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GALNT10 promotes the proliferation and metastatic ability of gastric cancer and reduces 5-fluorouracil sensitivity by activating HOXD13

G. XU, Y.-L. WU, N. LI, R. XU, J.-B. ZHANG, H. MING, Y. ZHANG

Department of Oncohaematology, The 960th Hospital of the PLA Joint Logistics Support Force, Zibo, China

Abstract. – OBJECTIVE: To investigate the expression and potential mechanism of GALNT10 in gastric cancer (GC).

PATIENTS AND METHODS: A total of 60 cases of GC tissues, as well as normal tissues were collected. The total RNA of GC specimens and cells were extracted by TRIzol method and the level of GALNT10 was examined by quantitative real-time polymerase chain reaction (qRT-PCR). In addition, the relationship between GALNT10 and clinical parameters and prognosis of GC patients was analyzed. Subsequently, Lentivirus was used to construct GALNT10 knockdown GC cell lines, and cell counting kit-8 (CCK-8) and transwell assays were applied to analyze the influence of GALNT10 on GC cell function. Bioinformatics and Luciferase assay was used to evaluate the relationship between GALNT10 and HOXD13. Furthermore, 5-fluorouracil (5-Fu)-resistant cells were used to detect the relationship between GALNT10 and 5-Fu sensitivity of GC cells.

RESULTS: qRT-PCR results revealed that GALNT10 level was markedly increased in tissues, as well as cell lines of GC. Statistical analysis suggested that GALNT10 expression was in close relation with the incidence of lymph node and distant metastasis along with poor prognosis in GC patients, but not with other indicators. CCK-8 and transwell migration experiment results indicated that GALNT10 silencing can inhibit the proliferative and migration ability of GC cells. Western blot results displayed that the HOXD13 level was remarkably decreased after GALNT10 knocking down. In addition, Luciferase gene assay indicated that GALNT10 could bind to HOXD13. Further rescue experiments showed that HOXD13overexpression can synergistically reverse the inhibitory effect of GALNT10 knockdown on GC cell proliferative and migration ability, which further demonstrated that GALNT10 could promote GC cell metastasis ability and reduce the sensitivity to 5-Fu by regulating HOXD13.

CONCLUSIONS: GALNT10 could regulate the proliferative and migration ability of GC cells and reduce the sensitivity to 5-Fu by enhancing the expression of HOXD13. Therefore, GALNT10 was expected to be a new therapeutic target for diagnosis of 5-fluorouracil resistance in GC.

Key Words:
GALNT10, HOXD13, Gastric cancer, Proliferation, Metastasis, 5-fluorouracil.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors in the world. About 170,000 people die of GC every year, which is close to 1/4 of the deaths of all malignant tumors. The morbidity and mortality rate still rank first in China, threatening people's health and life[1-3]. The traditional treatment for GC is surgical resection, chemotherapy, and radiotherapy. Chemotherapy can shrink the tumor, increase the patient's chance of surgery and cure, improve its long-term survival rate and the quality of life[4,5]. Therefore, it is currently the main treatment for GC. 5-Fluorouracil (5-Fu) is the first choice and the dominant combination for chemotherapy of GC[6,7]. However, tumor cells will become resistant to 5-Fu, making the chemotherapy effect of GC unsatisfactory. Therefore, the search for a low-toxicity, safe, small side reaction, high-efficiency chemotherapy sensitizer or a drug with antitumor activity itself has attracted the attention of scholars[8,9].

GALNT10 is a member of the N-acetylgalactosyltransferase family and an enzyme that catalyzes the first step in O-glycan synthesis. O-glycosylation is closely related to cell recognition,

Corresponding Author: Yan Zhang, BM; e-mail: 2412332090@qq.com

tumor cell growth, metastasis and adhesion[10,11]. GALNT10 can change the connection between cells and the communication environment between cells, thus achieving specific biological effects[11,12]. The structural changes of some glycosylation processes related to tumor growth of GALNT10 can affect the tumor migrate and invasive ability[12]. GALNT10 is highly expressed in many tumors and the tumor migration and invasion ability can be suppressed by inhibiting the expression of GALNT10[10-12]. However, the mechanism of GALNT10 in the malignant progression of GC and 5-Fu sensitivity has not been reported.

