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CDH17 is a downstream effector of HOXA13 in modulating the Wnt/β-catenin signaling pathway in gastric cancer

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Abstract. – OBJECTIVE: In this study, we investigated the mechanism underlying co-upregulation of HOXA13 and CDH17 in gastric cancer, the signaling pathway in which HOXA13 and CDH17 involve in and their functional role in gastric cancer cells.

MATERIALS AND METHODS: Relevant microarrays investigated the dysregulated genes in gastric cancer tissues were searched in ArrayExpress. The co-expression of HOXA13 and CDH17 was analyzed in the gastric cancer patient cohort in TCGA database using cBioportal and UCSC Xena. The regulative effect of HOXA13 on CDH17 expression was examined by dual luciferase assay. The involvement of HOXA13 and CDH17 in the Wnt/β-catenin signaling pathway was assessed by Western blotting. The functional role of HOXA13 and CDH17 in gastric cancer cells were studied by CCK-8 assay of cell growth, Transwell assay of cell invasion and flow cytometry of active caspase-3.

RESULTS: HOXA13 and CDH17 expression are upregulated and are highly correlated in gastric cancer tissues. HOXA13 overexpression significantly increased CDH17 mRNA and protein expression and also significantly increased the transcription activity of the luciferase reporter with integrate HOXA13 binding sites. HOXA13 shRNA and CDH17 shRNA had similar effect on reducing the expression of β-catenin, while shCDH17 abrogated HOXA13 induced upregulation of β-catenin. HOXA13 shRNA and CDH17 shRNA decreased cell proliferation and invasion and increased cell apoptosis in SGC-7901 cells.

CONCLUSIONS: HOXA13 can elevate CDH17 transcription via binding to its promoter. CDH17 is a downstream effector of HOXA13 in modulating the Wnt/β-catenin signaling pathway in gastric cancer cells. Both HOXA13 shRNA and CDH17 shRNA can decrease gastric cancer cell proliferation and invasion and increase their apoptosis.

Key Words:
CDH17, HOXA13, Wnt/β-catenin, Gastric cancer.

Introduction

Homeobox (HOX) genes, is a family of homeodomain-containing transcription factors with regulative effect on both embryogenesis and tumorigenesis[1,2]. Previous studies identified four HOX clusters in human genome located in different chromosomes, including HOXA at 7p15.2-p14.3, HOXB at 17p21.3, HOXC at 12q13.3, and HOXD at 2q31[3]. The homeobox A13 (HOXA13) gene is the most posterior of the HOX clusters in 7p15.2[3] and has oncogenic effect in several types of cancer, including hepatocellular carcinoma[4], glioma[5], prostate cancer[6] and gastric cancer[7]. HOXA13 can enhance gastric cancer cell invasion and epithelial-to-mesenchymal transition (EMT) via the TGF-β signaling pathway[7] and its upregulation is associated with poor prognosis in the gastric cancer patients[8]. Mechanistically, HOXA13 can transactivate the IGFBP-3 promoter via the HOX-binding site, while the subsequent IGFBP-3 activation can enhance the oncogenic potential and invasion activity of gastric cancer cells[9]. However, as a transcription factor, HOXA13 may involve in multiple signaling pathways in carcinogenesis. CDH17 (Cadherin 17 or LI-cadherin) is a novel oncogene that involved in tumor invasion and metastasis[10]. Recent studies[11,12] suggested that CDH17 is also an oncogene in gastric cancer; knockdown of endogenous CDH17 can reduce cancer cell proliferation and increase apoptosis partly via downregulating Wnt/beta-catenin signaling. However, the mechanism of its upregulation in gastric cancer is not well understood. In this study, by re-analysis of one publically available array, we found that HOXA13 and CDH17 are co-expressed and therefore we tried to explore the underlying mechanisms. Besides,

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we also investigated the signaling pathway in which HOXA13 and CDH17 may involve in and their functional role in gastric cancer cells.

