

Research Paper

β 3GnT8 Promotes Gastric Cancer Invasion by Regulating the Glycosylation of CD147

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Abstract

β 1, 3-N-acetylglucosaminyltransferase 8 (β 3GnT8) synthesizes a unique carbohydrate structure known as poly lactosamine, and plays a vital role in progression of various human cancer types. However, its involvement in gastric cancer remains unclear. In this study, we analyzed the expression and clinical significance of β 3GnT8 by Western blot in 6 paired fresh gastric cancer tissues, noncancerous tissues and immunohistochemistry on 110 paraffin-embedded slices. β 3GnT8 was found to be over-expressed in gastric cancer tissues, which correlated with lymph node metastasis and TNM stage. Forced the expression of β 3GnT8 promoted migration and invasion of gastric cancer cells, whereas β 3GnT8 knockdown led to the opposite results. Further studies showed that the regulated β 3GnT8 could convert the heterogeneous N-glycosylated forms of CD147 and change the poly lactosamine structures carried on CD147. In addition, our data suggested the annexin A2 (ANXA2) to be an essential interaction partner of β 3GnT8 during the process of CD147 glycosylation. Collectively, these results provide a novel molecular mechanism for β 3GnT8 in promotion of gastric cancer invasion and metastasis. Targeting β 3GnT8 could serve as a new strategy for future gastric cancer therapy.

Key words: β 3GnT8, CD147, invasion, gastric cancer, glycosylation

Introduction

Gastric cancer is one of the most common malignancies worldwide, particularly in Eastern Asia (Korea, Mongolia, Japan, and China) [1]. It is difficult to cure unless it is found at an early stage [2]. Owing to the lack of early detection markers and effective therapeutic strategies, it has often reached an advanced stage [3]. Most of gastric cancer patients die due to tumor recurrence and metastasis, and the 5-year survival rate is only 20-40% [4]. Thus, it is urgently needed for an improved understanding of the molecular mechanisms contributing to gastric cancer invasion and metastasis.

β 1, 3-N-acetylglucosaminyltransferase 8 (β 3GnT8) exerts its activity on tetraantennary N-glycans and

elongates poly lactosamine chains [5]. Initially, it had been reported that β 3GnT8 was cloned as β 3GalT7 from a human lung cDNA library [6]. Subsequently, it was named β 3GnT8 based on its position in a phylogenetic tree and enzymatic activity [5]. Hence, both β 3GalT7 and β 3GnT8 are authorized for this gene. It was indicated that β 3GnT8 expression in the cervix tumor tissues was obviously higher than that in normal tissues [7]. A correlation between β 3GnT8 and metastatic potential has been proposed base on its markedly enhanced expression in tumors such as colorectal cancer [8] and gliomas [9]. In a human gastric cancer cell line AGS, the expression of matrix metalloproteinase-2 (MMP-2) could be regulated by

β 3GnT8 [10]. In addition, the ability of β 3GnT8 to mediate CD147 signal transduction pathway has been confirmed in a gastric cancer cell line SGC-7901[11]. However, the regulatory mechanisms and biochemical properties of β 3GnT8 in gastric cancer invasion need further exploration.

In the present study, we demonstrated that β 3GnT8 was not only increased in gastric cancer tissues, but also was associated with clinical features, such as lymph node metastasis and TNM stage. We also confirmed that β 3GnT8 promoted gastric cancer cell invasion by regulating the N-glycosylation of CD147. Our findings provide novel insights into the crucial role of β 3GnT8 in gastric cancer progression and suggest β 3GnT8 as a potential target for the prevention of gastric cancer metastasis.

Materials and Methods

Tissue samples

110 human gastric cancer and 45 adjacent non-tumor tissues were collected from patients who underwent surgical resection between 2007 and 2014 at the Taihe Hospital, Hubei University of Medicine. These tissues were stored at -80°C immediately after surgical removal. 6 paired fresh gastric cancer tissues and noncancerous tissues were also obtained from the Taihe Hospital and stored in liquid nitrogen until use. None of the patients had received radiotherapy or chemotherapy before surgery. A written informed consent was obtained from each patient involved in this study and the study protocol was approved by the ethics committee of Hubei University of Medicine. All the clinicopathological parameters including age, gender, differentiation status, lymph node invasion and TNM stage were retrieved from patients' medical records. Investigations involving humans have been performed in accordance with the principles of Declaration of Helsinki.

