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HOXA13 upregulation in gastric cancer is associated with enhanced cancer cell invasion and epithelial-to-mesenchymal transition

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Abstract. – OBJECTIVE: In this study, we investigated the association between HOXA13 dysregulation and gastric cancer progression. We also explored the functional role of HOXA13 in invasion and epithelial-to-mesenchymal transition (EMT) of gastric cancer cells and the possible signaling pathway it might involve in.

MATERIALS AND METHODS: The microarray (E-GEOD-19826) examined the transcription profiles of 12 adjacent normal/tumor-matched gastric tissues was downloaded from the ArrayExpress and reanalyzed. Immunohistochemistry (IHC) staining was performed to assess HOXA13 expression in 23 stage I and 69 stage II/III/IV gastric cancer tissues. The human gastric cancer cell line AGS and SGC-7901 cells were transfected with HOXA13 siRNA and then were subjected to detection of epithelial and mesenchymal markers and cell invasion. The involvement of HOXA13 in TGF-β signaling was further studied.

RESULTS: HOXA13 is one of the most upregulated genes in gastric cancer tissues compared to adjacent normal tissues. Also, HOXA13 is further upregulated in the higher stage tumors. HOXA13 staining was significantly stronger in stage II/III/IV tumors than in stage I tumors. HOXA13 siRNA significantly restored the epithelial property and reduced the mesenchymal property of the cancer cells. Transwell assay showed that HOXA13 siRNA impaired the invasion capability of the cancer cells. The gastric cancer cells with HOXA13 knockdown had decreased expression of p-SMAD2 and p-SMAD3.

CONCLUSIONS: This study provides additional evidence about the association between HOXA13 upregulation and gastric cancer progression. Also, we showed that HOXA13 contributes to invasion and EMT of gastric cancer cells via the TGF-β signaling pathway.

Key Words:

Rectal adenocarcinoma, Differentially expressed HOXA13, Gastric cancer, EMT, TGF-β.

Introduction

Homeobox (HOX) genes are important transcriptional regulators in mammalian embryogenic development[1,2]. In human, four separate HOX clusters were identified on four different chromosomes, including HOXA at 7p15.2-p14.3, HOXB at 17p21.3, HOXC at 12q13.3, and HOXD at 2q31[3].

Recent studies[3] found that some HOX genes are dysregulated in tumorigenesis. HOXA9 and HOXB13 are the mostly reported dysregulated HOX genes in solid tumors. HOXA were reported to have a dysregulation in breast cancer, glioma, gastric cancer and ovarian cancers[3-5]. The homeobox A13 (HOXA13) gene is the most posterior of the HOX clusters in 7p15.2[3]. Its oncogenic effects were observed in some cancers, but the underlying mechanisms are still incompletely understood. HOXA13 upregulation is associated with high glioma stage and poor prognosis[6]. Higher HOXA13 expression was correlated with lymph node metastasis, poor histological differentiation, and decreased overall survival in patients with pancreatic ductal adenocarcinoma[7]. In gastric cancer, HOXA13 is also an oncogene associated with gastric cancer progression[8] and is considered as an independent prognostic marker of a worse outcome in gastric cancer patients[9]. Mechanistically, HOXA13 could trans-activate the insulin growth factor-binding protein 3 (IGFBP-3) promoter through the HOX-binding site, thereby stimulating the oncogenic potential and invasion activity of gastric cancer cells[10].

The progression of gastric cancer is a complex and multistep process that involves activation of oncogenes and silencing of tumor suppressive genes[11]. Epithelial-to-mesenchymal transition (EMT) has been elucidated as an important

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mechanism in gastric cancer progression, especially in tumor cell invasion and metastasis[11-13]. In this study, we further investigated the association between HOXA13 dysregulation and gastric cancer progression. We also explored the functional role of HOXA13 in invasion and EMT of gastric cancer cells and the possible signaling pathway it might involve in.

Materials and Methods

Bioinformatic Analysis

The normalized raw data of the array (E-GEOD-19826) was downloaded from the ArrayExpress. This microarray analyzed the transcription profiles of 12 adjacent normal/tumor-matched gastric tissues by using Affymetrix GeneChip Human Genome U133 Plus 2.0[14]. The raw data was reanalyzed to identify the most upregulated genes between tumor and adjacent normal tissues and between stage I and stage II/III/IV tumors by using Morpheus (https://software.broadinstitute.org/morpheus/).

