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ABOUT COVER

Editorial Board Member of *World Journal of Gastrointestinal Oncology*, Tomohide Hori, PhD, MD, FACS, is an Attending Surgeon at the Affiliated Hospital with Kyoto University (Kyoto, Japan). Dr. Hori obtained his medical doctor license from Tokyo Medical University (Japan) in 1997 and his Doctor of Philosophy degree from Mie University Graduate School of Medicine (Japan) in 2007. Dr. Hori is certified and qualified by many international and domestic societies, including the American College of Surgeons and Japan Society of Endoscopic Surgery. From 2009 to 2011, he worked at Mayo Clinic (Jacksonville, FL, United States). Dr. Hori's research career has spanned the fields of gastroenterology, hepatology, transplant immunology, and gastrointestinal, colorectal, hepatobiliary, pancreatic, transplant and pediatric surgeries, yielding more than 200 medical publications in international journals. (L-Editor: Filipodia)

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Basic Study

Decreased expression of the long non-coding RNA *HOXD-AS2* promotes gastric cancer progression by targeting *HOXD8* and activating *PI3K/Akt* signaling pathway

Lin Yao, Peng-Cheng Ye, Wang Tan, Ya-Jun Luo, Wan-Ping Xiang, Zi-Lin Liu, Zhi-Ming Fu, Fei Lu, Ling-Han Tang, Jiang-Wei Xiao

ORCID number: Lin Yao 0000-0002-1123-7789; Peng-Cheng Ye 0000-0003-3574-5128; Wang Tan 0000-0002-7780-8855; Ya-Jun Luo 0000-0002-8924-580X; Wan-Ping Xiang 0000-0003-1883-5180; Zi-Lin Liu 0000-0003-0113-9912; Zhi-Ming Fu 0000-0003-2468-899X; Fei Lu 0000-0002-1566-8746; Ling-Han Tang 0000-0001-8531-0304; Jiang-Wei Xiao 0000-0002-4288-7581.

Author contributions: Yao L and Xiao JW conceived and designed the experiments; Yao L and Ye PC did most of the experiments and data analysis; Yao L wrote the manuscript; Ye PC, Fu ZM, Lu F, and Tang LH collected the clinical samples; Tan W, Yao L, and Xiang WP performed data analysis; Xiao JW revised the manuscript; and all authors read and approved the final manuscript.

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Lin Yao, Department of Gastrointestinal Surgery, Clinical Medical College and The First Affiliated Hospital of Chengdu Medical College, Chengdu 610000, Sichuan Province, China, The Hepatobiliary Research Institute, North Sichuan Medical College, Nanchong 637000, Sichuan Province, China

Lin Yao, Peng-Cheng Ye, Zhi-Ming Fu, Fei Lu, Ling-Han Tang, Department of Gastrointestinal Surgery, The Affiliated Hospital of North Sichuan Medical College, The Hepatobiliary Research Institute, North Sichuan Medical College, Nanchong 637000, Sichuan Province, China

Wang Tan, Department of Gastrointestinal Surgery, Yaan People's Hospital, Yaan 625000, Sichuan Province, China

Ya-Jun Luo, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China

Wan-Ping Xiang, Department of Thoracic Surgery, Nanchong Central Hospital, Nanchong 637000, Sichuan Province, China

Zi-Lin Liu, Jiang-Wei Xiao, Department of Gastrointestinal Surgery, Clinical Medical College and The First Affiliated Hospital of Chengdu Medical College, Chengdu 610000, Sichuan Province, China

Corresponding author: Jiang-Wei Xiao, MD, PhD, Postdoc, Professor, Surgeon, Surgical Oncologist, Teacher, Department of Gastrointestinal Surgery, Clinical Medical College and The First Affiliated Hospital of Chengdu Medical College, No. 278 Baoguang Road, Xindu District, Chengdu 610000, Sichuan Province, China. xiaojiangwei2018@163.com

Abstract

BACKGROUND

Long non-coding RNAs (lncRNAs) have been shown to be associated with many tumors. However, the specific mechanism of lncRNAs in the occurrence and development of gastric cancer (GC) has not been fully elucidated.

AIM

accordance with the Declaration of Helsinki and with the approval of The Ethical Committee of The Affiliated Hospital of North Sichuan Medical College.

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To explore the expression level and molecular mechanism of *HOXD-AS2* in GC tissues and cells, and analyze its significance in the prognosis of GC.

METHODS

Real-time quantitative PCR was used to detect the expression of *HOXD-AS2* in 79 pairs of GC tissues and five cell lines. The pc*HOXD-AS2* plasmid vector was constructed and transfected into SGC-7901 and SNU-1 GC cells. Matrigel Transwell and wound healing assays were used to confirm the effect of *HOXD-AS2* on invasion and migration of GC cells. Cell counting kit-8 assay and flow cytometry were used to verify the effect of *HOXD-AS2* on the proliferation, cell cycle, and apoptosis of GC cells. The relevant regulatory mechanism between *HOXD-AS2* and *HOXD8* and PI3K/Akt signaling pathway was verified by Western blot analysis.

RESULTS

The low expression of lncRNA *HOXD-AS2* was associated with lymph node metastasis and tumor-node-metastasis stage in GC. *In vitro* functional experiments demonstrated that overexpression of *HOXD-AS2* inhibited GC cell progression. Mechanistic studies revealed that *HOXD-AS2* regulated the expression of its nearby gene *HOXD8* and inhibited the activity of the PI3K/Akt signaling pathway.

CONCLUSION

These results indicate that downregulation of *HOXD-AS2* significantly promotes the progression of GC cells by regulating *HOXD8* expression and activating the PI3K/Akt signaling pathway. *HOXD-AS2* may be a novel diagnostic biomarker and effective therapeutic target for GC.

Key Words: Long non-coding RNA; Gastric cancer; *HOXD-AS2*; *HOXD8*; PI3K/Akt; Progression

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Core Tip: In this study we found that the expression of long non-coding RNA *HOXD-AS2* was down-regulated in gastric cancer (GC) tissues and cells. The low expression of *HOXD-AS2* was associated with lymph node metastasis and tumor-node-metastasis stage in GC. Overexpression of *HOXD-AS2* inhibited the progression of GC cells. Decreased expression of *HOXD-AS2* promotes GC cell progression by targeting *HOXD8* and activating the PI3K/Akt signaling pathway. *HOXD-AS2* may be a novel diagnostic biomarker and effective therapeutic target for GC.

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INTRODUCTION

According to the latest statistics, in 2018 there were more than 1000000 new cases of gastric cancer (GC) and an estimated 783000 deaths from GC worldwide. The incidence rate of GC is fifth among all malignant tumors, and the mortality rate ranks third. GC seriously affects human health^[1]. China is one of the countries with a high incidence of GC, and with new cases of GC in China accounting for more than 40% of the world's GC cases, GC has become the second leading cause of cancer death in China^[2]. Because most patients with GC are asymptomatic at the early stage^[3], many are in advanced stage at the time of initial diagnosis, and the prognosis is often poor^[4]. At present, the therapeutic effects on GC are still not satisfactory. Therefore, further

understanding of the molecular mechanisms of gastric carcinogenesis, development, invasion, and migration and searching for new targeted drugs and methods are of great significance for the treatment of GC and improvement of the patient survival rate.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs that are more than 200 nucleotides in length and lack an open reading frame^[5]. lncRNAs have been shown to regulate RNA transcription and mRNA splicing, and play an important role in regulating the stability of RNA in the cytoplasm and the activity of microRNAs^[6,7]. An increasing number of studies have shown that abnormally expressed lncRNAs are involved in the process of tumor genesis and development and have a close relationship with tumor cell proliferation, apoptosis, invasion, and metastasis and poor prognosis^[8-12]. In recent years, many studies have also found that abnormally expressed lncRNAs are involved in the occurrence and development of GC. For instance, lncRNA *SLC7A11-AS1* is expressed at low levels in GC and significantly associated with tumor macroscopic type, distant migration, and tumor-node-metastasis (TNM) stage. *SLC7A11-AS1* can be used as a biomarker for the diagnosis and prognosis of GC, and provides a potential target for GC treatment^[13]. lncRNA *LINC01606* is highly expressed in GC. *LINC01606* can act as an endogenous competitive RNA (ceRNA) to adsorb miR-423-5p, attenuating the inhibitory effect of miR-423-5p on the *Wnt3a* gene, thereby activating the Wnt/ β -catenin signaling pathway and promoting the invasion and migration of GC cells^[14]. lncRNA *LINC01133* is downregulated in GC tissues and cells. Low expression of *LINC01133* is significantly associated with tumor size, depth of invasion, lymph node metastasis, and TNM stage and predicts poor prognosis. *LINC01133* can act as a ceRNA to adsorb miR-106a-3p and thereby regulate the expression of the *APC* gene and affect the Wnt/ β -catenin signaling pathway. The results suggest that *LINC01133* can be used as a potential biomarker for poor prognosis of GC^[15]. Although a major breakthrough has been achieved in the study of lncRNAs in the pathogenesis of GC, the specific mechanism of lncRNAs in the occurrence and development of GC has not been fully elucidated.

