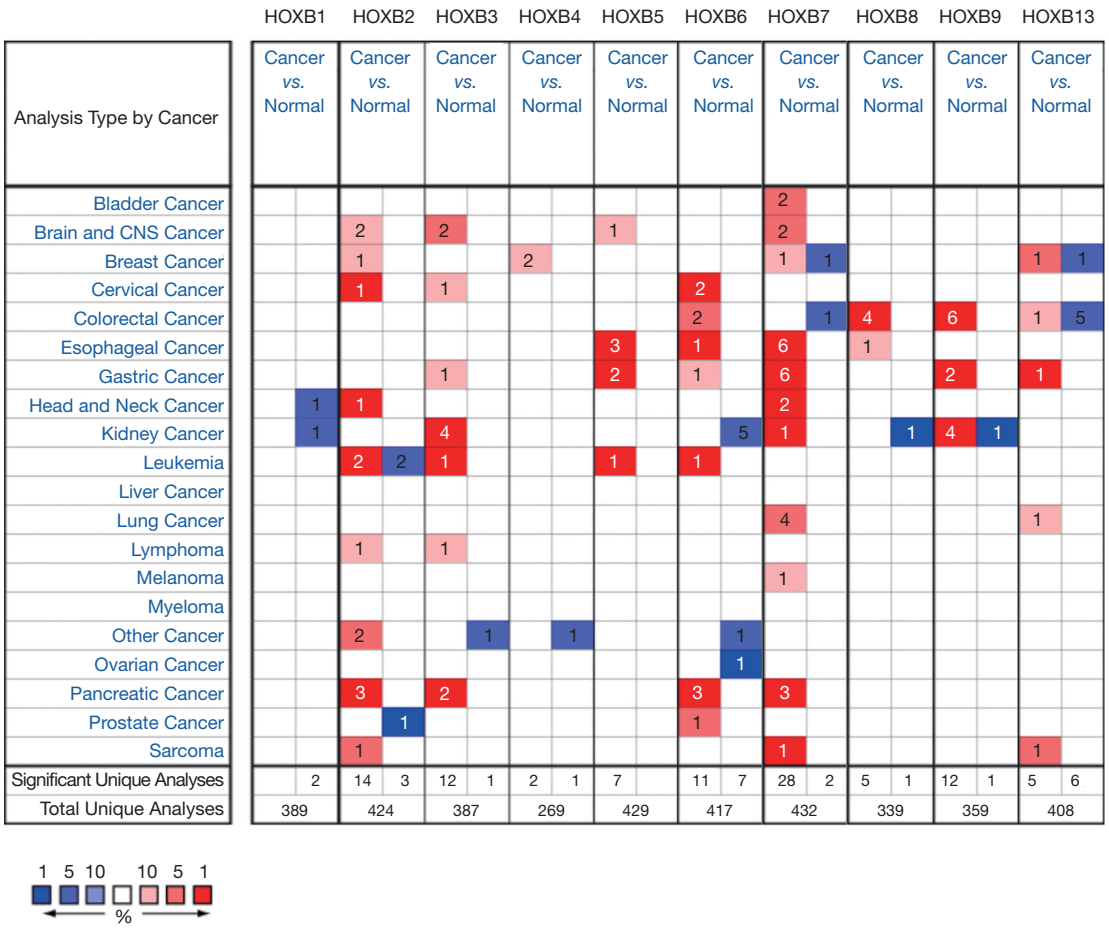


Figure 1 The transcription levels of *HOXB* factors in different types of cancers based on an analysis of the Oncomine database. The expression levels of *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB8*, *HOXB9*, and *HOXB13* in different types of cancers were analyzed using the Oncomine database. Cancer types are listed on the left, and the numbers of significant unique analyses, and total analyses are listed at the bottom. Red boxes and blue boxes indicate upregulated and downregulated expressions in cancer samples vs. normal tissue samples, respectively. The color intensity represents the extend of the differentiated expression, and darker colors indicate more differentiated expression. The values in the red and blue boxes indicate the numbers of data sources. *HOXB*, Homeobox B.

Similarly, as *Figure 2D* shows, the mRNA expression levels of *HOXB* family members were significantly correlated to tumor grades, and the mRNA levels of the *HOXBs* tended to be higher in higher grade tumor samples. Compared to normal tissues, tumor tissues of patients at grade 3 had the highest mRNA levels of *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13* (see *Figure 2D*), while the tumor tissues of patients at grade 2 had the highest mRNA expressions of *HOXB4*, *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13* (see *Figure 2D*). In addition, comparisons of *HOXB* expressions at different tumor grades revealed that the mRNA levels of *HOXB1*, *HOXB8*, and *HOXB9* from tumor tissues at grade 1 and 2 were obviously different. Taken together, these results indicate that mRNA expressions of *HOXB* family members are significantly associated with the clinicopathological parameters of GC patients.

The prognostic values of HOXB factors in GC

We further analyzed the effects of *HOXB* transcription levels on the survival of GC patients. The Kaplan-Meier plotter database was used to analyze correlations between the transcription levels of the mRNA of *HOXBs* in tumor tissues and the survival time of GC patients. The results revealed that the expressions of all the *HOXB* factors were

Table 1 The significant changes of *HOXB* expression in transcription levels between different types of stomach cancer (Oncomine database)

