

High expression of N-acetylglucosaminyltransferase V in mucinous tumors of the ovary

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Abstract. N-acetylglucosaminyltransferase V (GnT-V) is an enzyme that catalyzes β 1-6 branching of N-acetylglucosamine (GlcNAc) on asparagine-linked oligosaccharides of cell proteins. The present study aimed to investigate GnT-V expression and its prognostic significance in epithelial ovarian cancer. GnT-V expression was studied by immunohistochemistry in 83 surgically resected ovarian cancers, and the staining intensity was evaluated. High GnT-V expression in cancer cells was found in 17 (20.5%) of the 83 cases, and was positively correlated with early FIGO staging. In the histological type, mucinous adenocarcinoma showed significantly strong immunostaining compared to the non-mucinous type ($P<0.001$). In 36 mucinous tumors, the GnT-V immunostaining score was significantly higher in cancer than in benign and borderline tumors ($P<0.001$). NOM-1, a human ovarian mucinous adenocarcinoma cell line, expressed strong GnT-V protein and swainsonine treatment suppressed β 1-6GlcNAc branching and reduced migration ability significantly ($P<0.001$). These results suggested that GnT-V might be involved in the malignant potential of mucinous ovarian cancer.

Introduction

Epithelial ovarian carcinoma (EOC) is one of the most lethal malignancies of the female genital tract. Since EOC frequently remains clinically silent, the majority of patients with this disease have advanced intraperitoneal metastatic disease at diagnosis (1). Treatment for advanced EOC is difficult because of both the inability to completely resect

diffuse tumor involvement on the peritoneal surface and the eventual resistance of tumor cells to chemotherapy. Patient age, disease stage, tumor type and histological grade have been shown to have a prognostic value in EOC (2). Several studies have shown that the biological nature differs among histological types; chemosensitivity, major FIGO stage first diagnosed and prognosis (3). Although mucinous adenocarcinoma and clear cell adenocarcinoma are often diagnosed in the early stages, advanced cases have a poorer prognosis than other histopathologic subgroups due to the comparative resistance to antineoplastic agents (3-5); therefore, it is important to identify the histological character in order to develop strategies for improving the prognosis.

The glycosylation of cell-surface glycoproteins is widely accepted to play a key role in a variety of specific biological interactions (6). In particular, branching of asparagine-linked oligosaccharides is shown to regulate metastatic potential in cancer cells (7). N-acetylglucosaminyltransferase V (GnT-V, EC 4.1.15) catalyzes the addition of β 1-6 N-acetylglucosamine (GlcNAc) branching of N-glycans, which is attributable to tumor metastasis; therefore, high GnT-V expression was associated with poor clinical outcomes in human colorectal cancer, endometrial cancer, and gastric cancer (8-10). In contrast, low GnT-V expression was associated with the poor prognosis of non-small cell lung cancer and bladder cancer (11,12). Thus, GnT-V expression and its functional and prognostic significance in human cancer remain controversial; however, there have been no reports describing the clinical or pathologic significance of GnT-V in EOC. In the present study, we examined GnT-V expression by immunohistochemistry in surgically resected EOC and analyzed its biological and clinical importance.

Materials and methods

Patients and tissue samples. Eighty-three human EOC tissues were obtained from patients who underwent surgical treatment at Nagoya University Hospital between 1992 and 2006 after informed consent. The age of the patients ranged from 25 to 77 years, with a median age of 54 years. None of these patients had undergone neo-adjuvant chemotherapy before surgery. Surgical treatment consisted of total hysterectomy, bilateral

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salpingo-oophorectomy, omentectomy, and pelvic and para-aortic lymphadenectomy. The present series consisted of 83 carcinomas that were classified into the following histological types: 29 serous carcinomas, 14 mucinous carcinomas, 28 clear cell carcinomas and 12 endometrioid carcinomas. The histological cell types and histological grade (tumor differentiation) were assigned according to the criteria of the World Health Organization (WHO) classification (serous, endometrioid and mucinous type) or the Universal grading system (clear cell type) (13). Clinical stage was assigned based on the International Federation of Gynecology and Obstetrics (FIGO) staging system. In this series, 33 patients were diagnosed with FIGO stage I tumors, 12 with stage II, 29 with stage III and 9 with stage IV.

