Positive expressions of *N*-acetylglucosaminyltransferase-V (GnT-V) and ß1-6 branching *N*-linked oligosaccharides in human testicular germ cells diminish during malignant transformation and progression

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Abstract. *N*-acetylglucosaminyltransferase-V (GnT-V) is an enzyme that catalyzes ß1-6 branching of *N*-acetylglucosamine on asparagines (*N*)-linked oligosaccharides of cell proteins. We examined the implication of GnT-V and ß1-6 branching *N*-linked oligosaccharide expression in human testicular germ cells during malignant transformation and cancer progression. We analyzed immuhistochemically orchiectomy specimens of 130 patients with testicular germ cell tumors (TGCT) using anti-GnT-V monoclonal antibody, and compared GnT-V expression with clinicopathological features. *N*-linked oligosaccharide structural analysis was also performed to confirm the oligosaccharide profile produced by GnT-V. GnT-V was positive in all normal testis samples. This positive incidence declined in TGCT according to clinical stage; 16/71 (22.5%) in stage I, and 3/59 (5.1%) in stage II/III (p=0.015, χ^2 test). When divided into pathological subtypes, GnT-V positive incidences in stage I seminoma, stage II/III seminoma, stage I non-seminomatous germ cell tumor (NSGCT), and stage II/III NSGCT were 3/43 (7%), 0/22 (0%), 13/28 (46.4%), and 3/37 (8.1%), respectively. In stage I NSGCT, patients with GnT-V-negative tumor samples were at a significantly higher risk of recurrence than those with GnT-V-positive tumors (p=0.015, log-rank test). *N*-linked oligosaccharide structural analysis revealed that a normal testis has three kinds of ß1-6 branching *N*-linked oligosaccharides, all of which are downregulated in TGCT tissues. These results suggest that GnT-V and ß1-6 branching *N*-linked oligosaccharide expressions are downregulated during carcinogenesis and progression of human TGCT. GnT-V may be a promising recurrence predictor for stage I NSGCT.

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Introduction

Testicular germ cell tumor (TGCT) is the most common malignancy in young males, and the frequency has risen over the past few decades (1,2). TGCTs are broadly divided into two groups: seminomas and non-seminomatous germ cell tumors (NSGCT) (3). Their biological behavior, clinical features, and clinical management are quite different between the two distinct entities (2).

Aberrant glycosylation is commonly observed in human cancers (4-6). However, glycosylation status in testicular germ cell tumor (TGCT) has not been studied extensively, except for the role of glycosphingolipids (7). In an attempt to find a novel biomarker that represents clinical malignant potential, we analyzed previous studies on the role of glycosphingolipids in TGCT (8-10). We found that Gb3 glycosphingolipid expression was upregulated in TGCT (8) and that galactosyl-globoside (Gb5) may be a recurrence indicator for seminoma (10). The important roles of *N*-glycan oligosaccharides in TGCT, however, have not been reported.

Beta1,6 *N*-acetylglucosaminyltransferase (GnT-V), a key enzyme in the formation of ß1-6 branching of asparagines (*N*)-linked oligosaccharides, is tightly linked to tumor metastases (11-13). In colorectal cancer (14) and brain tumors (15), the expression of GnT-V correlated significantly with distant metastasis. In contrast, patients with hepatocellular carcinoma and those with low or no expression of GnT-V were more likely to show recurrence than cases with high expression of GnT-V (16). Moreover, GnT-V expression was inversely associated with prognosis and histology in patients

with non-small cell lung cancers (17) and bladder cancers (18,19). Therefore, the clinical implication of GnT-V expression may differ in each type of cancer.

This report demonstrates the downregulation of GnT-V and its product, ß1-6 branching *N*-linked oligosaccharide, during carcinogenesis and progression of human TGCT.

Materials and methods

Human testicular germ cell tumor specimens. One hundred and thirty patients with testicular germ cell tumor (TGCT) were consecutively enrolled in this study: 65 patients had seminoma and the other 65 had NSGCT. Formalin-fixed, paraffin-embedded tumor specimens obtained by routine orchiectomy were subjected to immunohistochemistry using anti-GnT-V monoclonal antibody (12). Four normal testis samples obtained adjacent of the testicular tumor were subjected to immunohistochemistry and *N*-linked oligosaccharide structural analysis. Informed consent was received from each patient. Clinical staging was determined according to the American Joint Committee on Cancer (AJCC) staging system (20).