Materials and Methods

Bioinformatic Data Mining

Data searching was performed in the ArrayExpress to identify relevant microarrays that investigated dysregulated genes in gastric cancer tissues. One Affymetrix GeneChip Human Genome U133 Plus 2.0 array (E-GEOD-19826) assessed the transcription profiles of 12 adjacent normal/tumor-matched gastric tissues[13]. The raw data of the array was downloaded and reanalyzed. The 100 most upregulated genes, including HOXA13 were loaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) database for analysis of the protein-protein interaction (PPI) network. The evidence level was set to > 0.7, which indicates experimentally validated interactions. The genes with positive correlation to HOXA13 expression were searched in the gastric cancer patient cohort in TCGA database using cBioportal (http://cbioportal.org)[14]. The heat map and the correlation between HOXA13 and the co-expressed genes in the same patient cohort were further verified and analyzed using UCSC Xena (http://xena.ucsc.edu/). The HOXA13 binding sites in CDH17 promoter were predicted by using the JASPAR Database (http://jaspar.genereg.net/).

Cell Culture and Transfection

The human gastric cancer cell line AGS and SGC-7901 cells were obtained from ATCC (Manassas, VA, USA) and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100-μg/mL penicillin, and 100 U/mL streptomycin.

Cell Transfection

The lentiviral human HOXA13 expression vector (lenti-HOXA13, RC209698L1V) and the empty control vector were purchased from OriGene (Rockville, MD, USA). HOXA13 lentiviral shRNA (shHOXA13, sc-45666-V), CDH17 lentiviral shRNA (shCDH17, sc-43014-V) and the empty control vector were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AGS and SGC-7901 cells were infected with the lentiviral particles or the negative controls in the presence of polybrene.

Dual Luciferase Assay

The CDH17 (NM_004063) promoter information was obtained from GeneCopoeia (HPRM17712). The promoter sequence information was given in supplementary Table I. The

Table I.

>HPRM17712 NM_004063; name=CDH17;Entrez_ID=1015;Genome=hg18;chr8-:95291048-95289760;TSS=95289986;
Upstream=1062,Downstream=226;Length=1289;
CCCACCAGTCCACCAAGGATGTTAACAGCTGGCATGCAACTAGATGGTTTCACTTGCAGG
GTCCTCAGGATAAATGAGGTCTATTCCCACAGTGGCTTAGAGCACTCATGAAACCTTCTC
ATGAAATCTGGAAAGGAGGGAGCCCTTCTAGAGAGTGGGCTGGGCTCTCTTGCTTTCCCT
CTGTCAGATGCACACCTGGAGAGGAATGGAAAACACAACCTCAGCTCAGGAGAAGCCAAG
AAATGAACATGAGAAGGTCCACAGAAACAGGGCCAAGTGTAACACACCAGACACAGACAT
GACATTGATGCTCACCTTCAGTGTGGATTCCCAGAGTGACCAAAGTGTGGAAATCAGGAA
TTGGTCTAAAAGAAGCCTTGACTTGAGAAATCTGGGGGCATTGGCTTTTATTGGTTAATC
AAAAACCTCATTTTGATTGGGGAAAAATAAGTTGTTCCTAGGTAAATCCATTCCCTGAAT
TGTGGGGGGGAAAAAAGGACTTGGTTTGTGTTTGGGGGAAATTTGCTATCGGTTGTTTCT
TGCAGACTTTGGAAGGGACCCTTGGATTTCAAAAGCAGGCACAGCCATATTGTGTCTTTC
CTCATGTCTTCTGAGTCATTTATGATATCTCATGTGGCTGGTTATCAATGAACTGTGTCA
TTGCTCAGACATGGCGCCCCTGCTCCGTCTTTTGTGTTTGAGATGGAATCTACATAAATA
TTTGCTGATCAAGTCTCCTGTGCTAAGTGTTGGGGGTACAAAATATGGAAAGTCTTTGAT
GTCATGAACCTCACACTCTAGAGATAGTTGGATACACAAAAACTTTTCCACTCTAACTTC
TTGTCTTTTCATTGACTCATCTACATTGAAAAATGTAGCAACCTGTTTTGAGATGGATTA
GATGTAAGTTAAACTTCTTTTGATACAAAGTCATTTCTTTCCTGGAGTAAAAGTAATGAC
ACTTTTTATGATACCCAGTGGCTCTCGAAGAGCAATAAAAAATGTTAATGGTTAATGTTT
GACTGAAGCTGAAGGGAGAGGCTGGGAGGCAAAGCAGGGAAGAGGGAGTGTTCCCGGGGG
AGATACTCCAGTCGTAGCAAGAGTCTCGACCACTGAATGGAAGAAAAGGACTTTTAACCA
CCATTTTGTGACTTACAGAAAGGTAAGGGCTGACATGTCTTAACTGTGTCAGTAACGTAT
TTATTCCAGAAGGACAAAGTAGATGGAGGGGGAGGGCACTTAAAAAGTTGCTTGACAAAT
TTGTTCCTTGAGGTGGGCTATGCAGATGC