The patients received postoperative chemotherapy with platinum plus cyclophosphamide and doxorubicin (before 1997), or platinum plus paclitaxel (after 1997) for high-risk early stage (stage I with grade 2-3, stage Ic and II) or advanced (stages III and IV) disease. Thirteen borderline mucinous tumors and 10 mucinous adenoma tissues were obtained from patients who underwent surgical treatment after informed consent. Tumor recurrence/progression was defined based on clinical, radiological or histological diagnosis. Patients with recurrence were treated with chemotherapy, local radiation therapy, or surgical tumor resection if possible.

Immunohistochemistry for GnT-V. Informed consent was obtained from individual patients for use of their tissue samples. Surgical specimens were fixed in 10% formalin and embedded in paraffin. Paraffin specimens were cut at a thickness of 4 μ m. For heat-induced epitope retrieval, deparaffinized sections were soaked in Target Retrieval Solution consisting of 10 mM Tris and 1 mM EDTA (Dako, Glostrup, Denmark), and treated at 95°C for 30 min in a microwave oven. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). Endogenous peroxidase activity was blocked by incubation with 0.3% H₂O₂ in methanol for 15 min, and non-specific immunoglobulin binding was blocked by incubation with 10% normal goat serum for 10 min. Sections were incubated at 4°C overnight with anti-GnT-V monoclonal antibody at 1:400 dilution (8). The sections were rinsed and incubated for 30 min with the biotinylated second antibody. After washing, the sections were incubated for 5 min with horseradish peroxidase-conjugated streptavidin, and finally treated with 3,3'-diaminobenzidine tetrahydrochloride (Nichirei) in 0.01% H₂O₂ for 3 min. The slides were counterstained with Meyer's hematoxylin. As a negative control, the primary antibody was replaced with normal mouse IgG at an appropriate dilution. As a positive control, tissue sections of normal placenta were used as previously reported (14). The GnT-V expression levels were classified semiquantitatively based on the total scores of the percent positivity of stained tumor cells and the staining intensity. Namely, the percent positivity was scored as 0 if <5% (negative), 1 if 5-30% (sporadic), 2 if 30-70% (focal) and 3 if >70% (diffuse) of cells stained, whereas staining intensity was scored relative to the known positive and negative controls as 0 if no staining, 1 if weakly to moderately stained and 2 if strongly stained. The final GnT-V

expression score was defined as follows: 'GnT-V low' if the sum of the percent positivity score and the staining intensity score was 0-2 and 'GnT-V high' if the sum was 3-5. In each case, at least three different areas were evaluated. The scoring procedure was carried out twice by two independent observers without any knowledge of the clinical data. The concordance rate was over 95% between observers. In the case of disagreement, the slides were reviewed simultaneously by these two observers, together with another observer, seated together at a multi-head microscope, in order to reach a consensus.

Cell line and culture. NOM-1 is a human ovarian mucinous adenocarcinoma cell line (15), and cells were grown in RPMI-1640 (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml) and 2 mM glutamine. Cultures were incubated at 37°C in a 5% CO₂ atmosphere. To suppress β 1-6GlcNAc branching, cells were incubated with 1 μ g/ml swainsonine (SW; Wako Pure Chemical Industries, Osaka, Japan), an inhibitor of GnT-V.

Western blot analysis. Cells were homogenized in lysis buffer consisting of a protease inhibitor mixture in radio-immunoprecipitation assay (RIPA) buffer. After centrifugation at 15,000 \times g for 20 min, the supernatant was obtained. Twenty micrograms of protein extract were separated by SDS 10% polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and immunoblotted with an anti-GnT-V monoclonal antibody (24D11) (16) at a dilution of 1:1000. Immunoreactive proteins were stained using a chemiluminescence detection system (ECL; Amersham, Arlington Heights, IL, USA).

