

# Expression of N-acetylglucosaminyltransferase V in gastric cancer correlates with metastasis and prognosis

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**Abstract.** N-acetylglucosaminyltransferase V (GnT-V) is an enzyme that catalyzes  $\beta$ 1-6 branching of N-acetylglucosamine on asparagine (N)-linked oligosaccharides (N-glycan) of cell proteins and the dysfunction of which is a common feature of various carcinomas. Nevertheless, the role of GnT-V remains controversial. Therefore, the clinical implication of GnT-V expression may differ in each cancer type. The implication of GnT-V status in patients with gastric cancer has not been studied extensively. In the present study, we examined GnT-V expression in gastric cancer specimen both at protein and mRNA levels. We compared GnT-V expression with clinical and pathologic variables. Kaplan-Meier survival curves were generated to show the cause-specific survival. Furthermore, the small interfering RNA was devised to downregulate the GnT-V mRNA expression in SGC7901 and BGC 823 cells. We characterized the function implication of GnT-V by cell proliferation and invasiveness analysis. Analysis in gastric cancer specimen revealed that GnT-V expression correlated with tumor grade and stage. The overall survival time of positive GnT-V expression in gastric cancer was significantly shorter than that of negative GnT-V expression. Moreover, the down-regulation of GnT-V expression by small interfering RNA resulted in a decrease of cell proliferation and invasiveness in SGC7901 and BGC 823 cells accompanied by morphological change. This supports that GnT-V correlates with metastasis and prognosis in gastric cancer. These results contribute to new insight into the underlying molecular mechanisms of GnT-V regulation in gastric cancer with potential translational clinical applications.

## Introduction

Although the incidence of gastric cancer has been substantially declining for several decades, gastric cancer is still the second most common cause of cancer-related death in the world, which accounts for 738,000 deaths annually (1-3). Due to its tendency to spread into the muscularis propria, the lymph node, and the vessel and the relative asymptomatic progression of early-stage, a number of cancer patients present with advanced diseases and have a poor long-term prognosis (4). In the past decades, intensive efforts have been made to identify specific markers to improve prognosis of gastric cancer, there is still a lack of molecular markers for targeting therapy. In clinical practice, clinicopathological features are mostly relied on to predict patients' outcome, however, these prognostic factors do not fully predict individual clinical outcome (5-7). As a result of these factors, there is a continued need for identifying more prognostic markers in order to guide clinical practice and improve the long-term prospect of survival for gastric cancer patients.

A substantial body of evidence has shown that the proliferation, invasion and metastasis of tumor cells, which are highly associated with N-acetylglucosaminyltransferase V (GnT-V), is one of the key factors reducing the effectiveness of cancer treatment (8-14). GnT-V is a key enzyme that catalyses the formation of 1, 6 N-acetylglucosamine (GlcNAc) through the action of adding antennae branching structures on a common core structure of Man3GlcNAc2 in the medial-Golgi apparatus (15-17). A previous study proposed that post-translational modification of cell surface glycoproteins through N-glycosylation, such as matrix metalloproteinase-9 (MMP-9) and E-cadherin (18,19), may have a role on the process of tumor invasion and metastasis, damage to the surrounding tissues, such as the extracellular matrix (ECM), the basement membrane and the vascular walls. The increased levels of GnT-V in human breast cancer tissues were reported (20,21). mRNA levels of GnT-V were elevated during hepatocarcinogenesis of rats (22,23), and increased expression of GnT-V in human hepatocellular carcinoma tissues was also reported and positive correlation to tumor size was observed (10,24). However, aberrantly expressed GnT-V has been reported in a variety of tumors. In neuroblastomas and colorectal cancer, the expression of GnT-V correlated significantly with distant metastasis. In contrast,

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hepatocellular carcinoma cases with low or no expression of GnT-V were more likely to show recurrence than cases with high expression. Moreover, GnT-V expression was inversely associated with prognosis and histology in non-small cell lung cancers. Thus, its clinical role in gastric cancer remains fragmentary. To explore its malignant influence and prognostic significance, we examined the GnT-V protein expression and localization of GnT-V in human cancer tissues as well as in normal tissues. The correlation between GnT-V and other clinical pathological parameters of gastric carcinomas including growth-pattern, invasion and metastasis were also analyzed in this study. Furthermore, we devised a knockdown approach, in which small interfering RNA (siRNA)-directed against GnT-V mRNA was used to downregulate GnT-V mRNA expression in two gastric cancer cell lines SGC7901 and BGC823 cells. In addition, the biological behavior was observed. The comparison of specific GnT-V expression in cancer tissues and conventional clinicopathological analysis as well as evaluation in gastric cancer lines raised a possibility of GnT-V expression as a novel poor prognostic factor and a new targeting marker for successful treatment of gastric cancer patients.

## Materials and methods

**Tumor samples and patient follow-up.** Surgical specimens of gastric cancer patients resected at surgery service in the Department of Surgery, Tongji Hospital, School and Medicine and Shanghai Pudong New Area Gongli Hospital were used as histological samples, with informed consent from the patients. Samples were fixed with 20% formalin in PBS for 72 h and embedded with paraffin. The conventional clinicopathological features and TNM staging were obtained by the pathologists according to the criteria of the TNM classification revised in 2003 by International Union against Cancer (UICC) and American Joint Committee on Cancer (AJCC). The patients were followed-up in outpatient clinic of our hospital for >36 months after surgery.