We screened for abnormally expressed lncRNA *HOXD-AS2* in GC by gene chip analysis. *HOXD-AS2*, which is located at 2q31.1 and is encoded by three exons, was mapped to chromosome 2 region 176134841-176137098. In the present study, we used quantitative polymerase chain reaction (qPCR) to detect the expression of *HOXD-AS2* in 79 pairs of GC tissues and 5 GC cell lines. Based on the clinical and pathological features of GC patients, we found that *HOXD-AS2* may be involved in the progression of GC. Then, a *HOXD-AS2* plasmid was constructed and transfected into SGC-7901 and SNU-1 cells. After transfection, the effect of *HOXD-AS2* on the progression of GC cells was analyzed. Furthermore, we illustrated a potential mechanism by which *HOXD-AS2* may modulate the expression of *HOXD8* and activate the PI3K/Akt signaling pathway in SGC-7901 and SNU-1 GC cells. The objective of our study was to explore the role of *HOXD-AS2* in the development and progression of GC and to elucidate its possible regulatory mechanisms.

MATERIALS AND METHODS

Patients and tumor tissues

A total of 79 human GC tissues and matched adjacent non-cancerous tissues (ANTs) (at a distance of 5 cm from the tumor margin) were obtained at the time of surgery from April 2015 to May 2017 at The Affiliated Hospital of North Sichuan Medical College (Sichuan, China). None of the patients received radiotherapy, chemotherapy, or other anti-tumor treatments before surgery. Following excision, the GC and non-cancerous tissues were immediately frozen in liquid nitrogen and preserved at -80 °C until use. The clinicopathological parameters of patients with GC were collected. All patients provided written informed consent, and the entire study protocol was approved by The Ethics Committee of the Affiliated Hospital of North Sichuan Medical College, Nanchong, China.

Cell culture and cell transfection

Five human GC cell lines, AGS, MGC-803, BGC-823, SNU-1, and SGC-7901, and the normal gastric mucosal cell line GES-1 were purchased from Shanghai Cell Bank (Shanghai, China). The results of short tandem repeat (STR) analysis showed that there was no tri-allelic phenomenon in any locus, and no cross-contamination of other human cells was found. STR analysis found a 100% match to the reference data in the

ATCC cell bank. Cells were cultured in DMEM or RPMI-1640 (Gibco BRL, Gaithersburg, MD, United States) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, United States) and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a humidified atmosphere with 5% CO₂.

The pc-*HOXD-AS2* plasmid vector was purchased from Beijing Syngentech (Beijing, China). The plasmid was transfected into SGC-7901 and SNU-1 cells using Lipofectamine 3000 (Invitrogen, United States). After 48 h, the efficiency of each transfection group was compared with regard to the expression of *HOXD-AS2*.

The PI3K inhibitor LY294002 was purchased from MedChemExpress (United States). LY294002 was dissolved in DMSO (100 mmol/L) and stored at -20 °C for 1 wk. Before use, LY294002 was quickly diluted into culture medium at a final concentration of 10 µmol/L.

Reverse transcription quantitative PCR

Total RNA was extracted from GC tissues, ANTs, and GC cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), and reverse transcription reactions were performed using the GoScript Reverse Transcription System (Promega, Madison, United States) according to the manufacturer's instructions. Real-time PCR was performed according to the instructions using standard Roche fluorescence quantitative PCR reagents (Roche, United States) and Roche fluorescent PCR instruments. *β-actin* or *GAPDH* was used as an internal reference. Each sample was detected three times. After the end of PCR, the dissolution profile of each specimen was stringently checked to ensure that there was only a single amplification product. *HOXD-AS2*, *HOXD8*, *GAPDH*, *β-actin*, *PI3K*, *Akt*, *MAM2*, and *p53* primers were purchased from Shanghai Biotech (Shanghai, China). The primer sequences are shown in [Supplementary Table 1](#). Analysis of the relative quantification of gene expression was performed using the classical 2^{-ΔΔCt} method.

Transwell migration and invasion assays

The invasive ability of GC cells (SGC-7901 and SNU-1) was detected by Matrigel Transwell assay. A 24-well Transwell plate was used with membranes separated by 8 µm pores (Costar, Cambridge, MA, United States), and 50 mg/L Matrigel (BD Pharmingen, San Jose, CA, United States) was added to the upper chamber. Then, the 24-well plate was incubated at 37 °C in a humidified incubator containing 5% CO₂ for 2 h. SGC-7901 and SNU-1 cells transfected for 48 h were inoculated into the upper chamber at 5 × 10⁵ cells per well, and complete medium containing 10% FBS was added to the lower chamber. After 48 h of incubation, the cells that did not invade the Matrigel were wiped off with a cotton swab, and the invaded cells attached to the lower surface of the membrane were fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO, United States), and stained with 1% crystal violet (Beyotime, Shanghai, China). The migration assay was similar to the invasion assay except that Matrigel was not added. Finally, three fields of view were randomly selected under a microscope for invasive and migratory cell counting. Three replicate wells were set in all experiments.

Wound healing assay

Cell migration ability was measured using a scratch wound healing assay. A straight line was marked with a marker in the middle on the back of a 3.5 cm dish. The SGC-7901 and SNU-1 GC cells transfected 24 h after each group were inoculated into a culture dish. After 24 h, a scratch was made on the bottom of the culture dish with a 200 µL tip, and the cell fragments were washed with sterile phosphate buffered saline (PBS). After incubation at 37 °C and 5% CO₂ for 48 h, the distance between the two edges of the scratch was observed under a microscope.

Cell proliferation assays

Cell counting kit-8 assay (CCK-8, Beyotime Institute of Biotechnology, Shanghai, China) was used to detect the cell viability. Cells were seeded at 5000 cells per well in a 96-well plate. After the cells were transfected with plasmids for 24 h, 10 µL of CCK-8 was added to each well at 0 h, 24 h, 48 h, and 72 h. The absorbance at 450 nm of each well was measured with a spectrophotometer. All experiments were performed in triplicate.

Flow cytometry assay

Cell apoptosis was analyzed with Annexin V-APC/7-AAD Apoptosis Detection kit (KeyGEN BioTECH, China) and a NovoCyte flow cytometer (ACEA, China). Forty-eight hours after cell transfection, the cells were digested with trypsin without EDTA

and washed twice with cold PBS. Then the digested cells were put into the flow sampling tube, and 500 μ L of Binding Buffer was added to the tube according to the ratio of 100:1, followed by the addition of 5 μ L Annexin V-APC and 5 μ L 7-AAD staining solution. After 15 min of reaction at room temperature and protection from light, samples were taken on the machine for apoptosis analysis. Each experiment was repeated three times.

For cell cycle detection, the cells were collected into a brown EP tube. Add pre-cooled 70% alcohol was added, the tube was shaken gently, and put in a refrigerator at 4 °C for 4 h. After the cell membrane was broken, 0.5 mL of propidium iodide staining solution was added to the tube for staining at room temperature in the dark for 30 min, and then the sample was subject to cell cycle analysis. This experiment was performed in triplicate.

Western blot analysis

Total protein was extracted, and the concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, United States). Sample lysates (10 μ g of protein) were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with specific antibodies against *HOXD8* (1:1000), *PI3K* (1:1000), *Akt* (1:2000), *MDM2* (1:500), or *p53* (1:1000) (Abcam, Cambridge, MA, United States) at 4 °C overnight, followed by incubation with secondary antibody. Protein levels were normalized to those of total *GAPDH*, which were detected using a monoclonal anti-*GAPDH* antibody (1:10000; Sigma-Aldrich Corporation, St. Louis, MO, United States). Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad).