Leukoagglutinating phytohemagglutinin blot analysis. Protein-blotted nitrocellulose filters were prepared in exactly the same way as described for Western blotting. After blocking with 5% skim milk for 30 min at room temperature, the filter was incubated in PBS containing 1:1000-diluted biotinylated leukoagglutinating phytohemagglutinin (L₄-PHA; Seikagaku, Tokyo, Japan), which preferentially recognizes β 1-6 branches of tri- or tetra-antennary sugar chains, for 1 h at room temperature. The filter was washed 3 times with PBS containing 0.05% Tween-20 (TPBS) for 10 min each. Substrate binding was detected with a 1:1000 dilution of avidin-peroxidase conjugate (ABC kit; Vector Res., CA, USA) in TPBS for 30 min at room temperature. The membrane was washed and then developed using ECL reagents.

In vitro cell proliferation assay. Cells were plated in triplicate at a density of 2×10^3 cells in 200 μ l volume in 96-well plates. Cell viability was determined by modified tetrazolium salt (MTS) assay using the Cell Titer 96 Aqueous One Solution Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Transwell migration assay. Migration assay was performed after 24-h incubation, as previously reported (17). Transwells (Corning Incorporated, Corning, NY, USA) with a filter of

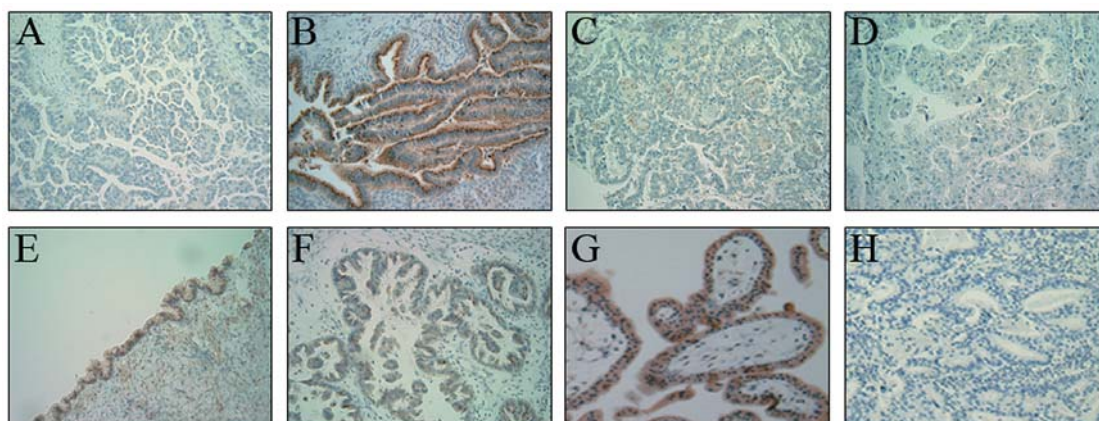


Figure 1. Immunohistochemical expression of GnT-V in ovarian carcinomas. (A) Serous adenocarcinoma; (B) mucinous adenocarcinoma; (C) endometrioid adenocarcinoma; (D) clear cell adenocarcinoma; (E) mucinous cystadenoma; (F) borderline mucinous tumor; (G) positive control (human placenta) and (H) negative control. Magnification, x200.

Table I. Correlation of GnT-V expression with clinicopathological factors in EOC.

	Patient no.	GnT-V expression		P-value
		Low (%)	High(%)	
Total	83	66 (79.5)	17 (20.5)	
Age				
<55	44	36 (81.8)	8 (18.2)	0.924
≥55	39	30 (76.9)	9 (23.1)	
FIGO stage				
I/II	45	32 (71.1)	13 (28.9)	0.04
III/IV	38	34 (89.4)	4 (10.5)	
Histological type				
Mucinous	14	1 (7.1)	13 (92.9)	<0.001
Non-mucinous	69	65 (94.2)	4 (5.8)	
Serous	29	29 (100)	0 (0)	
Endometrioid	12	11 (91.7)	1 (8.3)	
Clear	28	25 (89.3)	3 (10.7)	
Grade				
G1/G2	72	56 (77.8)	16 (22.2)	0.55
G3	11	10 (90.9)	1 (9.1)	

6.5 mm diameter and 8.0 μ m pore size were used. The number of cells was adjusted to 2.0×10^5 /ml in serum-free medium and a 100 μ l sample was added in triplicate to the upper wells and 600 μ l of conditioned medium with 10% FCS was added to the lower wells. The number of cells was counted under a microscope at x200 magnifications. Data were obtained from three individual experiments performed in triplicate.

