HOXA5 is a tumor suppressor gene that is decreased in gastric cancer

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Abstract. The abnormal expression of homeobox A5 (*HOXA5*) has been observed in breast and colon cancer; however, the clinical significance of *HOXA5* in gastric cancer (GC) is not yet clear. In this study, we found that *HOXA5* expression was decreased in GC tissues at the mRNA and protein level compared with paracancerous tissues using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis, respectively. Immunohistochemistry and Kaplan-Meier survival analysis confirmed that the under-expression of HOXA5 was associated with GC progression and indicated a poor prognosis of patients with GC. Given that proliferation-related genes may be potential target genes of HOXA5, we performed a series of experiments *in vitro* to examine the effects of HOXA5 on the proliferation of GC cells. A CCK-8 assay, colony formation assay and flow cytometry revealed that HOXA5 inhibited the abnormal proliferation of GC cells, and this finding was further supported by a 5-ethynyl-2'-deoxyuridine (EdU) assay. Further mechanistic investigation clarified that HOXA5 promoted the protein expression of p21 and inhibited the protein expression of c-Myc and Ki67. Additionally, the use of nude mouse models also verified that HOXA5 suppressed the proliferation of GC cells *in vivo*. Collectively, the findings of this study demonstrate that *HOXA5* acts as a tumor suppressor gene during the development and progresion of GC, possibly functioning by inhibiting the abnormal proliferation of cancer cells.

Introduction

Gastric cancer (GC) is the second most common malignant tumor in terms of morbidity and mortality. There are 900,000 new cases of GC and 700,000-related deaths worldwide each year (1). However, no biomarkers for GC are routinely used to predict its occurrence and prognosis.

Homeobox A5 (*HOXA5*) is located on chromosome 7, has a total length of 2,319 bp and is a member of the *HOX* family. Its encoded protein, HOXA5, contains 403 amino acids. HOXA5 is a transcription factor that is involved in regulating human embryonic development and adult stem cell differentiation (2,3). The role of HOXA5 in cancer progression has been gradually clarified. It has been demonstrated that HOXA5 inhibits the activity of the Wingless (Wnt) pathway through the regulation of β-catenin inhibitory proteins and forms a negative feedback loop with Wnt signals to inhibit the stemness characteristics of colorectal cancer cells (4). Retinoic acid (RA) is a promising anticancer drug, and it has been found that the enforced overexpression of HOXA5 induces the apoptosis and differentiation of breast cancer cells. Further mechanistic research has revealed that the RA response element upstream of the *HOXA5* promoter combines with RA receptor β (RARβ), thus mediating the anticancer effects of RA (5). F-actin reorganization and filopodia formation are known to promote cell movement, and HOXA5 in lung cancer cells has been shown to decrease the level of myosin-X proteins expressed on filopodia tips and to reduce the number of pseudopods, thereby preventing cancer cell metastases. Survival analysis has indicated that HOXA5 expression is associated with a better overall and disease-free survival of patients with non-small cell lung cancer expressing wild-type EGFR (6). These studies have suggested that *HOXA5* may be a broad-spectrum tumor suppressor gene with the potential for wide-ranging significance and applications.

A recent study found that *HOXA5* was significantly differentially methylated between GC tissues and adjacent non-cancer tissues (7). However, to the best of our knowledge, no information is available to date regarding the expression of HOXA5 and its function in human GC. Thus, in this study, in order to explore the vital role of HOXA5 in the tumorigenesis and progression of GC, the expression of HOXA5 in 81 patients with GC was detected by immunohistochemistry, and the association between HOXA5 expression and the clinicopathological characteristics of the patients with GC was analyzed. Additionally, the potential value of HOXA5 as a prognostic indicator was assessed in patients with GC. Finally, the effects of HOXA5 on cell proliferation were investigated *in vitro* and *in vivo*.

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Materials and methods

Patients and specimens. A total of 81 pairs of GC tissues and matched paracancerous tissues (≥6 cm away from the tumor) for immunohistochemistry and reverse transcription-quantitative PCR (RT-qPCR) were collected from patients with GC after surgical resection at the First Affiliated Hospital of Chongqing Medical University from January to October, 2009. Due to the long storage time, only 72 in 81 pairs of tissue samples were qualified for RT-qPCR. All patients had complete pathological parameters and follow-up information, including 53 males and 28 females with an average age of 64.1 years (range, 42-83 years). TNM staging was as follows: 5 cases of stage I, 23 cases of stage II, 48 cases of stage III and 5 cases of stage IV. In total, 30 pairs of GC and matched paracancerous specimens for western blot analysis were collected from patients with GC admitted to the First Affiliated Hospital of Chongqing Medical University from July to August, 2016. The patient cohort comprised 18 male and 12 female patients with an average age of 58.3 years (range, 31-83 years). All patients underwent total or subtotal gastrectomy and did not receive radiotherapy and chemotherapy prior to surgery. The use of human tissue samples and experimental protocols were approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University and written informed consent was obtained from all patients.

Cells and lentiviral transduction. The human GC cell line, SGC7901, was obtained from the Chinese Academy of Sciences Shanghai Cell Bank (Shanghai, China) and cultured in RPM-1640 (HyClone, Shanghai, China) supplemented with 10% fetal bovine serum (HyClone) in an incubator with 5% CO_2 at 37°C. The overexpression vector pLV-HOXA5-GFP-puro, the control vector pLV-GFP-puro and polybrene were obtained from Hanbio Technology (Shanghai, China). The SGC7901 cells were seeded at $1x10^5$/well in 6-well plates (1 ml/well). After 10 h, lentiviruses were added into the plates at a MOI of 40. Polybrene was added at a final concentration of 5 μg/ml to each well. The culture media were replaced after 15 h. After 72 h, the cells were examined to determine the transduction efficiency under a fluorescence microscope (Olympus, Tokyo, Japan) and puromycin (Beyotime, Shanghai, China) was then added at a final concentration of 2 μg/ml to each well. After selection for 2 weeks, puromycin was removed and the transduced cells were used in further experiments.

Antibodies and reagents. Rabbit anti-human HOXA5 (Cat. #ab82645), p21 (Cat. #ab227443) and c-Myc (Cat. #ab32072) antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-human Ki67 (Cat. #AP19895b) antibody was purchased from Abgent (San Diego, CA, USA). Rabbit anti-human GAPDH (Cat. #10494-1-AP), cyclin D1 (Cat. #12363-1-AP) and cyclin E (Cat. #11554-1-AP) antibodies and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cat. #SA00001-15) were purchased from Proteintech (Wuhan, China). Immunohistochemistry-related reagents, western blot analysis-related reagents, CCK-8 reagents and DAPI were purchased from Beyotime (Shanghai, China). All reagents used in RT-qPCR were purchased from Takara (Dalian, China). The 5-ethynyl-2'-deoxyuridine (EdU)

proliferation assay kit was obtained from Ribobio (Guangzhou, China). All primers used for RT-qPCR were obtained from Sangon Biotech (Shanghai, China).

RT-qPCR. RT-qPCR was used to assess the differences in *HOXA5* mRNA expression levels between the GC tissues and adjacent paracancerous tissues. Total RNA was extracted using TRIzol reagent (Takara, Dalian, China) and reverse transcribed into cDNA using the reverse transcription kit from Takara. The reverse transcription conditions were as follows: 37°C, 15 min; 85°C, 5 sec. Two-step RT-qPCR was performed using a SYBR-Green assay on a CFX96 PCR machine (Bio-Rad, USA) according to the instruction. The PCR conditions were as follows: Pre-denaturation at 95°C for 30 sec; 45 cycles of denaturation at 95°C for 5 sec and annealing at 60°C for 30 sec, extension at 65°C for 1 min. The data were analyzed using the $\Delta\Delta$Cq method (8). The primers used are shown in Table I.