Western blot analysis

Total proteins were extracted from the gastric cancer tissues and cells using the standard methods [9]. Proteins were quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Then samples were separated by 10% SDS-PAGE and transferred to PVDF membranes. The primary antibodies were anti- β 3GnT8 (1:1000), anti-CD147 (1:500; Santa Cruz, CA, USA), anti-annexin A2 (ANXA2) (1:1000; Santa Cruz), and anti-GAPDH (1:1000; Santa Cruz). The specificity of β 3GnT8 antibody has been verified in our previous study [7]. Bands on the membranes were visualized using an ECL detection kit obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

Immunohistochemical (IHC) staining

Formalin-fixed, paraffin-embedded tissue specimens were cut into $4\text{-}\mu\text{m}$ sections. Then sections were dewaxed in xylene and rehydrated through graded ethanols. To block endogenous peroxidase, 3% hydrogen peroxide in methanol was used. Sections were then incubated with the anti- β 3GnT8 antibody (1:100) or $4\mu\text{g/ml}$ biotinylated *Lycopersicon esculentum* agglutinin (LEL) (Sigma, St. Louis, MO, USA). After washing in phosphate-buffered saline (PBS), tissues were incubated with Horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody (Santa Cruz) or HRP-conjugated streptavidin (Sigma). Finally, the sections were visualized using diaminobenzidine and counterstain with hematoxylin (Beyotime Institute of Biotechnology). Sections were photographed on an Olympus photomicroscope (Inha, Japan). The degree of IHC staining was evaluated by two independent pathologists. Staining intensity was graded as "0" (negative), "1" (weak), "2" (moderate) and "3" (strong); staining percentage was graded as "0" (<5%), "1" (5-25%), "2" (25-50%), "3" (50-75%) or "4" (>75%)[12]. A final immunoreactivity scores (IRS) was calculated by multiplying the values of the staining intensity and staining percentage. The IRS value >4 was defined as high expression and IRS value ≤ 4 as low expression. ImagePro Plus (Media Cybernetics, Silver Spring, MD, USA) was used to quantitatively score the tissue sections.

Cell lines and transfection

Human gastric cancer cell lines AGS and NCI-N87 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). SGC-7901 cell line was purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (GIBCO BRL, Carlsbad, MD, USA) containing 10% fetal bovine serum (FBS) (HyClone, Waltham, UT, USA) in a humidified atmosphere with 5% CO_2 at 37°C . The pEGFP-C1 (Mock), pEGFP-C1- β 3GnT8 (T8S), pSilencircle- β 3GnT8Scr (T8Scr), and pSilencircle- β 3GnT8 (T8Si) plasmids were constructed by our laboratory [9]. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were collected 48 h after transfection for other assays.

RNA extraction and quantitative real-time PCR

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen). Reverse transcription of RNA was carried out using the reverse transcription kit (Invitrogen). Quantitative

PCR was conducted using SYBR-Green Real-Time PCR Master Mix kit (Toyobo, Osaka, Japan) and an ABI detection system (Applied Biosystems, Foster City, CA, USA). The PCR primers were designed for β 3GnT8 were 5'-GTCGCTACAGTGACCTGCTG-3' (forward) and 5'-GTCTTTGAGCGTCTGGTTGA-3' (reverse); for GAPDH were 5'-CCAACCGCG AGAAGATGA-3' (forward) and 5'-CCAGAG GCGTACAGGGATAG-3' (reverse). GAPDH was used as an internal control. The data were collected and analyzed using the comparative Ct (threshold cycle) method.

Lectin flow cytometry

Briefly, 5×10^5 cells were harvested with 0.25% trypsin, and permeabilized by 0.1% triton X-100 for 5 minutes at room temperature. Biotin-labeled LEL was added to a final concentration of 20 μ g/ml and incubated at 37°C for 2 h. Then phycoerythrin-conjugated streptavidin (Sigma) of 10 μ g/ml was used, and incubated for 1 h at 37°C in the dark. Fluorescent analyses were performed using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Only live cells were established as gates.

Lectin blot

The protein samples were extracted, quantified and subjected to 10% SDS-PAGE as described for western blot analysis. After blocking with Carbo-Free Blocking Solution (Vector Labs, Burlingame, CA, USA), the membranes were incubated with 2 μ g/ml of biotinylated LEL for 1 h. Then HRP-conjugated streptavidin (Sigma) was used as a secondary antibody. The blots were developed using an ECL substrate solution.

Cell migration and invasion assays

Wound-healing assay was performed to examine the migration rates of cultured cells. Briefly, the cell layer that reached confluence was scratched by a 20 μ l pipette tip. Then photographs were taken immediately (time zero) and 24 h after wounding. Cell migration was evaluated by measuring the difference in wound width. For cell invasion assay, cells in serum-free medium were plated on 24 well Transwell inserts (Corning Life Sciences, Tewksbury, MA, USA) precoated with Matrigel. Medium containing 10% FBS was added to the lower chamber. After 24 h culture, the cells that have moved through the pores to the lower side were stained with eosin staining solution (Beyotime Institute of Biotechnology). Invasion was evaluated by counting the number of the invaded cells.