Gene ID	Types of stomach cancer *vs.* normal	Fold change	P value	*t*-test	References
HOXB1	NA	NA	NA	NA	NA
HOXB2	NA	NA	NA	NA	NA
HOXB3	Gastric intestinal type adenocarcinoma *vs.* normal	2.481	3.28E-8	6.977	D'Errico gastric statistics (41)
HOXB4	NA	NA	NA	NA	NA
HOXB5	Gastric intestinal type adenocarcinoma *vs.* normal	3.064	3.82E-7	5.961	Cho gastric statistics (42)
	Diffuse gastric adenocarcinoma *vs.* normal	2.514	7.22E-7	5.585	Cho gastric statistics (42)
HOXB6	Gastric intestinal type adenocarcinoma *vs.* normal	3.278	3.43E-8	6.298	D'Errico gastric statistics (41)
HOXB7	GC *vs.* normal	4.894	9.08E-6	5.280	Wang gastric statistics (43)
	Gastric intestinal type adenocarcinoma *vs.* normal	2.801	9.31E-7	5.799	Cho gastric statistics (42)
	Gastric mixed adenocarcinoma *vs.* normal	3.147	2.12E-5	5.123	Cho gastric statistics (42)
	Diffuse gastric adenocarcinoma *vs.* normal	2.293	5.69E-5	4.259	Cho gastric statistics (42)
	Gastric intestinal type adenocarcinoma *vs.* normal	3.845	1.42E-11	8.328	D'Errico gastric statistics (41)
	Gastric mixed adenocarcinoma *vs.* normal	3.524	4.22E-5	6.678	D'Errico gastric statistics (41)
HOXB8	NA	NA	NA	NA	NA
HOXB9	GC *vs.* normal	39.316	3.87E-11	7.045	Cui gastric statistics (44)
	Gastric intestinal type adenocarcinoma *vs.* normal	2.305	1.53E-11	8.333	D'Errico gastric statistics (41)
HOXB13	GC *vs.* normal	27.758	4.25E-8	5.627	Cui gastric statistics (44)

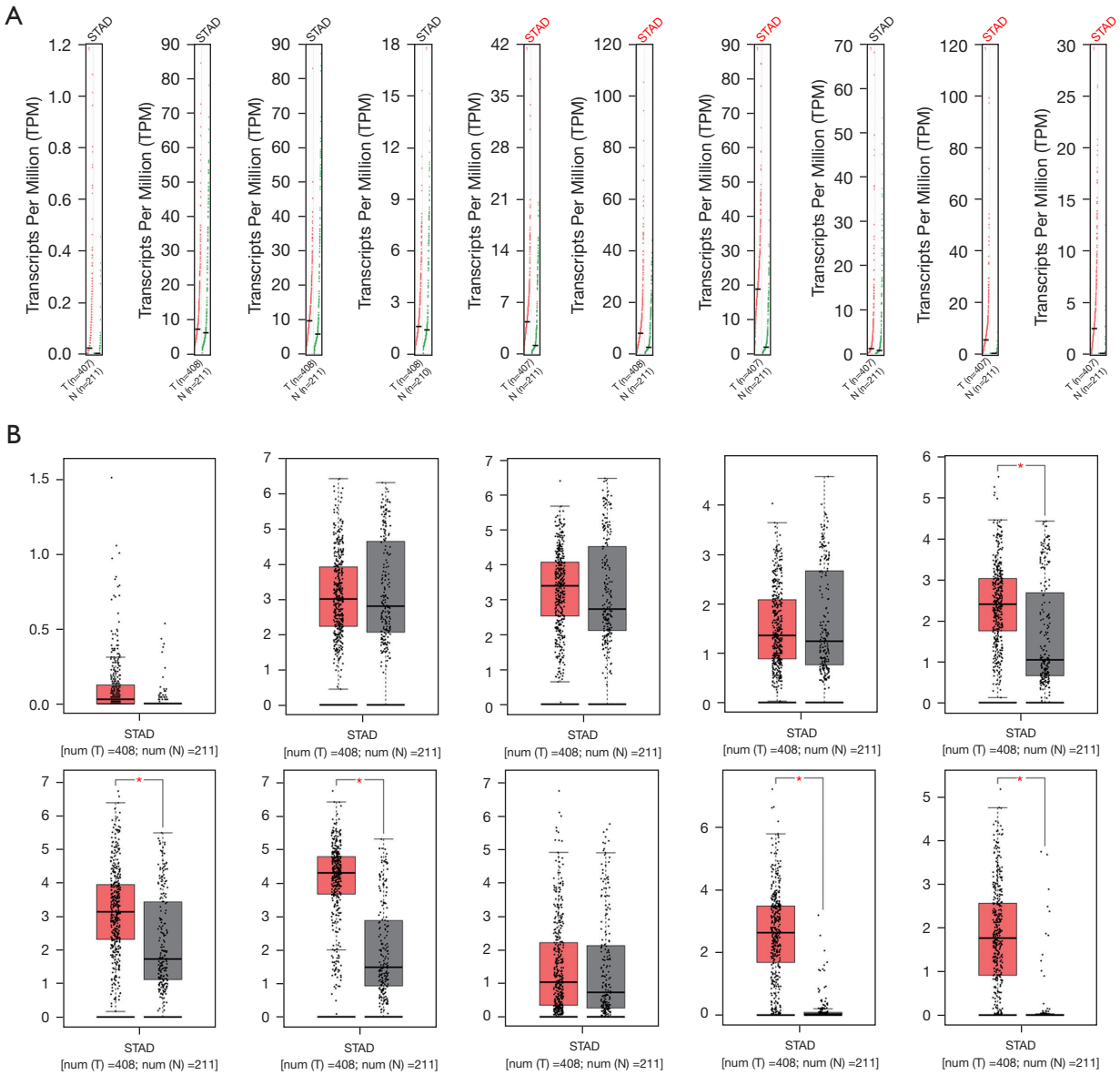
HOXB, Homeobox B; NA, not available; GC, gastric cancer.

significantly (P<0.05) associated with the OS, PFS, and PPS of patients with GC (see *Figure 3*). In particular, higher expressions of *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, and *HOXB9* were associated with worse OS in GC patients (see *Figure 3A*), and higher expressions of *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, and *HOXB9* were associated with worse PFS and PPS in GC (see *Figure 3B*,*3C*). Collectively, these results indicate that mRNA expressions of *HOXB* family members in tumors are significantly associated with the survival of GC patients, and *HOXB* factors are potentially useful biomarkers for predicting the prognosis of GC patients.

Correlation and gene-interaction networks among HOX members

We used GC transcriptomic data from the cBioPortal database (TCGA, Firehose Legacy) to analyze the mRNA transcription levels of *HOXB* genes and determine the Spearman correlation coefficients among *HOXBs* members. Our analysis of the Spearman correlation coefficients suggested that many *HOXB* factors, such as *HOXB5*, *HOXB6*, *HOXB9*, *HOXB8*, and *HOXB3*, had significant positive correlations with each other. In addition, the expressions of *HOXB8* or *HOXB9* were significantly correlated with the expressions *HOXB3*, *HOXB5*, *HOXB6*, and *HOXB7* (see *Figure 4A*).