**Immunohistochemistry and pathology review.** Immunohistochemical analysis of cancer tissue and adjacent non-cancerous mucosa was performed with a monoclonal antibody against GnT-V (Abcam, USA). Incubation with the primary antibody was followed by incubation with biotinylated goat anti-mouse IgG antibody. The specimens were analyzed by SABC method. The sections were examined by two independent observers without prior knowledge of the clinical status of the patients.

**RNA isolation from primary GCs.** Fresh, frozen tumor tissues and adjacent non-cancerous mucosa (different tissues from those used in immunohistochemical analysis) were sent to the Department of Gastroenterology, Tongji Hospital, Tongji University School of Medicine from Shanghai Minhang Central Hospital and Shanghai Pudong New Area Gongli Hospital with informed consent from the patients. All samples were obtained by surgery and were stored at -80°C. The RNA samples obtained from 43 patients with GC were subjected to quantitative real-time reverse transcription-PCR (RT-PCR) analyses. All of the patients were diagnosed clinically as well as pathologically. The tumors were staged according to standard methods, as stated above.

**Quantitative real-time PCR analysis of primary GCs.** The level of GnT-V mRNA was detected by quantitative real-time reverse transcription-PCR analysis (qRT-PCR). Reverse transcription reactions were performed with the PrimeScript RT Master Mix (Takara Biotechnology Co., Ltd.) and proceeded for 15 min at 37°C, followed by 5 sec at 85°C for complementary DNA (cDNA) synthesis. Real-time reactions were performed using the SYBR PrimeScript™ RT-PCR kit (Takara Biotechnology Co.) under the following conditions: 30 sec at 95°C for 1 cycle, 5 sec at 95°C, 20 sec at 60°C for 40 cycles, 95°C for 0 sec, 65°C for 15 sec and 95°C for 0 sec for melting curve analysis. The following primer sets were used: GnT-V, 5'-GATGCTTCTGCACTTTAC-3' and 5'-GCCTTGATGTACCTTTTG-3'; GAPDH, 5'-ATCACCATTGGCAATGAG-3' and 5'-AAGGTAGTTTCGTGGATG-3'. The relative mRNA expression level of GnT-V in each sample was calculated using the comparative expression level  $2^{-\Delta\Delta Ct}$  method. All experiments were carried out in triplicate for each data point.

**Cell culture.** SGC7901, BGC 823 cells and MKN45 cells were generously provided by the cell division of center laboratory in Tongji Hospital of Tongji University, Shanghai, China. Cells were cultured in 90% RPMI-1640 (Gibco) supplemented with 100 U/ml penicillin + streptomycin antibiotics (Gibco) and 10% fetal bovine serum (Gibco) at 37°C with 5% CO<sub>2</sub>.

**Construction of siRNA vector and retroviral infection.** Small interfering oligonucleotides specific for GnT-V were designed on the Takara Bio website (<http://www.takara-bio.co.jp/>) and the oligonucleotide sequences used in the construction of the siRNA vector were as follows: 5'-TGCTGAAAGAAGGC TCGCAGATGAGCGTTTTGGCCACTGACTGACGCTCAT CTGAGCCTTCTTT-3' and 5'-CCTGAAAGAAGGCTCAG ATGAGCGTCAGTCAGTGGCCAAAACGCTCATCTGCG AGCCTTCTTTC-3'. The oligonucleotides were annealed and then ligated into *Hind*III sites of the pcDNA6.2-GW/EmGFP-miR vector (Novo Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells (HEK293) using a pL-MIG retrovirus packaging system (Novo Bio). BGC823 and SGC7901 cells, two human GC cell lines, were infected with the viral supernatant, and the cells were then sorted by flow cytometry (BD). GFP-positive cells were confirmed by GnT-V gene and protein expression.

**Quantitative real-time PCR and western blot analysis of gastric cell line.** Quantitative real-time PCR analyses of GnT-V mRNA expression in cell lines were performed as described above.

The expression of GnT-V protein was detected by western blot assay. Cells (10<sup>7</sup>) were harvested and lysed with ice-cold lysis buffer (RIPA and a mixture of protease inhibitors, Beyotime Institute of Biotechnology). Protein concentration of the supernatant was determined by the BCA protein assay procedure. Equal amount of proteins were separated by 10% SDS-PAGE, respectively. Then, proteins were transferred to polyvinylidene difluoride membrane using a semi-dry transfer apparatus. The membrane was blocked in Tris-buffered saline (TBS) with 5% non-fat milk for 1 h at room temperature, followed by incubation with appropriate primary antibodies (1:500-diluted antibody of GnT-V, Abcam and