Immunoprecipitation(IP)

Total cell lysates were prepared with IP lysis buffer (10 mM Tris, 1% triton-X-100, 1 mM EDTA, 100 mM NaCl and a mixture of protease inhibitors) as previously described [13]. IP of indicated protein was performed with a 1:80 dilution of antibody overnight at 4 °C with constant rotation. Antibody-protein conjugates were pulled down by incubating samples with 10 μ l of Sepharose A beads (Thermo Fisher, Rockford, IL, USA) for 4 h at 4 °C. Finally, the beads were washed, boiled, centrifuged and the recovered samples were run on 10% SDS-PAGE for western blot or lectin blot as described above.

LC-MS/MS analysis

Coomassie brilliant blue R-250 (Sigma) staining was performed to visually detect specific immunoprecipitated bands of β 3GnT8 antibody. The bands were subjected to in gel digestion by trypsin(Sigma). Digested peptides were analyzed by nano-HPLC (Ultimate 3000, Dionex) coupled to a linear quadrupole ion trap-Orbitrap (LTQ Orbitrap XL)mass spectrometer (Thermo Fisher) equipped with a nano-ESI source[14]. A nonlinear gradient using 2% ACN in 0.1% formic acid in water and 0.1% formic acid in 98% acrylonitrile was used with a flowrate of 250 nl/min[14]. All measurements were performed in the positive-ion reflective mode at an accelerating voltage of 18 kV and delayed-pulsed ion extraction [15]. All MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.3.01) and searched against the latest NCBI nr protein database [16].

Statistical analysis

Data from the statistical analysis represent the mean \pm standard deviation (SD) from at least three independent experiments. Statistical differences between the two groups were examined by Student's *t*-test. Analyses of β 3GnT8 and polylectosamines expression with clinicopathologic parameters were performed using Fisher's exact test. *P* < 0.05 was considered statistically significant. All tests were performed by SPSS 16.0 software (SPSS Inc, Chicago, IL, USA).

Results

β 3GnT8 is highly expressed in gastric cancer tissues

We first examined the expression of β 3GnT8 using Western blot in 6 pairs of gastric cancer tissues and their corresponding nontumorous tissues. Gastric cancer tissues showed marked higher levels than did corresponding non-tumor tissues (*P*<0.05, Figure 1).

In order to investigate the clinical significance of $\beta 3\text{GnT8}$ in gastric cancer progression, immunohistochemical (IHC) staining was used to observe the expression of $\beta 3\text{GnT8}$ in 110 gastric cancer tissues. As expected, $\beta 3\text{GnT8}$ was mainly localized in the cytoplasm of gastric cancer cells, and its expression was much more dominant in tumor tissues than that in neighboring non-tumor tissues (Figure 2). $\beta 3\text{GnT8}$ is involved in the biosynthesis of polylysosamine chains. Using LEL staining, differential expression profiles of polylysosamines could be observed [17]. Polylysosamines were predominantly expressed in the membrane and cytoplasm of gastric cancer cells, and their expression was clearly higher in gastric cancer tissues (Figure 2).

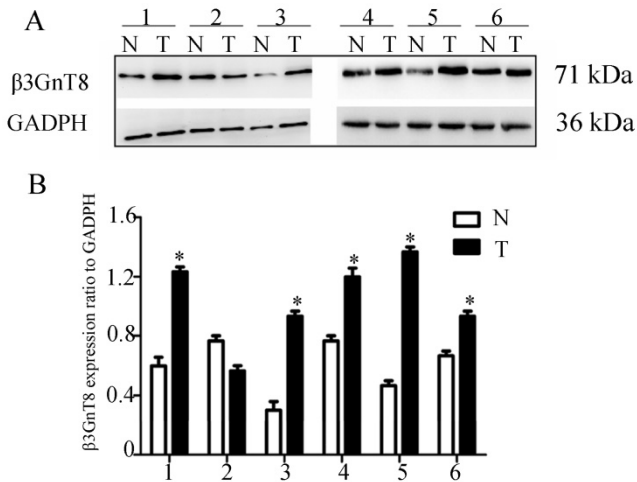


Figure 1. $\beta 3\text{GnT8}$ expression in human gastric cancer by western blot analysis. (A) The protein level of $\beta 3\text{GnT8}$ was high in 6 representative paired samples of gastric cancer tissue (T) compared with nontumorous adjacent tissues (N). GAPDH was used as a loading control. (B) The bar chart showed the ratio of $\beta 3\text{GnT8}$ protein to GAPDH. Bars represented mean \pm SD of three independent tests. (* $P < 0.05$ compared with nontumorous adjacent tissues).

Then the relationship between $\beta 3\text{GnT8}$, polylysosamine chains expression and clinicopathological features of gastric cancer was analyzed. As shown in Table 1, expression of $\beta 3\text{GnT8}$ was correlated with the increased clinical stage ($P < 0.05$), depth of invasion ($P < 0.05$) and lymph node metastasis ($P < 0.05$), but not statistically related to tumor differentiation, size, lauren classification, location or patients' gender and age. Polylysosamines were also closely related with lymph nodes metastasis ($P < 0.05$), depth of invasion ($P < 0.05$) and TNM staging ($P < 0.05$), whereas no correlation with other clinicopathological factors. Furthermore, we found that $\beta 3\text{GnT8}$ expression was positively correlated with polylysosamines expression in gastric cancer tissues ($P = 0.003$). These results suggest that $\beta 3\text{GnT8}$ may be of diagnostic and/or prognostic value as a biomarker in gastric cancer.

