

Polypeptide *N*-acetylgalactosaminyltransferase-6 expression in gastric cancer

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Abstract: Gastric cancer (GC) is one of the leading causes of cancer-related deaths, with limited improvement in its clinical outcome worldwide. Aberrant mucin-type *O*-glycosylation is a critical event widespread in the development of GC. Polypeptide *N*-acetylgalactosaminyltransferases (GALNTs) regulate the initial step and determine the sites of mucin-type *O*-glycoprotein biosynthesis. GALNT6 has considerable potential as a biomarker in various cancers. The roles of GALNT6 in GC were analyzed, and the results showed that GALNT6 expression markedly increased in GC tissues compared with those in adjacent gastric tissues. High intratumoral GALNT6 density was associated with the clinicopathological parameters of TNM stage and distant metastasis. GALNT6 was identified as an independent prognosticator for the poor prognosis of GC patients. Moreover, the high expression level of GALNT6 was significantly associated with the low expression levels of E-cadherin and β -catenin and the high expression levels of MMP9. These findings indicated that GALNT6 could provide new insights into the characterization of GC as well as contribute to the development of an efficient prognostic indicator and novel therapeutic modalities for GC.

Keywords: gastric cancer, *O*-glycosylation, GALNT6, prognosis

Introduction

Gastric cancer (GC) is characterized by high recurrence rates and a dismal prognosis, given the lack of early detection and effective treatment methods for this cancer type.¹ The development of novel biological markers and molecular-targeting drugs that could predict GC and improve clinical management is essential. Carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA19-9), and cancer antigen 72-4 (CA 72-4) are the most commonly used GC biomarkers.²⁻⁴ In this study, we identify and characterize *N*-acetylgalactosaminyltransferase-6 (GALNT6), which is infrequently reported in GC.

Mucin-type *O*-glycosylation is the most common and complex posttranslational modification of human proteins. This process involves several physiological events, such as the folding, stabilization, and targeting of various glycoproteins. Aberrant mucin-type *O*-glycosylation exists in a variety of epithelial cancers and has various effects on cell adhesion, invasion, and metastasis.⁵⁻⁹ Mucin-type *O*-glycosylation is initiated by a large family of polypeptide GALNTs.¹⁰ GALNTs transfer *N*-acetylgalactosamine (GalNAc) to serine or threonine residues during protein biosynthesis and determine *O*-glycosylation sites on proteins.^{11,12} Given their involvement in several features of tumor cell biology and behavior, the abnormal expression of GALNTs is a possible molecular mechanism that promotes malignant transformation and tumor progression.¹³⁻¹⁵ Accumulating evidence suggests that several GALNT family members are involved in various tumors of the breast, pancreas, and colon and in

neuroblastomas.^{16–19} GALNT6 expression has been mainly reported in breast carcinomas.^{16,20–24} Prior studies have revealed that GALNT6 expression is significantly higher in breast cancer tissues than in normal breast tissues. Furthermore, GALNT6 is exclusively upregulated in breast cancer cells but is not expressed in normal human vital organs. Therefore, GALNT6 may be an ideal therapeutic target for inhibitors in therapeutic modalities against various cancers, with minimal risks of adverse effects.¹⁶ GALNT6 expression in GC is rarely studied. Moreover, oncology studies on GALNT6 are confined to cytological studies. This study is a retrospective cohort study with a large number of samples and was designed to evaluate the relationship between GALNT6 protein expression and the clinicopathological characteristics of GC. Meanwhile, the molecular mechanism of GALNT6 was tentatively explored to understand its critical role in gastric carcinoma. The results of the present study could provide insight into the characterization of GC for the development of diagnostic and therapeutic strategies.