TSS site and the HOXA13 binding sites were labeled in red. The prediction indicates that the CDH17 promoter has a highly possible HOXA13 binding site located between -71 to -62 upstream the TSS site. To verify the predicted binding site, six pGL3-basic luciferase reporters carrying different truncated CDH17 promoter sequences (-1062 to +226, -700 to +226, -400 to +226, -100 to +226 and -50 to +226) were constructed. HEK-293 cells were seeded into 12-well plates (2×10^5 cells/well) and were further cultured overnight. Then, the cell was infected with lenti-HOXA13 or the empty control. 24 h later, the cells were further co-transfected with 1.5 μg reconstructed luciferase construct plasmid or the empty reporter vector and 0.05 μg phRL-TK by using Superfectin (Qiagen, Valencia, CA, USA). 24 h later, cells were lysed and the luciferase activity was measured by using the dual-luciferase reporter assay system with a luminometer (Promega, Madison, WI, USA) according to manufacturer's instruction.

Western Blot Assay

The cell samples were lysed to extract total protein. Then, equal amount of denatured protein samples were loaded and separated in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene fluoride (PVDF) membrane. Then, the membranes were incubated with primary antibodies against HOXA13 (ab106503, Abcam, Cambridge, MA, USA), CDH17 (ab199690, Abcam) β-catenin (ab6302, Abcam) and anti-β-actin (ab3280, Abcam). After the incubation, the membranes were washed and further incubated with secondary antibodies coupled to HRP. After washing, the protein bands were visualized by using the SuperSignal™ West Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA).

Cell Counting Kit-8 (CCK-8) Assay

24 h after transfection, SGC-7901 cells with or without knockdown of HOXA13 or CDH17 were plated onto 96-well cell culture plates at a density of 2×10^4 cells/well in 100 μl of culture medium. At 12, 24, 36, 48, 60 and 72 h, CCK-8 solution was added to each well and the plate was placed in a cell incubator for 4 h. Then, the plate was placed in a microplate reader (Bio-Rad, Hercules, CA, USA) to measure the absorbance at 450 nm. Relative OD values were used to draw cell growth curves.

Cell Invasion

The invasion capability of SGC-7901 cells with or without knockdown of HOXA13 or CDH17 was assessed using transwell assay. In brief, the transwell chambers (8.0 μm pore size; Corning, NY, USA) were coated on the upper surface with 50 μl (1.25 mg/ml) BD Matrigel™ Matrix (BD Biosciences, San Diego, CA, USA) and were placed onto 24-well plates. Then 1×10^5 cells were resuspended in 100-μl serum-free medium and added to the upper chambers. The lower chambers were filled with 300 μl of RPMI-l640 medium with 15% fetal bovine serum (FBS). After 24 h incubation, the cells on the lower surface of the filters were fixed in methanol, stained with 0.25% crystal violet for 15 min and counted in five random fields at a magnification of 100×.

Flow Cytometric Analysis of Active Caspase-3

24 h after transfection with HOXA13 shRNA or CDH17 shRNA, SGC-7901 cells were subjected to flow cytometric analysis of active caspase-3 using the GaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Biovision, Mountain View, CA, USA) according to the manufacturer's protocol. Fluorescence was examined by FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data were presented as means ± SD based on representative data from three repeated experiments. All analyses were performed with SPSS19.0 software package (SPSS Inc. Chicago, IL, USA). Data were analyzed for statistical significance by two-tailed Student's *t*-test or ANOVA with Student-Newman-Keuls test as a post hoc test. $p < 0.05$ was considered statistically significant.