Patient follow-up. Patients with stage I disease were followed up according to routine surveillance policy (i.e., observation alone after radical orchiectomy) until relapse was detected (21).

Immunohistochemistry. Imunohistochemical analysis using anti-GnT-V monoclonal antibody (12) was performed as previously described (18,19). Briefly, deparaffinized specimens were incubated with anti-GnT-V monoclonal antibody as the primary antibody. Anti-mouse immunoglobulin antibody conjugated with horseradish peroxidase (Nichirei, Tokyo, Japan) was used as the secondary antibody, and peroxidase activity was visualized with aminoethylcarbazol (AEC) solution (Nichirei, Tokyo, Japan). An experiment using a control was performed by omitting the primary antibody from the staining procedure. The results of immunostaining were evaluated in a blinded manner to clinical data. Based on the staining status of Golgi bodies, specimens possessing ≥10% positive cells were judged as GnT-V-positive.

N-linked oligosaccharide structural analysis. Four samples of normal testis and 6 of TGCT (4 seminomas and 2 NSGCTs) were subjected to structural analysis of *N*-linked oligosaccharides. Standard oligosaccharides were prepared from human transferrin and serum as previously described (22-24). Alpha-mannosidase, ß-N-acetylhexosaminidase, ß-galactosidase from jack bean, and pyridylaminated (PA) isomaltooligosaccharides were purchased from Seikagaku Co. (Tokyo, Japan).

N-linked oligosaccharide structural analysis was performed as previously described (18). Briefly, samples were treated with chloroform and methanol (2:1, v/v) to remove lipids and were treated with trypsin and chymotrypsin, *N*-glycosidase, and pronase sequentially and purified with Bio-Gel P-4 (24). Purified oligosaccharides were pyridylaminated (25,26).

PA-oligosaccharides were analyzed and isolated on HRC-ODS column 6x150 mm (Shimadzu, Kyoto, Japan) and TSKgel Amide-80 column 4.6x250 mm (Tosoh, Tokyo Japan) by NanoSpace SI-II (Shiseido, Tokyo Japan) high performance liquid chromatography (HPLC) systems. Elution on the ODS column was performed with 10 mM sodium phosphate buffer, pH 3.8, and 1-butanol at 55˚C. 1-Butanol concentration was increased from 0.1 to 0.25% (v/v) linearly in 60 min. Solvents on the amide column were 3% acetic acid triethylamine buffer, pH 7.3, and acetonitrile. The ratio of the buffer and acetonitrile was gradually changed from 35:65 to 44:66 by volume in 30 min at 40˚C. Flow rates were 1.0 ml/min and oligosaccharides were detected with fluorescence, Ex 320 nm, and Em 400 nm. Furthermore, isolated oligosaccharides were treated with sequential exoglycosidase digestions, and HPLC analysis was performed with standard oligosaccharides at each step of enzyme treatments (24).

Statistical analyses. χ^2 test was used to assess the association of the GnT-V expression with clinical stage. Statistical analysis for *N*-linked oligosaccharide structural analysis was performed by Mann-Whitney's U test. Recurrence-free survival for the patient with stage I NSGCT was estimated by Kaplan-Meier curves, and differences between groups were evaluated using the log-rank test.

Results

GnT-V expression is downregulated in TGCT and inversely correlated to clinical stage. Table I shows the results of immunohistochemical analysis obtained using anti-GnT-V monoclonal antibody. GnT-V was positively detected in all samples of normal testis. GnT-V showed clear positive staining on the Golgi apparatus in germ cells. Typical positive staining was found in primitive germ cells, for example, primary spermatocytes (Fig. 1A). In TGCT, GnT-V was found positive in 16/71 (22.5%) in stage I, and 3/59 (5.1%) in higher stage (II + III) (p=0.015). This difference was also found when patients were divided into seminoma and NSGCT according to histopathological classification (Table I). In seminoma, GnT-V-positive cases were found only in stage I. However, because of the small number of the samples, no statistically significant difference was demonstrated between stage I and stage II/III. GnT-V was positive in 13/28 (46.4%) of stage I NSGCT and 3/37 (8.1%) in stage II/III NSGCT (p=0.006). Photographic representation of immunohistochemistry is shown in Fig. 1.