Table 1. Relationship between $\beta 3\text{GnT8}$, polylysosamines expression and clinicopathological features of gastric cancer patients

Clinicopathological features	n	$\beta 3\text{GnT8}$		P	polylysosamines		P
		High	Low		High	Low	
Age							
<60	51	27	24	0.578	22	29	0.442
≥ 60	59	32	27		28	31	
Gender							
Male	57	28	29	0.616	25	32	0.756
Female	53	31	22		26	27	
Tumor Size							
<5cm	45	15	30	0.341	25	20	0.139
$\geq 5\text{cm}$	65	44	21		32	33	
Lauren classification							
Intestinal	62	30	32	0.445	27	35	0.217
Diffuse	48	29	19		24	24	
Tumor location							
Proximal	8	4	4	0.329	3	5	0.221
Middle	52	25	27		23	29	
Distal	50	30	20		26	24	
Differentiation							
Moderate-High	69	39	30	0.188	33	36	0.168
Low	41	18	23		20	21	
TNM stage							
I+II	47	28	19	0.013*	25	22	0.024*
III+IV	63	50	13		48	15	
Lymph node metastasis							
Positive	90	72	18	0.002*	65	25	0.011*
Negative	20	17	3		15	5	
Invasion depth							
T1+T2	23	19	4	0.036*	15	8	0.024*
T3+T4	87	68	19		61	26	

* $P < 0.05$

$\beta 3\text{GnT8}$ promotes the migration and invasion of gastric cancer cells

To further explore the role of $\beta 3\text{GnT8}$ in gastric cancer, we screened three gastric cancer cell lines, AGS, SGC-7901 and NCI-N87 for its expression. Among the cell lines, AGS had the highest expression of $\beta 3\text{GnT8}$ at both the mRNA and protein levels (Figure 3A). The expression of polylysosamines was also detected by flow cytometry with LEL (Figure 3B). Fluorescent intensity was computed as the median value of each staining. NCI-N87 cells showed lowest expression of polylysosamines, whereas AGS cells exhibited highest levels of polylysosamines. We therefore selected these two cell lines in the following experiments.

Then NCI-N87 cell line was transfected with a pEGFP-C1- $\beta 3\text{GnT8}$ plasmid to over-express $\beta 3\text{GnT8}$. AGS cell line was treated with a pSilencircle- $\beta 3\text{GnT8}$ plasmid to knockdown endogenous $\beta 3\text{GnT8}$ expression. The effect of ectopic expression and knockdown of $\beta 3\text{GnT8}$ in cells was confirmed by quantitative RT-PCR and western blot (Figure 3C). Compared with that of control cells, the migration

and invasion abilities were markedly stimulated in NCI-N87 cells that over-expressing $\beta 3\text{GnT8}$ as indicated by wound-healing and transwell assays ($P < 0.05$, Figure 3D and 3E). Likewise, knockdown of $\beta 3\text{GnT8}$ could apparently repress the

migration and invasion abilities of AGS cells ($P < 0.05$, Figure 3D and 3E). Taken together, these results suggest that the dynamic changes of $\beta 3\text{GnT8}$ may be common events during the progression of gastric cancer.