Materials and methods

Clinical specimens

Paraffin-embedded samples and corresponding clinical information were obtained from a retrospective cohort of patients (n=275) who were randomly selected and had a confirmed diagnosis of GC between 2001 and 2009 at the Tianjin Medical University Cancer Institute and Hospital. A total of 15 frozen GC and matched adjacent tissues were evaluated using quantitative real-time polymerase chain reaction (qPCR) and Western blot analysis. Written informed consent was provided by the patients whose specimens were used. This research was approved by the ethics committees of Tianjin Medical University.

qPCR and Western blot analysis

Total RNA was extracted from frozen tissue specimens using Trizol reagent in accordance with the manufacturer's protocol. Complementary DNA was synthesized by using a reverse transcription kit (TaKaRa Biotechnology Ltd, Dalian, People's Republic of China). The PCR primer sequences for GALNT6 were 5'-CCAGACATCGTCACCATCG-3' and 5'-AGGTCAGGCTCCAGTCAAAG-3'. The relative expression levels of GALNT6 were analyzed using the $2^{-\Delta\Delta Ct}$ method. GAPDH was applied as the calibration control in all specimens. Total proteins were extracted by using lysis buffer containing a protease inhibitor cocktail. Then, equivalent protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred

onto polyvinylidene fluoride (PVDF) membranes. GAPDH was used as a loading control.

Tissue microarray (TMA) construction

All candidate specimens were formalin-fixed paraffin-embedded (FFPE) tissue blocks and sections. FFPE sections were subjected to hematoxylin and eosin staining. TMAs were constructed using donor blocks taken from patients with GC. Representative tumor regions on slides were identified and marked by pathologists. The core selection of these donor blocks was guided by their corresponding marked slides. Tissue cores with a diameter of 2 mm were punched from the marked donor blocks. Then, recipient paraffin blocks were prepared. Finally, Quick-Ray was used to construct 15 TMA blocks representing 275 GC cases and their adjacent tissues.

Immunohistochemistry (IHC) analysis

TMA IHC staining was performed by using the EnVision two-step method in accordance with the manufacturer's instructions. The tissue sections were immunostained with GALNT6, E-cadherin, β -catenin, and MMP9 antibodies. A semiquantitative approach was used to score the immunostaining frequency of each sample as follows: 0 for negative sample or <5% stained tumor tissue, 1 for samples stained between 6% and 25%, 2 for samples stained between 26% and 50%, 3 for samples stained between 56% and 75%, and 4 for samples stained >75%. Staining intensity was scored as 1 (weak), 2 (moderate), and 3 (strong). The total immunostaining score of each sample was the product of both parameters as mentioned earlier.

Statistical analysis

All data were analyzed by SPSS software (version 17.0; SPSS Inc., Chicago, IL, USA). The chi-square test or Fisher's exact test was used for categorical variables. In addition, the analysis of continuous data was performed using the paired *t*-test or Wilcoxon matched-pairs test. Overall survival was defined as the time from diagnosis to the date of death or last follow-up. Cumulative survival time was calculated using the Kaplan–Meier method and performed with the use of log-rank test.

Results

Expression status of GALNT6 in GC

We utilized qPCR to assess GALNT6 mRNA levels in cancerous tissues from 15 matched patients with GC. qPCR results revealed that GALNT6 mRNA levels in GC tissues were at least twofold higher than those in corresponding adjacent

gastric tissues (Figure 1A). The expression of GALNT6 mRNA in GC samples was significantly higher than those in other groups ($P<0.001$). We also performed Western blotting using the same samples (Figure 1B). Western blot results generally conformed to qPCR results and showed that GALNT6 protein expression was dramatically higher in GC tissues compared with those in adjacent tissues ($P<0.001$).

IHC analysis of GALNT6 expression in patients with GC

IHC staining of GALNT6 was evaluated in tissues from randomly chosen patients. As shown in Figure 2, GALNT6 expression was negative or very weakly detectable in the glandular epithelium of adjacent gastric mucosa. Only 64 of 275 samples (23%) showed GALNT6 expression, whereas the positive rate of GALNT6 expression in GC was observed in 218 of 275 samples (Table 1). In these samples, GALNT6 expression was predominantly localized in the cytoplasm of tumor cells. The immunostaining pattern of GALNT6 was either diffused or perinuclear in GC cells. As illustrated in Table 1, GALNT6 expression in GC cases was significantly

stronger than that in adjacent tissues, and staining intensity usually varied. Therefore, GALNT6 might be a candidate regulator of GC initiation and progression.