Western blot analysis. The cancer tissues and matched paracancerous tissues were lysed using RIPA lysis buffer, and the protein concentration was measured using the BCA assay after the lysates were harvested by centrifugation (12,000 rpm) at 4°C. Subsequently, 20 μg of protein samples were separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C with HOXA5 antibody (1:1,000), p21 antibody (1:1,000), p53 antibody (1:1,000), cyclin D1 antibody (1:2,000), cyclin E antibody (1:500), c-Myc antibody (1:1,000) and Ki67 antibody (1:1,000) following blocking of the non-specific binding sites for 2 h in 5% non-fat milk. After washing with TBS-T, membranes were incubated with secondary antibody (1:2,000) at 37°C for 2 h. GAPDH was used as a loading control. An enhanced chemiluminescence kit (ECL) was used for visualizing immunobands and the signal intensity of each band was measured by Fusion software (Vilber Lourmat, Paris, France) to calculate protein levels.

Immunohistochemistry. Tissues were fixed by formalin and embedded in paraffin blocks, and a series of sections (4-mm-thick) were prepared. The tissue antigens were repaired by sodium citrate buffer (pH 6.0). The sections were incubated with rabbit anti-human HOXA5 antibody at 4°C overnight after non-specific binding was blocked with goat serum. The scoring criteria were as follows: 5 fields were randomly selected from each section under a light microscope (Olympus), and the percentage of positively stained cells to total cells was calculated. The sections were scored as follows: 0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. Additionally, the staining intensity was scored as follows: 0, no staining; 1, light yellow; 2, brownish yellow; and 3, brown. The total score was the sum of staining range and intensity, and was classified as low expression (≤3 points) or high expression (≥4 points).

CCK-8 and colony-forming assays. For the CCK-8 assay, the cells were seeded at $2x10^3$/well in 96-well plates (200 μl/well). After cell adherence, 20 μl CCK-8 reagent were added to each well on days 0, 1, 2 and 3. Following incubation at 37°C for 1.5 h, the OD value at 450 nm was measured using a

Table I. The sequences of the PCR primers used in this study.

Gene	Primer
GAPDH	F: 5'-CTTTGGTATCGTGGAAGGACTC-3'
	R: 5'-GTAGAGGCAGGGATGATGTTCT-3'
HOXA5	F: 5'-AGCCACAAATCAAGGACACA-3'
	R: 5'-GCTCGCTCACGGAACTATG-3'
E-cadherin	F: 5'-TGGCTTCCCTCTTTCATCTCC-3'
	R: 5'-TCATAGTTCCGCTCTGTCTTTGG-3'
N-cadherin	F: 5'-CGTGAAGGTTTGCCAGTGTGA-3'
	R: 5'-CCTGGCGTTCTTTATCCCG-3'
Vimentin	F: 5'-TCAATGTTAAGATGGCCCTTG-3'
	R: 5'-TGAGTGGGTATCAACCAGAGG-3'
CD44	F: 5'-TTACTCTGCTGCGTTGTCATTG-3'
	R: 5'-ACAACACCACGCCCAGAGGA-3'
EpCAM	F: 5'-CGCAGCTCAGGAAGAATGTGT-3'
	R: 5'-AGCCATTCATTTCTGCCTTCAT-3'
Lgr5	F: 5'-GAGCTGCCTTCCAACCTCAG-3'
	R: 5'-CCCGCAAGACGTAACTCCTC-3'
CCND1	F: 5'-ATGTTCGTGGCCTCTAAGATGA-3'
	R: 5'-CAGGTTCCACTTGAGCTTGTTC-3'
CCNB1	F: 5'-AATAAGGCGAAGATCAACATGGC-3'
	R: 5'-TTTGTTACCAATGTCCCCAAGAG-3'
Ki67	F: 5'-ATCGTCCCAGGTGGAAGAGTT-3'
	R: 5'-ATAGTAACCAGGCGTCTCGTGG-3'
MMP2	F: 5'-GACATACATCTTTGCTGGAGAC-3'
	R: 5'-TTCAGGTAATAGGCACCCTT-3'
MMP7	F: 5'-CGGATGGTAAGCAGTCTAGGG-3'
	R: 5'-AGGTTGGATACATCACTGCATTAG-3'
VEGFA	F: 5'-CACACAGGATGGCTTGAAGA-3'
	R: 5'-AGGGCAGAATCATCACGAAG-3'
VEGFR2	F: 5'-CCAGATGACAACCAGACGGA-3'
	R: 5'-AGCCTTCAGATGCCACAGACTC-3'
EGFR	F: 5'-AGGCACGAGTAACAAGCTCAC-3'
	R: 5'-ATGAGGACATAACCAGCCACC-3'
ABCC1	F: 5'-GTGATGGCCATGAAGACCAAGA-3'
	R: 5'-GCCAGCTCCCAGGCATAAAG-3'
ABCG2	F: 5'-TCTCTTCTTCCTGACGACCAA-3'
	R: 5'-AAACCACACTCTGACCTGCTG-3'
Caspase-3	F: 5'-TACCAGTGGAGGCCGACTTC-3'
	R: 5'-GCACAAAGCGACTGGATGAAC-3'
BCL2	F: 5'-CTGCACCTGACGCCCTTCACC-3'
	R:5'-CACATGACCCCACCGAACTCAAAGA-3'
BECN1	F: 5'-GGCTGAGAGACTGGATCAGG-3'
	R: 5'-CTGCGTCTGGGCATAACG-3'
p21	F: 5'-AGCGGAACAAGGAGTCAG-3'
	R: 5'-CGTTAGTGCCAGGAAAGAC-3'
p53	F: 5'-GCGTAAACGCTTCGAGATGTT-3'
	R: 5'-TTTTTATGGCGGGAAGTAGACTG-3'
C-Myc	F: 5'-ACACCCGAGCAAGGACGCGA-3'
	R: 5'-CGCGGGAGGCTGCTGGTTTTC-3'
β-catenin	F: 5'-GAGTGCTGAAGGTGCTATCTGTC-3'
	R: 5'-CTGAACAAGAGTCCCAAGGAGA-3'

F, forward; R, reverse.

microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), and the growth curve was then plotted. For the colony formation assay, the cells were seeded at 500/well in 6-well plates (1 ml/well) and cultured in complete medium. The culture medium was replaced every 4 days. After 15 days, the cells were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotime, Shanghai, China) at room temperature for 10 min. Images were obtained to calculate the number of clones.

Flow cytometry. At least $5x10^5$ cells were suspended and fixed with 70% ethanol for 2 h. Propidium iodide (IP) was used to stain the cells and the cell cycle distribution was detected using a flow cytometer (BD Biosciences, San Jose, CA, USA).

5-ethynyl-2'-deoxyuridine (EdU) assay. The cells were seeded at $2x10^3$/well in 96-well plates (200 μl/well) and were treated with 50 μM EdU for 2 h at 37°C. After washing with PBS and fixing with 4% paraformaldehyde for 30 min, the cells were permeabilized with 0.5% Triton X-100 for 5 min. The cells were then incubated with 100 μl 1X Apollo reaction cocktail for 30 min. DAPI was then used to stain the nuclei for 15 min. Images were obtained under a fluorescence microscope (Olympus) to calculate the proliferation rate.