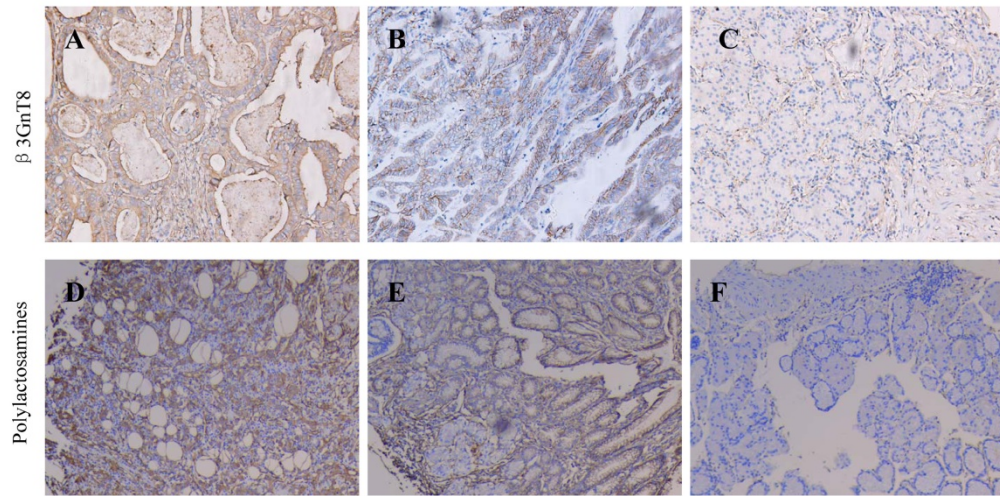


Figure 2. Immunohistochemical staining for the expression of $\beta 3\text{GnT8}$ and polylectosamines in gastric cancer and adjacent non-tumor tissues ($\times 200$). (A) High positive $\beta 3\text{GnT8}$ expression in gastric cancer. (B) Low positive $\beta 3\text{GnT8}$ expression in gastric cancer. (C) Negative $\beta 3\text{GnT8}$ expression in adjacent non-tumor tissues. (D) High positive polylectosamines expression in gastric cancer. (E) Low positive polylectosamines expression in gastric cancer. (F) Negative polylectosamines expression in adjacent non-tumor tissues.

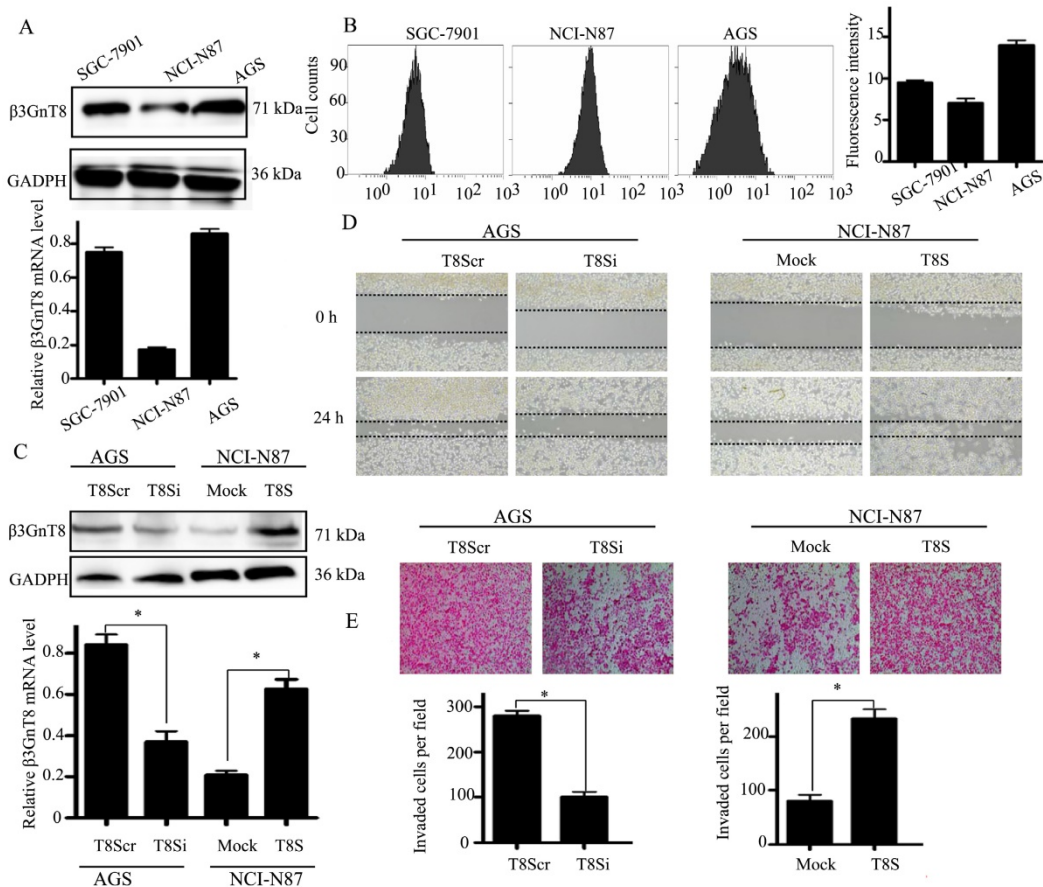


Figure 3. $\beta 3\text{GnT8}$ promotes gastric cancer cell migration and invasion. (A) Quantitative RT-PCR and western blot analysis of $\beta 3\text{GnT8}$ expression in three gastric cancer cell lines. (B) Lectin flow cytometry analysis of polylectosamine expression in three gastric cancer cell lines. (C) The mRNA and protein levels of $\beta 3\text{GnT8}$ were detected by quantitative RT-PCR and western blot in NCI-N87 and AGS cells transiently transfected with pEGFP-C1 (Mock), pEGFP-C1- $\beta 3\text{GnT8}$ (T8S), or pSilencircle- $\beta 3\text{GnT8}$ Scr (T8Scr), and pSilencircle- $\beta 3\text{GnT8}$ (T8Si) vectors, respectively. (D) Wound healing assay with gastric cancer cells. Microscopic observations were recorded after scratching the cell surface ($200\times$). (E) Transwell assay with gastric cancer cells ($200\times$). Histograms showed the numbers of invasion cells. Bars represented mean \pm SD of three independent tests, all $*P < 0.05$.