GnT-V expression inversely correlated with recurrence-free survival in stage I NSGCT. Patients with stage I disease were followed-up with surveillance policy after radical orchiectomy. For patients with stage I seminoma, predictive value of GnT-V for tumor recurrence was not assessed owing to the low incidence of GnT-V-positive cases (3/43). In 28 patients with stage I NSGCT, 1 of 12 (8.3%) patients whose tumor samples were positive for GnT-V relapsed. However, 8 of 16 (50%) patients who had positive GnT-V tumors relapsed during the mean follow-up period of 55.9 months (range 1-171). GnT-V-negative patients had a significantly higher risk for recurrence than GnT-V-positive patients. Kaplan-Meier plots and the log-rank test showed a significant difference in relapse-free survival between the two groups (p=0.015) (Fig. 2).

Table I. GnT-V expression in normal testis and testicular germ cell tumors.

GnT-V expression	All		Seminoma		NSGCT[a]	
	Positive/total	(%)	Positive/total	(%)	Positive/total	(%)
Normal testis	6/6	(100)				
Clinical stage[b]						
I	16/71	(22.5)	3/43	(7.0)	13/28	(46.4)
II + III	3/59	(5.1)	0/22	(0.0)	3/37	(8.1)
p-value	0.015		NS		0.006	

NS, not significant; [a]Non-seminomatous germ cell tumor; [b]AJCC, American Joint Committee on Cancer, 6th edition, 2002.

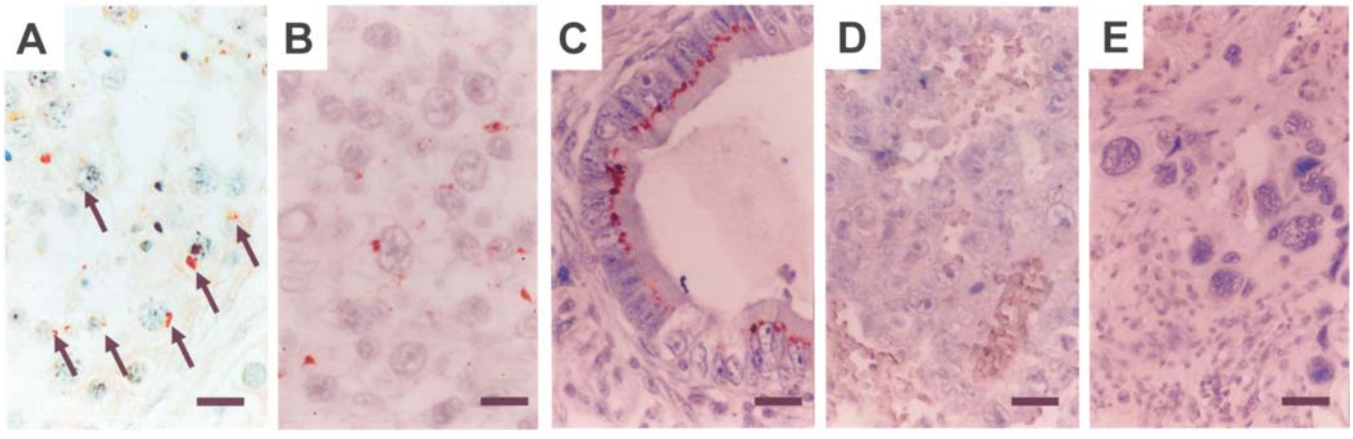

Figure 1. Immunohistochemistry of normal testis and human testicular germ cell tumor using anti-GnT-V. GnT-V showed positive staining in normal germ cells (a), seminoma (b), and teratoma with low-grade malignancy; negative in embryonal carcinoma (c) and choriocarcinoma (d), which have high malignant potential. GnT-V positive staining was clearly detected as a granular deposit around nuclei, which is consistent with its intracellular localization at Golgi bodies. Scale bar, 10 μm.