Results

HOXA13 is Upregulated in Gastric Cancer

To identify the dysregulated genes in gastric cancer tissues compared to the adjacent normal control, we re-analyzed the raw data of E-GEOD-19826[13], which compared transcription profiles of 12 adjacent normal/tumor-matched gastric tissues. Our reanalysis showed that HOXA13 is one of the most upregulated genes in the cancer tissues (Figure 1A, red arrow). To examine whether there is any known association

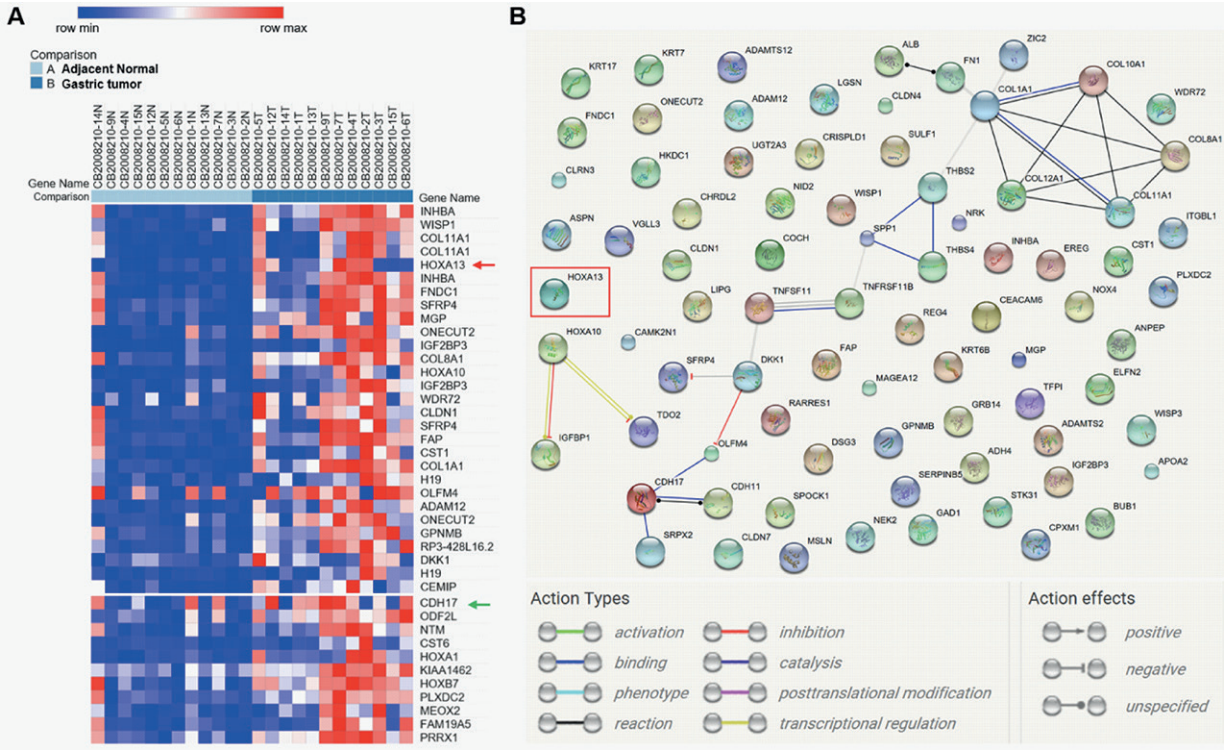

Figure 1. HOXA13 is upregulated in gastric cancer. ***A,*** Heat map of the most upregulated genes in 12 cancer tissues compared to adjacent normal tissues. Red: up-regulation; Blue: down-regulation. Image was obtained by re-analysis of the raw microarray data of E-GEOD-19826. ***B,*** PPI analysis of the known interactions among the 100 most upregulated genes in gastric cancer tissues.

between HOXA13 and the upregulated genes in the cancer tissues, the 100 most upregulated genes were uploaded into the STRING database for PPI analysis. By limiting the evidence using high-confidence score (> 0.7), we did not find any known association between HOXA13 and other upregulated genes (Figure 1B).