Statistical analysis. The association between GnT-V expression and clinicopathological parameters was evaluated using the χ^2 -test. The non-parametric Kruskal-Wallis test was performed to compare immunostaining scores among all

histological types, and three malignant levels of mucinous tumors. For data of *in vitro* experiments, statistical comparisons among groups were performed using Student's t-test. SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Differences were considered significant when $P < 0.05$.

Results

Immunohistochemical expression of GnT-V in epithelial ovarian cancer tissues. We examined GnT-V expression in ovarian cancer tissues by immunohistochemical staining using 83 surgical specimens. As shown in Fig. 1A-D, GnT-V

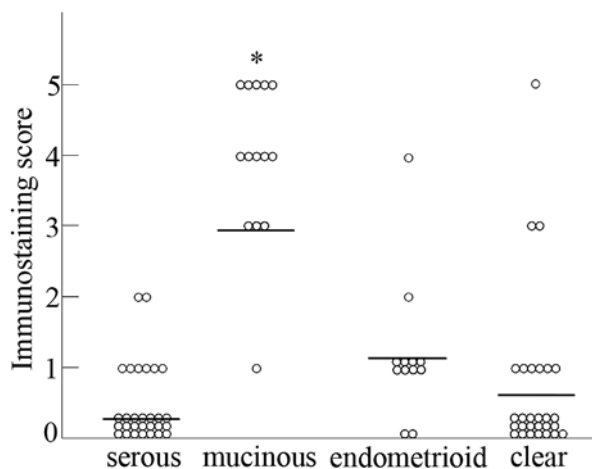


Figure 2. The immunostaining score of GnT-V expression in all ovarian cancers (n=83), including serous adenocarcinoma (n=29), mucinous adenocarcinoma (n=14), endometrioid adenocarcinoma (n=12) and clear cell adenocarcinoma (n=28). Each bar represents the mean of the immunostaining score. Statistical analyses were performed by the Kruskal-Wallis test. *P<0.001.

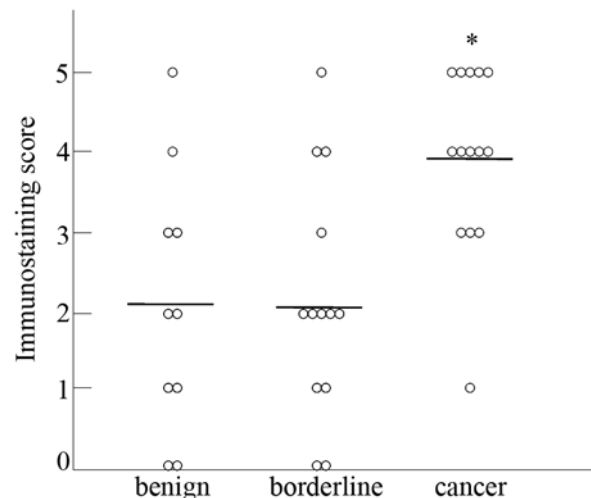


Figure 3. The immunostaining score of GnT-V expression in mucinous tumor (n=37). GnT-V expression was significantly higher in cancer (n=14) than in benign (n=10) and borderline (n=13) tumors. Each bar represents the mean of the immunostaining score. Statistical analyses were performed by the Kruskal-Wallis test. *P<0.001.