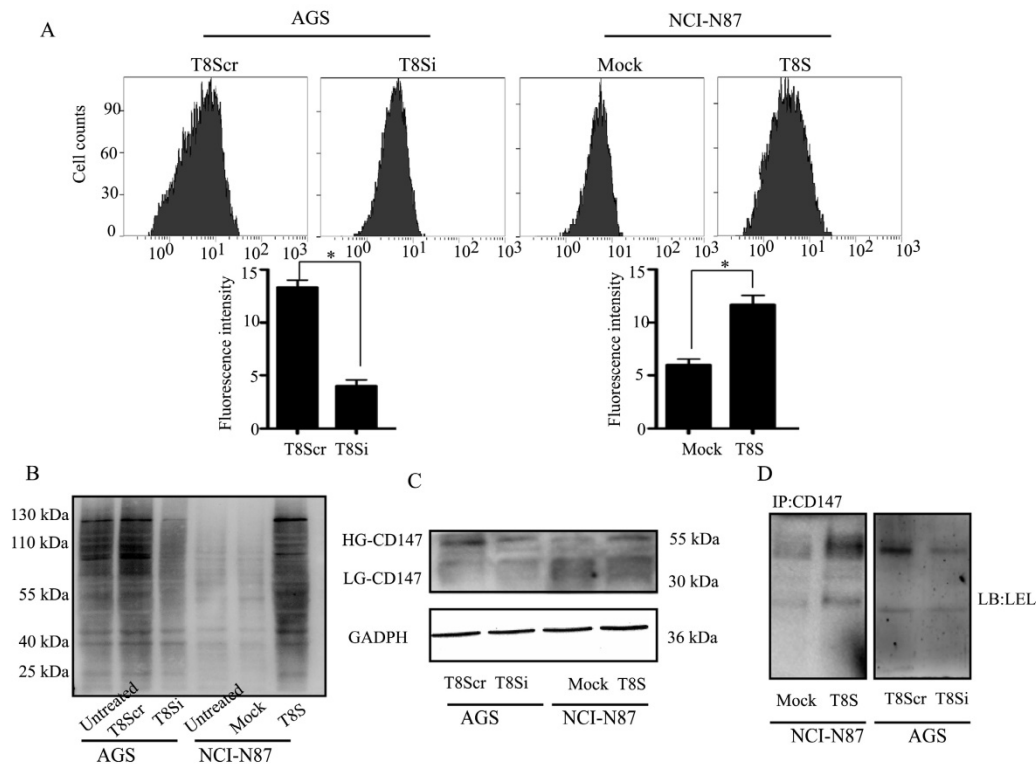


Figure 4. β 3GnT8 regulates the N-glycosylated forms of CD147 in gastric cancer cells. (A) Lectin flow cytometry analysis of poly-lactosamine expression in NCI-N87 and AGS cells transiently transfected with pEGFP-C1 (Mock), pEGFP-C1- β 3GnT8 (T8S), pSilencircle- β 3GnT8Scr (T8Scr), and pSilencircle- β 3GnT8 (T8Si) vectors, respectively. (B) Lectin blot assay with gastric cancer cells. (C) Western blot analysis of CD147 glycosylation in gastric cancer cells. (D) Immunoprecipitation plus lectin blot was performed. Bars represented mean \pm SD of three independent tests, all * $P < 0.05$. WCL, whole cell lysate.

Glycosylation of CD147 is involved in β 3GnT8 mediated gastric cancer cell invasion

CD147 is a glycoprotein that carries poly-lactosamine sugars on its N-glycosylation sites [18]. To further investigate if β 3GnT8 could affect CD147 glycosylation in gastric cancer cells, firstly, we analyzed the effect of β 3GnT8 on the synthesis of total poly-lactosamine chains. The results showed that knockdown of β 3GnT8 in AGS cells dramatically decreased total poly-lactosamine levels (Figure 4A). Conversely, poly-lactosamines were increased after ectopic expression of β 3GnT8 in NCI-N87 cells. Lectin blot analysis revealed that the poly-lactosamine units on glycoproteins were also altered by different β 3GnT8 levels (Figure 4B). Lectin blot analysis showed the consistent alteration of glycan structures with flow cytometry assay.

Furthermore, we observed that high glycosylated (HG)-CD147 was markedly increased by over-expression of β 3GnT8 in NCI-N87 cells (Figure 4C). Inversely, the expression of HG-CD147 was reduced when β 3GnT8 was down-regulated in AGS cells. After the IP with anti-CD147 antibody, lectin blot assay from total cell lysate protein revealed the similar results (Figure 4D). These data indicated that the regulated β 3GnT8 converted the heterogeneous N-glycosylated forms of CD147 in gastric cancer cells,

and significantly changed the poly-lactosamine structures on CD147.