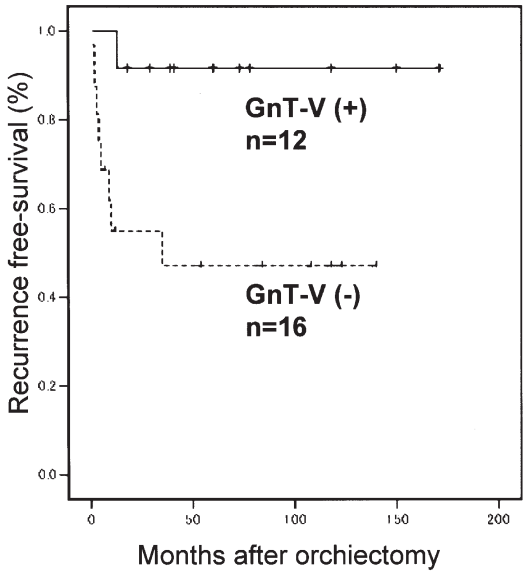

Figure 2. GnT-V expression and recurrence-free survival in stage I NSGCT. Patients with stage I NSGCT, GnT-V-positive cases had a significantly lower risk for recurrence (p=0.015, log-rank test).

Structural analysis revealed downregulation of ß1,6 branching N-linked oligosaccharides in human germ cell tumor. PA-oligosaccharide mixtures and samples were separated and analyzed after elution on ODS column. Each separated peak of sample was analyzed and separated on the respective amide column. The structures of isolated oligosaccharides were suggested by their elution positions on the ODS and amide columns, and their identities coincided with known oligosaccharide databases (24) (Fig. 3). To avoid any mis-interpretation, suggested structures were verified by analysis with standard oligosaccharides. Furthermore, to ensure these structures, oligosaccharides were treated with exo-glycosidases and analysis of their products were consistent with expected structures (data not shown). Their molar ratios were calculated from peaks obtained from HPLC analysis. In this way, oligo-saccharide structures and ratios were determined as shown in Table II. The average percentage of oligosaccharide of the normal testis was calculated.

The relative amounts of each oligosaccharide were plotted against those found in the normal testis (Fig. 4). TGCT samples, compared with normal testis, were poor in structure codes-310.18, 410.16, and 400.16 for which biosynthesis of

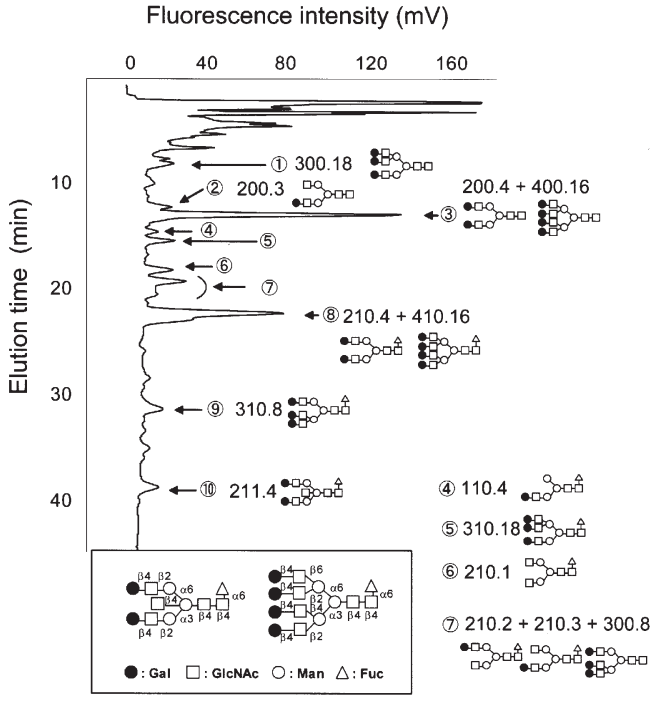

Figure 3. HPLC separations of PA-oligosaccharides on the ODS column and the suggested structure. The oligosaccharide structure corresponds to one eluted position on ODS column. Suggested structures were confirmed by analysis with standard oligosaccharides to avoid experimental misinterpretation. Furthermore, oligosaccharides were treated by exo-glycosidases and analysis of their products was consistent with the expected structures. Gal, galactose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose.