Statistical analysis

All experimental data were analyzed using SPSS20.0 (SPSS, Chicago, IL, United States) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, United States), and the measurement data are expressed as the mean \pm SD. Two-tailed Student's *t*-test was used to compare two groups of measurement data. The relationship between lncRNA expression levels and clinical indicators of patients was calculated by Chi-square test or Fisher's exact probability test (two-sided). Survival analysis for patients with GC was performed by the Kaplan-Meier method, and the differences between survival curves were estimated by the log-rank test. Spearman correlation analyses were performed to investigate correlations between gene expression levels. All tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Decreased expression of lncRNA *HOXD-AS2* is associated with a poor prognosis in patients with GC

As shown in **Figure 1A**, the relative expression level of lncRNA *HOXD-AS2* in 79 GC tissues was significantly decreased in comparison with that in the corresponding adjacent tissues ($P = 0.030$). As shown in **Figure 1B**, decreased expression of *HOXD-AS2* was observed in 49 (62.03%) out of 79 cases, while high expression was observed in the 30 remaining cases (37.97%). The relationship between the expression level of *HOXD-AS2* and the clinicopathological features of GC patients was analyzed. As shown in **Table 1**, low expression of *HOXD-AS2* was associated with lymph node metastasis ($P = 0.009$) and TNM stage ($P = 0.040$). Data from the Kaplan-Meier Plotter database (<http://kmplot.com/>) was used to analyze the relationship between *HOXD-AS2* expression and the overall survival rate of 348 patients with GC (109 patients with low *HOXD-AS2* expression and 239 with high *HOXD-AS2* expression). The prognosis of the *HOXD-AS2* low expression group was worse than that of the *HOXD-AS2* high expression group, and the difference was statistically significant ($P = 0.012$, **Figure 1C**).

lncRNA *HOXD-AS2* is downregulated in GC cells

As shown in **Figure 2A**, the relative expression level of lncRNA *HOXD-AS2* in the five GC cell lines SGC-7901, MGC-803, BGC-823, SNU-1, and AGS was lower than that in the normal gastric mucosal cell line GES-1 ($P = 0.005$, $P = 0.006$, $P < 0.001$, $P = 0.004$, and $P < 0.001$, respectively). The expression of *HOXD-AS2* was the lowest in SGC-7901 and SNU-1 cells, so we selected these two cell lines for subsequent experimental studies. As shown in **Figure 2B**, after transfection of SGC-7901 and SNU-1 cells, the expression of *HOXD-AS2* in the pc*HOXD-AS2* group was significantly increased compared with

Table 1 Association between *HOXD-AS2* expression and clinicopathological factors of human gastric cancer patients

Characteristic	Each group (n)	<i>HOXD-AS2</i> expression		P value
		High	Low	
All cases	79	30	49	
Age (yr, mean ± SD)	79	64.10 ± 9.323	62.04 ± 9.648	0.354
BMI	79	21.04 ± 2.797	21.90 ± 3.196	0.224
Gender	79			0.594
Male	63	23	40	
Female	16	7	9	
Smoking	79			0.179
Yes	32	15	17	
No	47	15	32	
Drinking alcohol	79			0.096
Yes	23	12	11	
No	56	18	38	
Maximum tumor diameter (cm, mean ± SD)	79	4.36 ± 1.856	4.87 ± 2.613	0.355
Histology	79			1.000
Undifferentiated	7	3	4	
Differentiated	72	27	45	
Depth of invasion	79			0.430
pT 1-2	9	5	4	
pT 3-4	70	25	45	
Lymph node metastasis	79			0.009
pN0	19	12	7	
pN1-3	60	18	42	
Distant metastasis	79			1.000
pM0	75	28	47	
pM1	4	2	2	
Tumor TNM stage	79			0.040
I-II	19	11	8	
III-IV	60	19	41	
Venous/lymphatic invasion	79			0.376
Positive	11	6	5	
Negative	68	24	44	
Nervous invasion	79			1.000
Positive	10	4	6	
Negative	69	26	43	
Liver metastasis	79			0.300
Absent	75	27	48	
Present	4	3	1	
Ascitic fluid	79			0.965
Negative	63	24	39	
Positive	16	6	10	

Fatty nodules	79		1.000
Positive	9	3	6
Negative	70	27	43

BMI: Body mass index; TNM: Tumor-node-metastasis.

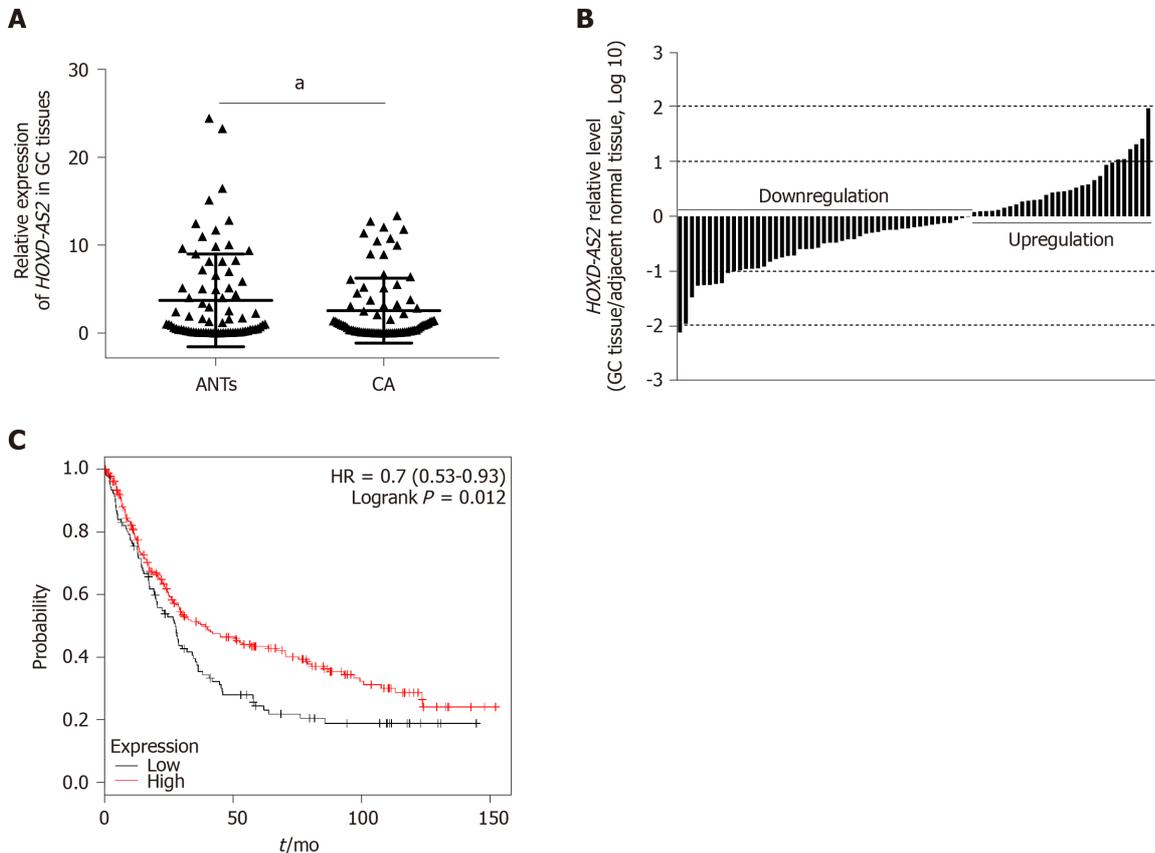


Figure 1 *HOXD-AS2* is downregulated in gastric cancer tissues and is associated with a poor prognosis in gastric cancer patients. A: The relative expression level of *HOXD-AS2* in gastric cancer (GC) tissues was significantly lower than that in adjacent non-cancerous tissues (ANTs; $P = 0.030$, $n = 79$); B: *HOXD-AS2* expression levels were decreased in human GC tissues and ANTs. Bars represent the ratio between the expression levels in GC tissues and ANTs (C/N, log scale) from the 79 patients. GC tissues expressed significantly lower levels of *HOXD-AS2* than ANTs in the majority of patients (62.03%); C: Kaplan-Meier survival analysis showed that patients with low *HOXD-AS2* expression had a poorer prognosis than those with high expression. Low: The expression level of *HOXD-AS2* is lower than that of ANTs. High: The expression level of *HOXD-AS2* is higher than that of ANTs. Expression levels were normalized to β -actin levels. The results are shown as the mean \pm SD. ^a $P < 0.05$, two-tailed Student's *t*-test. GC: Gastric cancer; ANTs: Adjacent non-cancerous tissues.

that in the control group ($P = 0.002$ and 0.002 , respectively).

Overexpression of *HOXD-AS2* inhibits the invasion and migration of GC cells SGC-7901 and SNU-1

The effects of *HOXD-AS2* on the invasion and migration of SGC-7901 and SNU-1 cells were detected by wound healing assay and Transwell assay. It was found that overexpression of *HOXD-AS2* inhibited the invasion and migration of SGC-7901 and SNU-1 cells (Figure 3A). We calculated the number of invasive and migratory cells in the pc-*HOXD-AS2* group and control group, and cell migration and invasion in the pc-*HOXD-AS2* group were significantly decreased compared with those in the control group (Figure 3A). The wound healing assay also confirmed the ability to inhibit the migration of SGC-7901 and SNU-1 cells after overexpression of *HOXD-AS2* (Figure 3B). These results show that *HOXD-AS2* is involved in the regulation of invasion and migration of GC cells.