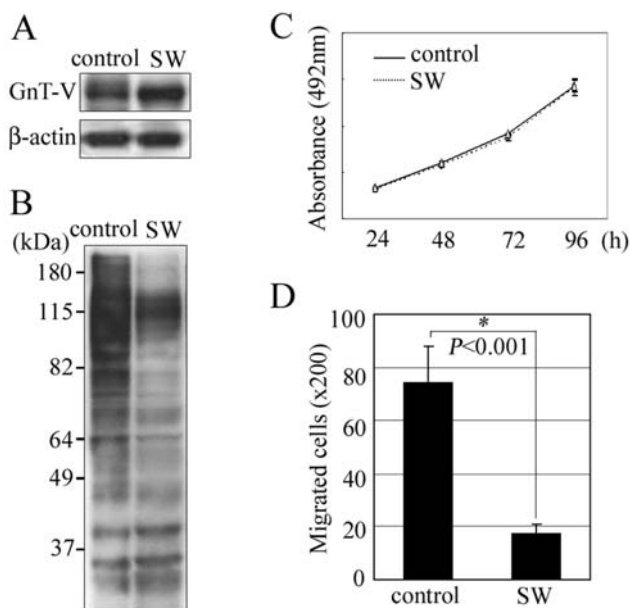


Figure 4. Swainsonine (SW) treatment leads to the suppression of B1-6 GlcNAc branching in NOM-1 cells and subsequent down-regulation of migration. (A) Western blot analysis showed that NOM-1 expressed GnT-V strongly with or without SW. (B) L₄-PHA lectin blot analysis revealed significantly reduced B1-6GlcNAc branching with SW. (C) Cell proliferation was not affected with SW for 96 h. (D) Graph depicting the significantly decreased cell migratory numbers in NOM-1 treated with SW, compared to the control. Each bar represents the mean migrated cell numbers compared to the control \pm SD. Data were obtained from triplicate experiments. *P<0.001.

immunoreactivity was detected at variable levels, and was found in the cytoplasm of cancer cells. In contrast, GnT-V immunoreactivity was very faint or absent in tumor stroma.

Correlation of GnT-V expression with clinicopathological parameters. Of the 83 specimens examined, 'low GnT-V expression' tumors were found in 66 (79.5%) cases, and

Table II. GnT-V expression in benign, borderline or malignant mucinous tumors of the ovary.

	Patient no.	GnT-V expression	
		Low (%)	High (%)
Benign	10	6 (60.0)	4 (40.0)
Borderline	13	9 (75.0)	4 (25.0)
Cancer	14	1 (7.1)	13 (92.9)

'high GnT-V expression' in 17 (20.5%) cases, respectively. The correlations of high GnT-V expression with various clinicopathological parameters in the 83 cases are summarized in Table I. High GnT-V expression was positively correlated with FIGO surgical stage (P=0.04), but not with age, histological grade. For histological type, high GnT-V expression was observed in no cases (0%) of 29 serous adenocarcinoma, 1 (8.3%) of 12 endometrioid adenocarcinoma, 3 (10.7%) of 28 clear cell carcinoma, and 13 (92.9%) of 14 mucinous adenocarcinoma; therefore, mucinous adenocarcinoma showed significantly strong GnT-V expression compared to non-mucinous type (P<0.001). In most non-mucinous-type tissues, the immunostaining scores were 0 and 1, and the median score was 0.34 in serous, 1.17 in endometrioid, and 0.61 in clear cell carcinoma, but 3.93 in mucinous adenocarcinoma (Fig. 2). Although overall survival (OS) and progression-free survival (PFS) curves were constructed using the Kaplan-Meier method, there was no significant difference of OS (P=0.39) and PFS (P=0.13) in patients among high and low GnT-V expression (data not shown).

GnT-V expression in mucinous ovarian tumor of various malignancies. Next, we investigated the difference in GnT-V



on among the level of malignancy of mucinous including mucinous cystadenoma (benign), borderline malignancy, and mucinous adenocarcinoma (Fig. 1B, E and F). GnT-V was expressed in both mucinous tumor cells of benign and borderline malignancy, but staining was weaker than in cancer cells. Fig. 3 shows the immunostaining scores of three groups and the score was significantly higher in carcinoma than in benign and borderline malignancy ($P < 0.001$). The median scores were 2.10 in benign, 2.15 in borderline malignancy and 3.93 in cancer groups.

GnT-V expression and β 1-6GlcNAc branching in a mucinous adenocarcinoma cell line. We examined GnT-V expression in the NOM-1 cell line, which was established from human ovarian mucinous cystadenocarcinoma. In NOM-1 cells, GnT-V protein was detected as approximately 110 kDa bands (Fig. 4A). To evaluate the level of β 1-6 branching, we also performed lectin blot analysis of total cellular proteins using L_4 -PHA, which preferentially binds to GlcNAc residues on β 1-6 branches of tri or tetra-antennary sugar chains (Fig. 4B). This analysis showed that GnT-V certainly catalyzed such specific glycosylation to target glycoproteins, whose major molecular sizes were approximately 60-200 kDa.