HOX family is a highly conserved family of transcription factors that play a key part in the formation of basic and secondary body axes during embryonic development[13,14]. HOXD13 is located at 2q31, containing 2 exons. HOXD13-encoded protein is mainly expressed in the heart, skeletal muscle, lung, prostate and other tissues[15,16]. The current research on HOXD13 gene and human diseases has been widely involved, but the relationship between HOXD13 gene and 5-Fu sensitivity of human GC has not been reported[17]. We aimed to explore the expression and significance of GALNT10 in GC by the targeting effect of GALNT10 and HOXD13, hoping to provide new ideas and explore new ways for cancer prevention and treatment.

Patients and Methods

Patients and Gastric Cancer Samples

60 gastric cancer specimens and their corresponding specimens were selected from surgically treated gastric cancer cases, and tissue samples of patients with invasive gastric cancer were collected and stored at -80°C. This investigation was approved by the Ethics Committee of The 960th Hospital of the PLA Joint Logistics Support Force. All patients have signed an informed consent form. Inclusion criteria: patients with no severe diseases in other organs, and none of patients had preoperative chemotherapy/radiotherapy or molecular targeted therapy. Exclusion criteria: patients with other malignancies, those with mental disease, those complicated with heart failure or other chronic diseases, or those previously exposed to radioactive rays. This study complies with the clinical practice guidelines of the Declaration of Helsinki. The postoperative pathological diagnosis comes from the Pathology Department of our hospital, and the diagnostic criteria are based on the NCCN guidelines; the tumor node metastasis (TNM) staging is based on the American Cancer Association AJCC seventh edition staging criteria.

Cell Lines and Reagents

Gastric cancer cell lines SGC-7901, AGS, MKN-45, BGC-823, and immortalized normal epithelial cells GES-1 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FSB) (Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. The blank control group (sh-NC) and the lentiviral vector containing the GALNT10 knockdown sequence (sh-GALNT10) were purchased from Shanghai Gene Pharma company (Shanghai, China).

Cell Proliferation Assays

Gastric cancer cells AGS and BGC-823 (5×10^3/well) were seeded into 96-well plates, and cells were stained with 100 μL of sterile Cell Counting Kit-8 (CCK-8) dye (0.5 mg/mL) at the designated time (Dojindo Molecular Technologies, Kumamoto, Japan), and the incubation continued 2 hours. The optical density of the detector (wavelength at 450 nm) is then checked with a microplate reader.

Transwell Assay

The serum-free medium diluted transfected cells (1×10^5) were inoculated into the upper layer of the matrix gel-containing chamber, and 600 μL of 20% FBS medium was placed as a chemical attractant in the lower compartment of the chamber. The transwell was incubated for 48 hours, then the lower layer penetrating cells were collected, fixed with 4% paraformaldehyde for 30 min, and stained with 0.5% crystal violet for 30 min. After washed with PBS, cells were subjected to penetrating cell counting in 5 random fields.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from tissues and cells. RNA reverse transcription was operated using Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) while real-time PCR was performed with the SYBR® Premix Ex

Taq™ kit (TaKaRa, Tokyo, Japan) on the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) system. The following primers were used in the qRT-PCR reaction (5'-3'): GALNT10: F: AAGACCATTGTGTGCCCGAT, R: GCCATCACGGGAGACTCAAA; HOXD13: F: CCCGGCTATATCGACATGGTG, R: TAGAGCCACATCCCCTGGAAA; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F: CCTGGCACCCAGCACAAT, R: GCTGATCCACATCTGCTGGAA.

Western Blot

Cells were lysed with cell lysis buffer. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). The primary antibodies were GALNT10, HOXD13 and GAPDH, and the secondary antibodies were anti-mouse and anti-rabbit, which were purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

Wild-type as well as the mutant-type binding sites were synthesized in the 3'-untranslated region (3'-UTR) of GALNT10 and subcloned into the HOXD13 basic plasmid vector. Dual-Luciferase reporter system (Promega, Madison, WI, USA) was used to assess Luciferase activity.

Statistically Analysis

GraphPad Prism 5 V5.01 software (La Jolla, CA, USA) was used for statistical analysis. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Each experiment was repeated at least three independent experiments, and the data were expressed as mean ± standard deviation (mean ± s). $p<0.05$ was considered statistically significant.

Results

GALNT10 Was Highly Expressed in GC Tissues As Well As Cell Lines

GALNT10 expression in 60 cases of GC tissues and their adjacent tissues was detected by qRT-PCR, which suggested that GALNT10 level was markedly increased in GC specimens than that of the adjacent ones (Figure 1A). In addition, GALNT10 was also markedly overexpressed in GC cell lines (Figure 1B). In addition, in order to explore the potential clinical value of GALNT10 in GC, we further analyzed the relationship between GALNT10 level and clinicopathological characteristics of GC patients. The statistical results suggested GALNT10 expression was closely related to the incidence of metastasis (lymph node and distant) of GC, but not with the age, sex, and pathological stage of tumor in patients with GC (Table I). Subsequently, follow-up data of patients

Table I. Association of GALNT10 expression with clinicopathologic characteristics of gastric cancer.