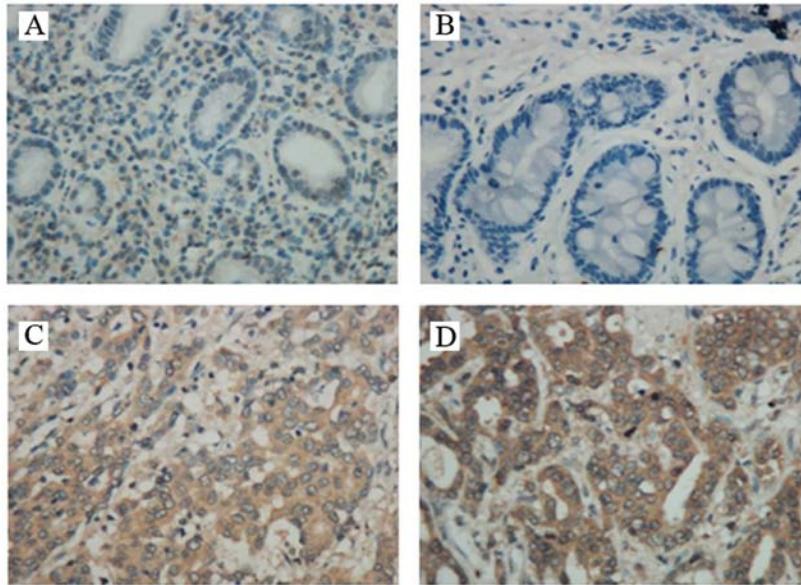


Figure 1. Immunohistochemical staining patterns for N-acetylglucosaminyltransferase V (GnT-V) in chronic gastritis tissues and gastric cancers. (A) Chronic superficial gastritis. (B) Chronic atrophic gastritis. (C) An early gastric cancer shows high GnT-V expression diffusely in the cytoplasm expression. (D) An advanced gastric cancer tissue.

1:2,000-diluted antibody of MMP-9, Abcam) at 4°C overnight. After washing in TBS-Tween-20 buffer, membranes were incubated for 2 h with the appropriate peroxidase-conjugated secondary antibodies, following washing in TBS-Tween-20 buffer, the protein bands on the membranes were visualized using ECL kit (Beyotime Institute of Biotechnology). The autographed film was scanned and processed with Odyssey Infrared Imaging system. Protein bands were quantified by Quantity One. The densitometric value of each protein band was normalized to GAPDH.

**Observation with transmission electron microscopy (TEM).** BGC823 parent and KD cells were collected after centrifuging (1500 rpm, 8 min), and fixed by 3%, pH 7.4 glutaraldehyde for 30 min at 4°C. The specimens were established using conventional methods. Cells were observed by TEM after double lead dyeing.

**Cell proliferation assay (CCK-8).** Cells were seeded in 96-well plates at  $2 \times 10^3$  cells/well. At the indicated times (d0-7), 10  $\mu$ l Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology) solution and 100  $\mu$ l RPMI-1640 + 10% FBS were added to each well. The cells were incubated for 60 min and absorbance at 450 nm was measured to calculate cell growth rates. Growth rate = (absorbance at 450 nm at dx - absorbance at 450 nm at d0) / (absorbance at 450 nm at d0).

**Cell cycle analysis.** Cells were harvested, washed with phosphate-buffered saline (PBS) twice and then fixed with 75% cold ethanol for 12 h. The fixed cells were spun down and re-suspended in PBS at  $1 \times 10^6$  cells/ml. After incubation with ribonuclease A at a final concentration of 3,000 U/ml at 37°C for 30 min, cells were filtered through a 40- $\mu$ m nylon mesh (BD Biosciences, USA). The cell suspension was stained with propidium iodide before analysis on a flow cytometer (BD Biosciences). Each test was repeated in triplicate.

**Cell apoptosis assay.** Cells were harvested, washed twice in ice-cold PBS solution, and re-suspended in binding buffer containing 7-AAD (7-amino-actinomycin D) for 10 min, followed by the addition of Annexin V-PE. Analysis of cell apoptosis was carried out using a flow cytometer (BD Biosciences).

**Cell invasion assay.** Using 24-well transwell units with 8- $\mu$ m pore size polycarbonate inserts (Matrigel™ invasion chamber, BD Biosciences). Cells that were suspended in RPMI-1640 + 10% FBS were added to each upper compartment of the transwell units. After being cultured for 24 h, cells migrating through the matrigel-coated polycarbonate membrane were fixed by 4% paraformaldehyde, then stained with Giemsa reagent and counted in five different fields. These fields were selected randomly.

**Statistical analysis.** The associations between GnT-V expression and clinical parameters were analyzed by the  $\chi^2$  test or Fisher's exact test, as appropriate. The survival curves were estimated using the Kaplan-Meier method and differences in survival distributions were evaluated by the generalized Wilcoxon test. Statistical comparisons of groups of cell lines were performed using one-way analysis of variance (ANOVA) and statistical significance was defined as  $P < 0.05$ . All the analyses were performed using SPSS13.0 software.

## Results

**Association between expressive levels of GnT-V protein and clinicopathology in primary gastric cancer tissues.** To assess the GnT-V protein expression of the gastric cancer (GC) tissues, we first performed immunohistochemistry analyses using 100 GC tissues and 100 chronic gastritis (CG) tissues. Typical immunostaining patterns for GnT-V in GC and CG tissue are shown in Fig. 1. As shown in Table I, positive GnT-V

Table I. GnT-V expression in gastric cancer and chronic gastritis tissues.

	GnT-V expression			P-value
	Negative (%)	Positive (%)	$\chi^2$	
Chronic gastritis tissues (n=100)	65 (65.0)	35 (35.0)	12.520	0.0004
Gastric cancer (n=100)	39 (39.0)	61 (61.0)		

expression was found in 61 (61%) GCs and 39 (39%) CGs. Positive expression of GnT-V was significantly more prevalent in tumors than CG tissues ( $P=0.0004$ ). In cancer cells, GnT-V expression was found diffusely in the cytoplasm or localized in the Golgi apparatus, as reported previously for colon cancers (8).