HOXA13 and CDH17 are Co-upregulated in Gastric Cancer

To further explore the potential regulation of HOXA13 in gastric cancer, we performed data mining in gastric cancer cohort in TCGA database using cBioPortal. Our data mining identified the top 15 genes positively correlated to HOXA13 expression (Figure 2A). Among the 15 genes, we found that CDH17 is also among the significantly upregulated genes in gastric cancer tissues compared to the adjacent normal control (Figure 2A and Figure 1A, green arrow). Regression analysis showed that HOXA13 and CDH17 had high correlation coefficients (Pearson's correlation=0.37; Spearman's correlation=0.55) (Figure 2B). To further verify the possible correlation between HOXA13 and CDH17, we examined the heap map of and co-expression between HOXA13 and CDH17 in the same data cohort using another tool, the Xena browser. Results showed that among 450 gastric cancer patients with gene expression examined by RNAseq, the expression of HOXA13 and CDH17 are highly correlated (Pearson's correlation=0.6258) (Figure 2C-D).

HOXA13 Enhances CDH17 Expression via Binding to CDH17 Promoter

Then, we decided to investigate whether HOXA13 had a direct effect on CDH17 expression and the possible underlying mechanisms in gastric cancer cells. AGS and SGC-7901 cells were firstly transfected with HOXA13 expression vector or the negative controls (Figure 3A). HOXA13 overexpression significantly increased CDH17 mRNA and protein expression (Figure 3B-C). Our bioinformatic analysis showed that the CDH17 promoter had a putative HOXA13 binding site (Figure 3D). To verify this predicted binding site, we constructed luciferase reporters carrying truncated CDH17 promoter sequences (-1062 to +226, -700 to +226, -400 to +226, -100 to +226 and -50 to +226). Following dual lucif-