Parameters	No. of cases	GALNT10 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.448
<60	28	15	13	
≥60	32	14	18	
Gender				0.611
Male	29	15	14	
Female	31	14	17	
T stage				0.427
T1-T2	32	17	15	
T3-T4	28	12	16	
Lymph node metastasis				0.017
No	34	21	13	
Yes	26	8	18	
Distance metastasis				0.019
No	32	20	12	
Yes	28	9	19	

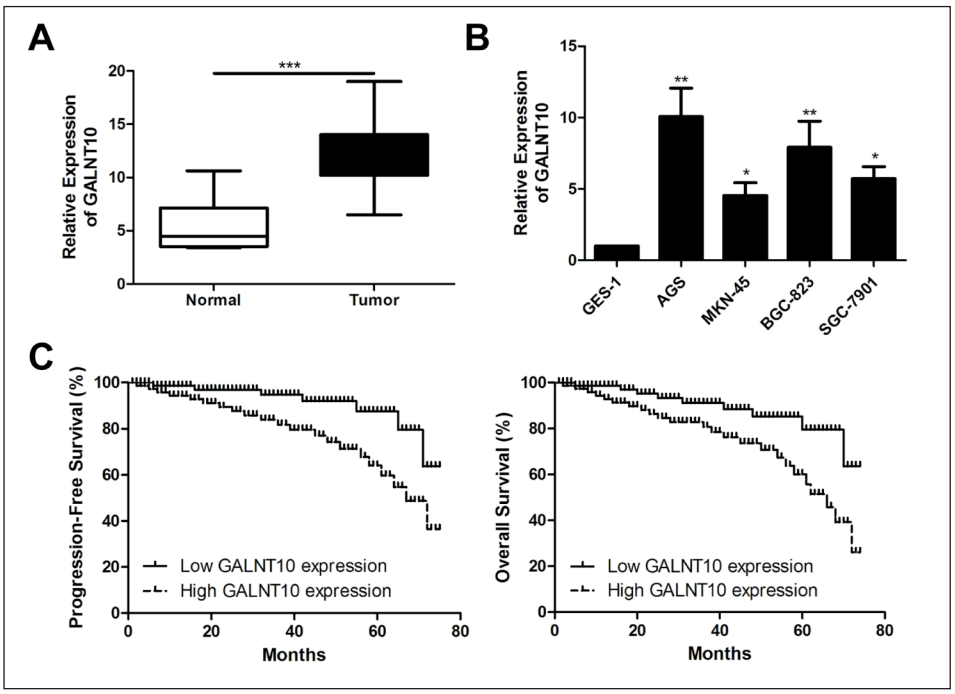

Figure 1. GALNT10 is highly expressed in GC tissues as well as cell lines. **A**, qRT-PCR analysis of GALNT10 expressions in GC tumor tissues and adjacent tissues; **B**, qRT-PCR analysis of GALNT10 expression levels in GC cell lines; **C**, Kaplan Meier was used to calculate the Disease-free survival curve and prognosis of patients in high GALNT10 expression group and low GALNT10 expression group; Data were average ± SD, *p<0.05, **p<0.01, ***p<0.001.

with GC were collected. Kaplan-Meier and log-rank test results suggested that patients with high expression of GALNT10 had remarkably shorter survival time (both disease-free and overall survival) (Figure 1C). Therefore, we speculated that GALNT10 may act as an oncogene in GC.

Knockdown of GALNT10 Inhibited the Proliferative Ability and Metastasis in GC Cell Lines

To evaluate the relationship between GALNT10 and malignant progression of GC, GALNT10 knockdown cell lines were established, which was confirmed by qRT-PCR (Figure 2A). Then CCK-8 assay suggested that the proliferation was markedly reduced after GALNT10 silencing (Figure 2B) while the number of transmembrane GC cells was also remarkably reduced after silencing GALNT10 (Figure 2C). Subsequently, Western Blot detection displayed that HOXD13 expression in GC cell lines was markedly reduced after silencing GALNT10 (Figure 2D). These above results demonstrated that knockdown of GALNT10 inhibited the proliferative ability and metastasis in GC.