*One hundred patients were analyzed in this study.* According to the standard of TNM classification revised in 2003 by International Union against Cancer (UICC) and American Joint Committee on Cancer (AJCC), lymph vessel invasion and venous invasion were assessed. The TNM classification was performed for the cases examined. As shown in Table II, GnT-V expression was associated with pTNM classifications. There was a significant difference in GnT-V-positive rate between TNM stages. For stage III and IV, GnT-V-positive rate was 74.6% (44/59), which was significantly higher than that (41.6%, 17/41) of stage I and II,  $P=0.0017$ . Furthermore, there was a significant correlation, respectively, between GnT-V and N factor (lymph node metastasis) and M factor (distant metastasis), other than T factor (tumor depth). GnT-V showed non-preferentially expressions in most of the tumors with tumor size  $>5$  cm (69.4%),  $P=0.2780$ . However, for T factor, higher positive expression rate of GnT-V was significantly found in T2-T4 phase (67.1%) compared to T1 phase tumors (38.1%),  $P=0.0300$ . For N factor, there was a significant difference in tissues with lymph node metastasis (76.3%) compared with non-lymph node metastasis (39.0%),  $P=0.0004$ . For M factor, difference were statistically found between M1 phase (89.2%) and M0 phase (50.0%),  $P=0.0007$ . Additionally, in 50 tumor tissues with poorly differentiated cells, 34 cases (68.0%) were GnT-V-positive, which was significantly higher than in 50 well-moderately differentiated cells (54.0%),  $P=0.0219$ . Forty-three patients were followed-up in outpatient clinic of our hospital for  $>36$  months after surgery. Positive GnT-V expression was found in 20 (90.1%) patients with overall survival time  $<3$  years, which was significant higher than the patients with overall survival time  $>3$  years,  $P=0.0009$ . Fig. 2 showed that 3-year overall survival rate for GnT-V-positive patients was 28.6%, which was significantly lower than that for the GnT-V-negative (86.7%),  $P=0.001$ .

*Higher expression levels of GnT-V mRNA in primary GC tissues than pericarcinomatous tissues.* We next analyzed the GnT-V mRNA expression in cancer cells. As shown in

Table II. Expression of GnT-V and clinicopathological features as well as TNM classification in gastric cancer.

Characteristics	n	Positive no. (%)	P-value
Age			
>60 years	27	17 (63.0)	0.157
$\leq 60$ years	73	44 (60.3)	
Sex			
Male	61	37 (60.7)	0.930
Female	39	24 (61.5)	
Tumor size			
$<5$ cm	64	36 (56.3)	0.278
$\geq 5$ cm	36	25 (69.4)	
Depth of infiltration			
T1	21	8 (38.1)	0.030
T2- T4	79	53 (67.1)	
T2-T3	65	41 (63.1)	0.186
T4	14	12 (85.7)	
Lymph node metastasis			
No	41	16 (39.0)	0.0004
Yes	59	45 (76.3)	
Distant metastasis <sup>a</sup>			
M0	72	36 (50.0)	0.0007
M1	28	25 (89.2)	
Histological differentiation			
Well-moderately	50	27 (54.0)	0.0219
Poorly	50	34 (68.0)	
TNM stages			
I-II	41	17 (41.6)	0.0017
III-IV	59	44 (74.6)	
Overall survival time			
$<3$ -year	22	20 (90.1)	0.0009
$\geq 3$ -year	21	8 (38.1)	

<sup>a</sup>Distant metastasis (liver, gallbladder, mesenteric, pancreas, ovarian and vascular carcinoma bolt, etc.).

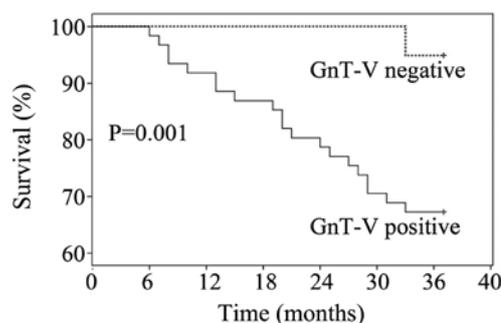


Figure 2. Kaplan-Meier survival curves for patients with gastric cancer. Survival curves for patients are stratified by positive and negative N-acetylglucosaminyltransferase V expression.

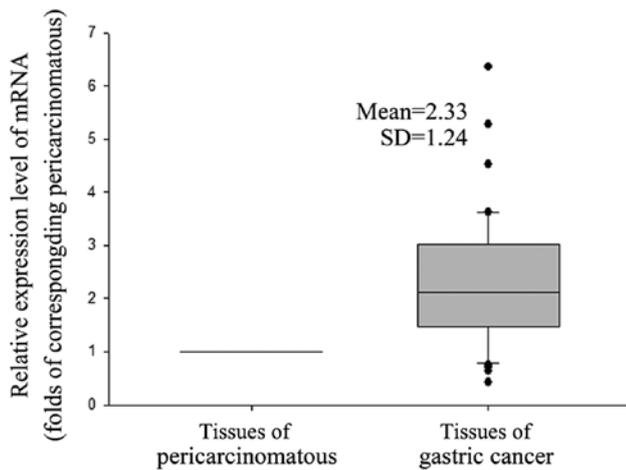


Figure 3. Box plot of gastric cancer patients by GnT-V mRNA expression. The GnT-V mRNA expression of GC and adjacent pericarcinomatous tissues were detected by qRT-PCR assay. The expression levels of gastric cancer tissues were  $2.33 \pm 1.24$ -fold that of adjacent pericarcinomatous tissue. There was a significant difference in GnT-V mRNA expression between GC and corresponding pericarcinomatous tissues ( $P=0.0000$ ).