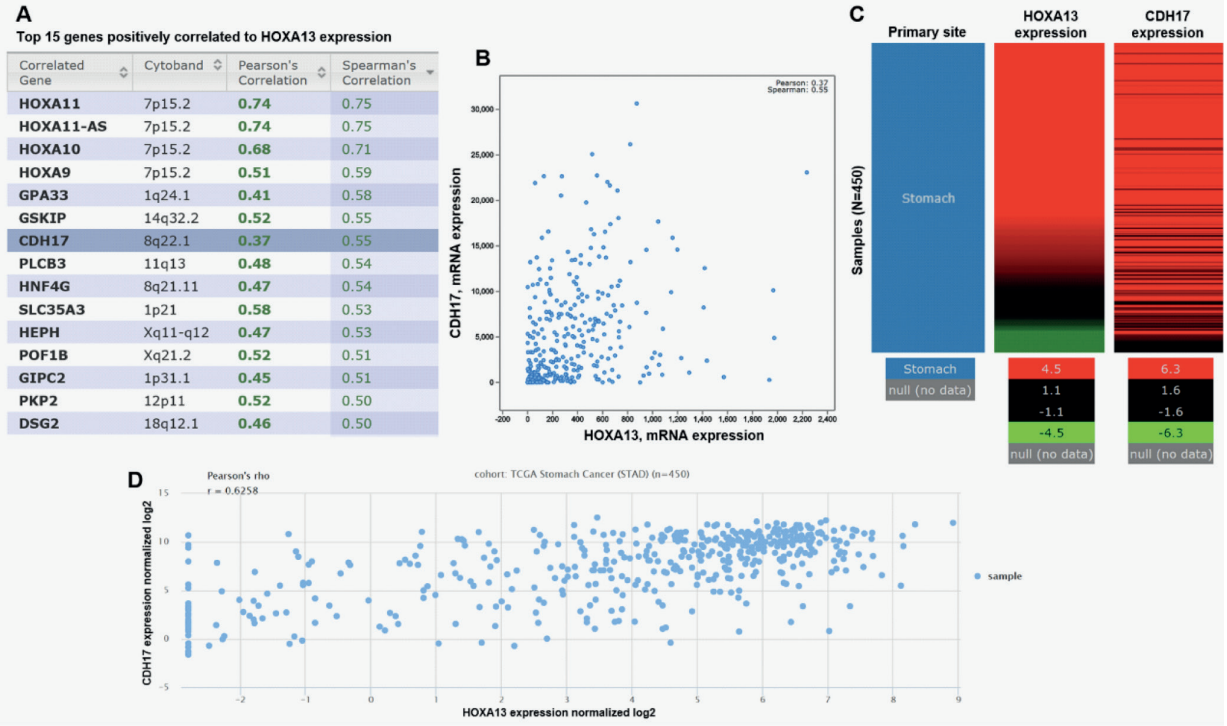

Figure 2. HOXA13 and CDH17 are co-upregulated in gastric cancer. ***A-B,*** The top 15 genes positively correlated to HOXA13 expression in gastric cancer cohort in TCGA database ***(A)***. Regression analysis between HOXA13 and CDH17 in gastric cancer cohort in TCGA database ***(B)***. Analysis was performed by using cBioPortal. ***C-D,*** The heat map ***(C)*** and Regression analysis ***(D)*** between HOXA13 and CDH17 in gastric cancer cohort in TCGA database. Analysis was performed by using UCSC Xena.

erase assay showed that HOXA13 overexpression significantly increased the transcription activity of the reporter with integrate HOXA13 binding sites (Figure 3E). This increase was fully abrogated by truncating the predicted binding site (Figure 3E).

CDH17 is a Downstream Effector of HOXA13 in the Wnt/β-catenin Signaling Pathway

In this study, we further investigated whether HOXA13 and CDH17 are involved in the same signaling pathways. SGC-7901 cells were transfected with HOXA13 shRNA or CDH17 shRNA. Western blot assay showed that knockdown HOXA13 significantly decreased CDH17 expression (Figure 4A). Interestingly, we also observed that HOXA13 shRNA and CDH17 shRNA had similar effect on reducing the expression of β-catenin (Figure 4A), while shCDH17 abrogated HOXA13 induced upregulation of β-catenin (Figure 4A). These results suggest that CDH17 is a downstream regulator of HOXA13 in the Wnt/β-catenin signaling pathway. Then, we investigated the functional role of HOXA13 and CDH17 in gastric cancer cells. Both HOXA13 shRNA and CDH17 shRNA caused a markedly reduced cell proliferation (Figure 4B) and reduced cell invasion capability (Figure 4C) in SGC-7901 cells. Also, we observed that HOXA13, shRNA and CDH17 shRNA led to increased proportion of cells with active caspase-3 (Figure 4D-E), which suggest increased cell apoptosis.

Discussion

HOXA13 has oncogene-like character in multiple cancers. HOXA13 expression might play an important role in tumor angiogenesis, progression and prognosis of hepatocellular carcinoma and its upregulation is associated with unfavorable overall survival (OS) and disease-free survival (DFS)[15]. HOXA13 can also promote glioblastoma progression partly via Wnt- and TGF-β-induced EMT and is considered as a potential diagnostic biomarker and an independent prognostic factor in high-grade glioma[5]. Several recent studies also revealed the oncogenic properties of HOXA13 in gastric cancer. He et al[7]