Nude mouse models. A total of 12 nude mice aged 4 weeks old (female, weighing 13 to 16 g) were obtained from the Animal Experimental Center of Chongqing Medical University and were maintained at 24±2°C under a 40-70% humidity with free access to food and water. The experiment was divided into SGC7901-NC group and SGC7901-HOXA5 group, with 6 mice in each group. When mice were 5 weeks old, $2x10^6$ cells suspended in 100 μl PBS were injected subcutaneously into the left flank. The tumor volume was monitored by measuring diameters and calculated as $LxS^2/2$ (L indicates the long side and S indicates the short side) every 3 days. After 3 weeks, the nude mice were sacrificed by ether anesthesia combined with carbon dioxide asphyxiation. The flow rate of the gas was 20% of the chamber volume per minute and the death of the mice was judged according to breathing, heartbeat, nerve reflex and muscle relaxation after 5 min. The tumor tissues were stained with hematoxylin and eosin (H&E) for pathological confirmation. The animal experimental protocols were approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University.

GEPIA analysis. Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn), an online cancer microarray database, was used to analyze the effect of HOXA5 on the overall survival of GC patients (9). Patients were divided into high expression group and low expression group according to the median expression level of *HOXA5* mRNA (Normalized by GAPDH) and the overall survival was assessed by a Kaplan-Meier survival plot.

Statistical analysis. All experiments were repeated 3 times and the data were analyzed using SPSS 19.0 software. Measurement data are expressed as the means ± standard deviation (SD). Comparisons were made between two groups using the Student's t-test. Wilcoxon signed-rank tests were used
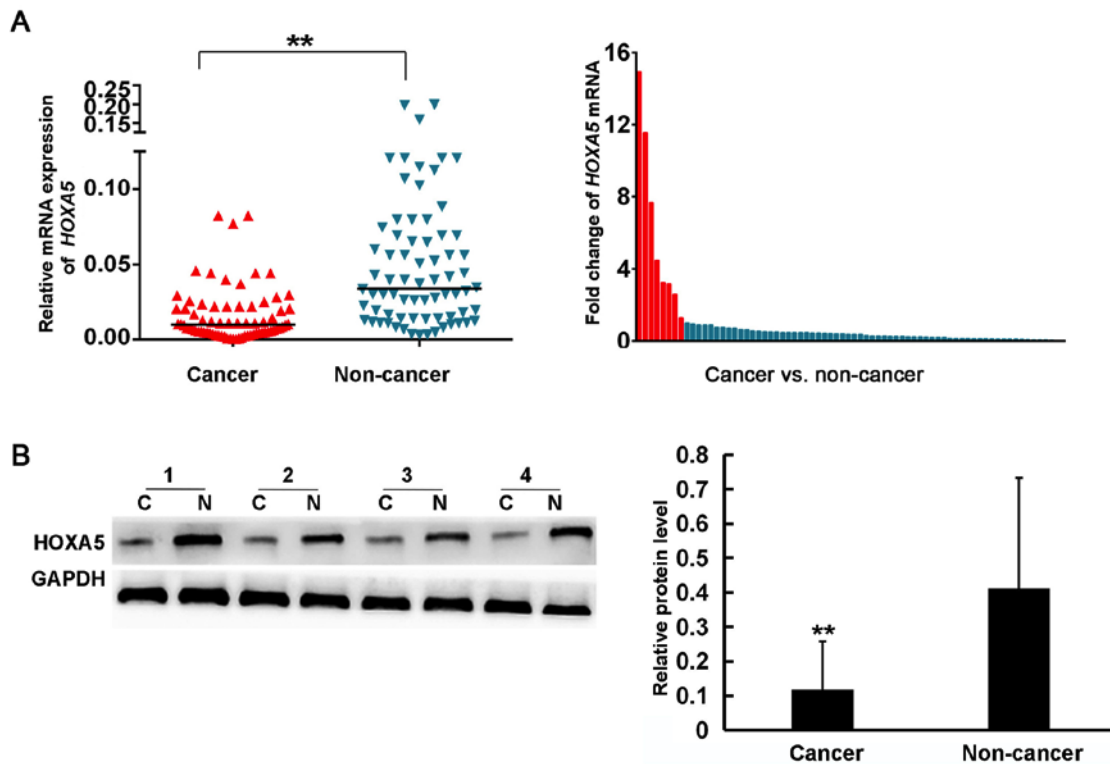
Figure 1. Homeobox A5 (HOXA5) expression is downregulated in gastric cancer (GC) tissues. (A) The mRNA expression levels of *HOXA5* in GC and adjacent non-cancer tissues of 72 patients were detected by RT-qPCR. *GAPDH* was used as an endogenous control (left panel), [**]P<0.01; The fold change is expressed as the ratio of *HOXA5* mRNA expression in GC tissue to the expression of non-cancer tissues (right panel). (B) The protein expression of HOXA5 in GC tissues and adjacent non-cancer tissues was detected by western blot analysis.

to assess the differences in *HOXA5* mRNA expression levels between the GC tissues and adjacent paracancerous tissues of 72 patients. The correlation between HOXA5 protein expression and each clinicopathological parameter was examined using χ^2 tests. Survival curves for different expression levels of HOXA5 were plotted according to the Kaplan-Meier method, and prognostic significance of all factors was calculated by the log-rank test. Significant parameters (P<0.05) in the univariate model were further evaluated using multivariate Cox regression, after which the independence and hazard ratio (HR) of each prognostic factor could be calculated. Differences were considered statistically significant at a P-value <0.05. The correlation between the expression of *HOXA5* mRNA and 23 genes related to malignant biological behavior in 30 GC tissues was determined using Spearman's correlation analysis.

Results

Decreased expression of HOXA5 in human GC tissues. To examine the role of HOXA5 in GC, RT-qPCR was used to detect the mRNA expression of *HOXA5* in 72 pairs of GC issues and adjacent paracancerous tissues. The results demonstrated that 63 patients (87.5%) exhibited a lower mRNA expression of *HOXA5* in GC tissues compared to the matched non-cancer tissues (Fig. 1A). The average mRNA expression level of *HOXA5* in the cancer tissues was 3.23-fold lower than that in the adjacent non-cancer tissues. Subsequently, western blot analysis was performed to determine whether HOXA5 was also downregulated at the protein level in 30 GC patients. As shown in Fig. 1B, the average protein expression level of

HOXA5 in the GC tissues was 3.73-fold lower than that in the non-cancer tissues (0.11±0.14 vs. 0.41±0.32, respectively). These results suggested that HOXA5 expression was decreased in GC.

HOXA5 acts as a tumor suppressor gene in GC. To further illuminate the role of HOXA5 in the progression of GC, we used immunohistochemistry to detect the expression of HOXA5 in samples from 81 patients with GC and analyzed the association between HOXA5 and clinical parameters. We found that HOXA5 staining was mainly located in the nucleus and cytoplasm (Fig. 2). Compared with a 75.3% positive expression of HOXA5 (61/81) in the adjacent non-cancer tissues, the positive expression in the GC tissues was only 35.8% (29/81).

The GC specimens were then divided into a high expression group and a low expression group, according to the immunohistochemistry score for HOXA5 expression. As shown in Table II, a decreased HOXA5 expression was significantly associated with tumor size and histological grade. These results thus suggest that *HOXA5* acted as a tumor suppressor gene in the progression of GC.