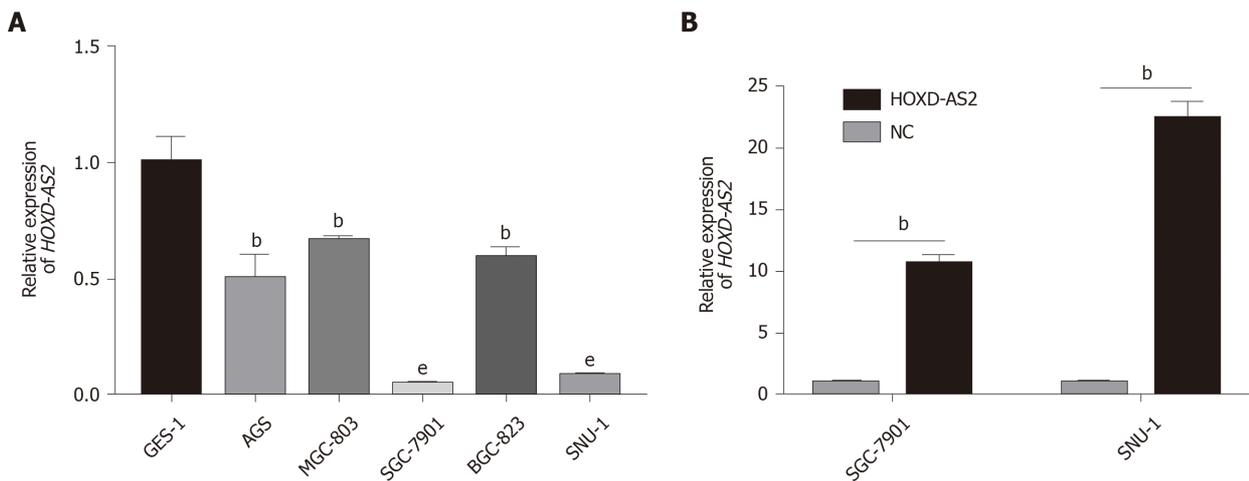


Figure 2 *HOXD-AS2* is downregulated in gastric cancer cells. A: The expression of *HOXD-AS2* was downregulated in gastric cancer AGS, MGC-803, SGC-7901, BGC-823, and SNU-1 cells compared with gastric epithelial GES-1 cells; B: The expression of *HOXD-AS2* in SGC-7901 and SNU-1 cells after transfections. The results are shown as the mean \pm SD. ^b $P < 0.01$, ^e $P < 0.001$.

***HOXD-AS2* regulates the proliferation, cell cycle progression, and apoptosis of SGC-7901 and SNU-1 cells**

The cell proliferation activity was detected by the CCK8 experiment, and it was found that overexpression of *HOXD-AS2* can inhibit the proliferation ability of SGC-7901 and SNU-1 cells (Figure 4A and B). We examined the effect of *HOXD-AS2* on the apoptosis of SGC-7901 and SNU-1 cells by flow cytometry. The results showed that the apoptosis rate of the *HOXD-AS2* overexpression group was higher than that of the control group (Figure 4C). Cell cycle detection results showed that in SGC-7901 and SNU-1 cells, overexpression of *HOXD-AS2* can increase the number of G0/G1 phase cells, reduce the number of S phase cells, and block the cell cycle in G0/G1 phase (Figure 4D).

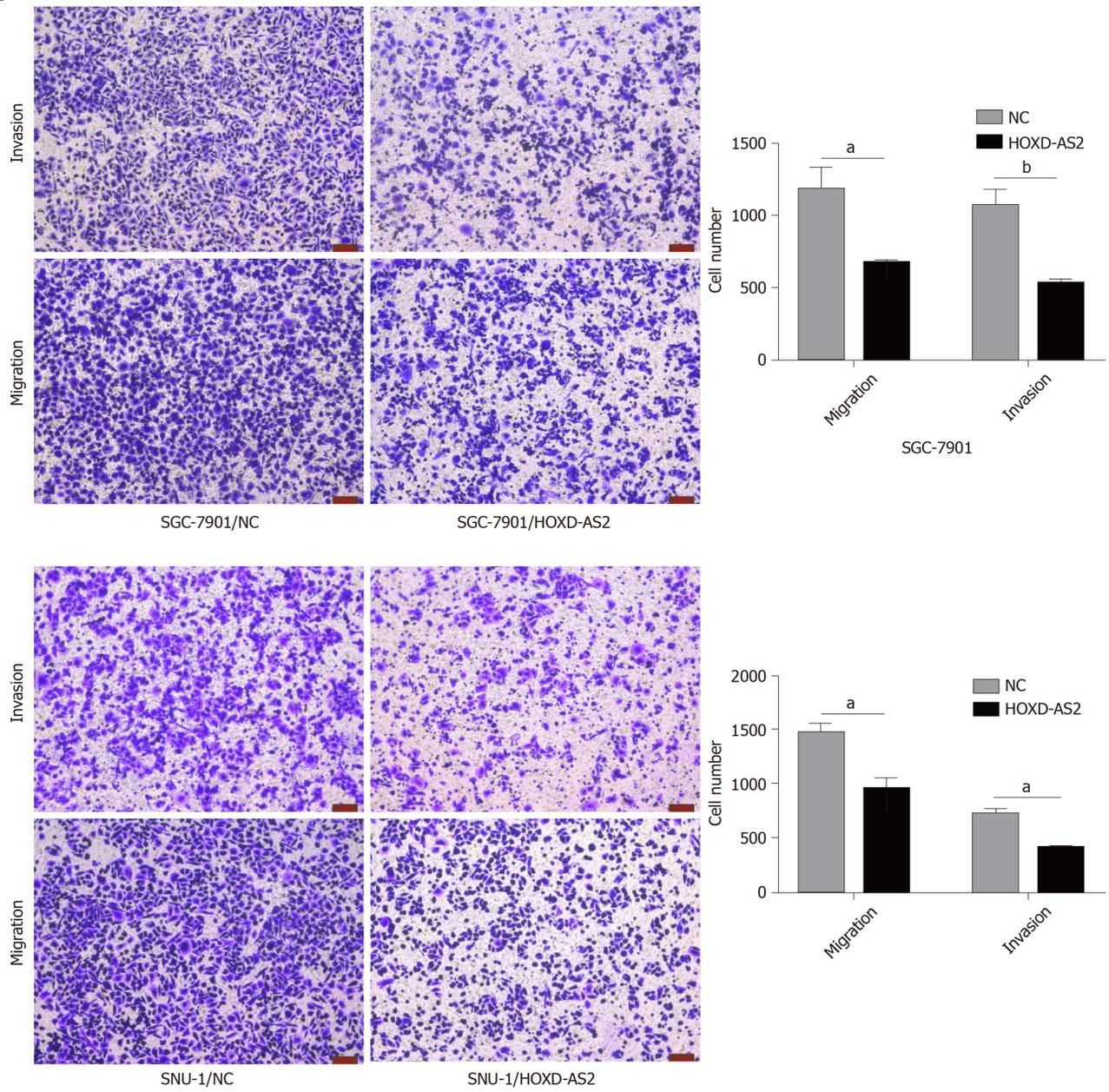
***HOXD8* is downregulated in human GC tissues and positively correlated with *HOXD-AS2* expression**

To explore the regulatory mechanism of *HOXD-AS2* in GC, we predicted the co-expressed genes associated with lncRNA *HOXD-AS2* via circIncrNAnet (<http://app.cgu.edu.tw/circlnc/>) and found that *HOXD-AS2* was positively correlated with its nearby gene *HOXD8* ($r = 0.785$, $P < 0.05$) (Figure 5A). Then, we included our qPCR data in the analysis and confirmed that *HOXD-AS2* had a positive correlation with *HOXD8* ($r = 0.9157$, $P < 0.05$) (Figure 5B). As shown in Figure 5C, G, and H, the relative mRNA expression level of *HOXD8* in 79 GC tissues was significantly decreased in comparison with that in the corresponding adjacent tissues ($P = 0.048$), and the protein expression levels of *HOXD8* in six GC tissues was significantly decreased in comparison with that in the corresponding adjacent tissues ($P < 0.001$). As shown in Figure 5D, E, and F, the relative mRNA and protein expression levels of *HOXD8* in the five GC cell lines SGC-7901, MGC-803, BGC-823, SNU-1, and AGS were lower than those in the normal gastric mucosal cell line GES-1 ($P < 0.001$). In SGC-7901 and SNU-1 cells, we overexpressed *HOXD-AS2* and found that *HOXD8* protein levels also increased (Figure 6A, D, and E). According to these results, we speculate that *HOXD-AS2* is likely to participate in the progression of GC cells via interaction with *HOXD8*.