GALNT10 Was Bound to HOXD13

Bioinformatics experiments suggested that GALNT10 can combine with HOXD13. In order to further verify the targeting effect of GALNT10 on HOXD13, the Luciferase reporter gene experiment was performed, which confirmed that GALNT10 can be targeted by HOXD13 (Figure 3A). In addition, HOXD13 expression in GC tumor tissue was markedly increased compared with adjacent ones (Figure 3B). Besides, qRT-PCR analysis revealed that the expressions of GALNT10 and HOXD13 showed a significant positive correlation in tumor specimens of GC patients (Figure 3C).

GALNT10 Decreased the Sensitivity of GC Cell Lines to 5-Fu

To establish a 5-Fu-resistant GC cell line, we performed CCK-8 assay and found that the inhibition of proliferation increased dependently of the 5-Fu drug concentration (Figure 4A). Besides, the cell proliferative rate in 5-Fu-resistant cell was remarkably lower than that of normal GC cells; meanwhile, after knocking down GALNT10, the cell proliferative rate in both normal GC cells and 5-Fu-resistant GC cells was markedly reduced (Figure 4B, 4C).

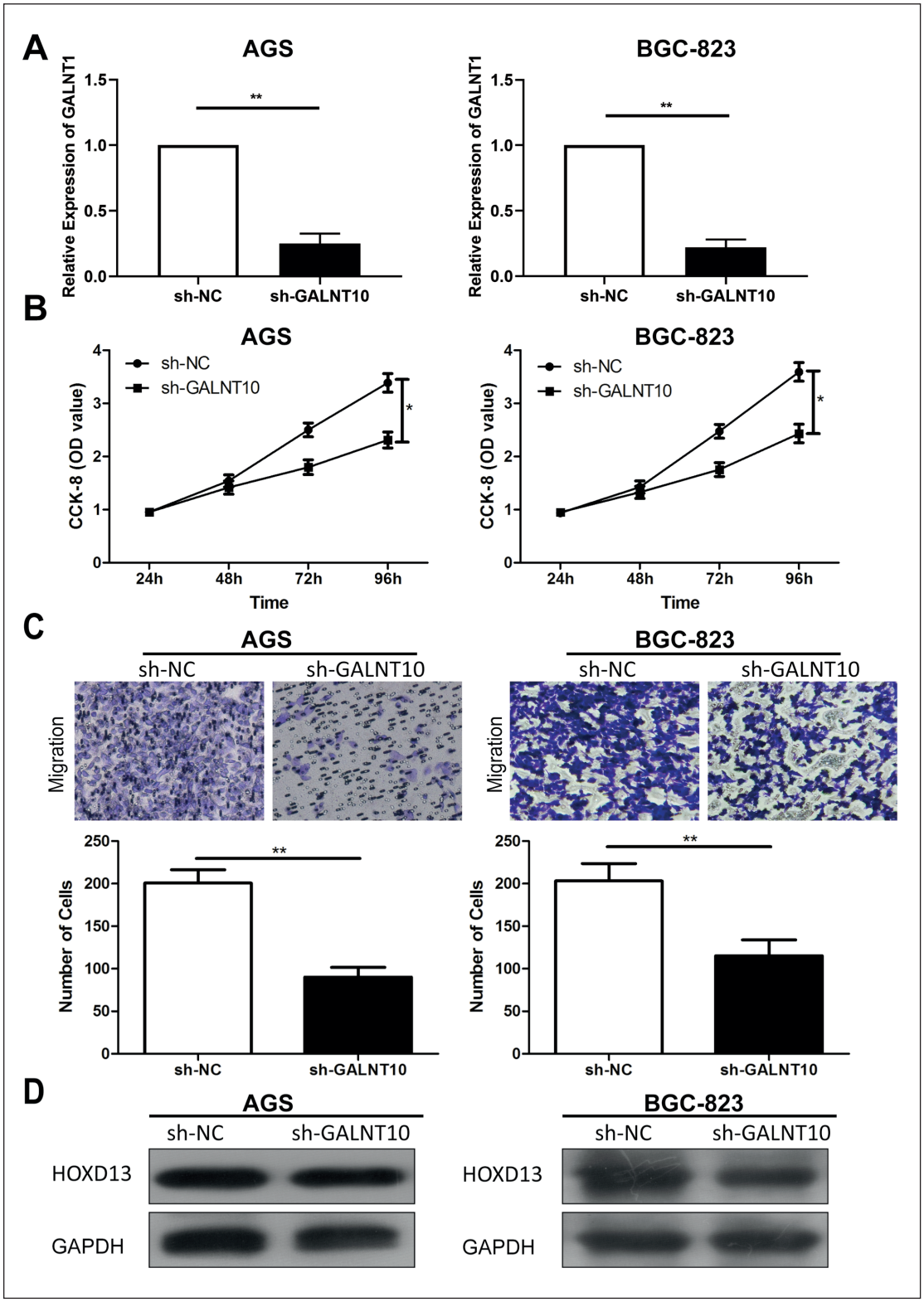

Figure 2. Silencing GALNT10 can inhibit the proliferation and migration of GC cells. **A**, qRT-PCR analysis of the interference efficiency of GALNT10 after transfection of GALNT10 in GC cell lines AGS and BGC-823; **B**, CCK-8 was used to detect the cell proliferation of GC cell lines AGS and BGC-823 after knocking down GALNT10; **C**, Transwell migration was applied to detect the migration of GC cell lines AGS and BGC-823 after knockdown of GALNT10 (magnification: 40×); **D**, Western Blot analysis of the expression level of HOXD13 in AGS and BGC-823 after GALNT10 knockdown. Data were average ± SD, *p<0.05, **p<0.01.

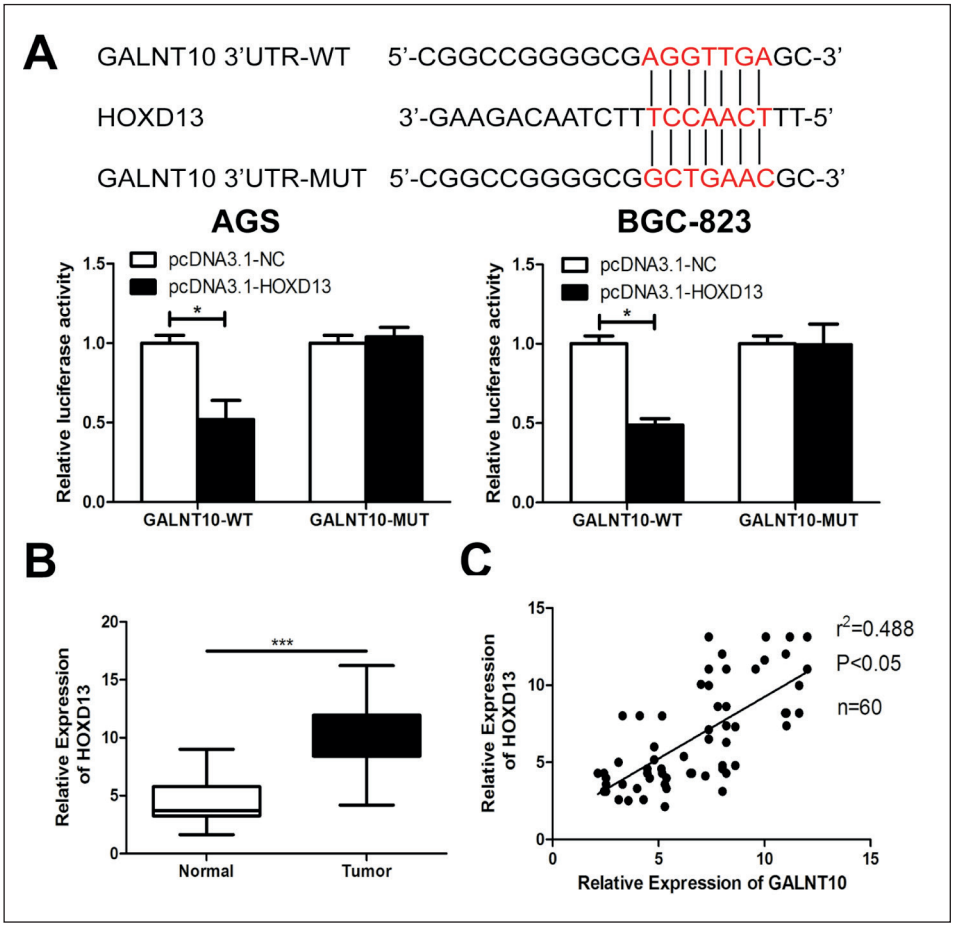

Figure 3. The interaction of GALNT10 and HOXD13. **A**, Dual-Luciferase reporter gene experiment was performed to verify the direct targeting of GALNT10 and HOXD13; **B**, qRT-PCR analysis of the expression of HOXD13 in GC tumor tissues and adjacent tissues; **C**, The correlation between GALNT10 and HOXD13 in GC tissues. Data were average ± SD, *p<0.05, ***p<0.001.