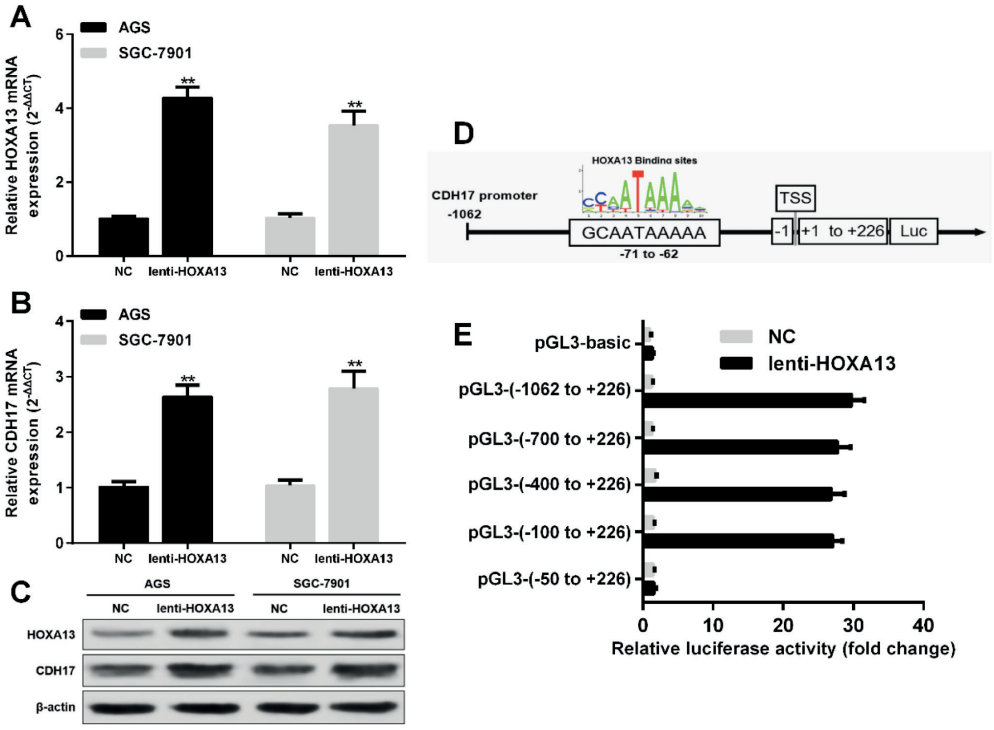

Figure 3. HOXA13 enhances CDH17 expression via binding to CDH17 promoter. ***A-C,*** QRT-PCR analysis of HOXA13 ***(A)*** and CDH17 ***(B)*** mRNA expression and Western blotting of HOXA13 and CDH17 protein expression ***(C)*** in AGS and SGC-7901 cells 24 h after transfection with lentiviral HOXA13 expression particles or the empty control (NC). ***D,*** Predicted HOXA13 binding site in CDH17 promoter ***(D)***. ***E,*** The luciferase reporter constructs carrying truncated CDH17 promoter sequences were introduced into HEK-293 cells transfected with lentiviral HOXA13 expression vector or the empty control. Luciferase activity was measured 24 h post-transfection. **, $p < 0.01$.