Identification of interaction partners of β 3GnT8 in gastric cancer cells by proteomic analysis

To explore the mechanism underlying β 3GnT8-mediated glycosylation of CD147 in gastric cancer cells, a proteomic approach by IP of β 3GnT8 followed by Nano-HPLC and LC-MS/MS analysis was applied. And a negative control using serum IgG was used to distinguish non-specifically bound proteins. We thereby identified total of 9 proteins from the analyses, which were immunoprecipitated together with β 3GnT8 but not with the control. Table 2 shows the most abundant hits also indicating the number of biological repeats in AGS and NCI-N87 cells. A careful review of the literature revealed that there exist interaction effects between ANXA2 and CD147 [19]. As expected, we found that ANXA2 specifically bound to CD147 in gastric cancer cells (Figure 5A). We therefore selected ANXA2 as a candidate molecule.

In order to validate the interaction between ANXA2 and β 3GnT8, we undertook co-IP assay. Protein expression of ANXA2 in AGS and NCI-N87 cells was detected by western blot. As shown in

Figure 5B, the expression of ANXA2 was much higher in AGS cells than that in NCI-N87 cells. ANXA2 was then found to co-immunoprecipitate with β 3GnT8 in AGS and NCI-N87, indicating that ANXA2 and β 3GnT8 interact in their native form in gastric cancer cells (Figure 5C). However, directly binding between the CD147 and β 3GnT8 was not found in this study. ANXA2 and β 3GnT8 may act as a functional complex, which plays an important role in CD147 glycosylation.

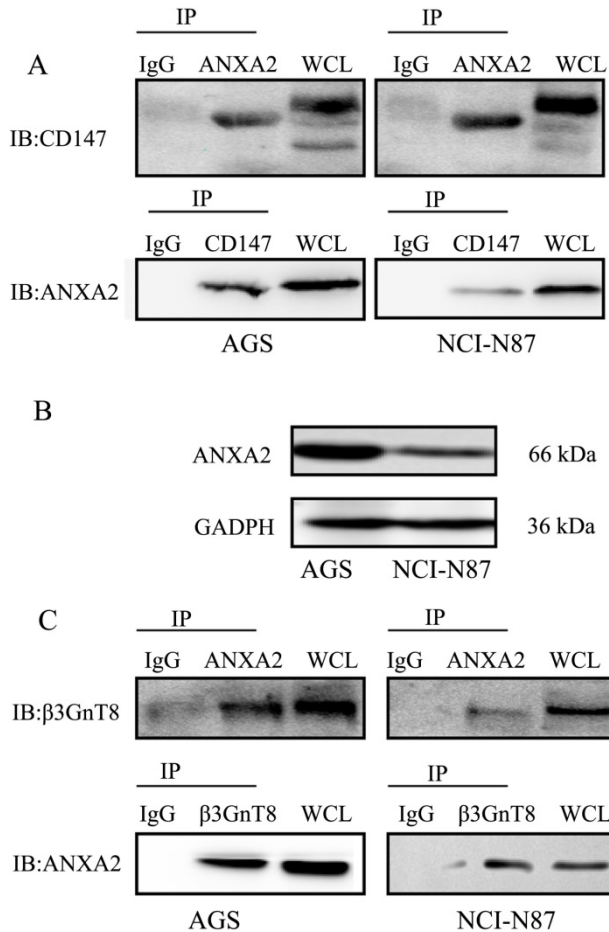


Figure 5. Co-immunoprecipitation of β 3GnT8 and ANXA2 in gastric cancer cells. (A) CD147 immunoprecipitated with ANXA2 in NCI-N87 and AGS cells. (B) The protein expression of ANXA2 was detected by western blot in NCI-N87 and AGS cells. (C) β 3GnT8 immunoprecipitated with ANXA2 in NCI-N87 and AGS cells. Serum IgG was used as a negative control.

Discussion

Glycosylation controls diverse protein functions and regulates various cellular phenotypes. Alterations in cell surface glycosylation are believed to play crucial roles in tumor progression, metastasis, and therapeutics[20]. Recent studies reveal that decreased core-fucosylation of N-glycans [21, 22] and reduced gland mucin-specific O-glycans [23] contribute to malignancy in gastric cancer. Identifying these

biomarkers could provide new insights for understanding the mechanisms of gastric cancer invasion, and designing better therapeutic strategies. β 3GnT8 transfers GlcNAc to the non-reducing terminus of the Gal β 1-4GlcNAc of tetra-antennary N-glycan to form polylectosamine structures [5]. As β 3GnT8 expression was associated with aggressive tumor phenotype, we identified and functionally characterized β 3GnT8 as an important regulator in gastric cancer invasion. In addition, it provides a potential therapeutic target for the future treatment of gastric cancer.