Association of GALNT6 expression with the clinicopathological features of patients with GC

To identify associations between GALNT6 expression and clinicopathological characteristics, the valuable variables of GC are listed in Table 2. GALNT6 expression was divided into two groups of low and high expression levels based on the criteria for immunostaining. The relationships between the clinicopathological parameters of GC patients and GALNT6 expression levels are also summarized in Table 2. Statistical analysis was performed to determine the correlations between clinicopathological parameters and GALNT6 expression. GALNT6 expression showed a significant correlation with clinical stage and distant metastasis ($P<0.05$). GALNT6 tended to be highly expressed with more advanced clinical stages. In addition, GC metastasis was more frequent among patients in the strong GALNT6 expression

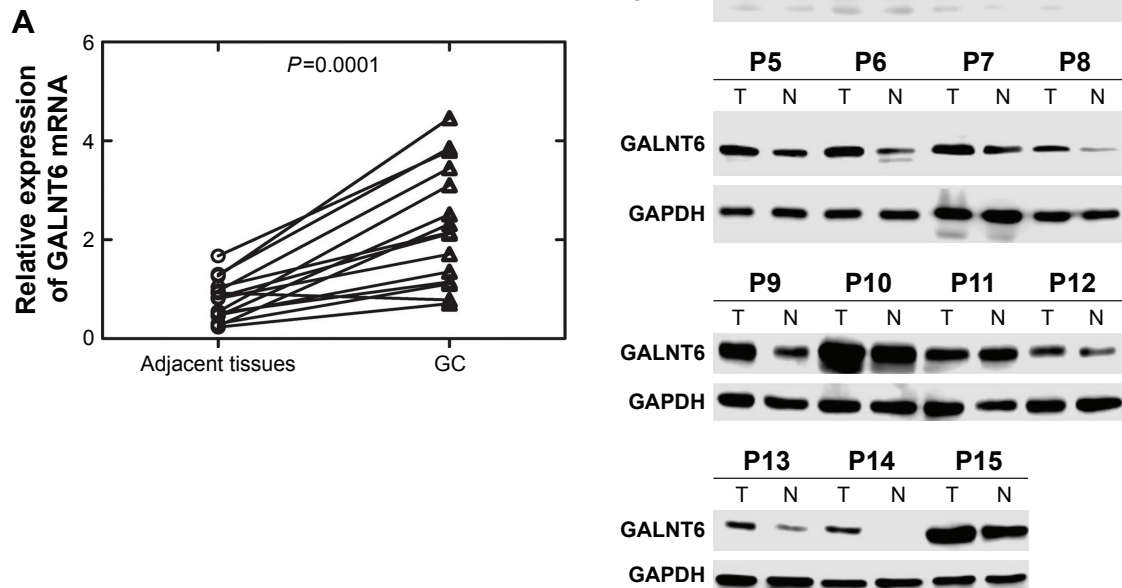


Figure 1 Expression levels of GALNT6 mRNA and protein in GC.

Notes: (A) The expression of GALNT6 mRNA from 15 paired patients (P1–P15) with GC was analyzed by using qPCR. Relative mRNA levels were normalized to GAPDH. GALNT6 expression in GC tissues was significantly higher than those in adjacent tissues ($P<0.001$). (B) The expression levels of GALNT6 protein were quantified via Western blotting. A total of 15 paired cases of the Western blot results are shown in B. The expression levels of GALNT6 protein dramatically increased in GC tissues compared with those in adjacent gastric tissues.

Abbreviations: GC, gastric cancer; N, nontumor adjacent tissues; qPCR, quantitative real-time polymerase chain reaction; T, tumor tissues.