Decreased expression of HOXA5 protein as a prognostic marker for GC. To investigate the prognostic value of HOXA5 for GC, survival analysis from GEPIA database was obtained. *HOXA5* mRNA was not associated with the overall survival of patients with GC (Fig. 3A). Given that HOXA5 functions as a transcription factor, we further assessed the association between HOXA5 protein expression and survival according
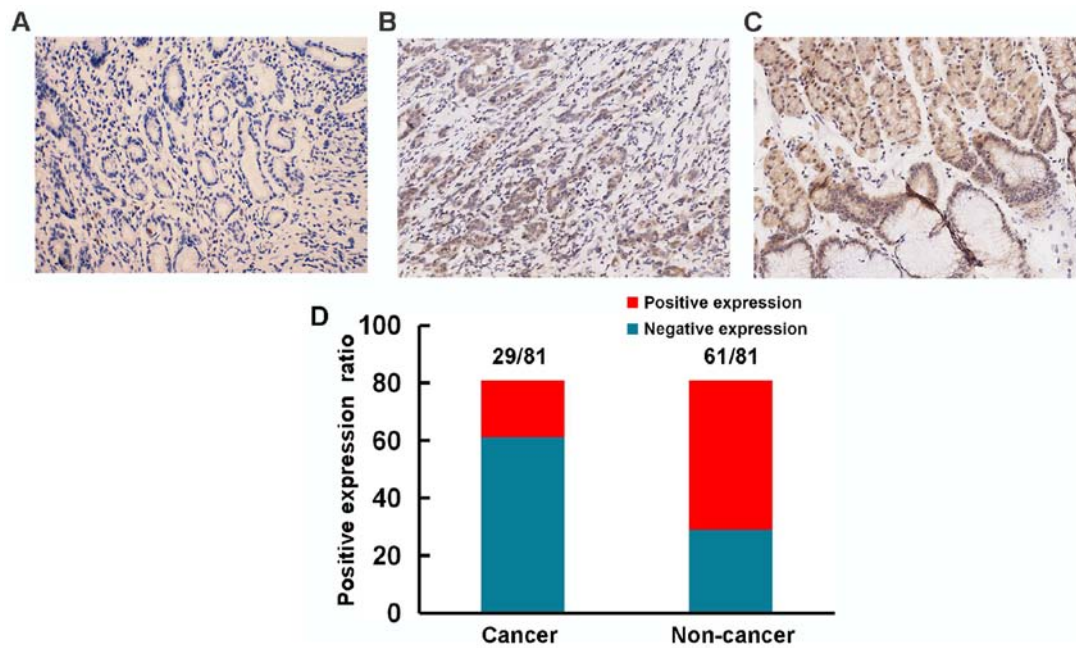
Figure 2. Representative immunohistochemical images showing the expression of homeobox A5 (HOXA5) in paraffin-embedded sections (magnification, x200). (A) A gastric cancer sample without HOXA5 expression. (B) A gastric cancer sample with a high expression level of HOXA5. (C) A non-cancer sample with a high expression level of HOXA5. (D) The positive HOXA5 staining ratio in GC samples and adjacent non-cancer samples.
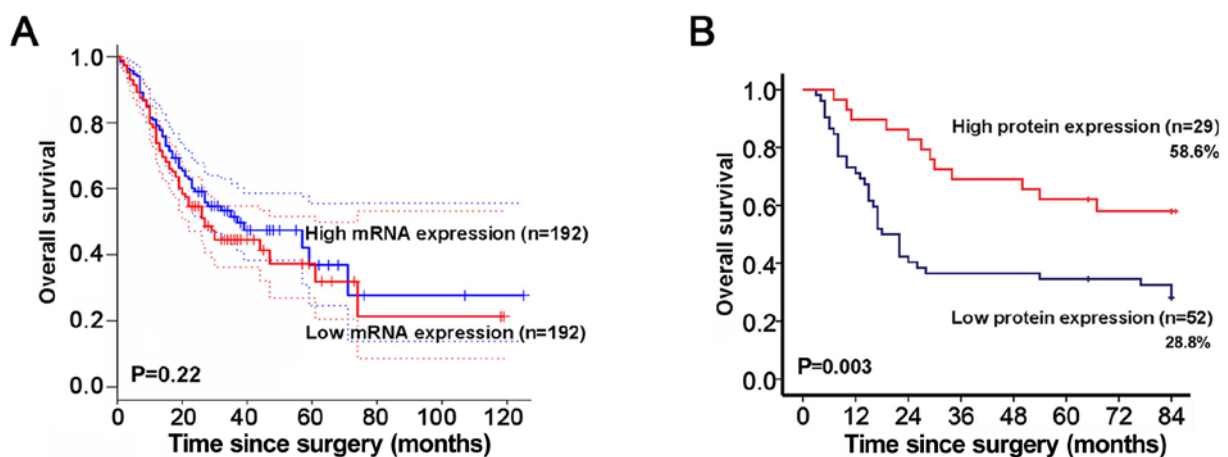

Figure 3. Decreased expression of homeobox A5 (HOXA5) protein indicates a poor prognosis of patients with gastric cancer (GC). (A) The overall survival of patients with GC was compared between the patients with a high *HOXA5* mRNA expression and those with a low *HOXA5* mRNA expression by Kaplan-Meier survival curve analysis. (B) The overall survival of patients with GC was estimated by Kaplan-Meier survival curve analysis and compared between the patients with high HOXA5 protein expression and those with low HOXA5 protein expression using the log-rank test.

to the immunohistochemistry score. It is worth noting that the 75% survival duration of the patients in the low protein expression group was 10 months, which was significantly shorter than that of 30 months for the patients in the high protein expression group. Patients with a low protein expression level of HOXA5 had an overall survival rate of 28.8% in comparison to 58.6% for patients with a high protein expression level (Fig. 3B).

According to a univariate analysis of the prognostic factors of GC, the overall survival was found to be significantly associated with each of the following: Histological grade (P=0.031), tumor size (P<0.001), lymph node involvement (P<0.001), TNM stage (P<0.001), distant metastasis (P<0.001), and decreased HOXA5 expression (P=0.003). To investigate

whether a decreased HOXA5 is an independent prognostic marker in patients with GC, a multivariate Cox regression analysis was performed. We found that tumor size, lymph node involvement, TNM stage, distant metastasis and a decreased HOXA5 expression were significantly associated with overall survival after controlling for other prognostic factors in the multivariate Cox regression analysis (Table III). These data suggested that a decreased HOXA5 expression was an independent risk factor for the prognosis of GC and could be a prognostic marker for patients with GC.

Identification of potential target genes of HOXA5. The progression of GC involves a series of cellular biological events, including the loss of control of cell

Table II. Association between the expression of HOXA5 and clinical parameters of patients with gastric cancer (GC).

Index	Case	HOXA5 +	HOXA5 -	χ^2	P-value
Sex				0.974	0.324
Male	53	21	32		
Female	28	8	20		
Age (years)				0.822	0.365
<60	31	13	18		
≥60	50	16	34		
Histological grade				7.814	0.005[b]
Well-differentiated	39	20	19		
Poorly-differentiated	42	9	33		
Tumor size (cm)				10.65	0.001[b]
<5	39	21	18		
≥5	42	8	34		
TNM stage				2.486	0.115[a]
1+2	38	17	21		
3+4	43	12	31		
Lymph node involvement				0.510	0.475
No	24	10	14		
Yes	57	19	38		
Vascular invasion				2.523	0.112
Absent	16	3	13		
Present	65	26	39		
Distant metastasis				0.008	0.928
No	78	28	50		
Yes	3	1	2		

[a]P<0.05, [b]P<0.01; Chi-square test.

Table III. Kaplan-Meier univariate survival analysis and multivariate Cox regression analysis of prognostic factors in gastric cancer (GC) for overall survival.