Fig. 3, the expression levels of gastric cancer tissues were  $2.33 \pm 1.24$ -fold on average that of the adjacent pericarcinomatous tissue,  $P=0.0000$ . Furthermore, the clinical features of the 43 gastric cancer patients were analyzed (Table III). Among the tumors with size  $>5$  cm /  $<5$  cm, GnT-V mRNA expression was elevated by  $2.18 \pm 1.45$ - and  $2.43 \pm 1.21$ -fold that of pericarcinomatous tissues, respectively,  $P>0.05$ . GnT-V mRNA expression was found in association with pTNM classifications, which was consistent with the results of GnT-V protein detection by IHC. For stage III and IV, GnT-V mRNA was elevated by  $2.98 \pm 1.36$ -fold ( $n=21$ ), which was significantly higher than of stage I and II ( $1.70 \pm 0.69$ -fold,  $n=22$ ),  $P=0.00018$ . Correlation between GnT-V mRNA levels and T factor (tumor depth), N factor (lymph node metastasis) and M factor (distant metastasis) had a significant difference, respectively. For T factor, the levels of GnT-V mRNA were elevated by  $4.26 \pm 1.71$ -,  $2.15 \pm 0.93$ - and  $1.53 \pm 0.77$ -fold in T1 ( $n=5$ ), T2-T3 ( $n=33$ ) and T4 ( $n=5$ ), respectively, with a significant difference between T1-T3 and T4 group,  $P=7.91 \times 10^{-5}$ . For N and M factor, GnT-V expression was significantly increased in tumors with lymph node metastasis ( $P=0.0000$ ) and with distant metastasis ( $P=0.0000$ ). However, no obvious difference in GnT-V expression was found between poorly and well-moderately differentiated GCs,  $P=0.1760$ .

**Expression of GnT-V in various human GC cell lines.** To further explore the underlying mechanism of GnT-V in gastric cancer, we examined the expression of GnT-V in various human GC cell lines and the gastric epithelial cell line GES-1. As shown in Fig. 4, each cell line expressed GnT-V at distinct levels. GnT-V expression was elevated in gastric cancer cells compared to gastric normal epithelial cells. The BGC823 cells showed the highest GnT-V mRNA and protein expression among the three GC cell lines. It is known that the BGC823 and MKN45 cell lines are poorly differentiated, and SGC7901 cell line is moderately differentiated. Hence, the levels of GnT-V expression may not predict

Table III. Correlation of GnT-V mRNA expression with clinic pathological features in gastric carcinoma.

Clinicopathological features	n	GnT-V mRNA	
		Mean $\pm$ SD	P-value
<b>Tumor size</b>			
<5 cm	18	$2.18 \pm 1.45$	0.265
$\geq 5$ cm	25	$2.43 \pm 1.21$	
<b>Depth of infiltration</b>			
T1	5	$1.53 \pm 0.77$	0.065
T2-T4	38	$2.43 \pm 1.26$	
T2-T3	33	$2.15 \pm 0.93$	$7.91 \times 10^{-5}$
T4	5	$4.26 \pm 1.71$	
<b>Lymph node metastasis</b>			
No	24	$1.55 \pm 0.64$	$4.98 \times 10^{-8}$
Yes	19	$3.30 \pm 1.12$	
<b>Distant metastasis</b>			
M0	32	$1.76 \pm 0.70$	$3.80 \times 10^{-9}$
M1	11	$3.80 \pm 1.11$	
<b>Histological differentiation</b>			
Well-moderately	19	$2.52 \pm 1.44$	0.176
Poorly	24	$2.16 \pm 1.04$	
<b>TNM stages</b>			
I-II	22	$1.70 \pm 0.69$	0.00018
III-IV	21	$2.98 \pm 1.36$	

the degree of differentiation in cell lines, which was consistent with previous results.

**Downregulation of GnT-V expression in BGC823 and SGC7901 cells.** We next prepared a retroviral siRNA vector containing a small hairpin construct capable of generating a duplex RNAi oligonucleotide corresponding to human GnT-V. SGC7901 and BGC823 cell lines were selected to further examination due to the relatively higher expression of GnT-V mRNA than that of MKN45 cells. After retroviral infection, SGC7901 and BGC823 cells were sorted by flow cytometry. The GnT-V expression was effectively downregulated by 38.93 and 88.07%, respectively, compared with those in parent or mock cells (Fig. 5).

**Observation of biological behavior in BGC823 and SGC7901 KD cells.** To confirm our clinical observations *in vitro*, we examined the biological behavior in cells after GnT-V gene silencing. We first used transmission electron microscopy (TEM) to observe the BGC823 and KD cells. As shown in Fig. 6, the size of the nuclei was decreased visibly, with image of shrinkage and irregularity in KD cells. Moreover, cytoplasmic enrichment, nuclear heterochromatin edge setting and reduction of organelles were present in KD cells, in contrast, there were clear and complete nuclear membrane structure and enriched cell organelles in cytoplasm of the BGC823 cells.