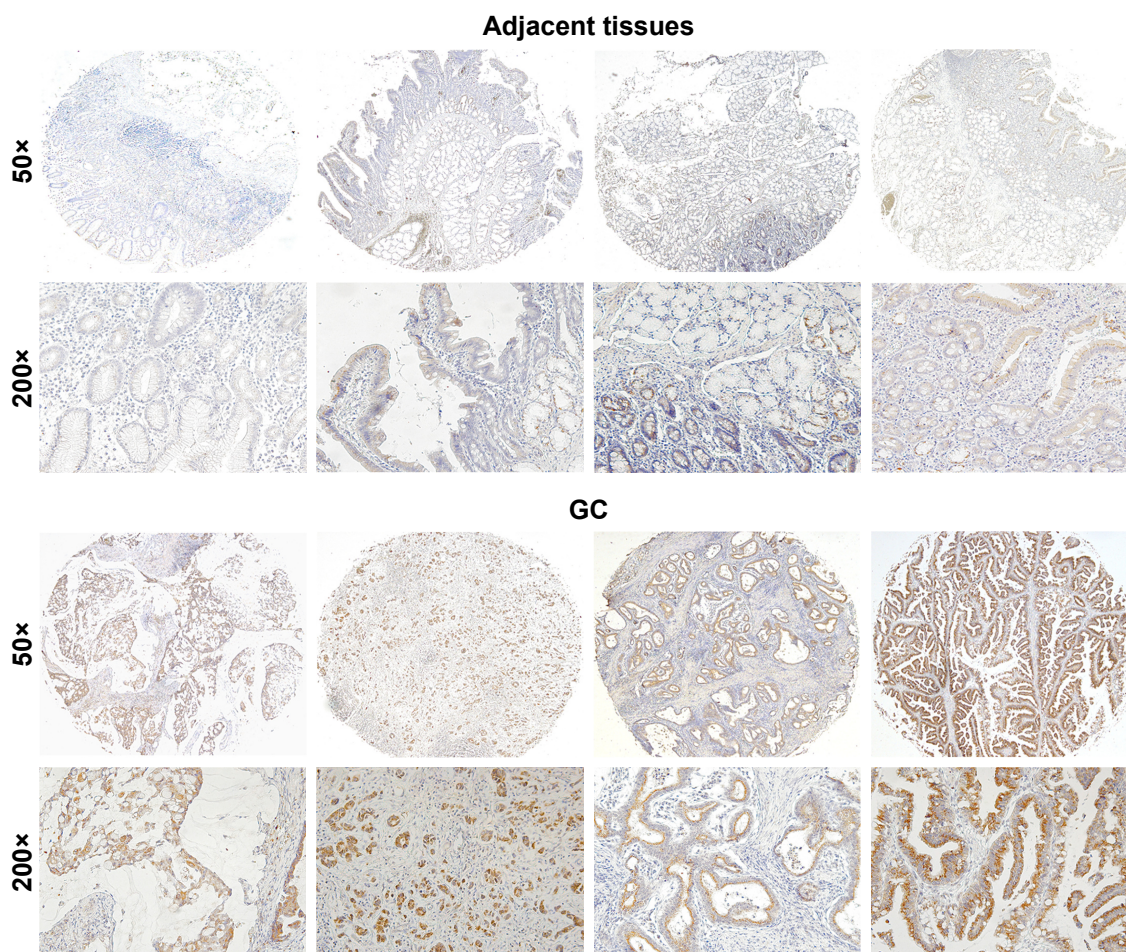


Figure 2 Representative images of GALNT6 IHC staining in gastric tissues.

Notes: Adjacent gastric tissues and GC tissues were subjected to IHC evaluation. GALNT6 expression was absent or very weakly detectable in normal gastric mucosa. GALNT6 was strongly expressed in GC tissues, and predominantly localized in the cytoplasm of tumor cells. The immunostaining pattern of GALNT6 was diffused or perinuclear in GC cells.

Abbreviations: GC, gastric cancer; IHC, immunohistochemistry.

group (42/73), compared with those in other groups (31/73). Our results showed that GALNT6 expression in GC was not correlated with the age, gender, tumor size, and histologic grade of patients. Thus, the expression level of GALNT6 in patients has a crucial impact on clinical outcome.

Influence of GALNT6 expression level on the survival of patients with GC

Kaplan–Meier survival analysis was employed to evaluate the correlation of GALNT6 expression with patient prognosis. The survival curves demonstrated that patients with high expression levels of GALNT6 had significantly shorter overall survival time (Figure 3). Therefore, GC patients with high positive staining for GALNT6 had a poorer prognosis than those with a low positive rate. Furthermore, the high expression level of GALNT6 was associated with poorer prognosis and shorter disease survival in GC patients.

GALNT6 expression may be a useful marker to predict the overall survival of patients with GC.