Overexpression of *HOXD-AS2* inhibits the PI3K/Akt signaling pathway in SGC-7901 and SNU-1 cells

In our previous research, through Gene Ontology (GO) analysis and KEGG signaling pathway enrichment analysis of GC-related signaling pathways, we identified the top 10 most enriched signal pathways in GC^[14]. In this study, we found that lncRNA *HOXD-AS2* may play an important role in regulating the progression of GC cells through the PI3K/Akt signaling pathway. The results showed that in SGC-7901 and SNU-1 cells, the protein levels of p-PI3K, p-Akt, and p-MDM2 in the pc-*HOXD-AS2* group were lower than those in the control group, while the level of p53 was higher than that in the control group (Figure 6A, D, and E). To further demonstrate whether

A



B

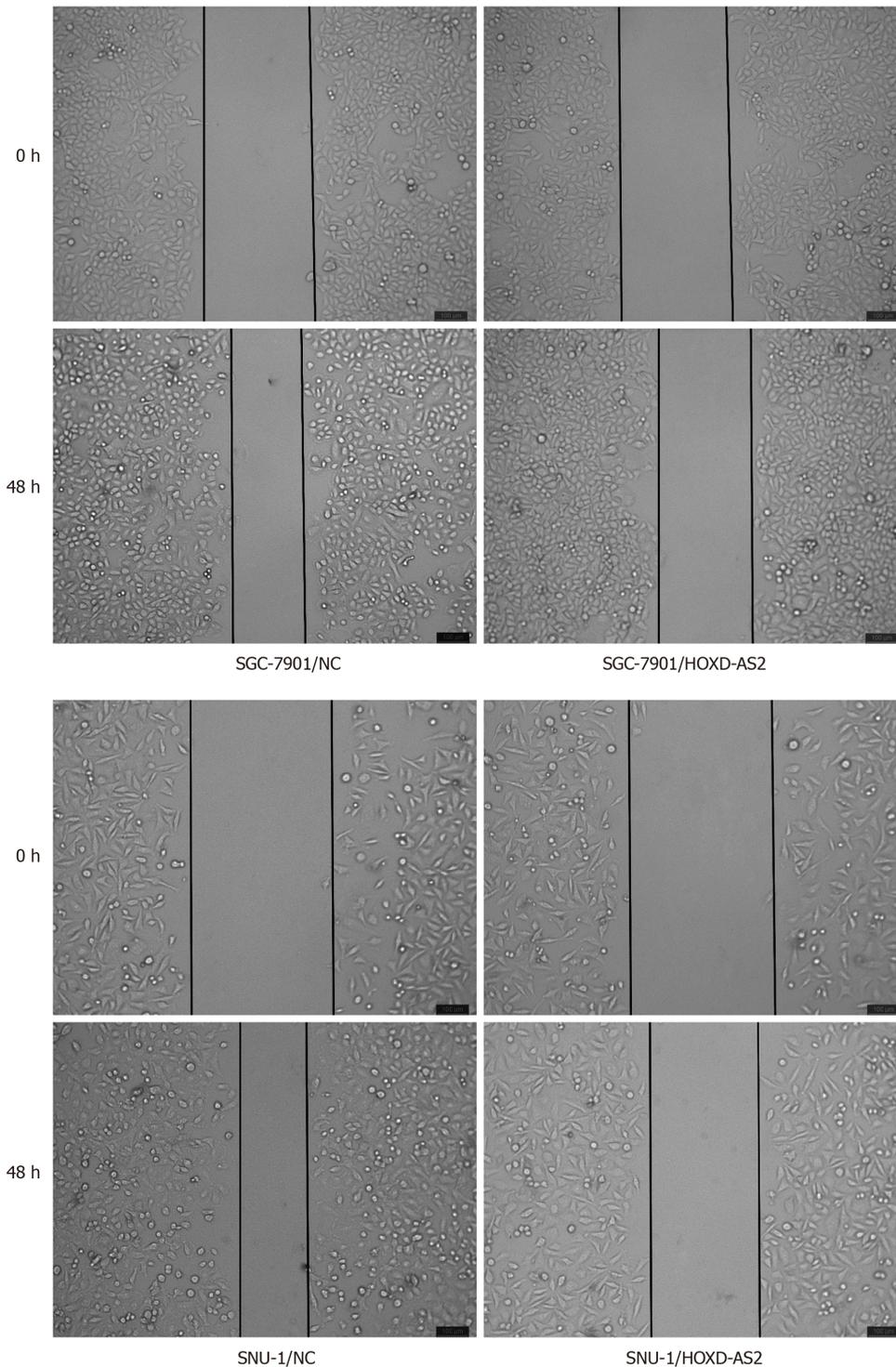
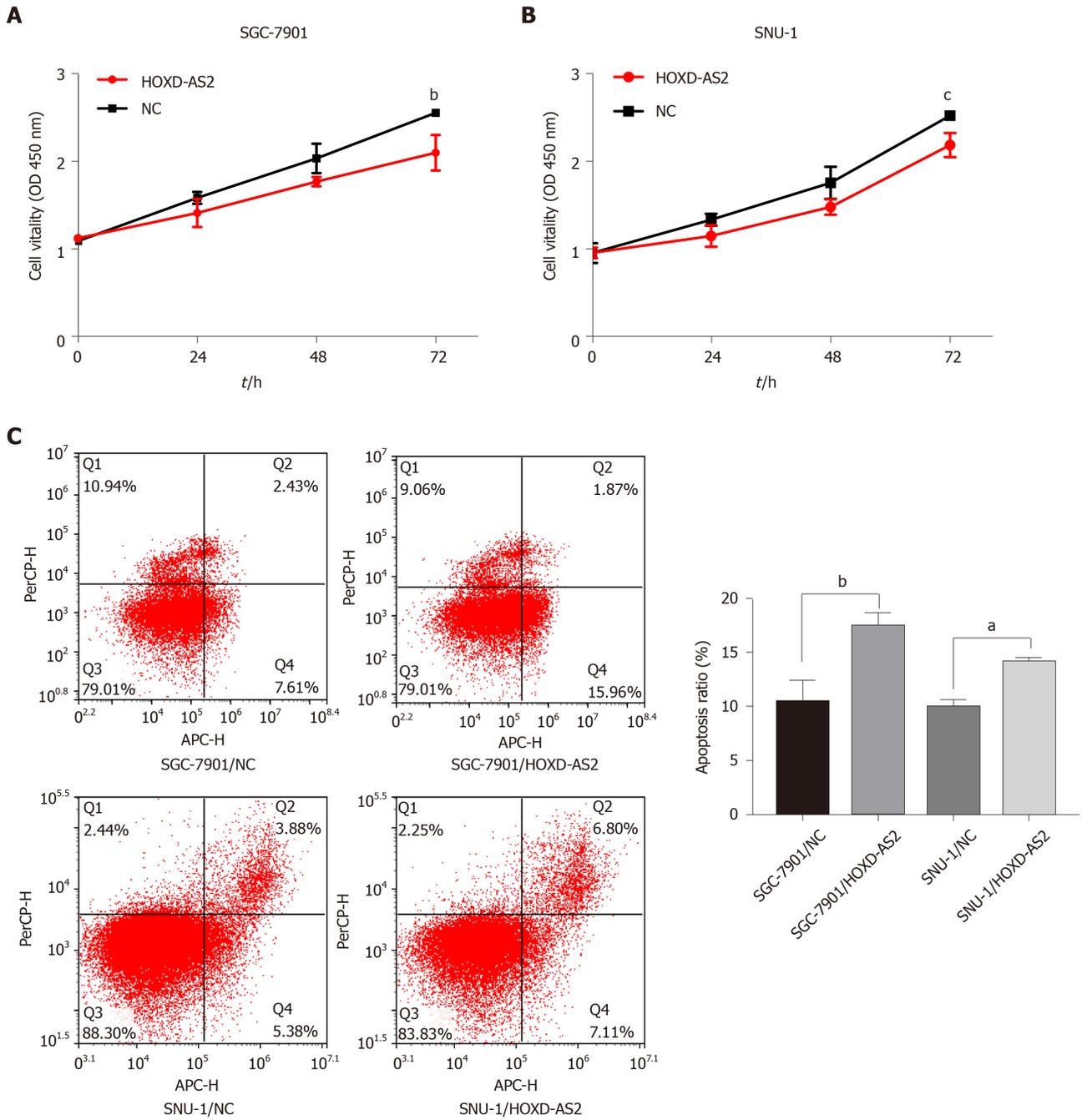


Figure 3 Overexpression of *HOXD-AS2* inhibits gastric cancer cell migration and invasion *in vitro*. A: Transwell assays were performed to detect the effect of *HOXD-AS2* on the migration and invasion of SGC-7901 and SNU-1 cells. The results revealed that overexpression of *HOXD-AS2* inhibited the migration and invasion of SGC-7901 and SNU-1 cells; B: Wound healing assay further showed that overexpression of *HOXD-AS2* inhibited gastric cancer cell migration and invasion. ^a*P* < 0.05, ^b*P* < 0.01. Data are shown as the mean ± SD. All the experiments were performed in triplicate.