Cell Culture and Transfection

The human gastric cancer cell line AGS and SGC-7901 cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cancer cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 U/mL streptomycin. HOXA13 siRNA (5'-GCGGACAAGUACAUGGAUATT-3')[6] were synthesized and obtained from Ribobio (Guangzhou, China). AGS and SGC-7901 cells were transfected with 100 nM HOXA13 siRNA or the negative control using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

QRT-PCR Analysis

Total RNA was extracted from cell samples using the TRIzol reagent (Invitrogen) and then cRNA was reversely transcribed using a reverse transcription kit (Promega, Madison, WI, USA). QRT-PCR was performed using the Quantifast SYBR green PCR kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instruction. Amplification curves and gene expression were normalized to GAPDH. The primers for HOXA13 were: forward, 5'-CCTCTGGAAGTCCACTCTGC-3'; reverse, 5'-GGTATAAGGCACGCGCTTC-3'.

Immunohistochemistry (IHC) Staining of HOXA13

The study was approved by the Human Research Ethics Committee of the Qingdao Central Hospital, China. 92 cases of formalin-fixed paraffin-embedded gastric adenocarcinoma cancer tissues were collected from the tissue bank of the Department of Gastroenterology of the hospital. Among the tissue samples, there were 23 stage I cases and 69 stage II/III/IV cases (N=23 in stage II, III and IV respectively). IHC staining was performed following the methods described in one previous study[15]. In brief, the tumor tissue blocks were sectioned and the sections (5 μm) were de-paraffinized, rehydrated and processed for antigen retrieval using Antigen Retrieval Solution (Dako, Carpinteria, CA, USA). After that, the sections were treated with 3% H_2O_2 for 5 min to inactivate the peroxidases and then were subjected to pre-treatment with antibody diluent solution containing 1% bovine serum albumin (BSA), followed by 40 min incubation at room temperature with primary antibodies for HOXA13 (ab106503, 1:100 dilution, Abcam, Cambridge, UK). Labeling was performed with biotinylated secondary antibodies and streptavidin-HRP using Biotinylated Link Antibody kit (Dako), AEC substrate chromogen, and counterstained with hematoxylin for 5 min. Sections were mounted with aqueous media, examined and imaged using Olympus IX81 microscope (Tokyo, Japan). Negative control tests were conducted with samples in the absence of primary antibody.

Western Blot Analysis

Cell samples were lysed using a lysis buffer (Beyotime, Shanghai, China) and the protein concentration in the lysate was measured using a BCA protein assay kit (Beyotime). The samples containing 20 μg of proteins were subjected to separation in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Primary antibodies used include anti-HOXA13 (ab106503, Abcam, Cambridge, MA, USA), anti-E-cadherin (ab15148, Abcam), anti-N-cadherin (ab18203, Abcam), anti-Vimentin (Ab8978, Abcam), anti-SMAD2 (ab40855, Abcam), anti-SMAD3 (ab40854, Abcam), anti-p-SMAD2 (ab53100, Abcam) and anti-p-SMAD3 (ab52903, Abcam). After that, the membranes were incubated with the correspond-

ing HRP conjugated secondary antibodies. The blot signals were visualized using the ECL Western blotting substrate (Promega, Madison, WI, USA).

Transwell Assay

Briefly, 1×10^5 AGS or SGC-7901 cells 24 h after transfection of HOXA13 siRNA or the negative controls were suspended in 200 µL serum-free RPMI-1640 medium and then plated into the upper chamber. The lower chamber was filled with RPMI-1640 supplemented with 20% FBS to create a chemoattractant environment. After 24 h incubation in a cell incubator, cells on the top surface of the insert were removed. The cells on the bottom surface were fixed with 4% polyoxymethylene and the number of invading cells was counted after staining with 0.1% crystal violet.