In addition, transwell migration results showed GALNT10 knockdown remarkably reduced the number of transmembrane GC cells in 5-Fu-resistant cell (Figure 4D). These results suggested that GALNT10 decreased the sensitivity of GC cells to 5-Fu.

GALNT10 Modulated HOXD13 in GC Cell Lines

We further explored the interaction between GALNT10 and HOXD13 in GC cell lines. Briefly, GALNT10 knockdown vector and HOXD13 overexpression vector were co-transfected in 5-Fu-resistant GC cell lines AGS and BGC-823. Western Blot analysis revealed that the co-transfection markedly increased GALNT10 expression (Figure 5A). Subsequently, CCK-8 assay suggested that overexpression of HOXD13 could resist the inhibitory effect of silencing GALNT10 on the proliferative ability of GC cells (Figure 5B). In addition, transwell migration results indicated that overexpression of HOXD13 can resist the inhibitory effect of silencing GALNT10 on the migration of GC cells (Figure 5C). In sum, these results recommended that GALNT10 modulated HOXD13 in GC cell lines.

Discussion

GC accounts for the second leading cause of cancer-related death in the world. Chemotherapy is currently the main treatment for advanced metastatic GC, which is also an important auxiliary treatment before and after surgery[1-4]. As a member of the N-acetylgalactosyltransferase family, GALNT10 can transfer N-acetylgalactose to serine or acid-acid residues in the side chains of specific proteins in the Golgi body, thereby changing its biological properties to achieve specific biological effects[10,11]. In addition, GALNT10, as a glycosylation en-