Variables	Univariate P-value	Multivariate analysis HR	Multivariate analysis 95% CI	Multivariate analysis P-value
Sex	0.307			
Age (years)	0.943			
Histological grade	0.031[a]	0.683	0.379-1.234	0.206
Tumor size	<0.001[b]	2.697	1.309-5.558	0.007[b]
TNM stage	<0.001[b]	3.083	1.474-6.447	0.003[b]
Lymph node	<0.001[b]	3.787	1.308-10.962	0.014[a]
Vascular invasion	0.284	0.714	0.334-1.526	0.385
Distant metastasis	<0.001[b]	9.263	2.068-41.502	0.004[b]
HOXA5 expression	0.003[b]	0.429	0.202-0.912	0.028[a]

[a]P<0.05, [b]P<0.01; log rank test and cox regression.

proliferation, an enhanced angiogenesis, the acquisition of epithelial-mesenchymal transition (EMT) and stemness, and sustained invasion and drug resistance. Our data suggested that HOXA5 inhibited the progression of GC. In order to further explore the underlying mechanisms, we used RT-qPCR to detect the expression of the following genes in
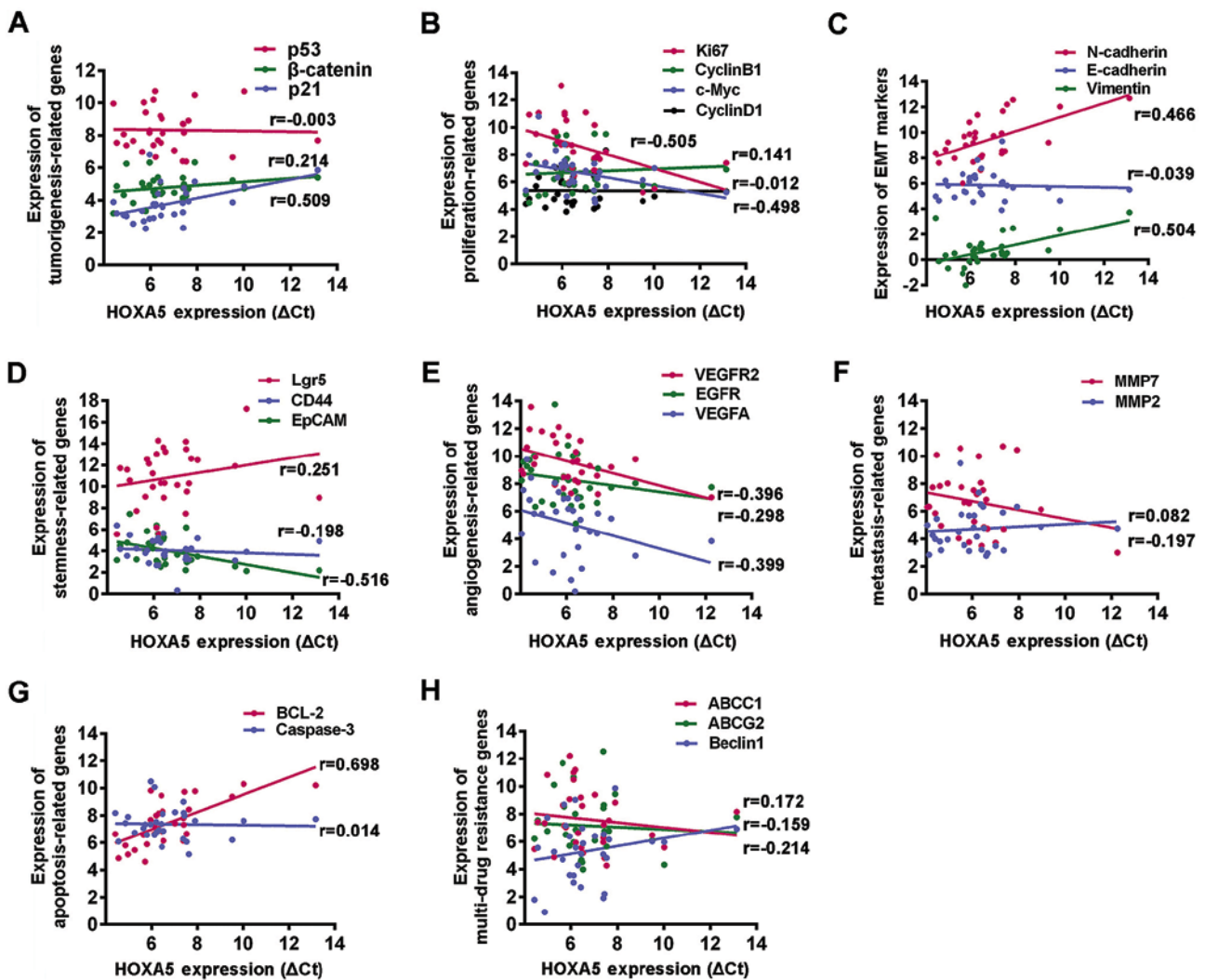
Figure 4. Correlation between homeobox A5 (*HOXA5*) and cancer malignant behavior-related genes. *HOXA5* and the mRNA expression of 23 typical malignant behavior-related genes were detected using RT-qPCR in 30 GC tissues. *GAPDH* was used as a loading control and the relative expression levels were expressed by ΔΔCq. Spearman's correlation analysis was used to determine the correlation *HOXA5* and (A) tumorigenesis-related genes, (B) proliferation-related genes, (C) EMT markers, (D) stemness markers, (E) angiogenesis-related genes, (F) metastasis-related genes, (G) apoptosis-related genes, and (H) multi-drug resistance genes.

30 GC tissues, including tumorigenesis-related genes (*p21*, *p53* and *β-catenin*), proliferation-related genes (*cyclin B1*, *cyclin D1*, *c-Myc* and *Ki67*), angiogenesis-related genes [vascular endothelial growth factor (*VEGF*)*A*, *VEGFR2* and epidermal growth factor (*EGFR*)], metastasis-related genes [matrix metalloproteinase (*MMP2*) and (*MMP7*)], apoptosis-related genes (*BCL2* and *caspase-3*), EMT markers (vimentin, N-cadherin and E-cadherin), stemness markers (*Lgr5*, *CD44* and *EpCAM*) and multi-drug resistance genes (*ABCC1*, *ABCG2* and Beclin1) (10-15). A Spearman's correlation model was used to examine the correlation between each of these genes and *HOXA5* mRNA expression. We found that *HOXA5* mRNA expression positively correlated with the tumor suppressor gene, *p21*, and negatively correlated with the proliferation-related genes, *c-Myc* and *Ki67*, the angiogenesis genes, *VEGFA* and *VEGAFR2*, and the stemness marker, *EpCAM*. No correlation was observed with the other genes examined. Surprisingly, although HOXA5 exhibited a role in inhibiting the progression of GC, its mRNA expression positively correlated with the mesenchymal factors, *vimentin*

and *N-cadherin*, which indicated the acquisition of EMT; it also positively correlated with the anti-apoptotic molecule, *BCL2*, which is known to play an important role in apoptosis resistance (Fig. 4 and Table IV).