GnT-V is required. Other than these alterations, TGCT samples were rich in 210.1 and 310.8 oligosaccharides, which were not detected in normal testis. Decrease of bisecting *N*-linked oligosaccharide, code 211.4, in TGCT was also detected.

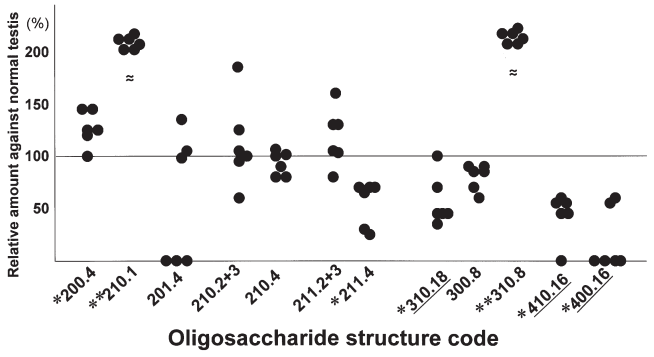

Figure 4. Relative amount of oligosaccharide in testicular germ cell tumor (TGCT) against average amount of normal testis. When compared with the normal testis, TGCT express smaller amounts of structure codes 310.18, 400.16, and 410.16, for which biosynthesis GnT-V is responsible (underlined). It is also noted that TGCT express codes 210.1 and 310.8, which could not be detected in normal testis. *Oligosaccharide structure with significantly different amount compared with normal testis; $p<0.05$. **Oligosaccharide structure with significantly different amount compared with the normal testis; $p<0.01$.

There were no significant changes between histopathological subtypes.

Discussion

In this study, we have demonstrated the downregulated expression of GnT-V in TGCT compared with normal testis. GnT-V-positive rate in normal testis was 100%, while it was 3/65 in seminoma and 16/65 in NSGCT. In seminoma, GnT-V was positive only in stage I cases. In NSGCT, the GnT-V-positive rate in stage I was significantly higher than that in stage II/III. The data suggest that GnT-V is down-regulated in TGCT carcinogenesis and progression.

Table II. *N*-linked oligosaccharide composition of normal testis and testicular germ cell tumors.

Code	N1	N2	N3	N4	S1	S2	S3	S4	NS1	NS2
200.4	23.13	30.78	30.44	22.6	32.5	31.8	40.21	27.2	33.34	34.11
201.4	0.00	1.91	1.53	2.63	1.46	0.00	2.17	0.00	1.74	0.00
210.1	0.00	0.00	0.00	0.00	3.83	2.85	5.03	12.68	6.44	3.35
210.2+3	5.64	6.07	5.64	8.05	5.94	4.01	6.12	11.64	7.98	6.88
210.4	36.77	27.34	26.93	26.2	28.3	32.1	19.66	26.39	22.98	32.42
211.2+3	2.78	2.79	2.36	5.15	4.22	3.63	4.28	3.72	5.37	2.51
211.4	16.32	15.4	14.75	18.3	10.9	9.89	11.09	5.21	11.34	4.57
300.8	5.46	6.45	7.27	4.83	4.56	3.52	5.22	5.52	5.35	5.76
310.18	**6.46**	**4.82**	**5.54**	**5.05**	**2.41**	**5.41**	**1.62**	**2.41**	**2.02**	**3.87**
310.8	0.00	0.00	0.00	0.00	4.11	5.24	3.35	3.88	2.08	3.73
400.16	**1.32**	**1.92**	**2.44**	**3.62**	**0.00**	**0.00**	**0.00**	**0.00**	**1.35**	**1.33**
410.16	**2.08**	**2.5**	**3.11**	**3.57**	**1.77**	**1.66**	**1.34**	**1.34**	**0.00**	**1.52**
Total (%)	100.00	100.00	100.00	100.00	100.0	100.00	100.00	100.00	100.00	100.00

N, normal testis; S, seminoma; NS, non-seminomatous germ cell tumor. Structure codes in bald, GnT-V products.