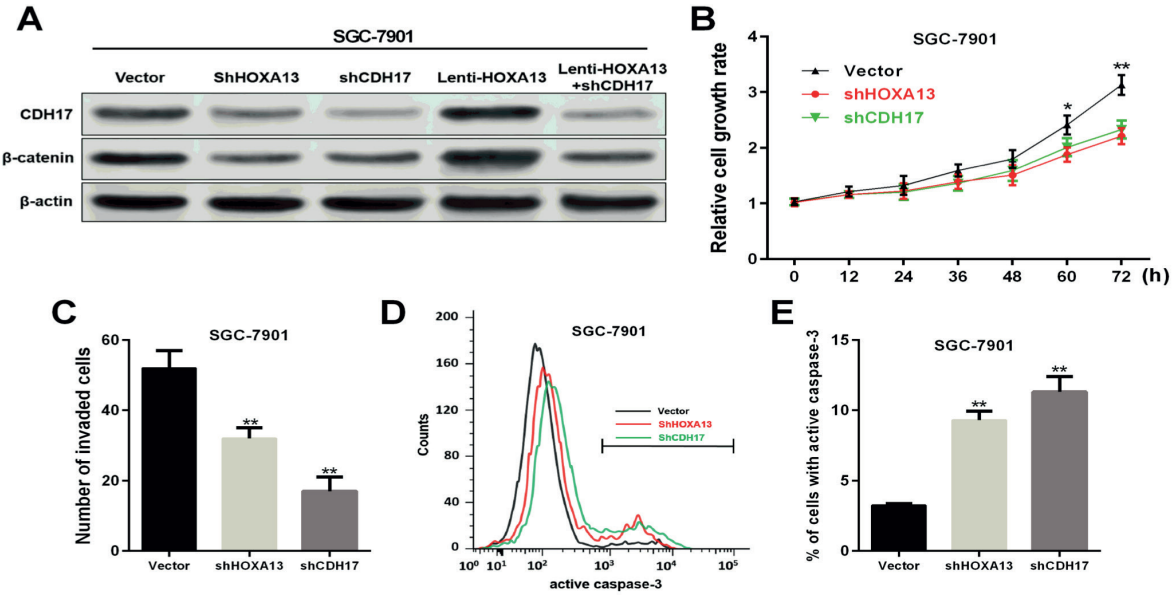

Figure 4. CDH17 is a downstream effector of HOXA13 in the Wnt/β-catenin signaling pathway. ***A,*** Western blot analysis of CDH17 and β-catenin in SGC-7901 cells 24 h after transfection of HOXA13 shRNA, CDH17 shRNA, HOXA13 expression lentiviral particles or the combined HOXA13 expression lentiviral particles and CDH17 shRNA. ***B-E,*** 24 h after transfection of HOXA13 shRNA or CDH17 shRNA, the indicating SGC-7901 cells were subjected to CCK-8 assay of relative growth rates ***(C)***. Transwell assay of cell invasion capability ***(D)***; flow cytometric analysis of the proportion of cells with active caspase-3 ***(D-E)***. **, $p < 0.01$.

reported that HOXA13 staining was significantly stronger in stage II/III/IV tumors than in stage I tumors, while HOXA13 siRNA significantly restored the epithelial property and reduced the mesenchymal property of the gastric cancer cells. The association between HOXA13 upregulation and worse DFS and OS in gastric cancer was also observed[8], but the role of a transcription factor is quite complex and can modulate multiple signaling pathways during carcinogenesis. It is quite meaningful to further explore the possible oncogenic mechanism of HOXA13 in gastric cancer. In the current study, we found that CDH17 is co-upregulated with HOXA13 in gastric cancer tissues. In both AGS and SGC-7901 cells, we observed that HOXA13 overexpression resulted in increased CDH17 expression at mRNA and protein levels. Notably, our bioinformatic analysis predicted a highly possible HOXA13 binding site in the CDH17 promoter. Therefore, we hypothesized that HOXA13 might elevate CDH17 transcription via binding to its promoter. By using luciferase reporters carrying truncated CDH17 promoter sequences, we found that HOXA3 significantly enhanced transcription activity of the reporters with integrate HOXA13 binding sites, but had no effect on the reporter without the binding site. Therefore, these findings confirmed our hypothesis. CDH17 has been reported as an oncogene in gastric cancer. Inhibition of CDH17 can reduce proliferation and increase apoptosis of gastric cancer cells both *in vitro* and *in vivo*[12]. CDH17 was also an independent prognostic factor in patients with stage I or node-negative gastric cancer[16]. Since we confirmed the direct regulative effect of HOXA13 on CDH17 expression, we further investigated the signaling pathway in which they both involve in. Previous studies[5] found that HOXA13 can enhance the Wnt/β-catenin signaling pathway in glioma, while knockdown of CDH17 may impair the Wnt/β-catenin signaling pathway in gastric cancer[11]. By performing Western blot assay, we found that both HOXA13 shRNA and CDH17 shRNA reduced the expression of β-catenin, while shCDH17 abrogated HOXA13 induced upregulation of β-catenin. Therefore, we infer that CDH17 is a downstream effector of HOXA13 in modulating the Wnt/β-catenin signaling pathway. In SGC-7901 cells, we demonstrated that HOXA13 shRNA and CDH17 shRNA decreased cell proliferation and invasion and increased cell apoptosis. These results further confirmed the oncogenic properties of HOXA13 and CDH17.

Conclusions

HOXA13 can elevate CDH17 transcription via binding to its promoter. CDH17 is a downstream effector of HOXA13 in modulating the Wnt/β-catenin signaling pathway in gastric cancer cells. Both HOXA13 shRNA and CDH17 shRNA can decrease gastric cancer cell proliferation and invasion and increase their apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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