Relationship between GALNT6 expression and MMP9, E-cadherin, and β -catenin protein

To understand the role of GALNT6 in GC, we assessed the correlation of GALNT6 expression with several molecules detected by IHC (Table 3). Our data showed that the staining

Table 1 Correlation of GALNT6 expression with GC and adjacent gastric tissues

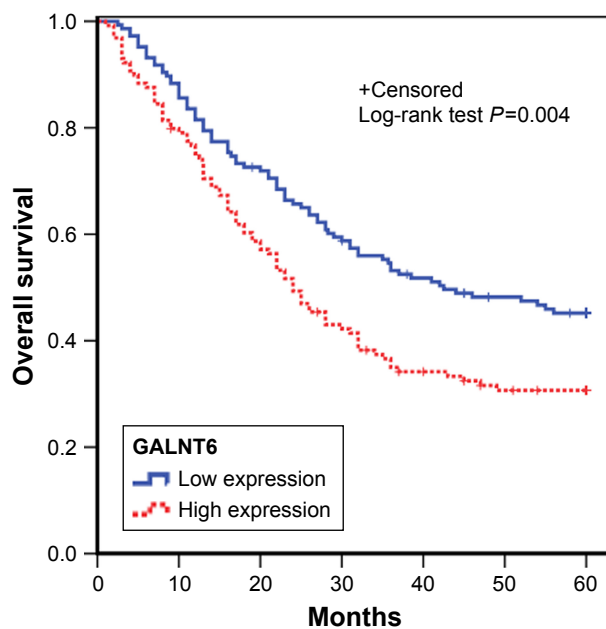
Tissue type	GALNT6 expression		P-value
	Positive, n (%)	Negative, n (%)	
GC	218 (79.3)	57 (20.7)	<0.001
Adjacent gastric tissues	64 (23.3)	211 (76.7)	

Abbreviation: GC, gastric cancer.

Table 2 Relationship between GALNT6 expression and clinical characteristics

Characteristics	Cases, n	GALNT6 expression		
		Low, n	High, n	P-value
Gender				0.677
Male	212	114	98	
Female	63	32	31	
Age				0.446
≤60 years	117	59	58	
>60 years	158	87	71	
Tumor size				0.367
≤5 cm	138	77	61	
>5 cm	137	69	68	
Histologic grade				0.264
Moderate/well	99	57	42	
Poor	176	89	87	
Tumor stage				0.026
I-II	92	59	33	
III	79	40	39	
IV	104	47	57	
Lymphatic metastasis				0.064
N0	81	50	31	
N1	194	96	98	
Distant metastasis				0.034
M0	202	115	87	
M1	73	31	42	

intensity of GALNT6 was associated with increased MMP9 expression. The expression levels of GALNT6 and MMP9 were statistically and significantly associated. In addition, the relative expression of GALNT6 was negatively

**Figure 3** Analyses of overall survival based on the expression of intratumoral GALNT6 in patients with GC (n=275).

Note: The Kaplan–Meier survival curves demonstrated that a high level of GALNT6 expression was significantly associated with poor overall survival.

Abbreviation: GC, gastric cancer.

Table 3 Relationship between GALNT6 expression and protein molecules of MMP9, E-cadherin, and β -catenin

Characteristics	Cases, n	GALNT6 expression		
		Low, n	High, n	P-value
MMP9				<0.001
Low	85	59	26	
High	190	87	103	
E-cadherin				0.038
Low	227	114	113	
High	48	32	16	
β -catenin				0.047
Low	202	100	102	
High	73	46	27	

correlated with the expression of E-cadherin and β -catenin. Cases A and B were representative cases for the illustration of the correlation among GALNT6 and related protein molecules based on relative expression (Figure 4A). Meanwhile, the representative case A and case B were subjected to Western blotting (Figure 4B), which revealed correlations between cases A and B.

Discussion

Molecular biomarkers and targeted therapies have been widely developed and implemented for various tumors. CEA, CA 72-4, and CA19-9 are widely used as biomarkers for GC. The low sensitivity and high false-positive rate of these biomarkers, however, have greatly limited their clinical value. Combining these biomarkers could improve their sensitivity and detection rate.^{4,25} In this study, we identified a promising molecule that could be used as an efficient prognostic indicator for the development of novel GC treatment strategies. Previous studies have shown that GALNT6 is a potential biomarker in breast cancer, as confirmed by cytological experiments.^{16,20,21} Park et al¹⁶ considered that the knockdown of GALNT6 suppresses the growth of breast cancer cells. Subsequently, the knockdown of GALNT6 significantly decreases cell viability in pancreatic cancer cells. These results indicated that GALNT6 may be essential for the survival of cancer cells.²⁶ In this study, we evaluated several hundreds of tissue samples from GC patients to investigate the role of GALNT6 in gastric carcinoma. qPCR, Western blot, and IHC results revealed that GALNT6 was upregulated in GC. In addition, the expression levels of GALNT6 and several clinicopathological parameters were significantly associated. As shown in Table 2, patients with GC and with high expression levels of GALNT6 exhibited advanced TNM stages and a higher possibility of distant metastases. Furthermore, intratumoral GALNT6 expression could influence the overall survival of