In addition, in patients with stage I NSGCT without metastatic lesions, the recurrence-free rate of GnT-V-positive patients was significantly lower than that of GnT-V-negative patients. Generally, in patients with stage I NSGCT followed by surveillance policy, the recurrence rate was reported up to 30% (27,28). Thus, it is of vital importance to identify recurrent risk factors (29). Conventional clinicopathological risk factors, however, have some limitations for clinical application (29). GnT-V expression may be a reliable indicator of tumor recurrence in stage I NSGCT. In stage I seminoma, however, the GnT-V-positive incidence was low (3/43, 7%). This is the reason why we did not plot Kaplan-Meier curves for patients with stage I seminoma.

It is sometimes noted that certain glycosyltransferase expression is not always accompanied by a specific oligosaccharide structure that the enzymatic biosynthesis can account for. Thus, immunohistochemically proven findings should be validated by precise structural analysis. In our *N*-linked oligosaccharide structural analysis, it was demonstrated that normal testis samples had significantly higher amounts of GnT-V products than TGCT samples. These results are consistent with those obtained by immunohistochemical analysis using by anti-GnT-V monoclonal antibody.

Other than the alteration of ß1-6 branching *N*-linked oligosaccharides, the present structural analysis also revealed increase of codes 210.1 and 310.8 oligosaccharides: code 210.1 is bi-antennary *N*-linked oligosaccharide, code 310.8 is ß1-4 branching *N*-linked oligosaccharides for which GnT-IV is accountable. Because these oligosaccharides were not detected in normal testis, studies relating to these structures are of great interest. Decrease of bisecting *N*-linked oligosaccharide, code 211.4, in TGCT was also detected. It is well known that bisecting *N*-acetylglucosamine structure affects the conformation of sugar chains. Once GnT-III acts on bi-antennary sugar chains, the other glycosyltransferases, such as GnT-II, GnT-IV, and GnT-V are no longer able to act on the bi-antennary sugar chains (12). Competition of GnT-III and GnT-V *in vivo* leads to suppression of cancer metastasis (30). However, present oligosaccharide structural analysis revealed that both these enzymes are downregulated in human TGCT. A remarkable increase of bi-antennary (code 210.1) and ß1-4 branching (310.8) *N*-linked oligosaccharides was also observed. To summarize *N*-linked oligosaccharide alteration of human TGCT, upregulation of GnT-IV, and downregulations of GnT-V and III may be key events.

In malignant tumors, clinicopathologic implication of GnT-V and ß1-6 branching *N*-linked oligosaccharides may vary in each cancer. In colon cancer (14), glioma (15), esophagus cancer (31), and breast cancer (32) malignant potentials were associated with ß1-6 branching *N*-linked oligosaccharide expressions. On the other hand, GnT-V is associated with favorable features in non-small cell lung cancer (16), hepatocellular carcinoma (17), and neuroblastomas (33). Our previous study demonstrated that GnT-V and ß1-6 branching *N*-linked oligosaccharides were closely related to good prognosis for patients with bladder cancer (18). Li and Roth (34) provided an interesting hypothesis for these complex phenomena. Almost all epithelial cell types of normal human and rat tissues, including bronchial epithelial cells and alveolar pneumocytes express ß1-6 branching *N*-linked oligosaccharides. Exceptions were epithelia of the colon, esophagus, and resting mammary gland. In cancers derived from these epithelia, GnT-V expression has been shown to be linked to malignant transformation, invasion, and metastatic potential. We support their hypothesis, because in our structural analysis normal germ cells express ß1-6 branching *N*-linked oligosaccharide. Recently, it has been postulated that GnT-V may cause these tumors to regress by increasing their susceptibility to apoptosis (33). Further studies are required to ensure that this apoptotic mechanism ensues with other kind of cancers.

In conclusion, GnT-V and its products ß1-6 branching *N*-linked oligosaccharides are downregulated during malignant transformation and progression of human TGCT. GnT-V expression may be a promising predictor of recurrence for stage I NSGCT.

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