Cell migration was decreased by β 1-6GlcNAc branching suppression in NOM-1. To investigate the functional effect of GnT-V in mucinous ovarian cancer cells, we used swainsonine (SW), which inhibits Golgi α -mannosidase II and ultimately inhibits N -linked β 1-6 oligosaccharide formation upstream of the action of GnT-V. GnT-V expression was not changed with 1 μ g/ml SW treatment for 48 h (Fig. 4A); however, L_4 -PHA lectin blotting showed that the level of β 1-6GlcNAc branching was significantly decreased (Fig. 4B). We assessed the effect of β 1-6GlcNAc branching decrease on cell proliferation by MTS proliferation assay. The number of NOM-1 cells increased after 96 h; however, this was not significantly different in SW treatment cells (Fig. 4C). In contrast, migration analyses revealed that NOM-1 cells had significantly lower potential to migrate with SW treatment (17.3 ± 3.9) than the control (74.3 ± 13.9 , $P < 0.001$) (Fig. 4D). These results suggested that GnT-V might promote the migratory capabilities of mucinous ovarian cancer cells.

Discussion

In the present study, we demonstrated the expression of GnT-V in EOC using 83 surgical specimens, and found that GnT-V expression was significantly stronger in mucinous adenocarcinoma than in the other histological types. High GnT-V expression was also positively correlated with the FIGO surgical stage in EOC, but this was because 84.6% (11 cases) of high GnT-V expression in stage I/II was the mucinous type. Furthermore, 92.9% (13 cases) of high GnT-V expression was mucinous adenocarcinoma; therefore, OS and PFS showed no significant differences between low and high GnT-V in EOC. The GnT-V immunostaining score was lower in the non-mucinous type of EOC, compared to benign and borderline mucinous tumors. These results suggested that

GnT-V expression is a specific feature of the mucinous type among human EOC. Previous reports on the relationship between GnT-V expression and the prognosis in various cancers were controversial (8,11,12,17,18), but our data strongly suggested that it depends on the histological type, as well as the original organ of the cancer.

In mucinous ovarian tumors, GnT-V was increased in cancer cells compared to benign and atypical borderline tumor cells. Although there was no significant difference in GnT-V expression between benign and borderline tumors, the recurrence rate was 50% in high GnT-V expression (2 cases of 4 patients) compared to 11.1% in low GnT-V expression (1 case of 9 patients) in the borderline group; however, in mucinous carcinoma, the mean of the immunostaining score was higher in disease-free patients (4.22 in 9 cases) than in recurrent and dead patients (2.8 in 5 cases). GnT-V increase in the pre-cancer stage was previously reported in hepatocellular carcinoma and esophageal cancer (19,20). These results suggested that GnT-V might be involved in the early step of carcinogenesis in mucinous tumor.

Our *in vitro* experiments using a mucinous adenocarcinoma cell line showed that the suppression of β 1-6GlcNAc branching, which is catalyzed by GnT-V, reduced cell migration, but cell proliferation was not affected. L_4 -PHA lectin blotting revealed that the major target glycoproteins of GnT-V in mucinous ovarian carcinoma were 60-200 kDa in molecular size. Previous reports indicated several specific substrates for GnT-V glycosylation and changes in the biological characteristics of cancer cells; matriptase (21), lamp-1 (22), N-cadherin (23), α 5 β 1-integrin (14,16), and epidermal growth factor receptor (EGFR) (24). In these substrates, α 5 β 1-integrin is a protein heterodimer of 150 and 130 kDa, and is associated with migration. α 5 β 1-integrin was reported to be involved in the implantation of ovarian cancer cells into the peritoneal mesothelium by the recognition of mesothelial-associated fibronectin or by the formation of multicellular spheroids (25,26). These results suggested that a target molecule of GnT-V in mucinous ovarian cancer could be α 5 β 1-integrin, but further studies are needed.

In conclusion, we demonstrated that high GnT-V expression correlated with the histological type, especially mucinous carcinoma, in EOC. GnT-V was expressed in most mucinous tumors, and was significantly increased in cancer compared to benign and low malignant potential tumors. Furthermore, SW-treated cells decreased β 1-6GlcNAc branching and cell migration. These results suggested that GnT-V might be involved in the malignant potential of mucinous ovarian cancer.

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