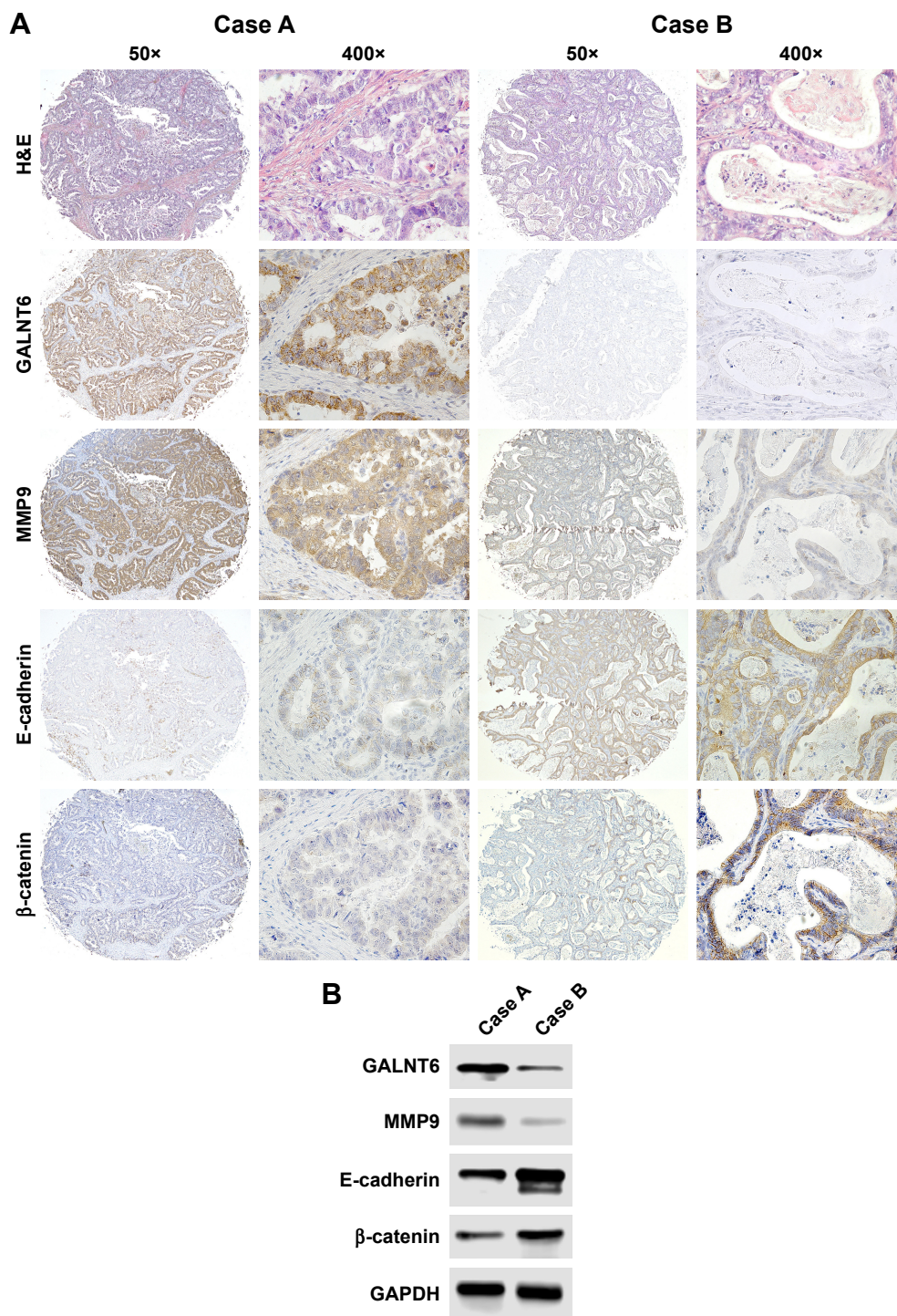


Figure 4 Expression of GALNT6 and related proteins in GC tissues in the validation study.