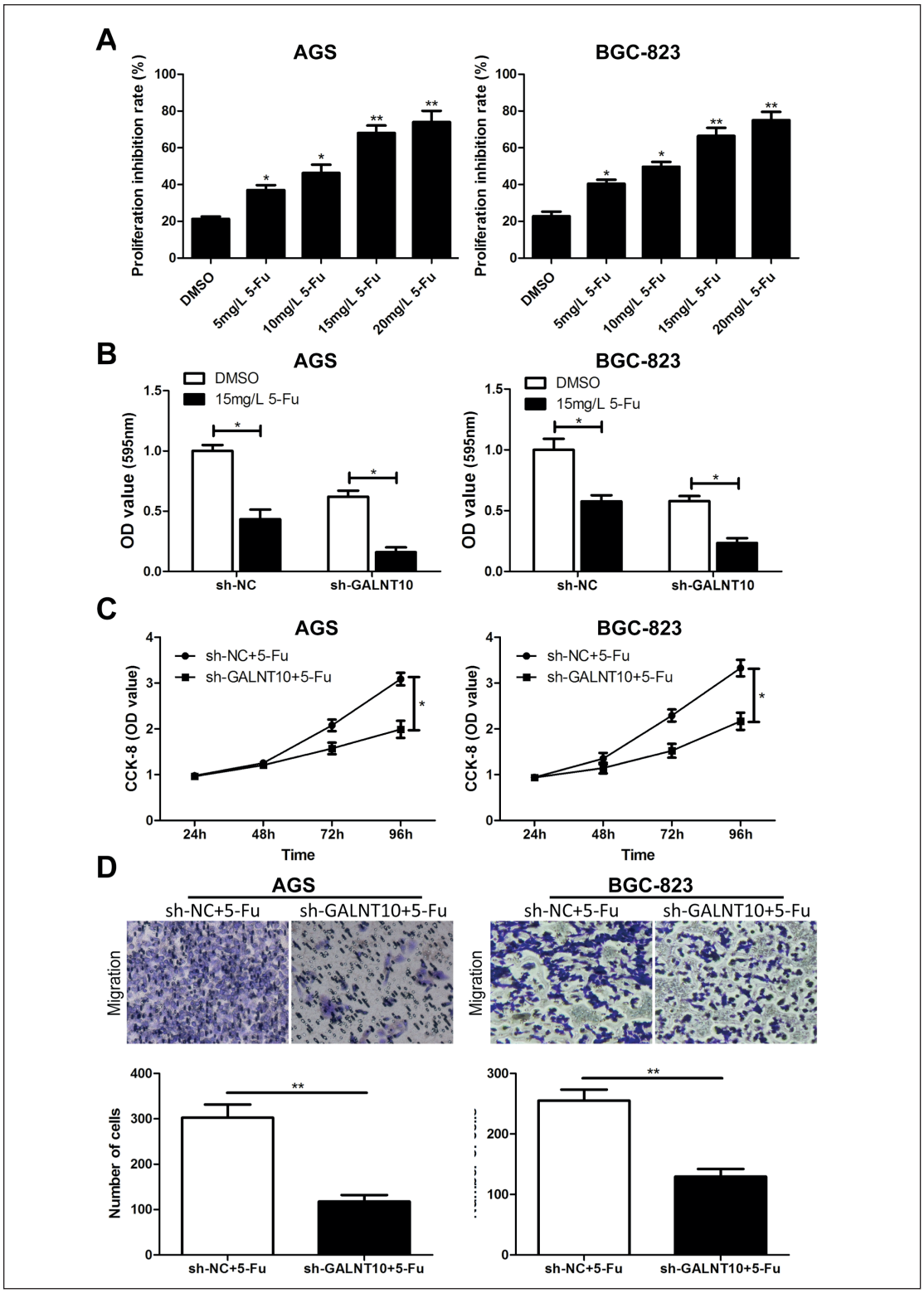

Figure 4. GALNT10 can reduce the sensitivity GC cell lines to 5-Fu. **A**, CCK-8 was used to evaluate the cell proliferation rate of GC cell lines AGS and BGC-823 at different concentrations of 5-Fu; **B**, CCK-8 analysis of normal GC cell lines and 5-Fu resistant GC cell lines after GALNT10 knockdown; **C**, CCK-8 detection of cell proliferation in 5-Fu resistant GC cell lines AGS and BGC-823 after GALNT10 knockdown; **D**, Transwell migration assay was performed to detect the migration ability of 5-Fu resistant AGS and BGC-823 cells after GALNT10 knockdown (magnification: 40×). Data were average ± SD, *p<0.05, **p<0.01.