Enforced overexpression of HOXA5 suppresses the aberrant proliferation of GC cells. The immunohistochemistry results revealed that HOXA5 expression negatively correlated with the size of the tumor. Moreover, correlation analysis revealed that *Ki67*, *c-Myc* and *p21* (these 3 genes are involved in the regulation of tumor proliferation) may be potential target genes of HOXA5. This indicated that HOXA5 may exert its anticancer effects through the negative regulation of cancer cell proliferation. To examine the function of HOXA5 in the progression of GC, we used HOXA5 overexpression vectors to transfect GC SGC7901 cells, which express a low level of HOXA5, and detected its effect on cell proliferation using a CCK-8 assay (Fig. 5A). The growth curve indicated that HOXA5-overexpressing cells (SGC7901-HOXA5) exhibited a significantly decreased proliferation compared with the
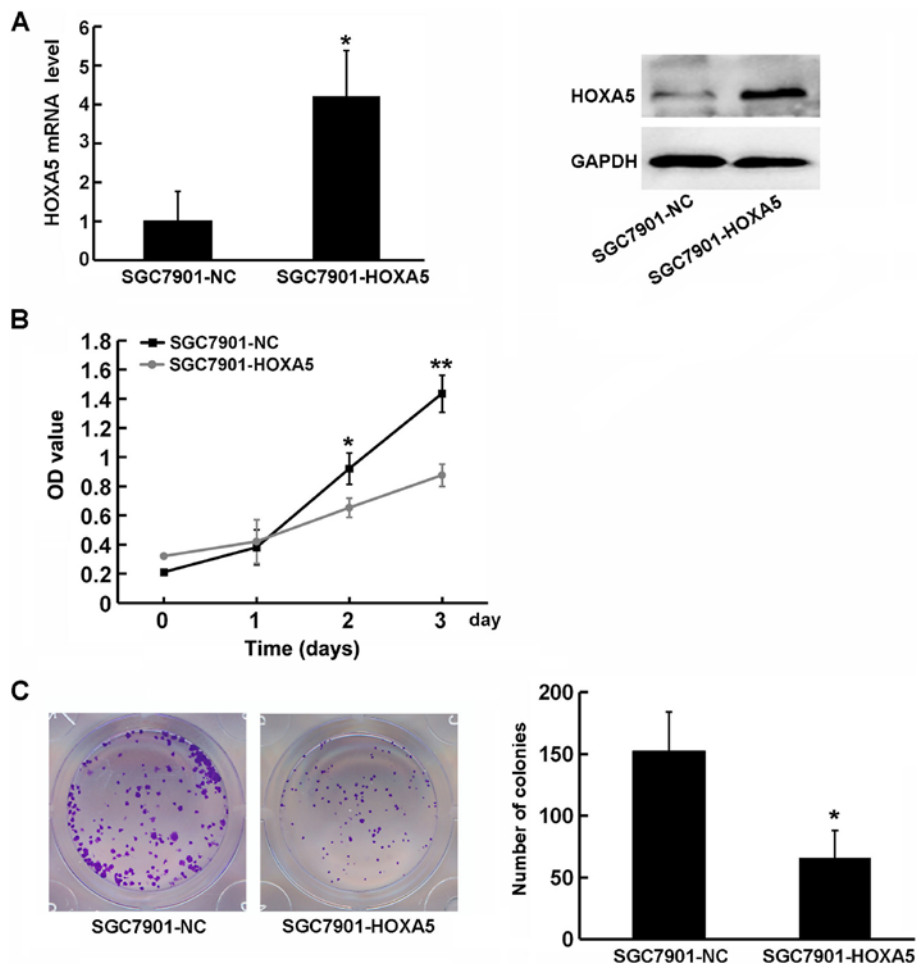
Figure 5. Homeobox A5 (HOXA5) attenuates the aberrant proliferation of gastric cancer (GC) cells. (A) SGC7901 cells were transduced with lentiviruses containing HOXA5 overexpression plasmids. After 72 h, the overexpression efficiency was confirmed by RT-qPCR (left panel) and western blot analysis (right panel). (B) The growth curves of SGC7901-NC and SGC7901-HOXA5 cells were plotted according to the CCK-8 assay. (C) A colony formation assay was used to investigate the colon formation ability of SGC7901-NC and SGC7901-HOXA5 cells.

negative control cells (SGC7901-NC) (Fig. 5B). Similarly, a colony formation assay revealed that HOXA5 overexpression resulted in an impairment of SGC7901 colony formation (Fig. 5C). These results indicated that the upregulation of HOXA5 in GC cells was beneficial in inhibiting cell growth, and that HOXA5 suppressed the proliferation of GC cells.

Enforced overexpression of HOXA5 decelerates the G_1-S phase transition of GC cells. Cell proliferation depends on the proper progression of the cell cycle, particularly in the G_1-S phase transition. As shown in Fig. 6A, the results from flow cytometry revealed that compared with the SGC7901-NC cells, the SGC7901-HOXA5 cells exhibited a significantly increased percentage of cells in the G_0/G_1 phase (38.7% vs. 50.8%), but an decreased percentage of cells in the S phase (48.6% vs. 32.7%). We then employed an EdU staining assay to detect the cell proliferation rate of the cells in the SGC7901-NC and SGC7901-HOXA5 groups. Fluorescence microscopy revealed a lower proliferative activity in the SGC7901-HOXA5 group (19.2%) than that noted in the SGC7901-NC group (55.7%), which was consistent with the flow cytometry data (Fig. 6B). These results indicated that the enforced overexpression of HOXA5 decelerated the G_1-S transition of the GC cells.

HOXA5 regulates the expression of cell cycle-related proteins. Our data suggested that HOXA5 decelerated the cell cycle, thus inhibiting the proliferation of GC cells. In order to further clarify the underlying mechanisms, we used western blot analysis to detect the effect of HOXA5 on a series of cell cycle-related proteins. We found that HOXA5 promoted the expression of p21 and inhibited the expression of c-Myc and Ki67 at the protein level, although it had no marked effect on the expression of p53, cyclin D1 and cyclin E (Fig. 7). This may be the underlying mechanism through which HOXA5 exerts its inhibitory effect on GC cell proliferation.

HOXA5 suppresses the proliferation and promotes the differentiation of GC cells in vivo. Xenograft experiments *in vivo* are the gold standard to determine cell proliferation. Thus, we injected SGC7901-NC or SGC7901-HOXA5 cells subcutaneously into nude mice to observe their different effects on proliferation. As shown in the tumor growth curve, the SGC7901-HOXA5 group began to display a smaller tumor volume than that of the SGC7901-NC group from day 15 (Fig. 8A). In addition, H&E staining revealed that compared with the NC group, the tumors in the SGC7901-HOXA5 group were loosely arranged, with more mesenchymal and inflammatory cell infiltration and
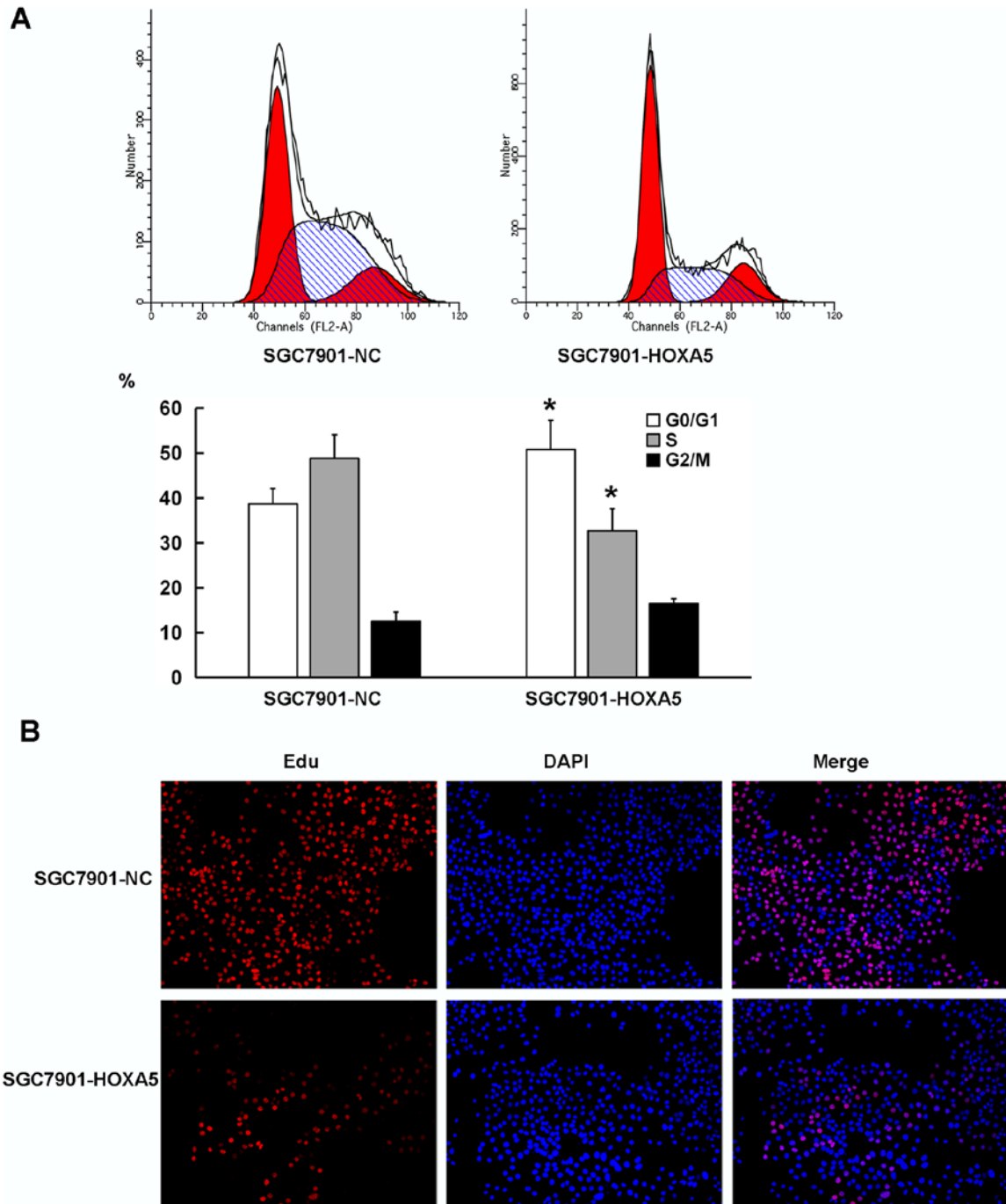
Figure 6. Effect of homeobox A5 (HOXA5) on cell cycle progression in gastric cancer (GC) cells. (A) The cell cycle distributions of SGC7901-NC and SGC7901-HOXA5 cells were evaluated by flow cytometry. Representative images of cell cycle distribution (high) and cell cycle analysis (low). (B) EdU staining was performed to determine the cell proliferation rate of SGC7901-NC and SGC7901-HOXA5 cells (magnification, x200). The proliferation rate was calculated using EdU-stained cell (red) number compared to the total cell number (DAPI-stained, blue). *P<0.05.