Immunofluorescent Staining

24 h after transfection of HOXA13 siRNA or the negative control, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and then were permeabilized in 0.1% Triton X-100 and blocked with 1% BSA. Then, the cells were incubated with primary antibodies against N-cadherin (ab18203, Abcam), SMAD2 (ab40855, Abcam), SMAD3 (ab40854, Abcam), p-SMAD2 (ab53100, Abcam) and p-SMAD3 (ab52903, Abcam) at 4°C overnight. Then, the cells were incubated with secondary Anti-Rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 555 Conjugate) (#4413, Cell Signaling, Danvers, MA, USA) for 1 h at room temperature in the dark. Nuclei were stained using Prolong® Gold Antifade Reagent with DAPI (#8961, Cell Signaling, Tokyo, Japan). Immunofluorescent images were obtained using an Olympus IX81 inverse microscope.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0. The difference between groups was evaluated by unpaired, two-tailed Student *t*-test. $p < 0.05$ indicates statistical significance.

Results

HOXA13 Upregulation is Associated High Stage of Gastric Cancer

One previous gene array analyzed transcription profiles of 12 adjacent normal/tumor-matched gastric tissues by using Affymetrix GeneChip Human Genome U133 Plus 2.0[14]. The raw data of the array was downloaded from the ArrayExpress (E-GEOD-19826) for re-analysis. The MA plot data of the array showed that hundreds of genes were dysregulated in gastric cancer tissues *vs.* adjacent normal tissues (Figure 1A, red plots). By reanalyzing the raw data, we found that HOXA13 is among the most significantly upregulated genes in gastric cancer tissues vs. adjacent normal tissues (Figure 1B, red arrow). Among the 12 tumor tissues, there were 3 stage I, 3 stage II, 3 stage III and 3 stage IV tissues. Then, we further analyzed the most upregulated genes in stage II/III/IV tissues (n = 9) compared to stage I (n=3) tissues. Interestingly, we observed that HOXA13 is further increased in the high stage tissues (Figure 1C, red arrow). Therefore, we decided to further investigate the association between HOXA13 upregulation and the stage of gastric cancer. We examined IHC staining in 92 cases of gastric adenocarcinoma cancer tissues obtained from the tissues bank of the Qingdao Central Hospital, among which there were 23 stage I cases and 69 stage II/III/IV cases. The results showed that the stage II/III/IV tumor tissues had significantly stronger HOXA13 staining than the stage I tumor tissues (Figure 2A-B). 10/23 (43.5%) stage I tumor tissues had moderate to strong HOXA13 staining, while the rate in stage II/III/IV tumors were 60/69 (87.0%) ($\chi^2 = 22.6$, $p < 0.01$) (Table I). However, the quantity of HOXA13 staining was similar in both groups (Figure 2A and C, Table I).

HOXA13 Modulates Invasion and EMT of Gastric Cancer Cells

Then, we further analyzed the functional role of HOXA13 in EMT and invasion of gastric cancer cells. Both AGS and SGC-7901 were transfected with HOXA13 siRNA. HOXA13 siRNA significantly decreased HOXA13 expression at both mRNA and protein levels in AGS and SGC-7901 cells (Figure 3A-C). By performing transwell assay, we observed that AGS and SGC-7901 cells with HOXA13 knockdown had significantly decreased invasion capability (Figure 3D). Since EMT is an important mechanism of enhanced cancer cell invasion, we further detected the epithelial and mesenchymal markers in AGS and SGC-7901 cells with HOXA13 knockdown. Western blotting data showed that HOXA13 siRNA significantly restored epithelial marker, E-cadherin expression, and also markedly decreased the mesenchymal markers, including N-cadherin and Vimentin (Figure 3E). By using immunofluorescent staining, we