HOXD-AS2 regulates the progression of GC cells *via* the PI3K/Akt signaling pathway, we added 10 μmol/L PI3K inhibitor to SGC-7901 and SNU-1 cells. After the use of the PI3K-specific inhibitor LY294002, a similar effect was obtained as with transfection of the *HOXD-AS2* plasmid. The results also showed that the expression of *HOXD-AS2* increased and the expression of HOXD8 protein increased, which further demonstrated that *HOXD-AS2* regulates the progression of GC cells by inhibiting the activation of the PI3K/Akt signaling pathway (Figure 6B, C, F, and G).



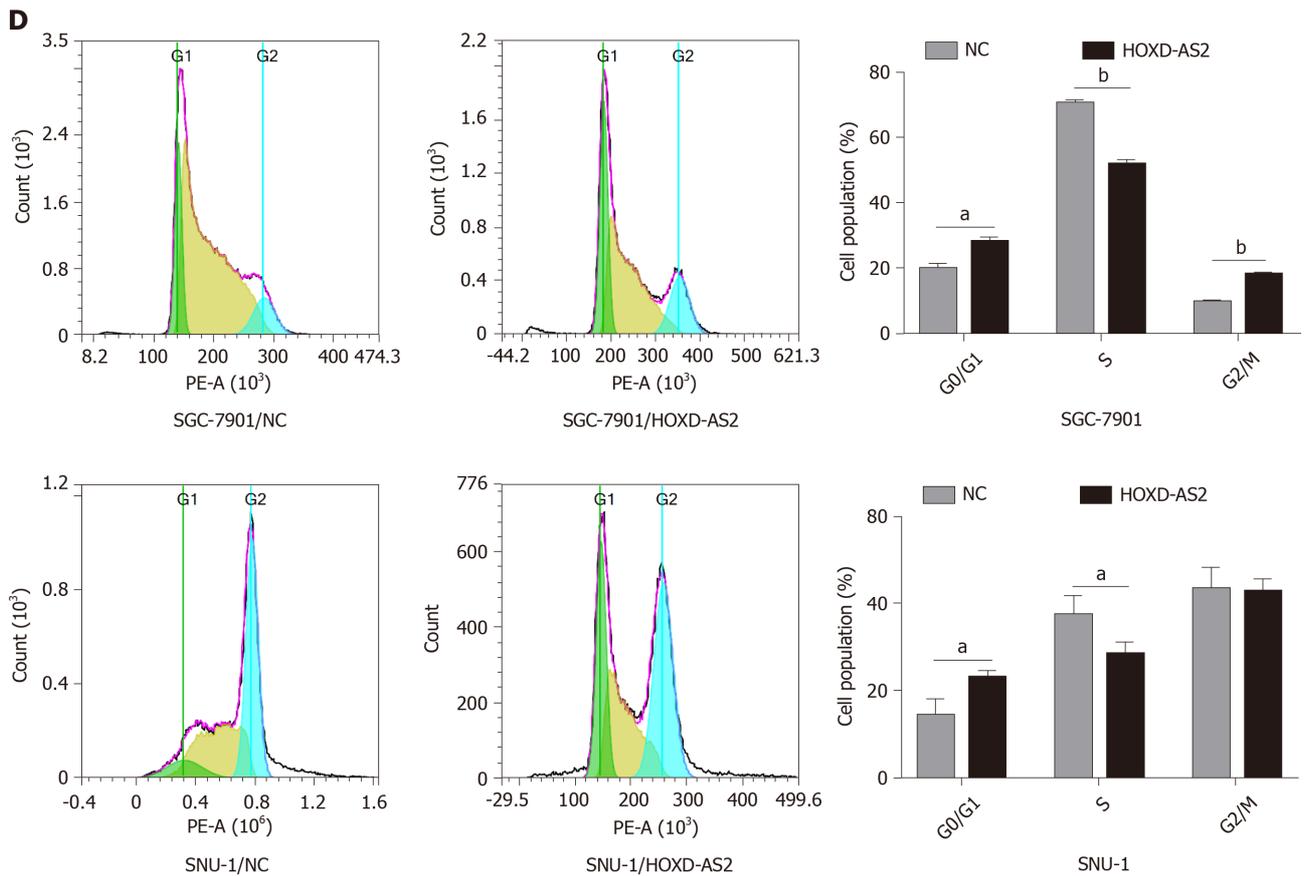
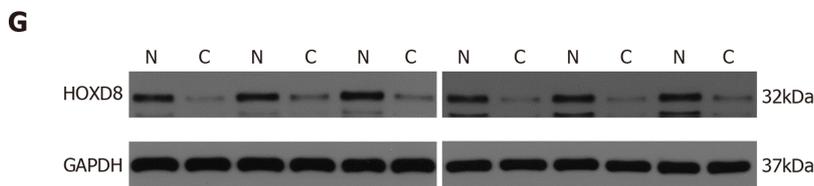
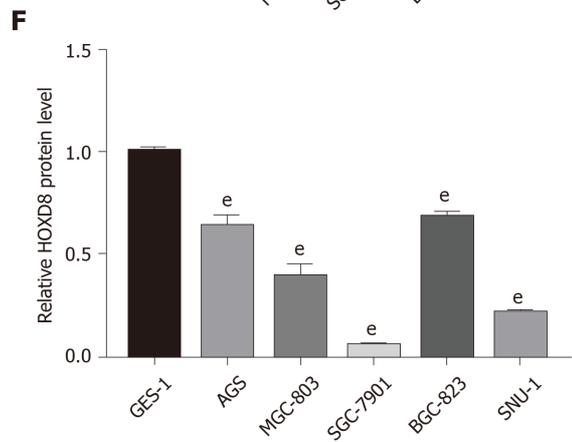
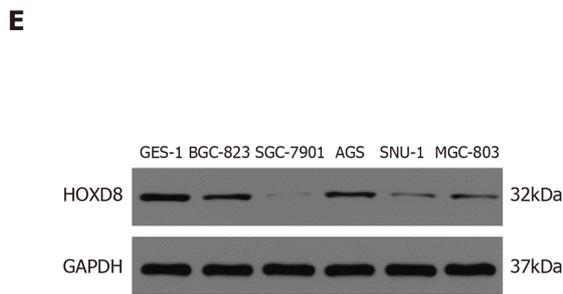
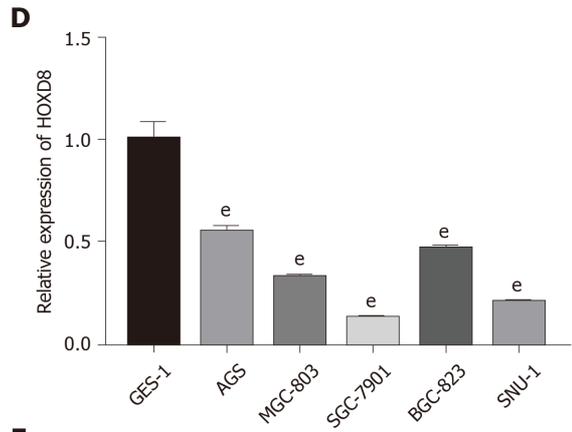
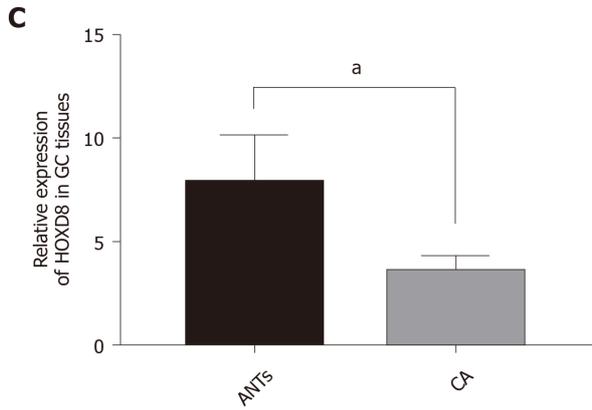
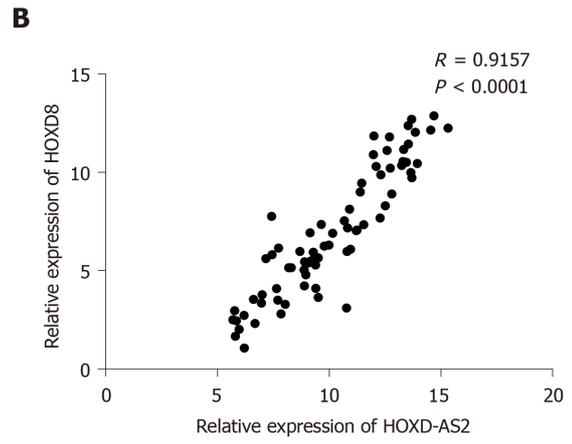
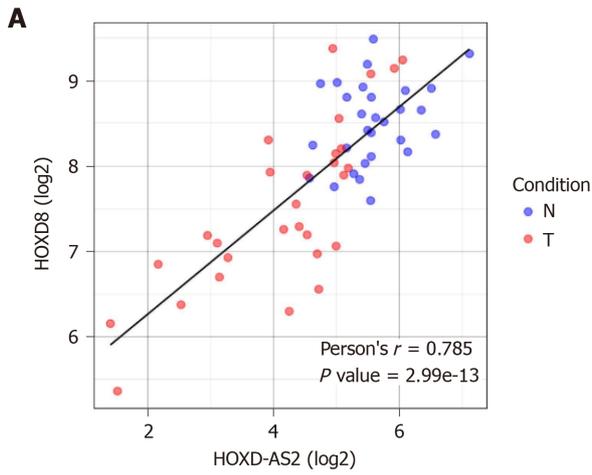