Using the GeneMANIA online analysis tool, *HOXB* family members were located at the central node, and 20 related nodes were selected outside the circle (see *Figure 4B*). These gene nodes were closely related to the *HOXBs* in terms of inter-gene interactions, co-expressions, predictions, co-localizations, and genetic interactions. The closely related genes included Src kinase associated phosphoprotein 1 (*SKAP1*), Homeobox A7 (*HOXA7*), Homeobox A10 (*HOXA10*), Homeobox A5 (*HOXA5*), and Homeobox C6 (*HOXC6*). In addition, the functional analysis of these proteins showed that they were closely related to the negative regulation of myeloid cell differentiation (false discovery rate =1.21E-4). Additionally, these proteins were associated with the regulation of myeloid cell differentiation, blood vessel morphogenesis, myeloid cell differentiation,



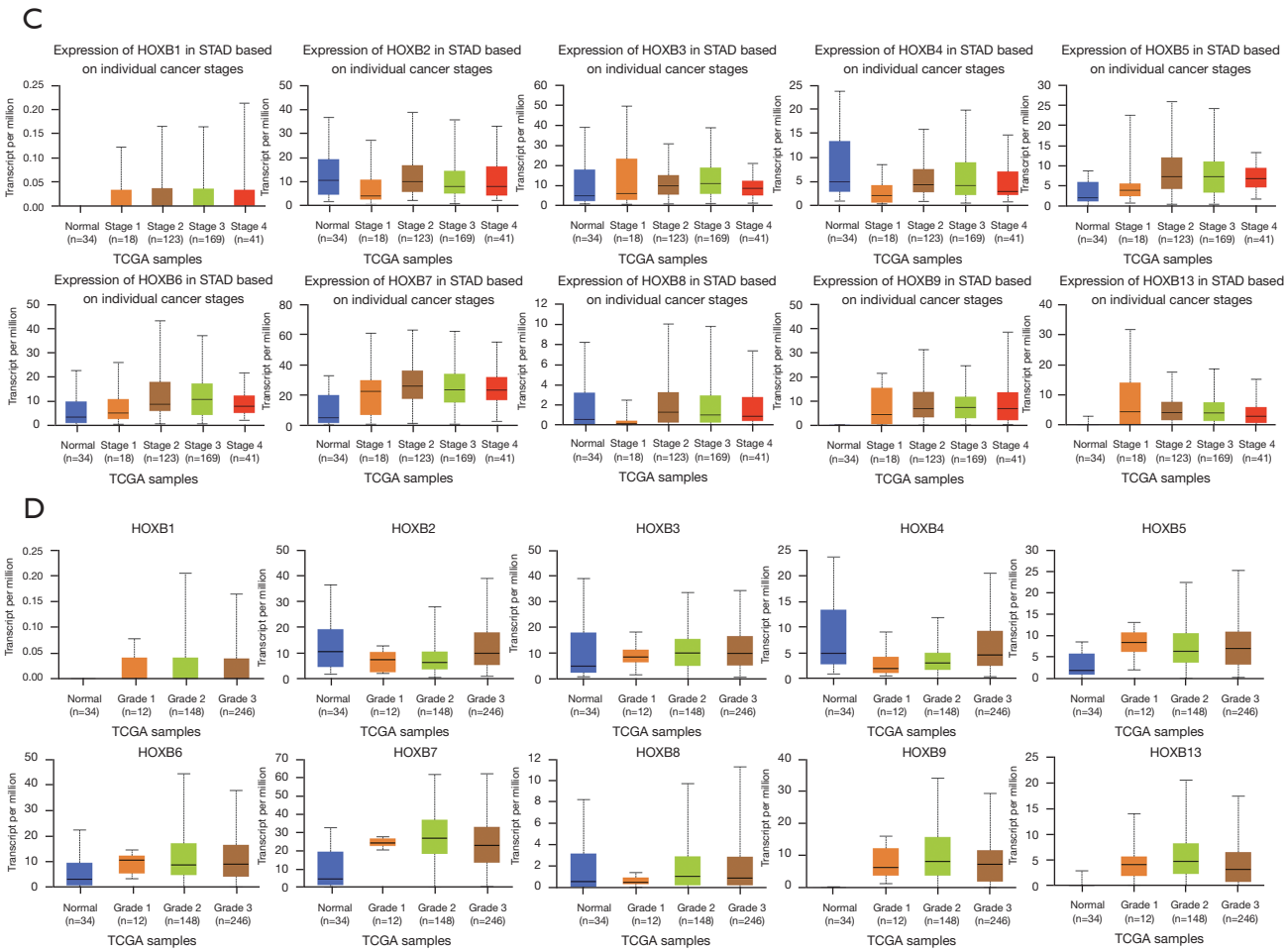


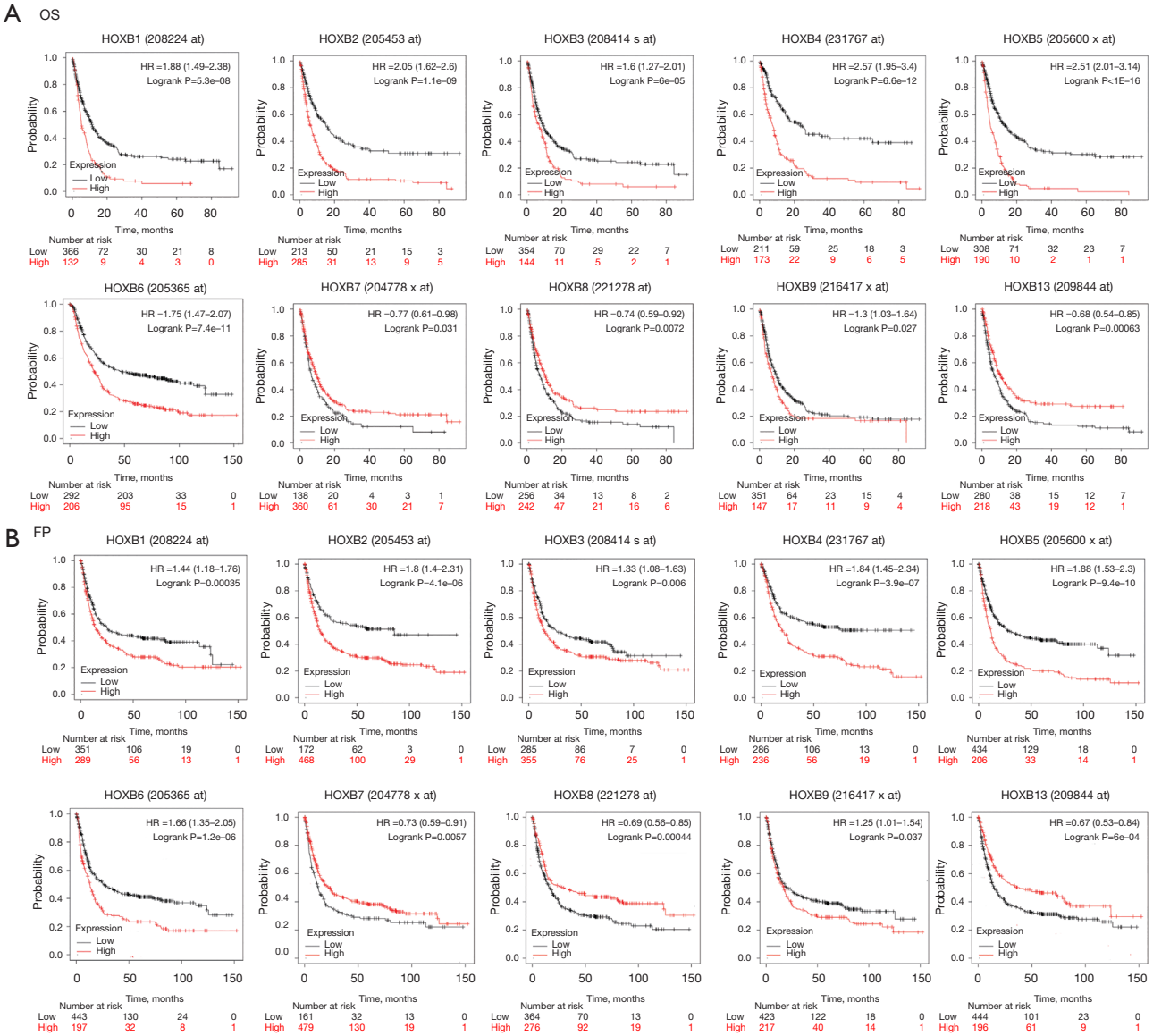
Figure 2 The expressions of HOXBs in GC samples and their correlation with tumor stages and grades. (A,B) The expression levels of *HOXBs* in gastric tumor samples and normal tissue samples were analyzed using the GEPIA data set. The data are presented in scatter diagrams (A) and box plots (B). (C,D) The correlations between *HOXB* expression levels and tumor stage (C) or tumor grade (D) of GC patients were analyzed using the UALCAN platform. *, P<0.05. *HOXB*, Homeobox B; GC, gastric cancer; GEPIA, Gene Expression Profiling Interactive Analysis.

blood vessel development, angiogenesis, and the negative regulation of cell differentiation (see *Figure 4B*).

Functional and pathway enrichment analyses of HOXB9