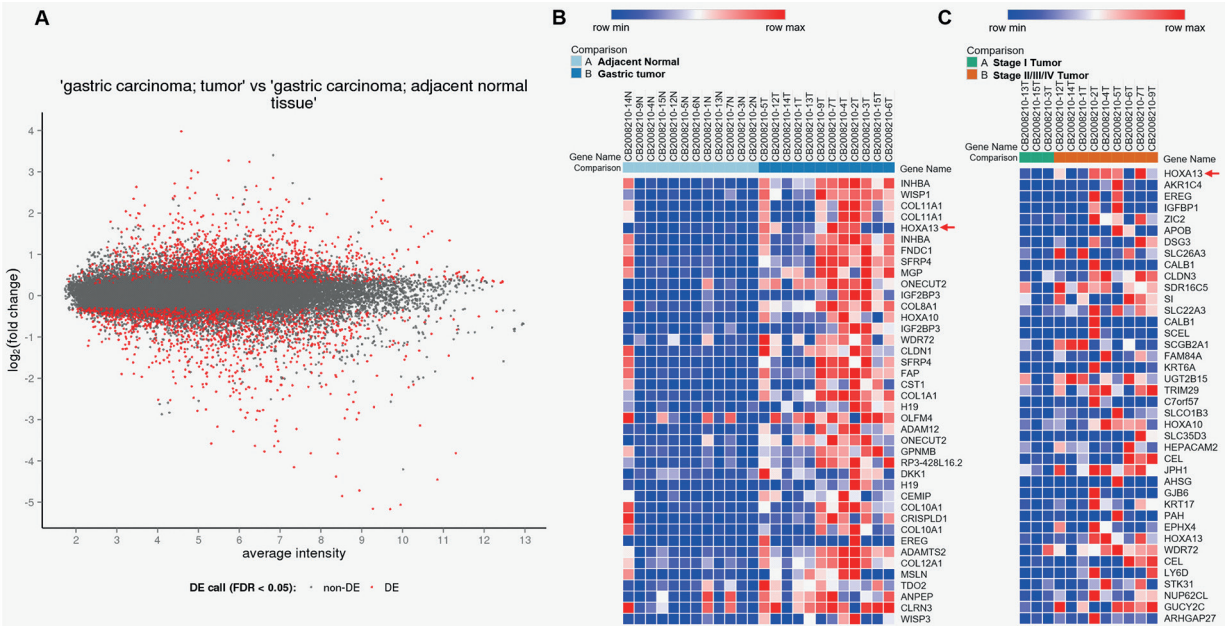

Figure 1. Microarray analysis of the gene transcription profiles of 12 adjacent normal/tumor-matched gastric tissues. ***A,*** The MA plots of gene expression in gastric cancer and adjacent normal tissues. Red plots indicate dysregulate genes. ***B,*** Heat map of the top 40 upregulated genes in 12 cancer tissues compared to adjacent normal tissues. Red: up-regulation; Blue: down-regulation. ***C,*** Heat map of the top 40 upregulated genes in 9 stage II/III/IV cancer tissues compared to 3 stage I cancer tissues. Red: up-regulation; Blue: down-regulation. Raw microarray data was obtained from the ArrayExpress (E-GEOD-19826).