Figure 4 Overexpression of *HOXD-AS2* inhibits gastric cancer cell proliferation and cell cycle progression and promotes apoptosis *in vitro*. A and B: The effect of *HOXD-AS2* on cell proliferation was determined by cell counting kit-8 assay. Cell proliferation was decreased after overexpression of *HOXD-AS2* in SGC-7901 ($P = 0.0019$) and SNU-1 ($P < 0.001$) cells; C: Flow cytometry was used to detect the percentage of apoptotic cells in the pc-*HOXD-AS2* and the control groups. The percentage of apoptotic cells was increased after overexpression of *HOXD-AS2* in SGC-7901 ($P = 0.007$) and SNU-1 ($P = 0.021$) cells; D: The effect of *HOXD-AS2* on cell cycle was measured by flow cytometry after 7-AAD staining in SGC-7901 and SNU-1 cells. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

DISCUSSION

LncRNAs have diverse biological functions and mainly regulate gene expression at three levels: Epigenetic, transcriptional, and post-transcriptional. Regulation at the epigenetic level is mainly through DNA methylation and demethylation. Transcriptional regulation mainly affects the expression of genes through promoters and enhancers. Regulation at the post-transcriptional level is mainly through the regulation of post-transcriptional processing and modification processes such as mRNA splicing, editing, and degradation^[16-18]. Many studies have found that lncRNAs are closely related to GC, and lncRNAs can act as tumor suppressor genes or oncogenes to play an important role in the regulation of malignant biological behaviors in GC such as proliferation, apoptosis, invasion, and metastasis^[19]. However, the specific molecular mechanism of lncRNAs in GC has not been fully elucidated. In our previous study, we constructed an lncRNA expression chip to compare differentially expressed lncRNAs in GC tissues and matched paracancerous tissues^[13,14,20]. *HOXD-AS2* has great expression differences between GC tissues and matched paracancerous tissues, but its specific role in GC remains unclear. The purpose of this study was to detect the expression of *HOXD-AS2* in GC tissues and cells, analyze its relationship with the clinicopathological features of GC patients, and explore its role and specific molecular regulatory mechanism in the occurrence and development of GC.

In the present study, we verified the expression of *HOXD-AS2* in 79 GC tissues and 5 GC cell lines by qRT-PCR. The relationship between the expression of *HOXD-AS2* and the clinicopathological features of GC patients was analyzed. It was found that low expression of *HOXD-AS2* was significantly associated with lymph node metastasis and TNM stage. We analyzed data from the Kaplan-Meier plotter database and found that the *HOXD-AS2* low expression group had a lower overall survival rate and worse prognosis than the *HOXD-AS2* high expression group. Lymph node metastasis and



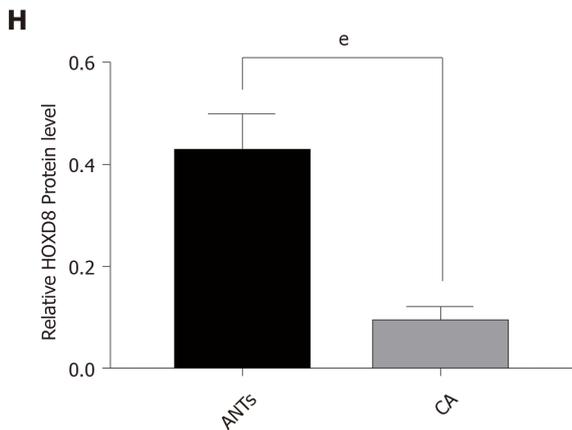


Figure 5 *HOXD8* is downregulated in human gastric cancer tissues and positively correlated with *HOXD-AS2* expression. A: CircIncrNA.net database analysis results show that *HOXD-AS2* is positively correlated with *HOXD8* ($r = 0.785$, $P < 0.05$); B: Bivariate correlation analysis of the relationship between *HOXD-AS2* and *HOXD8* expression levels, and the resulting Spearman correlation coefficient was calculated as 0.9157 with $P < 0.0001$ ($n = 79$); C and D: The relative mRNA expression level of *HOXD8* was decreased in human gastric cancer (GC) tissues ($P = 0.048$); E and F: The relative protein expression levels of *HOXD8* were decreased in human GC cells ($P < 0.001$); G and H: The relative protein expression level of *HOXD8* was decreased in human GC tissues ($P < 0.001$). The results are shown as the mean \pm SD. ^a $P < 0.05$, ^e $P < 0.001$. GC: Gastric cancer; ANTs: Adjacent non-cancerous tissues.

TNM stage are closely related to the progression of tumors. We speculated that *HOXD-AS2* may be involved in the biological behavior of GC cells. To confirm our hypothesis, we specifically upregulated the expression of *HOXD-AS2* in GC cells *in vitro* and found that the invasion, migration, and proliferation ability of GC cells was significantly inhibited. All the results indicated that *HOXD-AS2* may play an important regulatory role in the progression of GC.

Many studies have found that the natural antisense transcript types of lncRNAs can regulate the expression of their nearby genes. For example, lncRNA *FOXP4-AS1* can act as a ceRNA to adsorb miR-3184-5p, attenuating the inhibitory effect of miR-3184-5p on its target gene *FOXP4*, thereby promoting prostate cancer cell proliferation^[21]. LncRNA *SLC7A11-AS1* is negatively regulated by its adjacent gene *SLC7A11*. Decreasing *SLC7A11-AS1* can increase the expression of *SLC7A11*. *SLC7A11-AS1* can also regulate the proliferation of GC cells by activating the ASK1-p38MAPK/JNK signaling pathway^[13]. LncRNA *MACC1-AS1* is highly expressed in GC tissues, and it can upregulate the expression of *MACC1* by enhancing the stability of *MACC1* mRNA, thereby enhancing the glycolysis process and antioxidant capacity of GC cells to promote their proliferation, invasion, and migration. *MACC1-AS1* can be used as a biomarker for the diagnosis and prognosis of GC^[22]. Similarly, when we predicted the co-expressed genes related to *HOXD-AS2* through an online database, we found that the expression of the nearby gene *HOXD8* was positively correlated with the expression of *HOXD-AS2*. Therefore, we speculated that *HOXD-AS2* may be involved in the regulation of *HOXD8* through a ceRNA mechanism and may play a role in regulating the process of GC cells. We used qRT-PCR assay to further verify that there was a significant positive correlation between the expression of the two genes in clinical GC tissues and GC cells. After overexpression of *HOXD-AS2*, we found that the protein level of *HOXD8* was higher than that of the control group, further confirming that *HOXD-AS2* can regulate the expression of *HOXD8*. However, little is known about the role of *HOXD8* in tumorigenesis and development and the specific regulatory mechanisms. *HOXD8*, which is located at 2q31.1 and contains two exons, has been mapped to chromosome 2 region 176129705-176132695. The expression of *HOXD8* is decreased in colorectal cancer. Overexpression of *HOXD8* can inhibit the proliferation, invasion, and migration of colorectal cancer cells and promote their apoptosis^[23]. *HOXD8* is highly expressed in non-small cell lung cancer. Overexpression of *HOXD8* can upregulate the expression of proliferation-related genes *p53*, *PTEN*, and *p21* and promote the proliferation of non-small cell lung cancer cells. In addition, miR-520a-3p can inhibit the proliferation of non-small cell lung cancer cells by downregulating the expression of *HOXD8*^[24]. The specific regulatory mechanism of *HOXD8* in tumors still needs further exploration and confirmation.