We believe that HOXB9 can be used as a marker for the prognosis of GC. To further explore the biological function of HOXB9, the DAVID was used for the functional enrichment analysis. The KEGG pathway analysis results (see *Figure 5A*) showed that the main related pathways of the genes co-expressed with *HOXB9* were cell cycle, DNA replication, RNA transport, ribosome biogenesis in eukaryotes, homologous recombination, the Fanconi anemia pathway, and spliceosome, which are closely related to the occurrence and development of tumors.

The results of the GO enrichment analysis of biological processes showed that DNA replication, chromosome segregation, sister chromatid segregation, DNA conformation change, mitotic sister chromatid segregation, nuclear chromosome segregation, mitotic nuclear division, organelle fission, and DNA-dependent replication were regulated by *HOXB9* transcription in GC tissues (see *Figure 5B*). The results of the GO enrichment analysis of the cellular components showed that the chromosomal region condensed chromosome, chromosome, centromeric region, kinetochore, nuclear chromosome part, condensed



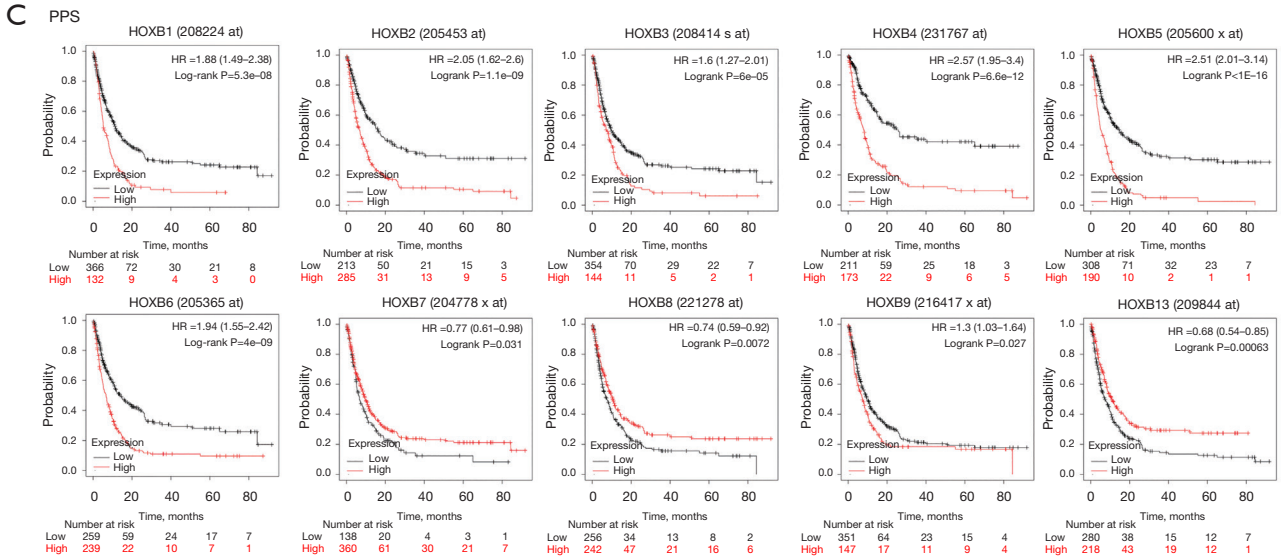


Figure 3 The prognostic value of the mRNA levels of *HOXB* factors in GC patients were explored using the Kaplan-Meier plotter database. The OS (A), PFS (B), and PPS (C) of patients with GC were analyzed by the Kaplan-Meier plotter database. The patients were grouped according to the expression levels of the indicated *HOXB* factors in tumor. mRNA, messenger RNA; *HOXB*, Homeobox B; GC, gastric cancer; OS, overall survival; PFS, progression-free survival; PPS, post-progression survival; HR, hazard ratio.