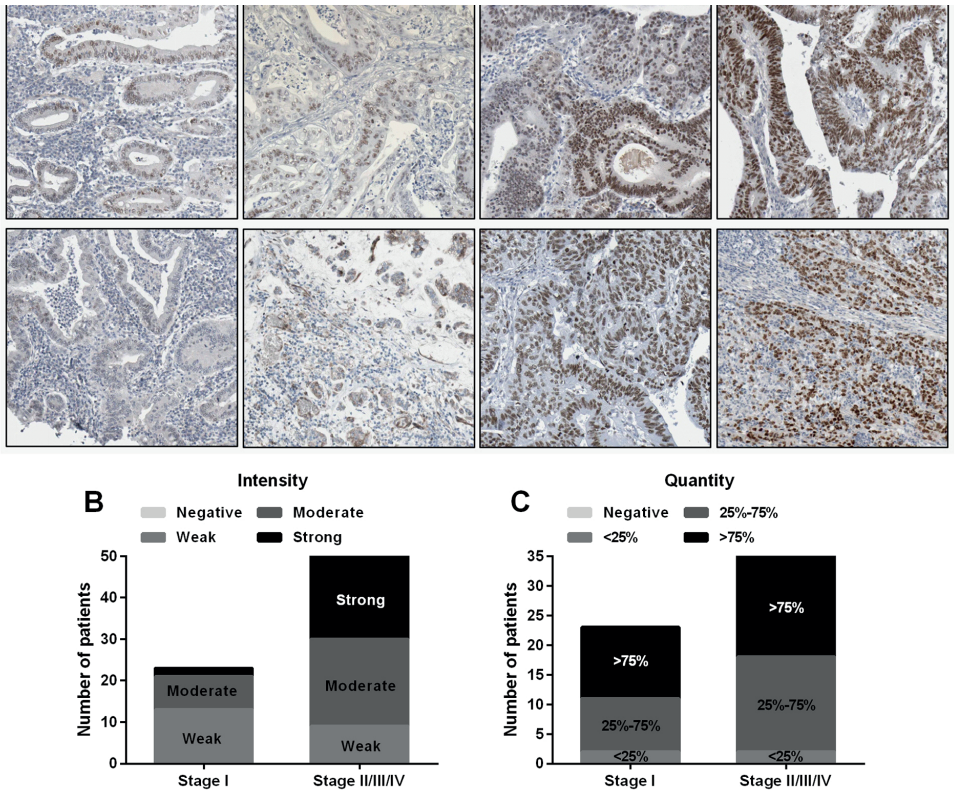

Figure 2. HOXA13 upregulation is associated high stage of gastric cancer. ***A,*** Representative IHC staining images of HOXA13 expression in stage I, II, III and IV gastric cancer tissues. ***B-C,*** Quantitation of HOXA13 staining intensity ***(B)*** and quantity ***(C)*** in 23 stage I cases and 69 stage II/III/IV cases.

Table I. Characteristics of the IHC staining of the tissue samples from patients with gastric adenocarcinoma cancer.

	Stage I	Stage II/III/IV	χ^2	*p*-value
No.	23	69		
Age (mean ± SD)	42.3 ± 5.2	43.2 ± 6.2		N.A.
HOXA13 (intensity)				
Weak	13	9	22.6	< 0.01
Moderate	8	21		
Strong	2	39		
HOXA13 (quantity)				
< 25%	2	2	4.1	0.13
25%-75%	9	16		
> 75%	12	51		

further confirmed downregulation of N-cadherin in AGS and SGC-7901 cells with HOXA13 knockdown (Figure 3F).

Knockdown of HOXA13 Reduced TGF-β Signaling in Gastric Cancer Cells

The TGF-β/SMAD signaling pathway plays an important role in EMT of gastric cancer[16]. Also, HOXA13 has been demonstrated as an enhancer of the TGF-β/SMAD signaling pathway in some cancers[6,17]. Therefore, we decided to further investigate how HOXA13 siRNA modulates this signaling pathway in gastric cancer cells. Western blotting data showed that the levels of SMAD2 and SMAD3 were not changed after HOXA13 knockdown (Figure 4A). But the level of phosphorylated SMAD2 and SMAD3 (p-SMAD2 and p-SMAD3) were all markedly