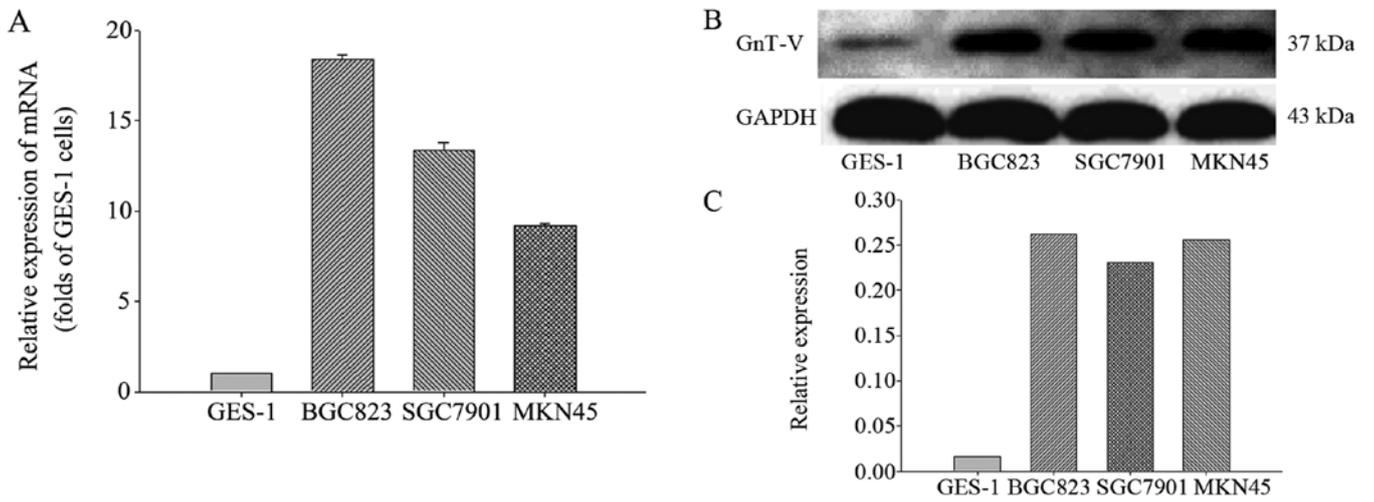


Figure 4. Expressions of GnT-V in various human GC cell lines. (A) mRNA expression of GnT-V in human gastric cancer cell lines was detected by qRT-PCR. The expression of GnT-V mRNA in BGC823, SGC7901 and MKN45 cell lines was 18.41±0.23-, 13.38±0.38- and 9.17±0.16-fold that of GES-1 cells, respectively. (B) Protein expression of GnT-V in human gastric cancer cell lines was determined by western blot assay. (C) Protein bands were quantified by Quantity One. The densitometric value of each protein band was normalized to GAPDH. The result is displayed with a bar diagram. Compared with GES-1 cells, GnT-V protein was 15.85±0.01-, 13.99±0.00- and 15.48±0.01-fold in BGC823, SGC7901 and MKN45 cells, respectively.

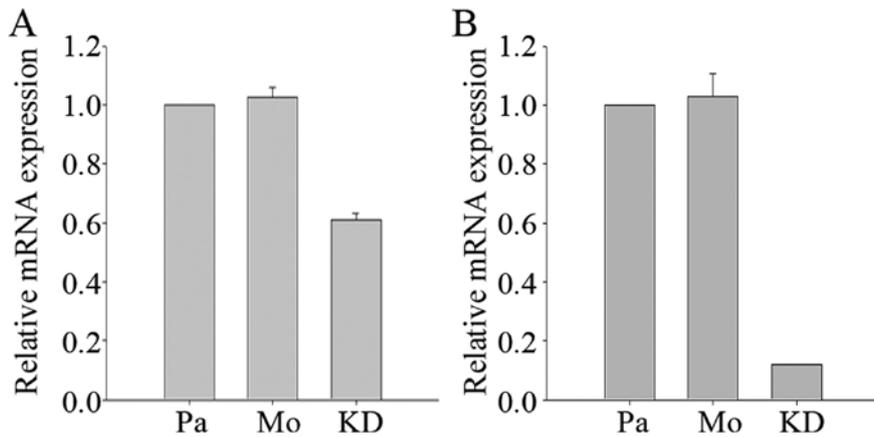


Figure 5. mRNA expression levels in siRNA-mediated GnT-V-knockdown cells. (A) mRNA expression of GnT-V knockdown SGC7901 cells. (B) mRNA expression of GnT-V knockdown BGC823 cells. Quantitative analysis was performed by real-time PCR. Pa, parent cells. Mo, mock cells. KD, GnT-V-knockdown cells.