chromosome kinetochore, spindle, chromatin, spindle pole were also significantly controlled by *HOXB9* alterations (see *Figure 5C*). The results of the GO enrichment analysis of molecular functions showed that DNA-dependent adenosine triphosphatase (ATPase) activity, chromatin binding, helicase activity, DNA helicase activity, catalytic activity, acting on DNA, ATP-dependent DNA helicase activity, ATP-dependent helicase activity, purine NTP-dependent helicase activity, ATPase activity, ATPase activity-coupled were also significantly controlled by the *HOXB9* alterations (see *Figure 5D*).

Higher levels of HOXB9 in GC were associated with higher stages and worse survival in a new cohort of patients

To further validate the role of *HOXB9* in GC progression, we investigated the association between *HOXB9* expression in tumors, the stage, and survival in an independent new cohort of GC patients enrolled at our hospital. The cohort comprised 48 male patients and 22 female patients, and there was no significant difference in age between the two groups (P>0.05). According to the degree of *HOXB9* immunohistochemical staining in gastric tissue sections of patients with GC, patients with GC were divided into two groups. There were significant differences between the groups with high and low expressions of *HOXB9* in relation to the degree of lymph node metastasis (P=0.006) and distant metastasis (P<0.001). However, *HOXB9* expression level was not significantly correlated with sex, age, or tumor (T) stage (P>0.05; see *Table 2*).

Seventy patients with GC were selected for follow-up observation. The last follow-up date was March 10, 2019. Among these 70 patients, 53 patients (75.71%) had complete follow-up information, and 17 lost patients (24.29%) had incomplete follow-up information. The follow-up period for all patients commenced on the day of their operation, and continued to the deadline. The deadline for the lost patients was the date of their last visit. The survival analysis showed that the survival rate of patients with a high expression of *HOXB9* was significantly lower than that of patients with a low expression of *HOXB9* (P<0.0001; see *Figure 6A*). A Cox regression model was used to analyze the age, gender, tumor, lymph node metastasis, and distant metastasis of all patients with GC according to the expression of *HOXB9*. The results of the univariate Cox regression analysis showed that there was a significant correlation between OS and *HOXB9* expression (P<0.001) and distant metastasis (P<0.001). The results of the multivariate Cox regression analysis showed that the high expression of *HOXB9* (P=0.002) was an independent risk factor for the survival and prognosis of

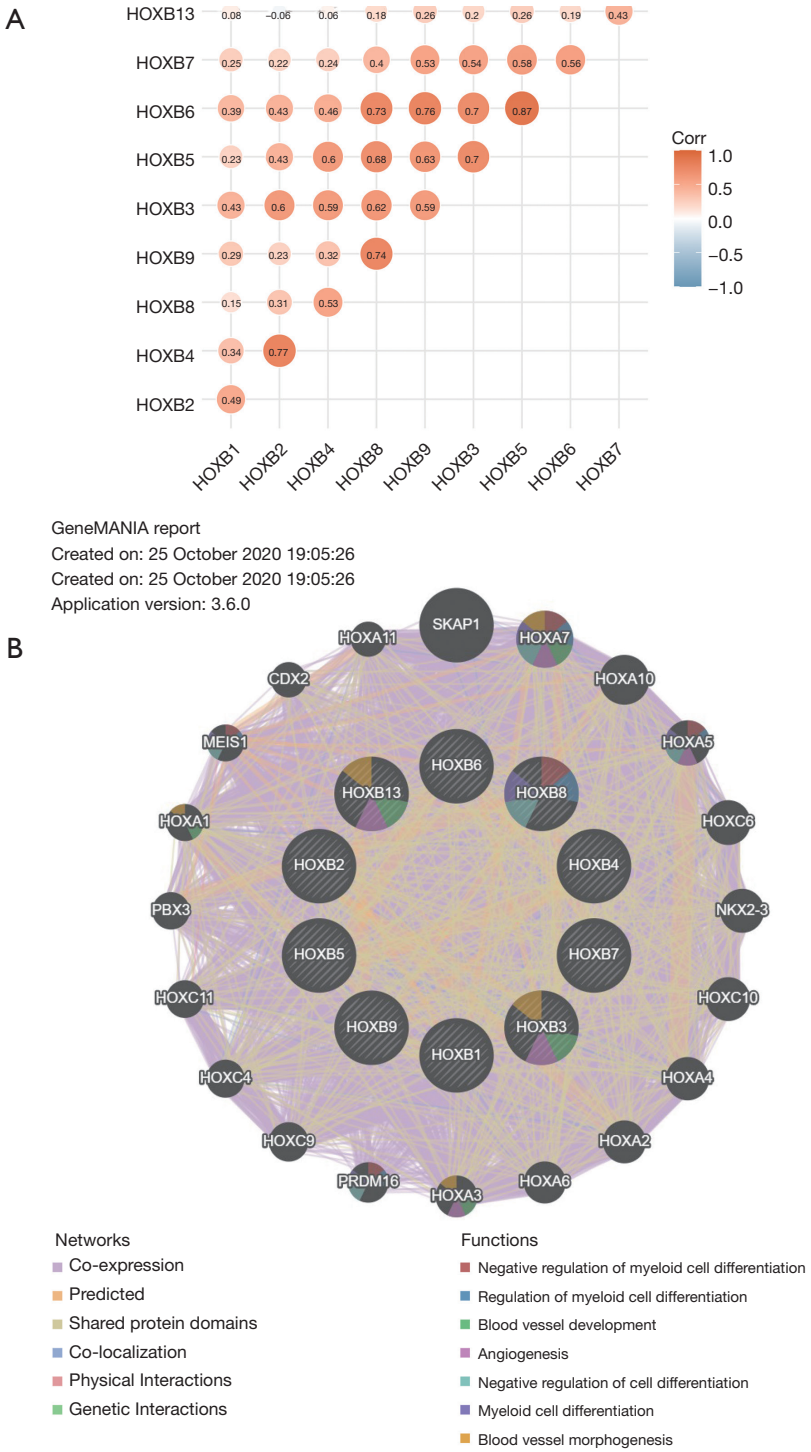


Figure 4 Correlation and gene interaction networks among *HOXB* members. (A) The Spearman correction method was applied to the correlation coefficients among the *HOXBs* members. A correlation coefficient >0.4 indicates a strong correlation. Blue is negative; red is positive. The shade of the color indicates a change in the degree of correlation. (B) Gene-gene interaction networks among members of the *HOXB* family. The names of different genes are at the nodes, the radius of the nodes represents the intensity of the interaction, the color of the nodes represents the possible biological function of each gene, and the color of the line between nodes represents the type of interaction between the genes. *HOXB*, Homeobox B.