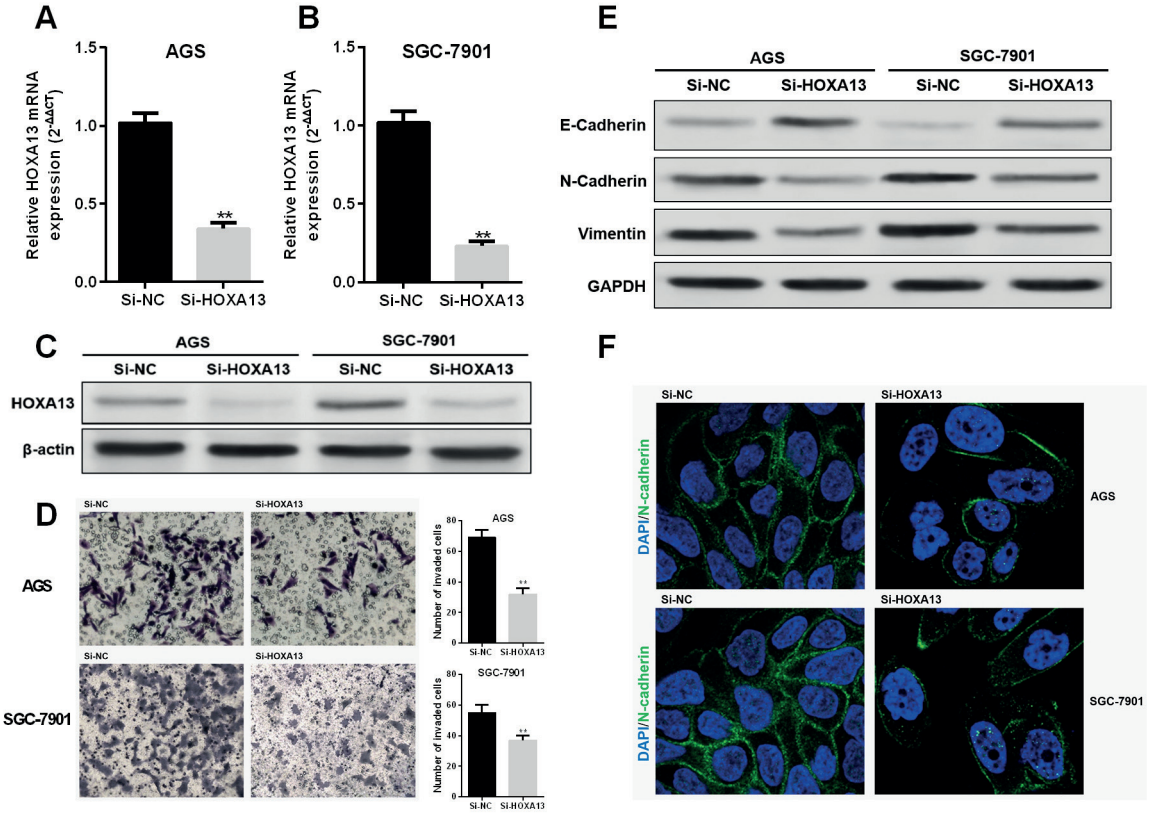

Figure 3. HOXA13 upregulation is associated high stage of gastric cancer. ***A,*** Representative IHC staining images of HOXA13 expression in stage I, II, III and IV gastric cancer tissues. ***B-C,*** Quantitation of HOXA13 staining intensity (B) and quantity (C) in 23 stage I cases and 69 stage II/III/IV cases.

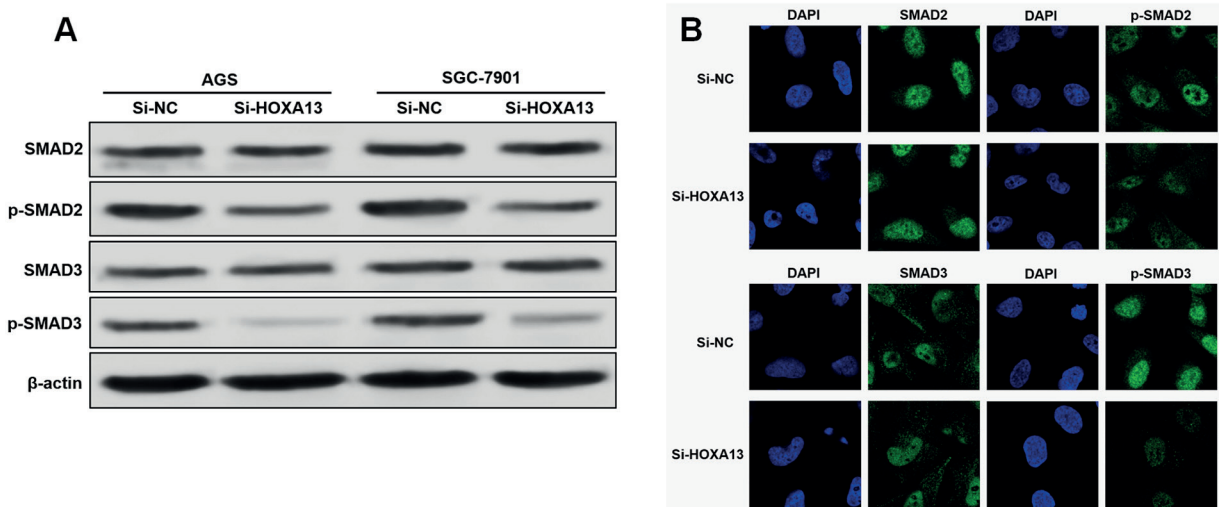

Figure 4. HOXA13 modulates invasion and EMT of gastric cancer cells. ***A,*** Western blotting images of SMAD2, SMAD3, p-SMAD2 and p-SMAD3 expression in AGS and SGC-7901 cells 24 h after transfection with HOXA13 siRNA or the negative control. ***B,*** Immunofluorescent staining of SMAD2, SMAD3, p-SMAD2 and p-SMAD3 in SGC-7901 cells 24 h after transfection with HOXA13 siRNA or the negative control.