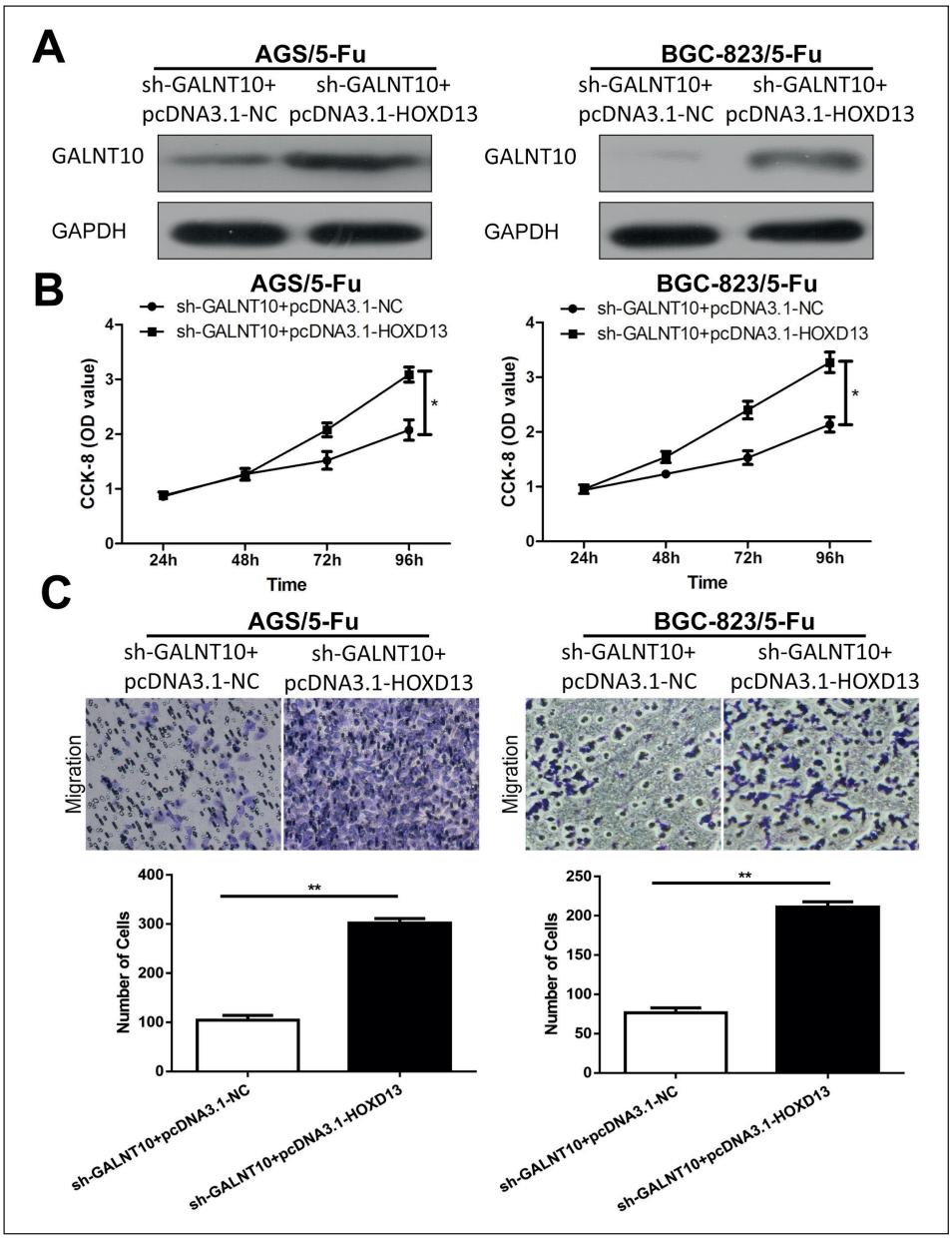

Figure 5. GALNT10 can regulate the mechanism of action of HOXD13 in 5-Fu-resistant GC cell lines. **A**, Western blot detection of the expression level of GALNT10 after co-transfection of GALNT10 knockdown and HOXD13 overexpression vectors in 5-Fu-resistant GC cell lines AGS and BGC-823; **B**, CCK-8 analysis of GC cell line AGS and BGC-823 after co-transfection of GALNT10 knockdown and HOXD13 overexpression vectors; **C**, Transwell migration assay was used to detect the migration ability of AGS and BGC-823 cells after cotransfection of GALNT10 knockdown and HOXD13 overexpression vectors (magnification: 40×). Data are average ± SD, *p<0.05, **p<0.01.

zyme, participated in the first step of protein O-glycosylation. Many members of the N-acetylgalactosyltransferase family participate in the glycosylation of protein structures[11,12]. Abnormal glycosylation usually predicts human tumorigenesis and is closely related to metastasis, invasion, proliferation, adhesion, etc.[12]. Although research has revealed that protein glycosylation plays a key role in tumor composition, it is still unknown how GALNT10 exerts its regulatory function in tumors[10-12]. In our study, qRT-PCR suggested that GALNT10 gene expression was markedly increased in GC cell lines compared to normal gastric mucosal cells. In addition, GC tissue verification revealed that GALNT10 expression in tumor specimens of GC patients was remarkably higher

than that of paracancerous tissues, and its expression was markedly in correlation with metastasis incidence and poor prognosis in GC. Besides, silencing GALNT10 in GC cell lines could inhibit the proliferative and migration ability of GC. The above results suggested that GALNT10 acted as an oncogene in GC. Bioinformatics experiments suggested that GALNT10 can bind to HOXD13, which was further verified by Luciferase gene experiments. Western Blot analysis displayed that HOXD13 expression was remarkably reduced after silencing GALNT10 in GC cell lines. Additionally, qRT-PCR analysis indicated that the expressions of GALNT10 and HOXD13 in GC tumor tissues showed a positive correlation.

Infinite proliferative and migration activity of tumor cells are two important characteristics of GC[18]. Inhibiting the proliferative and migration ability of tumor cells has become the best treatment for GC. Therefore, studying the pathological characteristics of GC and its related mechanisms will provide new clues for the discovery of potential therapeutic targets[18,19]. 5-Fu is a cell cycle-specific drug that mainly acts on S-phase cells. 5-Fu could be converted into fluorouracil deoxynucleotide in the body and specifically bind to thymine nucleotide synthase, thus affecting the cellular DNA synthesis and eventually leading to cell death[6-9]. In this study, GC cell lines were treated with 5-Fu so as to establish 5-Fu-resistant GC cell lines. Furthermore, CCK-8 and transwell experiments suggested that silencing GALNT10 could inhibit the proliferative and migration ability of 5-Fu-resistant GC cells. In addition, we found that HOXD13 overexpression can lead to a significant increase in GALNT10 expression, thereby jointly promoting the development of GC. Further recovery experiments suggested that overexpression of HOXD13 can restore the inhibitory effect of GALNT10 silencing on the proliferative and migration ability of 5-Fu resistant GC cells. Therefore, the above results indicated that HOXD13 might regulate the transcription activity of GALNT10, which further promoted the malignant progression of GC and reducing 5-Fu sensitivity.

Conclusions

In summary, GALNT10 could regulate the proliferative ability and migration of GC cells and reduce 5-fluorouracil sensitivity by enhancing the expression of HOXD13. Therefore, GALNT10 is expected to serve as a new molecular target for clinical diagnosis of 5-fluorouracil resistance in GC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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