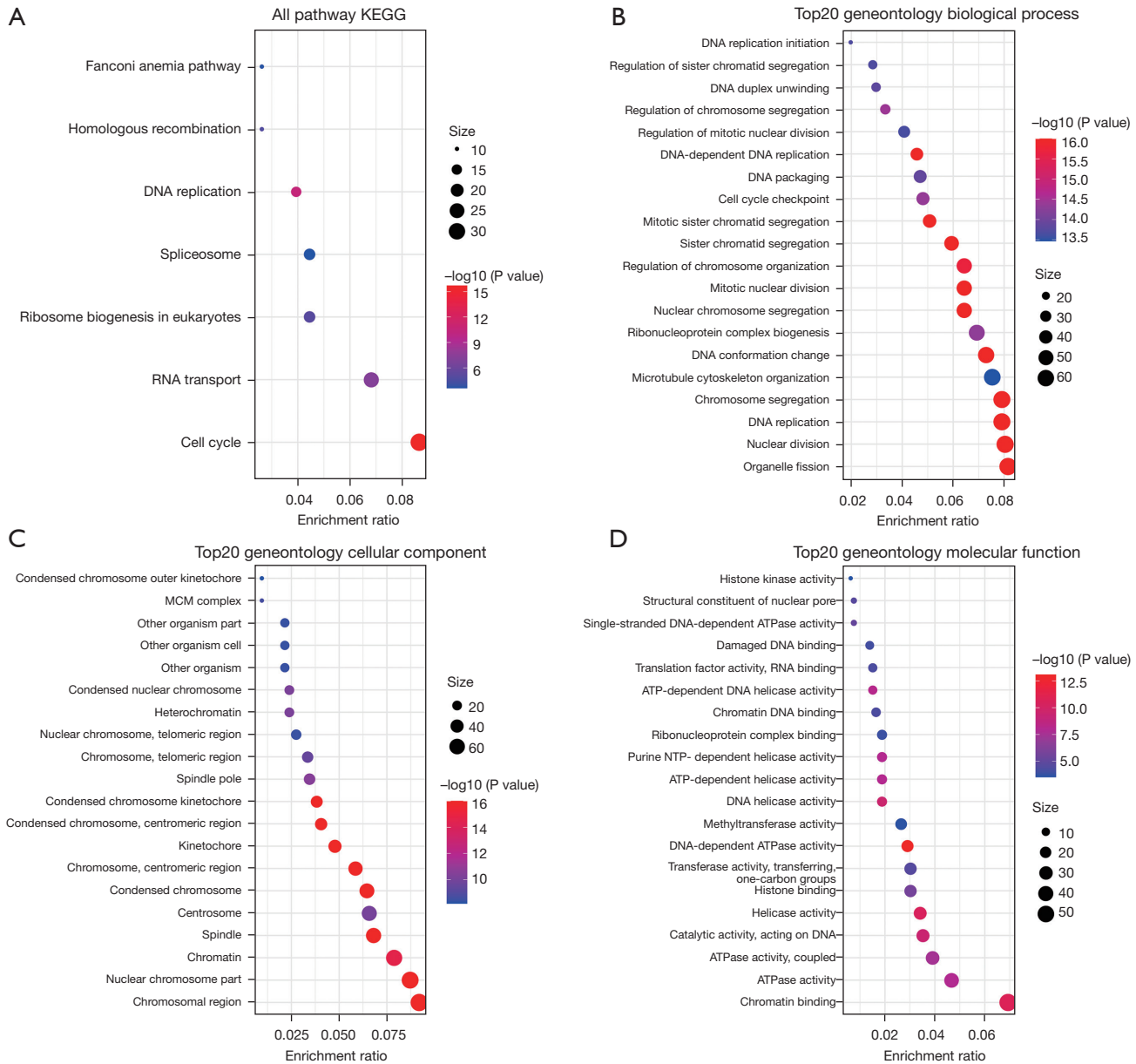


Figure 5 The function of *HOXBs* and genes significantly associated with *HOXB9* alterations. (A) Gene function and pathway enrichment analysis of *HOXB9* co-expression. A Spearman correlation coefficient >0.4 was used as the selection criteria. Nine hundred and sixty genes co-expressed with *HOXB9* were obtained from the cBioPortal database. The bubble chart was constructed with the ggplot2.R software package. (B-D) GO pathway enrichment analyses were subsequently conducted to identify genes and pathways in terms of the biological processes (B), cellular components (C), and molecular functions (D). HOXB, Homeobox B; GO, Gene Ontology.

patients with GC (see *Figure 6B*).

Immunohistochemical staining for HOXB9 and relation between HOXB9 expression and clinicopathologic factors

HOXB9 expression in patients with GC. The expression of *HOXB9* was evaluated in 70 tumor specimens. Immunohistochemical staining for *HOXB9* (*Figure 7*) was performed. Staining intensity was graded as none, weak and intense. According to relation between *HOXB9* expression and clinicopathologic factors in 70 GC patients (*Table 3*), a total of 36 tumors (51.4%) were positive for *HOXB9*

Table 2 The expression of *HOXB9* in GC is related to the clinical characteristics of patients with GC

Characteristics	N	*HOXB9* expression		P value
		Low (n)	High (n)	
Age (years)				0.071
<60	27	16	11	
≥60	43	16	27	
Gender				0.288
Male	48	24	24	
Female	22	8	14	
T stage				0.187
T1	2	1	1	
T2	10	7	3	
T3	16	9	7	
T4	42	15	27	
Lymph node metastasis				0.006
N0	10	8	2	
N1	28	16	12	
N2	24	7	17	
N3	8	1	7	
Metastasis				0.000
No	35	29	6	
Yes	35	3	32	

HOXB, Homeobox B; GC, gastric cancer.

expression. *HOXB9* staining was observed in the nucleus of GC cells.