Notes: (A) Representative case A showed high expression levels of GALNT6 and MMP9 and low expression levels of E-cadherin and β -catenin. Case B showed low expression levels of GALNT6 and MMP9 and high expression levels of E-cadherin and β -catenin. (B) GALNT6, MMP9, E-cadherin, and β -catenin expression levels in the same representative tissue samples, as quantified by Western blotting. Higher expression levels of GALNT6 were correlated with higher lower expression level MMP9 and lower expression levels of E-cadherin and β -catenin.

Abbreviation: GC, gastric cancer.

patients with GC. Kaplan–Meier analysis showed that GC patients with high expression levels of GALNT6 had a poor survival rate. GALNT6 is a member of the GALNT family that regulates the initiation of *O*-linked protein glycosylation.

The considerable increase in GALNT6 expression in GC leads to the production of abnormal *O*-glycans, which influence cell biological activities and contribute to the invasion and metastases of GC.^{27,28} We previously demonstrated that GALNT6

expression is associated with certain clinicopathological characteristics of patients with GC. In summary, the results presented in the previous study provided clinicopathological evidence that verifies the hypothesis that GALNT6 is associated with tumor invasion and metastases in GC.^{14,16,20,26}

GALNT6 expression is a potential independent predictor for the prognosis of patients with GC. Thus, GC patients who present high expression levels of GALNT6 should receive particular attention from oncologists for more aggressive treatment. An increasing number of studies on the important roles of GALNT6 have been reported. However, the exact mechanism of action of GALNT6 in tumor progression remains unclear. A recent study showed that the knockdown of GALNT6 by small interfering RNA slows down breast cancer cell growth by increasing cell adhesion.¹⁴ More interestingly, another study demonstrated that the knockdown of GALNT6 accelerates the cell adhesion complex, including E-cadherin and β -catenin, thus attenuating the invasiveness of pancreatic cancer cells.²⁶ To confirm previous results and further characterize the biological significance of GALNT6, we examined and analyzed the status of E-cadherin and β -catenin in the regulation of cell morphology and carcinogenesis. Results showed that E-cadherin expression was inversely associated with the expression level of GALNT6. In addition, another cell membrane molecule, β -catenin, which mediates cell adhesion along with E-cadherin, presented a consistent relationship with GALNT6 similar to that with E-cadherin. Moreover, the upregulated expression of GALNT6 enhanced the transformational potentials of epithelial cells and epithelial–mesenchymal transition (EMT)-like cellular alterations through *O*-glycosylation.²⁹ Prior studies presented similar conclusions that the deregulation of GALNT6 and *O*-glycosylation affects cellular adhesion and invasion in different carcinomas.^{30,31} GALNT6 regulates the biochemical properties of cell surface proteins to maintain cellular polarity and adhesion. Our findings further revealed that the high expression of GALNT6 was accompanied by the strong staining of MM9. Furthermore, we selected representative cases to illustrate the relationship of GALNT6, E-cadherin, β -catenin, and MMP9 in GC tissues. These results are also consistent with those of a study on GALNT13 in cancer metastasis, where the high expression of the GALNT13 gene generated trimeric Tn antigen on Syndecan 1; this expression pattern could increase cell adhesion to fibronectin under the reduced expression of monosialotetrahexosyl ganglioside. This phenomenon enhances invasion and metastasis via the formation of a molecular complex that consists of integrin α 5 β 1,

Syndecan 1, and MMP9 in glycolipid-enriched microdomain/rafts.³² Thus, the abnormal expression of GALNT6 in GC may induce changes in the adhesive and invasive functions of cells. The present cohort study shows that GALNT6 is an independent and efficient negative biomarker of clinical management in GC. Moreover, our results contribute to the development of a target molecular inhibitor that could facilitate early diagnosis and treatment.

Conclusion

We demonstrated that GALNT6 expression was markedly increased in GC. High intratumoral GALNT6 density was associated with several clinicopathological parameters. Furthermore, it can be used as an independent prognosticator for poor prognosis of GC patients. These findings indicate that GALNT6 could provide new insights into GC characterization and contribute to the development of efficient prognostic indicator and novel therapeutic modalities.

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Disclosure

The authors report no conflicts of interest in this work.

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