fewer abnormal cell mitoses. The overexpression of HOXA5 promoted the tumor cells to enter a more differentiated state (Fig. 8B). These results suggested that HOXA5 suppressed the proliferation and promoted the differentiation of GC cells *in vivo*.

Discussion

In the present study, we demonstrated that HOXA5 was down-regulated in GC and that the underexpression of HOXA5 was associated with the progression of GC. In addition, we also demonstrated that the underexpression of HOXA5 was an independent prognostic factor for patients with GC. Furthermore, we revealed that HOXA5 may exert its anticancer effects through the inhibition of the abnormal proliferation of GC cells.

GC is the second most common malignant tumor world-wide and is associated with a poor prognosis. Despite the existence of comprehensive treatments based on surgery and chemotherapy, the 5-year survival rate of patients with
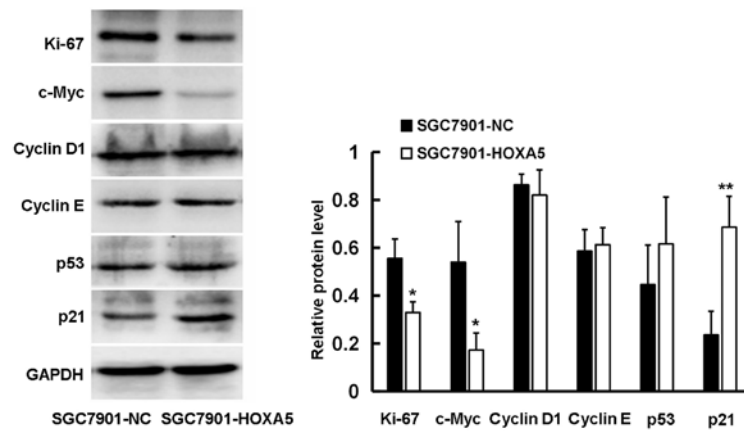
Figure 7. Homeobox A5 (HOXA5) regulation of cell cycle-related proteins. The protein expression levels of p21, p53, cyclin D1, cyclin E, c-Myc and Ki67 in SGC7901-NC and SGC7901-HOXA5 cells were detected by western blot analysis. GAPDH was used as a loading control. $^{*}P<0.05$, $^{**}P<0.01$.
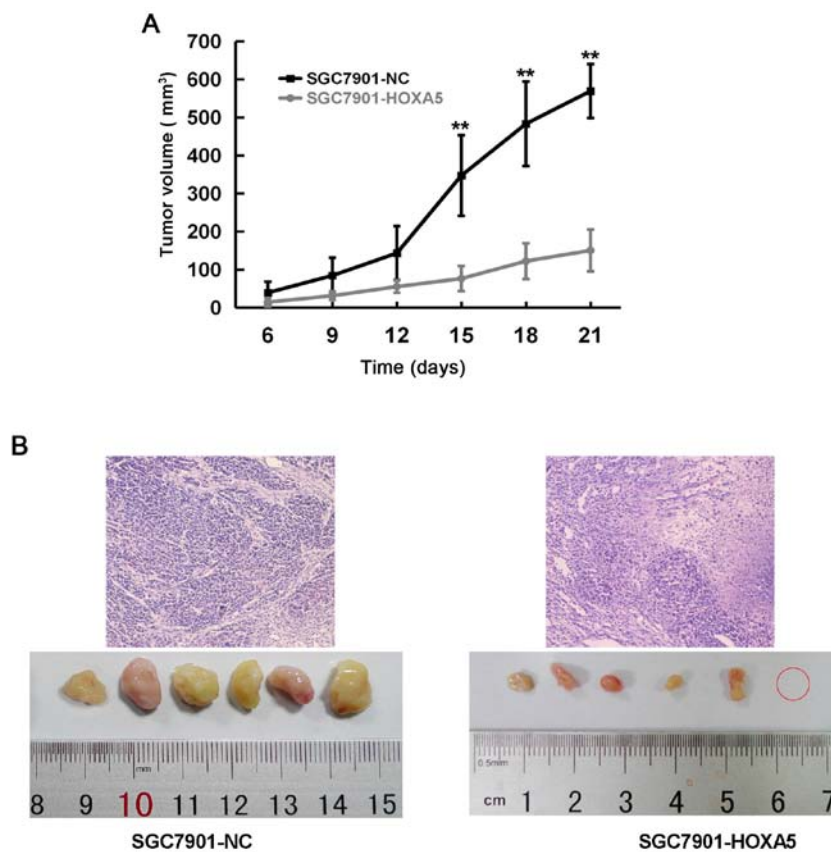

Figure 8. *In vivo* assessment of homeobox A5 (HOXA5) on the proliferation of gastric cancer (GC) cells. (A) Cells ($2x10^6$) were subcutaneously injected into the left flank of nude mice, with 6 mice in each group. The tumor growth curve was plotted on the basis of the tumor volume monitored every 3 days. (B) After 3 weeks, the mice were sacrificed and the tumor tissues were stained for hematoxylin and eosin (H&E) for pathology confirmation. $^{**}P<0.01$.

GC has not improved significantly. It is thus of great clinical significance to find an effective molecular target for the early diagnosis and treatment of GC.