decreased after transfection of HOXA13 siRNA in both AGS and SGC-7901 cells (Figure 4A). To further confirm the changes, we performed immunofluorescence staining to evaluate the changes in SGC-7901 cells after HOXA13 depletion. The results showed that HOXA13 siRNA resulted in a significantly less fluorescence signal intensity of p-SMAD2 and p-SMAD3 compared to the control group. The fluorescence intensity of SMAD2 and SMAD3 were similar in the two groups (Figure 4B). Therefore, we infer that HOXA13 may contribute to an enhanced invasion ability of gastric cancer cells via the TGF-β signaling pathway.

Discussion

HOX genes are essential for embryogenesis and involve in regional specification of internal tubular organs, such as the gastrointestinal tract[18]. Also, the expression of different HOX clusters presents region-specific expression patterns in each subdomain of developing gut[18]. A series of HOX genes were dysregulated in gastric cancer. For example, HOXA4, HOXA5, HOXA7, HOXA9, and HOXA13 were highly expressed in gastric cancer cells and might involve in gastric carcinogenesis[19]. Two previous studies based on clinical tumor samples showed that HOXA13 and HOXC6 upregulation were associated with poor survival in gastric cancer patients[9,20]. Mechanistically, HOXB5 can induce invasion and migration through direct transcriptional up-regulation of β-catenin in human gastric carcinoma[21]. Knockdown of HOXA13 resulted in downregulation of long non-coding RNA HOTTIP and IGFBP-3) genes in gastric cancer cells, while HOXA13 can trans-activate the IGFBP-3 promoter via the HOX-binding site[10]. Activation of IGFBP-3 significantly enhanced the oncogenic potential and invasion activity of the cancer cells[10].

In this study, by reviewing one available microarray data, we also confirmed that HOXA13 is one of the most upregulated genes in gastric cancer tissues compared to adjacent normal tissues. Notably, by comparing the gene array data between stage I and stage II/III/IV tumors, we observed that HOXA13 is further upregulated in the higher stage tumors. By performing IHC staining based on 92 cases of gastric adenocarcinoma cancer tissues, we confirmed that HOXA13 staining was significantly stronger in stage II/III/IV tumors than in stage I tumors. Several previous studies explored the association between HOXA13 and tumor invasion. Knockdown of HOXA13 resulted in inhibited pancreatic cancer cell growth, invasion, and EMT[7]. In glioma, HOXA13 can increase cell proliferation and invasion and inhibited apoptosis[7]. Therefore, we decided to further investigate the whether HOXA13 exerts similar functional role of in gastric cancer cells. In both AGS and SGC-7901 cells, we observed that HOXA13

siRNA significantly restored the epithelial property and significantly reduced the mesenchymal property of the cancer cells. Transwell assay showed that HOXA13 impaired the invasion capability of the cancer cells. One recent study reported that HOXA13 depletion decreased β-catenin, phospho-smad2, and phospho-smad3 in the nucleus and increased phospho-β-catenin in the cytoplasm in the glioma cells, suggesting that HOXA13 may regulate the Wnt- and TGF-β signaling pathways[7]. The TGF-β signaling has been shown to play an important role in inducing EMT[22], while SMAD2 and SMAD3 are two of the key transcription factors in the pathway[23]. In this study, we demonstrated that gastric cancer cells with HOXA13 knockdown had decreased expression of p-SMAD2 and p-SMAD3, with no significant change in the total SMAD2 and SMAD3. Mechanistically, HOXA13 can interact with the MH2 domain of receptor-regulated SMAD (R-SMAD, include SMAD2 and SMAD3) and enhance their activation[17]. Therefore, we infer that HOXA13 may activate TGF-β signaling by increasing the level of phosphorylated-SMAD2/3.

Conclusions

This study provides additional evidence about the association between HOXA13 upregulation and gastric cancer progression. We also showed that HOXA13 contributes to invasion and EMT of gastric cancer cells via the TGF-β signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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