Discussion

In addition to cancer genetics, the abnormal regulation of epigenetics is also involved in the occurrence, development, and prognosis of GC. As an important part of the epigenetic regulatory complex, *HOXB* family proteins are involved in the occurrence of many cancers, including GC (8,21). Some members of the *HOXB* family have been shown to play a key role in GC; however, the unique roles of *HOXB* family members in GC require clarification. In the present study, we analyzed the expressions and prognostic values of multiple *HOXB* family members in GC using large-scale published data sets, and validated the relationship between high levels of *HOXB9* expression and survival in a new cohort of patients with GC.

The results of our study showed that the mRNA expression levels of *HOXB3*, *HOXB5*, *HOXB6*, *HOXB7*, *HOXB9*, and *HOXB13* were higher in tumor tissues than normal tissues, and the mRNA expression of *HOXBs* was significantly associated with cancer stages and tumor grades in GC patients. Additionally, higher mRNA expression levels of *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, and *HOXB9* were significantly associated with shorter OS in GC patients, while higher mRNA expressions of *HOXB6*, *HOXB7*, *HOXB8*, *and HOXB13* were significantly related to favorable OS in GC patients. Thus, *HOXB1*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, and *HOXB9* function more like oncogenes, while *HOXB6*, *HOXB7*, *HOXB8*, and *HOXB13* are putative tumor suppressors of GC. We also analyzed the functions and pathways of the mutations in *HOXBs* and their 20 frequently altered neighbor genes in GC patients, and found that the 5 genes most associated with the *HOXB* family were *SKAP1*, *HOXA7*, *HOXA10*, *HOXA5*, and *HOXC6*. Further research on these *HOXB*-correlated genes in the progression of GC needs to be conducted to extend our understanding of the cellular and molecular mechanisms underlying the altered levels of these *HOXBs* in the pathogenesis of GC.

Our comprehensive analyses indicated that *HOXB9* has the greatest correlation with the prognosis of GC patients, and is closely related to the tumorigenesis and development of GC. Thus, the *HOXB9* gene was selected for a follow-up study to further explore the biological processes and related signaling pathways involved in its co-expressed genes. *HOXB9* was previously identified as an oncogenic factor in human breast cancer (25). The ability of *HOXB9* to modulate damage responses may involve EMT induced by the transcription target transforming growth factor-beta (45). Additionally, the positive roles of *HOXB9* in promoting cellular proliferation, angiogenesis, and the migration and invasion of PCa cells were recently reported (27). Lymph node metastasis and lymphatic invasion are related to lymphogenic metastasis, a specific phenomenon of GC progression (46). The experimental results of Kato *et al.* (47) showed that the lymphangiogenic factor VEGF-D increased in TMK-1 cells transfected with *HOXB9* gene. *HOXB9* gene was overexpressed in human GC TMK-1 cells. That was the first study suggesting that *HOXB9* promoted lymphangiogenesis. Therefore, we concluded that *HOXB9* affects lymphangiogenesis of tumor cells *in vitro* by promoting the expression of

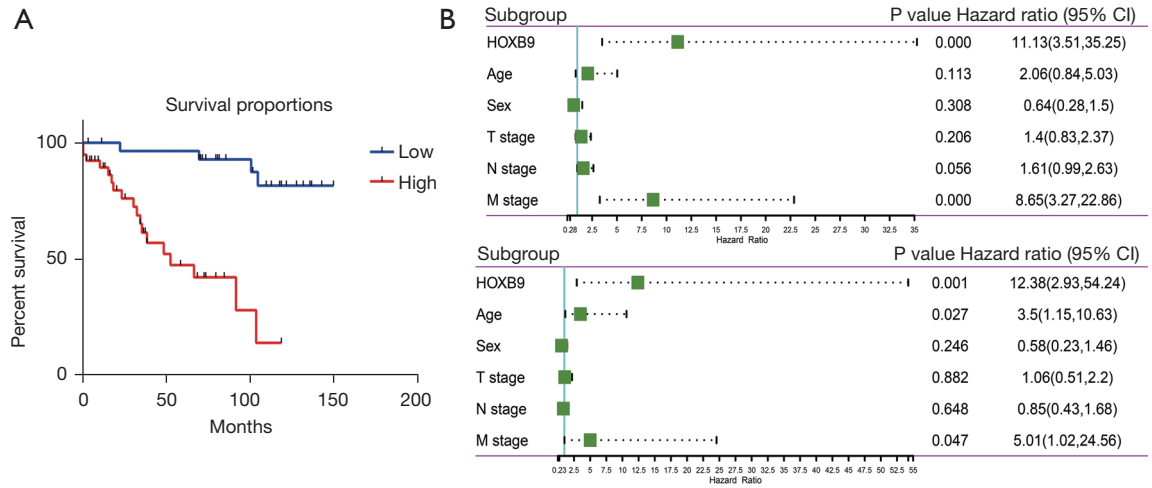


Figure 6 The expression of *HOXB9* in GC and the survival of patients with GC and the factors affecting prognosis. (A) Relationship between the expression of *HOXB9* in tumor tissues of patients with GC and patient survival. (B) Forest map of univariate and multivariate Cox regression analyses based on the survival data of patients with GC. *HOXB*, Homeobox B; GC, gastric cancer; CI, confidence interval.