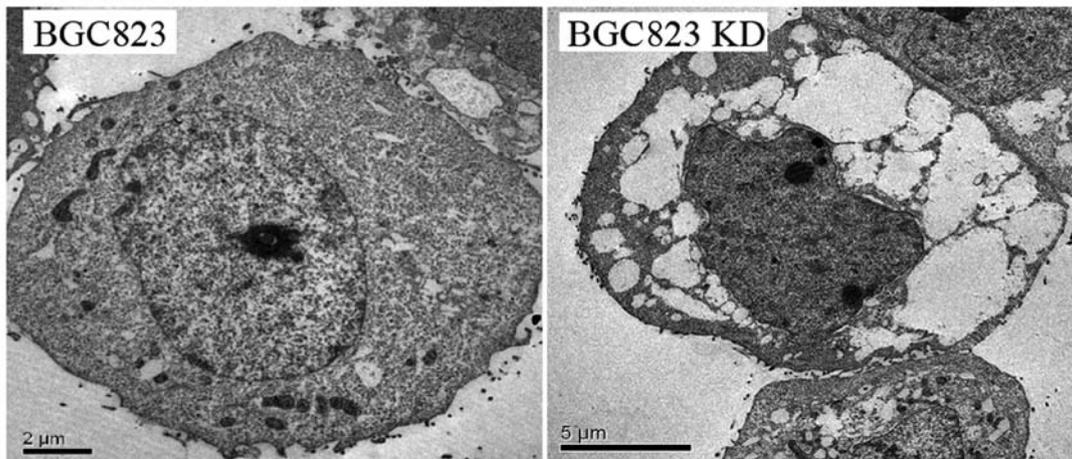


Figure 6. BGC823 and KD cells were observed by transmission electron microscopy. In KD cell, the size of nuclei was decreased visibly, with image of shrinkage and irregularity and cytoplasmic enrichment, nuclear heterochromatin edge recruitment and reduction of organelles were present, on the contrary, in BGC823 cells, there were clear and complete nuclear membrane structure and enriched cell organelles in the cytoplasm.

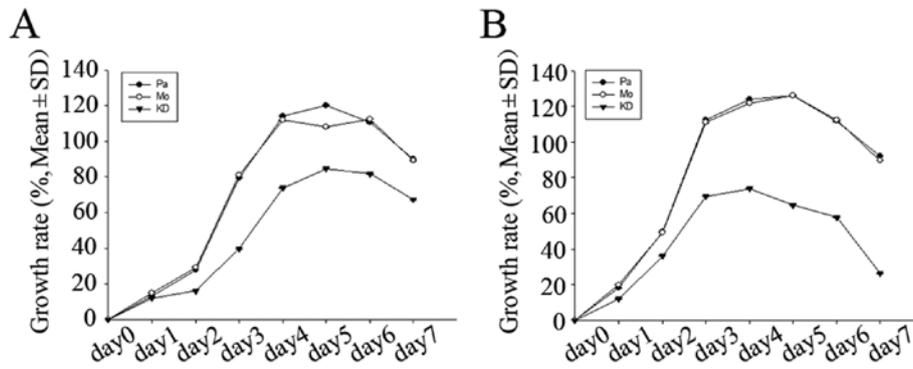


Figure 7. Comparison of growth rates among parent, mock and KD cells. The parent, mock and KD cells of SGC7901 and BGC823 were counted over a 7-day period. Growth rate = (absorbance at 450 nm at dx - absorbance at 450 nm at d0) / (absorbance at 450 nm at d0).

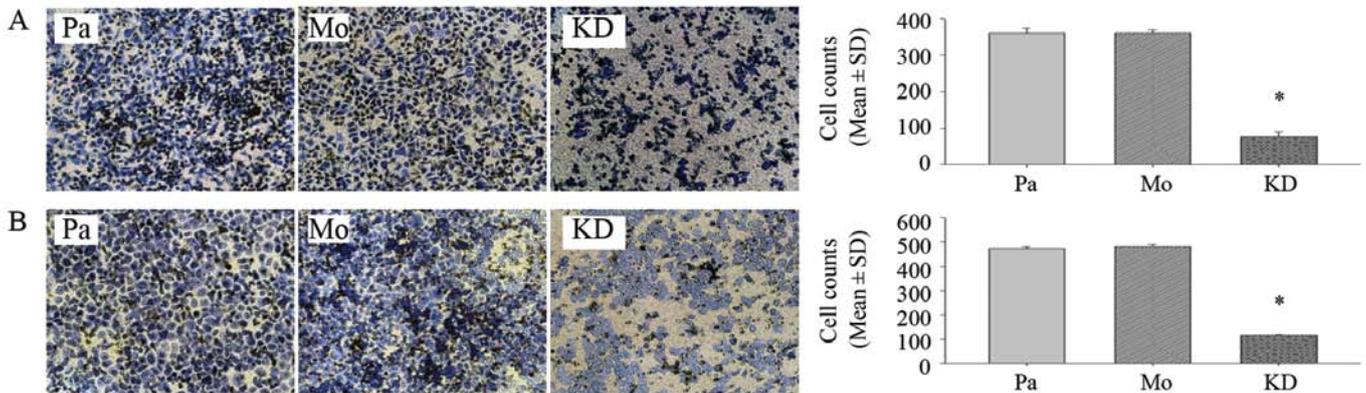


Figure 8. Invasion ability assay of cells. (A) SGC7901 (B) BGC823. Pa, parent cells. Mo, mock cells. KD, GnT-V-knockdown cells. \*Statistically significant.

Table IV. Cell cycle distribution and apoptosis rate in parent, mock and KD cells.

Cell cycle	Cell line			Apoptosis rate (%)
	G1	S	G2/M	
SGC7901-Pa	44.19±2.61	32.58±0.13	23.23±0.17	3.75±0.98
SGC7901-Mo	46.09±3.03	33.12±0.64	20.79±0.21	3.69±0.11
SGC7901-KD	75.47±0.31	14.33±2.47	10.20±0.67	18.12±2.08
BGC823-Pa	47.51±1.02	36.14±1.09	16.31±0.88	3.44±0.55
BGC823-Mo	46.87±1.38	36.41±1.04	16.72±1.02	3.70±0.47
BGC823-KD	79.81±1.45	14.83±1.08	5.36±1.24	23.54±0.87

We investigated the cell growth ability using proliferation assay by CCK-8 and cell cycle as well as apoptosis rate analysis by flow cytometry (FCM). As shown in Fig. 7, growth rates of KD cells were lower than those of parent and mock cells over a 7-day period. Cell cycle proportion in parent, mock and KD cells is presented in Table IV. There was no significant difference in G1, S and G2/M proportion between parent and mock cells ( $p > 0.05$ ). Compared with parent and mock cells, G1 proportion in KD cells significantly increased ( $P < 0.05$ ), whereas, the S and G2/M proportion decreased ( $P < 0.05$ ). This indicated that downregulated GnT-V expression may decrease S, G2/M proportion, and increase G1 proportion. The cell

apoptosis rates were all increased in KD cells compared with the parent and mock cells ( $P < 0.05$ ) (Table IV).