Table 2. Identification of potential interaction partners of β 3GnT8 by LC-MS/MS analysis in different gastric cancer cells

Protein	Description	Uniprot accession number	AGS ^a	NCI-N87 ^a
ACTB	Actin, cytoplasmic 1	P60709	18	19
JUP	Junction plakoglobin	P14923	23	13
DCD	Dermcidin	P81605	12	16
ANXA2	Annexin A2	P07355	22	29
PIP	Prolactin-inducible protein	P12273	21	27
HRNR	Hornerin	Q86YZ3	8	5
TET1	Methylcytosine dioxygenase TET1	Q8NFU7	17	14
AGPAT1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha	Q99943	4	2
HSPD1	Heat shock 60kDa protein 1	P10809	2	3

^aNumber of unique peptides identified.

Polylectosamine, which can be incorporated into both N- and O-linked glycans, is a unique glycan composed of repeating N-acetyllectosamine units[24]. This polymers carried on N-glycans are believed to aid metastasis by making the cells more invasive [25-27]. Inhibition of polylectosamines using different strategies always results in the loss of the metastatic ability. Conversely, induction of their expression has been shown to result in acquisition of the metastatic phenotype. Lectin staining with LEL, which recognizes polylectosamines, was undertaken to obtain expression profiles of these structures[28, 29]. In the present study, we showed that polylectosamine chains were significantly up-regulated in gastric cancer tissues compared with the adjacent non-cancerous tissues. Moreover, polylectosamine levels were significantly correlated with aggressive tumor characteristics (lymph node metastasis and TNM stage). We also confirmed that the expression levels of β 3GnT8 were positively correlated with polylectosamines expression in gastric cancer tissues and cell lines. Over-expression and knockdown of β 3GnT8 could markedly increase and reduce polylectosamines in gastric cancer cells. To our knowledge, this is the first report on the role of polylectosamines in gastric cancer progression.

However, to further confirm the functions of β 3GnT8 and poly-lactosamines, a larger number of gastric cancer samples than available would be required.

In addition, we explored the mechanisms behind the association between β 3GnT8 and gastric cancer metastasis and identified downstream effectors contributing to this process. CD147, a cell surface transmembrane glycoprotein, has been reported to be correlated with gastric cancer aggressiveness. For example, CD147 expression was associated with gastric cancer invasion, metastasis and TNM stage [30]. Up-regulated CD147 could enhance gastric cancer cell invasion and angiogenesis [31]. Because of the heterogeneous N-glycosylation, CD147 shows both high glycosylated (HG)-CD147 (~40-60 kDa), and a low glycosylated (LG) -CD147 (~32 kDa) form [32]. Elevated glycosylation of CD147, yielding HG-CD147, is attributable to high poly-lactosamine content [33]. Our results showed that knockdown of β 3GnT8 could efficiently inhibit HG-CD147 in AGS cells. In contrast, up-regulation of β 3GnT8 significantly enhanced HG-CD147 expression in NCI-N87 cells. β 3GnT8 associates with HG-CD147 and enhances the biosynthetic conversion of LG-CD147 to HG-CD147. This is in line with our previous study that β 3GnT8 could regulate the metastatic potential of colorectal cancer cells by altering the glycosylation of CD147 [8]. It is so far not well understood how these processes are regulated and which interaction partners are involved.

We performed a proteomic screen for investigation of the interactome of β 3GnT8 in gastric cancer cells. We thereby identified a number of interaction proteins. We confirmed ANXA2 as a general binding partner for β 3GnT8 by co-IP assay. ANXA2 is a calcium-dependent phospholipid binding protein that is mainly located on the cell membrane [34]. Its aberrant expression enhanced the malignant properties of cancer cells [35]. Up-regulated ANXA2 was associated with lymph node metastasis, advanced TNM stage in patients with gastric cancer [36, 37]. Here, we found that AGS cells expressed more ANXA2 protein than NCI-N87 cells. Changes in the expression of ANXA2 were closely associated with the invasion phenotype of different gastric cancer cell. β 3GnT8 and ANXA2 may act as a functional complex during the invasion and migration of gastric cancer cells.

Recent studies have revealed that ANXA2 and CD147 interact with each other in the same signal transduction pathway [19]. ANXA2 was found to be co-localized and co-immunoprecipitated with CD147 in hepatocellular carcinoma cells [38, 39]. In this study, the two molecules were also found to co-immunoprecipitate with each other in gastric

cancer cells. According to our data, we suggest that ANXA2 might act as a linker between CD147 and β 3GnT8, and then regulate β 3GnT8-induced glycosylation of CD147.

In conclusion, our data highlight the molecular etiology and clinical significance of β 3GnT8 in gastric cancer. β 3GnT8 promotes gastric cancer cell migration and invasion by functionally converting the poly-lactosamine chains carried on CD147. ANXA2 is an essential interaction partner of β 3GnT8 during CD147 glycosylation. Thus, targeting β 3GnT8 may represent a new therapeutic strategy for the treatment of gastric cancer patients.

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Competing Interests

The authors have declared that no competing interest exists.

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