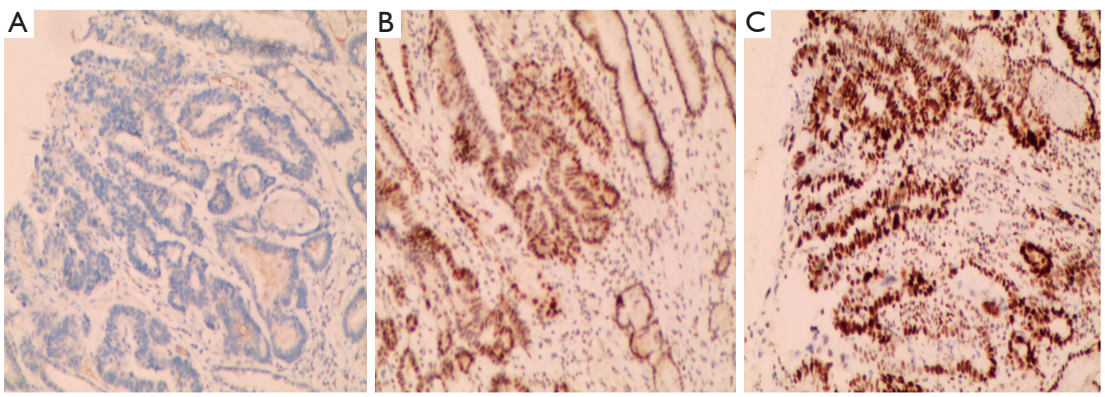


Figure 7 Immunohistochemical staining for homeobox B9 expression in human GC tissues. Staining intensity was graded as (A) no reaction, (B) weak reaction and (C) intense reaction (original magnification, ×400).

lymphangiogenic factor. *HOXB9* expression was positively correlated with GC progression and lymphangiogenesis marker expression (44). Consistent with previous findings, our results also suggest that *HOXB9* plays an oncogenic role in the tumorigenesis of GC, implying the conservative functions of *HOXB9* in human malignancies regardless of cancer types.

Various databases were used to further verify the correlation between *HOXB9* expression and GC development. The Oncomine database showed that the expression level of *HOXB9* mRNA in GC was significantly upregulated. As the GC stage progressed, the expression level of *HOXB9* mRNA increased significantly. The results of the prognostic analysis showed that the upregulation of *HOXB9* mRNA expression was most closely related to poor OS, PFS, and PPS in GC patients. We also analyzed the relationship between *HOXB9* expression and clinicopathological parameters, survival rate, and prognosis in GC patients. Patients with high expressions of *HOXB9* mostly had advanced GC and a poor prognosis. The Cox regression analysis showed that the high expression of *HOXB9* is an independent risk factor for survival and the prognosis of patients with GC. Thus, *HOXB9* is a potential biomarker for early diagnosis, and can be used to evaluate the survival and prognosis of patients with GC.

The functions and pathways associated with *HOXB9*

Table 3 Relation between *HOXB9* expression and clinicopathologic factors in 70 GC patients

Characteristics	No. of patients	Negative (n=34)	Positive (n=36)	P value
Gender				0.7239
Male	48	24	24	
Female	22	10	12	
Age				0.1563
<60	27	16	11	
≥60	43	18	25	
T feature				0.0574
T1–2	29	18	11	
T3–4	41	16	25	
Lymph node metastasis				0.0442
Negative	12	9	3	
Positive	58	25	33	
Lymphatic invasion				0.0140
Negative	23	16	7	
Positive	47	18	29	
Vascular invasion				0.2379
Negative	24	14	10	
Positive	46	20	26	

HOXB, Homeobox B; GC, gastric cancer; T, pathological depth of tumor invasion.

co-expressed genes that were identified using cBioPortal database were analyzed. The results showed that these pathways are involved in the regulation of DNA replication and dissociation. As *HOXB9* has been reported to modulate the damage response in breast cancer cells (48), research needs to be conducted to determine whether *HOXB9* modulates the irradiation-induced DNA damage responses of GC cells. In addition, it is reasonable to believe that *HOXB9* and its co-expressed genes may promote the occurrence and development of GC via a complex regulatory network.

We used the GeneMANIA database to construct the interaction network between genes to further explore the mechanism of *HOXBs* in GC. The results showed that *HOXBs* interacted closely with *SKAP1*, *HOXA7*, *HOXA10*, *HOXA5*, and *HOXC6*. *SKAP2* and its related family member *SKAP1* (mainly in T cells) have been shown to participate in cell adhesion by binding to integrin and actin (49). *HOXA7* plays a key role in tumor invasion and metastasis by inducing EMT (50). *HOXA10* aggravates GC by activating the *Jak1/STAT3* signaling pathway (51). The persistent endothelial expression of *HOXA5 in vivo* affects pathological angiogenesis and tumor progression (52). *HOXC6* promotes tumor development through the indirect activation of the Notch and Wnt signaling pathways (53). Thus, genes interacting with *HOXB* family members jointly promote the occurrence and development of tumors, and in-depth basic experiments need to be conducted to study the specific mechanism of action.

Our comprehensive analysis showed that *HOXB9* is highly expressed in GC tissues, and the high expression of *HOXB9* promotes the occurrence and development of GC, and is positively correlated with the poor prognosis of GC patients. Thus, we believe that *HOXB9* can be used as a novel biomarker for the clinical diagnosis, therapeutic treatment and prognosis of GC patients. At the same time, we further analyzed the clinicopathological and molecular characteristics of GC. The previous studies of Wang *et al.* showed that copy number gains (CNGs) at 20q11.21-13.12 occurred frequently in hepatoid adenocarcinoma of

the stomach (HAS), nearly 50% of HAS tumors harbored at least one gene with a CNG at 20q11.21-13.12. This CNG tended to be related to more adverse biobehavioral, including poorer differentiation, greater vascular and nerve invasion, and greater liver metastasis. Pathway enrichment analysis revealed that the HIF-1 signaling pathway and signaling pathways regulating stem cell pluripotency were specifically enriched in HAS. The survival analysis showed that a preoperative serum alpha-fetoprotein (AFP) level ≥500 ng/mL was significantly associated with poorer OS (P=0.007) and tended to be associated with poorer disease-free survival (DFS) (P=0.05) (54). Previous study showed that *HOXB9* can promote adrenal tumor progression in a sex-dependent manner and have identified HOX factors as potential drug targets, leading to novel therapeutic approaches in adrenocortical carcinoma (ACC) (55). They identified *HOXB9* as a critical regulator of metastatic prostate cancer stem cells (mPCSC) behavior. This occurs through altering the expression of a panel of CSC growth- and invasion/metastasis-related genes via TGF-β signaling. Thus, targeting *HOXB9* is a potential novel therapeutic PCa treatment strategy.

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Footnote

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