Matrigel-coated transwell assay was applied to detect cell invasion ability. Less cells penetrated the matrigel-coated membrane in KD cells than in parental and mock cells,  $P < 0.05$  (Fig. 8).

## Discussion

Various mechanisms have been shown to underlie elevated transcription of GnT-V in cancer, which is induced by direct effects on the GnT-V promoter by the Ets family of tran-

scriptional activators, which are upregulated by a cellular proliferation signaling pathway. This pathway begins with growth factor receptors that activate tyrosine kinases at the cell surface and proceeds through *src*, *ras*, *raf* and proto-Ha-*ras* oncogenes (25-27). GnT-V shows a biological and functional effect of invasiveness and metastatic potential *in vitro*. The upregulating cell motility of 92-1, Mel202 and IGR-39 compared to FM55P cells, melanoma cell lines, was found directly associated with an increased level of  $\beta$ 1-6 branched N-oligosaccharides as the result of hyperactivity of GnT-V (28). The mobility of Mv1Lu, mink lung epithelial cells, was elevated by the transfection of the GnT-V gene (29). A specific increase in  $\beta$ 1-6 branching due to an elevation in GnT-V expression increases metastatic potential of mouse mammary lung cancer cells (30). However, the role of GnT-V in different cancers remains controversial. In the case of colon cancer and hepatocarcinoma, for instance, high GnT-V expression is associated with a poor prognosis (8,10). In contrast, low GnT-V levels are linked to a poor prognosis in lung, bladder carcinomas and neuroblastoma patients (11-13). The function of GnT-V with gastric cancer and its invasion behavior has scarcely been studied. Tian *et al* (14) reported that high GnT-V expression was observed in 46% (23/50) gastric cancer tissues and was significantly correlated with lymph node metastases, peritoneal dissemination and liver metastases, respectively. Altogether, these data supported the positive correlation in our report between metastasis and GnT-V expression in gastric cancer patients.

The mechanisms underlying this relationship can be demonstrated by recent advances in glycoprotein biology. GnT-V is a Golgi located enzyme participating in the synthesis of multi-antennary asparagines linked glycans (N-glycans) during the processing of glycoproteins. It catalyses the transference of GlcNAc residue from UDP-GlcNAc to the  $\alpha$ 1,6 mannoside of C<sub>2</sub>C<sub>2</sub> biantennary or C<sub>2,4</sub>C<sub>2</sub> triantennary N-glycans, and produce a  $\beta$ 1,6GlcNAc branching structure in the products, C<sub>2</sub>C<sub>2,6</sub> tri- or C<sub>2,4</sub>C<sub>2,6</sub> tetra-antennary N-glycan (31-33). Cancer invasion and metastasis is associated with changes in cell growth control and morphology. For example, expression of epidermal growth factor receptor (EGFR) family members in breast cancer correlates with aggressive tumor behavior (34). EGFRs are generally N-glycosylated transmembrane proteins, and the residency at the surface is dependent in part on the dynamics of membrane remodeling. Endogenous lectins, such as galectins, can cross-link glycoproteins at the cell surface forming lattices that enhance residency time at the cell surface (35). The level of EGFR expression is correlated with poor survival in gastric cancer patients and highly metastatic phenotype of gastric cancer (36,37). Based on these points, GnT-V may induce tumor metastasis through controlling EGFR distribution on tumor cell surface.

In line with the discrepancy of the relationship of GnT-V activity and tumor size assessed by the TNM classification in pancreatic carcinoma and hepatocellular carcinoma as well as colon carcinoma (10,16,39), our data demonstrated no significant relationship between tumor size and GnT-V expression.

The overall survival rate of GnT-V-positive patients was significantly less than that of GnT-V-negative patients. The relationship between GnT-V expression and concomitant poor prognosis in gastric cancer patients may provide insights into

the decision whether adjuvant chemotherapy is necessary or not for those GnT-V-positive patients. The expression of GnT-V in resected specimen will not only help such decision but also give information on patient prognosis so that intensive follow-up may be done.

Based on the knowledge of histologic characteristics of GnT-V in gastric cancer, we assumed that GnT-V is a valuable targeting marker for inhibiting metastasis of gastric cancer. In the present study, downregulation of GnT-V mRNA in gastric cancer cell line SGC7901 and BGC823 support the hypotheses we assumed. Intriguingly, ongoing clinical trials are proceeding to test the possibility of swainsonine, an inhibitor for expression of  $\beta$ 1-6 branched oligosaccharides, as an anti-cancer drug (38,39), but not in gastric cancer yet. Screening of GnT-V expression in resected cancer tissues may be able to identify post-operative patients eligible for such an inhibitor.

Overall the present study provides the possibility of GnT-V expression as a predictor for the prognosis of gastric cancer, contributing therefore to improve the diagnosis, prognosis and perhaps the therapeutic stratification of the patients.

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