HOXA5 is a member of the homeobox gene family, and it encodes the 29 kDa HOXA5 protein, which functions as a critical master regulatory factor in controlling embryonic development and adult stem cell differentiation. Recently, accumulating evidence has revealed that the human HOXA5 protein limits the aggressiveness of breast cancer and colon cancer (4,5). To clarify the associatoin between HOXA5 and

GC, we detected the expression of HOXA5 using RT-qPCR and western blot analysis in 30 patients with GC and found that HOXA5 expression was significantly lower in GC tissues than that in adjacent non-cancer tissues. This result was consistent with a previous finding, in that *HOXA5* was hypermethylated in GC tissues (7). A recent study reported that HOXA5 was a marker of a good prognosis in patients with colon cancer (4), although another study demonstrated that the overexpression of HOXA5 was associated with a poor prognosis in non-small cell lung cancer (16). In this study, we

Table IV. Spearman's correlation analysis of the correlation between the expression of *HOXA5* and malignant behavior-related genes.

Variables	Spearman's correlation analysis	
	r value	P-value
Carcinogenesis-related genes		
p21	0.509	<0.01[b]
p53	-0.003	0.987
β-catenin	0.214	0.256
Proliferation-related genes		
CCNB1	0.141	0.456
CCND1	-0.012	0.951
Ki67	-0.505	<0.01[a]
C-Myc	-0.498	<0.01[a]
EMT markers		
E-cadherin	-0.039	0.836
N-cadherin	0.466	0.01[a]
Vimentin	0.504	<0.01[a]
Stemness markers		
Lgr5	0.251	0.181
CD44	-0.198	0.295
EpCAM	-0.516	<0.01[a]
Angiogenesis-related genes		
VEGFA	-0.399	0.029[b]
VEGFR2	-0.396	0.03[b]
EGFR	-0.298	0.109
Metastasis-related genes		
MMP2	0.082	0.668
MMP7	-0.197	0.296
Apoptosis-related genes		
BCL2	0.698	<0.01[a]
Caspase-3	-0.014	0.941
Multi-drug resistance genes		
ABCC1	-0.214	0.256
ABCG2	-0.159	0.401
Beclin1	0.172	0.364

[a]$P<0.05$, [b]$P<0.01$; Spearman's correlation analysis.

found that a decreased HOXA5 expression in GC tissues was associated with larger tumors that were poorly differentiated and a higher TNM stage.

Although that the mRNA level of *HOXA5* was not directly associated with the prognosis of patients with GC, patients with a low HOXA5 protein expression had a significantly shorter overall survival than patients with a high expression. One possible explanation for this is that HOXA5 is a transcription factor which functions as a protein and there may be post-transcriptional modifications during the process of translation of *HOXA5* mRNA. Further multivariate analysis confirmed that a decreased HOXA5 expression, together with tumor size, lymph node involvement, distant metastasis and

TNM stage, were independent prognostic factors in patients with GC. Therefore, our results support the notion that a decreased HOXA5 expression promotes the progression of GC and is an indicator of a poor prognosis of patients with GC.

As is widely known, cancer progression involves a series of cellular biological events, including the loss of control of cell proliferation, angiogenesis, EMT, stemness, invasion and drug resistance (17-20). In this study, we found that the mRNA expression of *HOXA5* positively correlated with the broad spectrum tumor suppressor gene, *p21*. Although it has been shown that HOXA5 promotes apoptosis by the transcriptional regulation of p53 in breast cancer cells expressing wild-type *p53* (21), our study did not show an association of *HOXA5* with *p53*. In addition, *HOXA5* was found to negatively correlate with the proliferation-related genes, *c-Myc* and *Ki67*, the angiogenesis-related genes, *VEGFA* and *VEGAFR2*, and the stemness marker *EpCAM*, suggesting that HOXA5 may suppress GC progression by inhibiting abnormal proliferation, angiogenesis and the acquisition of stemness. These results are consistent with previous findings on breast cancer and colon cancer (4,5). It is worth noting that *HOXA5* exhibited a significant positive correlation with the mesenchymal factors, *N-cadherin* and *vimentin*, and the anti-apoptotic molecule, *BCL2*. It is well known that the upregulation of mesenchymal molecules can induce cytoskeletal remodeling, polarity loss and an increased migratory capacity, which are early markers of metastasis in epithelium-derived tumors (22). However, taking into account that HOXA5 is expressed in the mesenchyme and regulates organ patterning through signal pathways such as the hedgehog (Hh) and transforming growth factor-β (TGF-β) pathways in the processes of respiratory and digestive tract development, it is not difficult to envision that HOXA5 also increases N-cadherin and vimentin expression in GC (23). Although HOXA5 may increase *BCL2* expression according to our data, the association between HOXA5 and apoptosis remains to be further verified given the presence of multiple complex signaling pathways in the regulation of apoptosis. Our results suggested that HOXA5 may inhibit the proliferation, angiogenesis and the stemness of GC through mediators or signaling pathways. Despite possibly facilitating EMT, HOXA5 exerted an inhibitory effect on the progression of GC.

Previous studies have reported that HOXA5 inhibits the proliferation of breast and lung cancer cells (24,25). Moreover, in this study, the results from immunohistochemistry also indicated that HOXA5 may exert its anticancer effect through the negative regulation of cancer cell proliferation. To further confirm the role of HOXA5 in GC cell proliferation, we overexpressed HOXA5 by the transduction of lentiviral vectors in SGC7901 cells. As was suspected, the enforced overexpression of HOXA5 significantly inhibited the proliferation of GC cells, as shown by CCK-8 and colony formation assays. The results from flow cytometry and EdU assay revealed that HOXA5 overexpression induced arrested cell cycle at the G_1 phase. The G_1-S transition is regulated by a series of factors (26,27).

In this study, we demonstrated that HOXA5 upregulated p21 and downregulated c-Myc and Ki67, although the expression of two important G_1 phase proteins, cyclin D1 and cyclin E, was not altered. The cyclin-dependent kinase inhibitor, p21, inhibits the activity of the cyclin D1-CDK4 and

cyclin E-CDK2 complexes and prevents the cells from entering the S phase from the G_1 phase, which is the most important pathway to block the G_1-S transition (28,29). By contrast, the proliferation-related proteins, c-Myc and Ki67, contribute to the progression of the cell cycle. It is worth noting that the correlation model in tissues revealed that the correlation between these three genes and *HOXA5* was unimpressive. Our function assays in cells demonstrated that the overexpression of HOXA5 can cause significant p21 upregulation and Ki67, c-Myc downregulation. One possible explanation is that HOXA5 is a type of transcription factor and can function through downstream signaling pathways (27), or direct transcriptional activation or inhibition of target genes (30) in the form of a protein. Once HOXA5 protein in the GC cells was upregulated, the downstream target genes revealed obvious changes. Therefore, we hypothesized that HOXA5 suppressed the aberrant proliferation of GC cells through the regulation of p21, c-Myc, and Ki67. This may be the mechanism through which HOXA5 exerts its anti-tumor effects on GC. Finally, nude mouse models also verified that HOXA5 suppressed the proliferation of GC cells *in vivo*.

In conclusion, in this study, we demonstrated that *HOXA5* was a tumor suppressor gene and was decreased in GC. Its underexpression may be used as a direct prognostic indicator of a negative outcome. Our research may provide an opportunity for developing a novel therapeutic target as well as a prognostic marker in GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and are freely available to any researchers.

Authors' contributions

ZW conceived and designed the experiments; XP, LZ and AC performed the experiments and analyzed the data; and XP wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The use of human tissue samples and experimental protocols were approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University and written informed consent was obtained from all patients. The animal experimental protocols were approved by the Medical Ethics Review Committee of the First Affiliated Hospital of Chongqing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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