The PI3K/Akt signaling pathway has been well documented in a number of studies as a key mechanism regulating tumor cell processes, including proliferation, apoptosis, invasion, and migration^[25-27]. Many lncRNAs have also been found to be closely related to the PI3K/Akt signaling pathway in GC. Huang *et al*^[28] found that

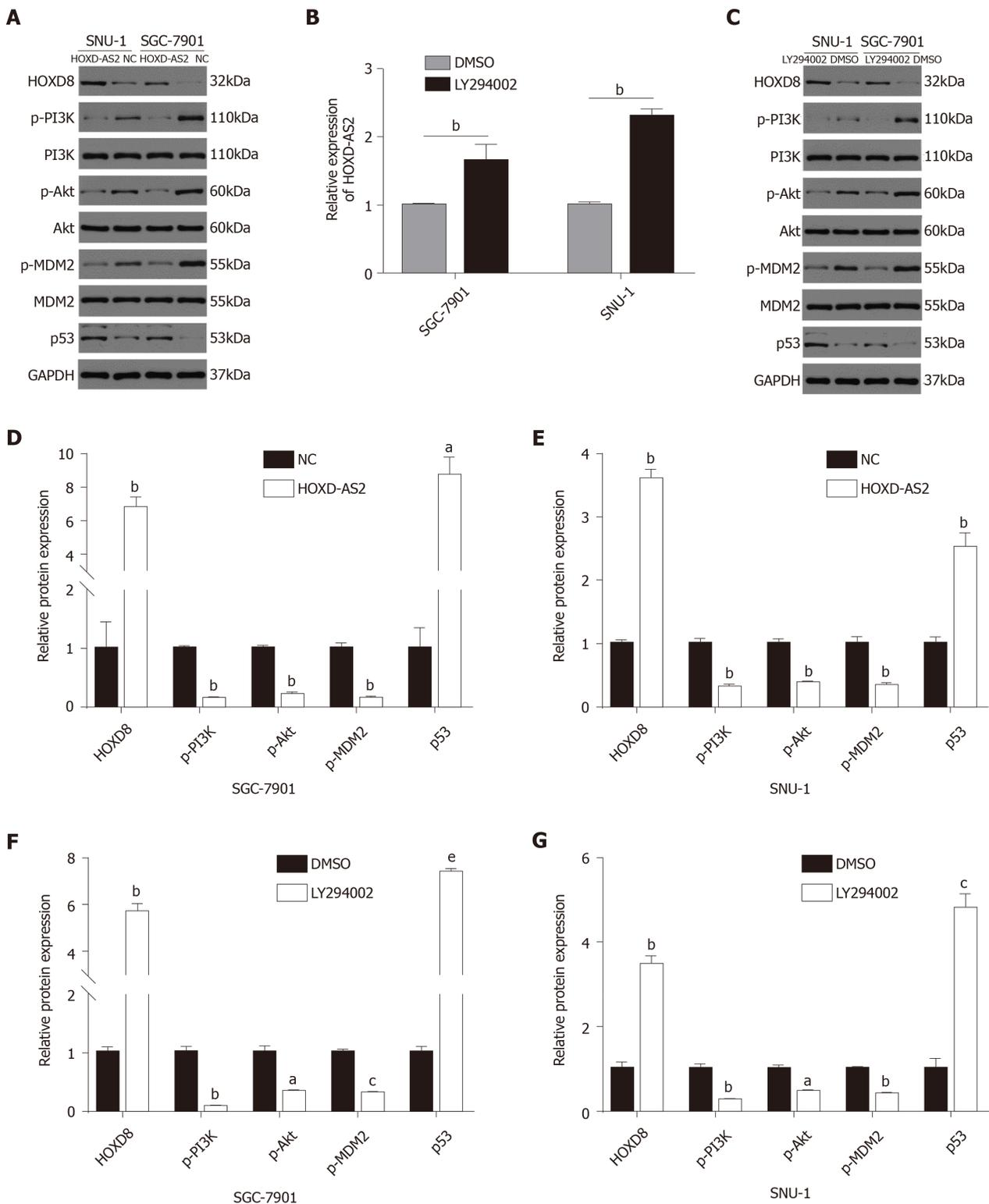


Figure 6 Overexpression of *HOXD-AS2* inhibits the PI3K/Akt signaling pathway. A, D, and E: Overexpression of *HOXD-AS2* reduced p-PI3K, p-Akt, and p-MDM2 protein levels, and increased p53 and HOXD8 protein levels; B: *HOXD-AS2* mRNA levels were significantly increased following treatment with LY294002 in SGC-7901 ($P = 0.009$) and SNU-1 ($P = 0.001$) cells for 48 h. Expression levels were normalized to GAPDH levels. The results are shown as the mean \pm SD; C, F, and G: HOXD8 and p53 protein levels were significantly increased but p-PI3K, p-Akt, and p-MDM2 protein levels were significantly reduced following treatment with LY294002 in SGC-790 and SNU-1 cells for 48 h. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$.

lncRNA *AK023391* promotes the development and progression of GC by activating the PI3K/Akt signaling pathway. Cheng *et al*^[29] found that downregulation of lncRNA *HOTAIR* can upregulate the expression of miR-34a, inhibit the PI3K/Akt and Wnt/ β -catenin signaling pathways, and attenuate the resistance of GC cells to cisplatin. In this study, we found a possible link between the PI3K/Akt signaling pathway and GC by GO and KEGG analysis. After overexpression of *HOXD-AS2* in GC cells SGC-7901 and

SNU-1, we found that the PI3K/Akt signaling pathway is inhibited. *In vitro*, after we specifically overexpressed *HOXD-AS2* in GC cells SGC-7901 and SNU-1, we found that the expression levels of several key genes in the PI3K/Akt signaling pathway were downregulated and that the signaling pathway was inhibited. In the positive control group, we obtained similar results after adding the PI3K inhibitor LY294002. We found that the expression level of *HOXD-AS2* increased, the protein level of *HOXD8* also increased, and the PI3K/Akt signaling pathway was inhibited. The invasion, migration, and proliferation ability of GC cells also decreased. Based on the above findings, we speculate that the low expression of *HOXD-AS2* may regulate the activation of the PI3K/Akt signaling pathway *via HOXD8* through a ceRNA mechanism, which may promote the progression of GC.

CONCLUSION

In summary, our current results demonstrate that *HOXD-AS2* might play an important role in suppressing the progression of GC cells by regulating *HOXD8* gene expression and inhibiting the PI3K/Akt signaling pathway. These results suggest that *HOXD-AS2* may be a key molecule in tumor development and a potential target for the treatment of GC.

ARTICLE HIGHLIGHTS

Research background

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs with a length of more than 200 nucleotides and lack an open reading frame. They mainly regulate RNA transcription and mRNA splicing and play an important role in regulating the stability of RNA in the cytoplasm and the activity of microRNAs. More and more studies have shown that abnormally expressed lncRNAs are involved in all aspects of tumor occurrence and development, and are closely related to tumor proliferation, apoptosis, invasion, metastasis, drug resistance, and poor prognosis. In recent years, more and more studies have found that abnormally expressed lncRNAs are involved in the occurrence and development of gastric cancer (GC), and they are expected to become new biomarkers for the diagnosis and treatment of GC.

Research motivation

GC is one of the most common malignant tumors in the world. The incidence and mortality of GC are in the forefront of all malignant tumors. Although a major breakthrough has been achieved in the study of lncRNAs in the pathogenesis of GC, the specific mechanism of lncRNAs in the occurrence and development of GC has not yet been fully elucidated. Exploring new lncRNAs can help to understand the molecular mechanism of GC more deeply.

Research objectives

The main purpose of this study was to explore the effect of downregulation of *HOXD-AS2* on the biological behavior of GC cells SGC-7901 and SNU-1 and the underlying mechanism. Studies have found that the downregulation of *HOXD-AS2* can regulate the expression of its neighboring gene *HOXD8*, and can also activate the PI3K/Akt signaling pathway, thereby promoting the progression of GC cells. The results of this study may provide a new idea for the treatment of GC.

Research methods

The pc*HOXD-AS2* plasmid vector was constructed and transfected into SGC-7901 and SNU-1 GC cells. Matrigel Transwell and wound healing assays were used to confirm the effect of *HOXD-AS2* on invasion and migration of GC cells. Cell counting kit-8 assay and flow cytometry were used to verify the effect of *HOXD-AS2* on proliferation, cell cycle, and apoptosis of GC cells. The relevant regulatory mechanism between *HOXD-AS2* and *HOXD8* and PI3K/Akt signaling pathway was verified by Western blot analysis.

Research results

In this study, we found that the low expression of lncRNA *HOXD-AS2* was associated

with lymph node metastasis and tumor-node-metastasis stage in GC. *In vitro* functional experiments demonstrated that overexpression of *HOXD-AS2* inhibited GC cell progression. Mechanistic studies revealed that *HOXD-AS2* regulated the expression of its nearby gene *HOXD8* and inhibited the activity of the PI3K/Akt signaling pathway.

Research conclusions

These results indicate that downregulation of *HOXD-AS2* significantly promotes the progression of GC cells by regulating *HOXD8* expression and activating the PI3K/Akt signaling pathway. *HOXD-AS2* may be a novel diagnostic biomarker and effective therapeutic target for GC.

Research perspectives

This study combines basic experimental research and bioinformatics results to reach a relatively novel conclusion. To further confirm the results of this study, siRNA and animal experiments may be better.

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