

Co-expression of matriptase and *N*-acetylglucosaminyltransferase V in thyroid cancer tissues—its possible role in prolonged stability *in vivo* by aberrant glycosylation

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UDP-*N*-acetylglucosamine:α-mannoside β-1,6-*N*-acetylglucosaminyltransferase (GnT-V) catalyzes the formation of β-1-6 GlcNAc branches on asparagine-linked oligosaccharides, which is directly linked to tumorigenesis. Our recent studies indicate that the secretion of matriptase from cancer cells is increased via the action of GnT-V, as evidenced by the fact that matriptase-bearing β-1-6 GlcNAc branching is dramatically inhibited. In this study, we report on an investigation of the expression of GnT-V and matriptase in thyroid neoplasm tissues to determine the clinical significance on the co-expression of these two proteins in thyroid cancer. Although neither GnT-V nor matriptase was expressed in normal thyroid tissue, positive staining for matriptase and GnT-V was observed in 52/68 and 66/68 cases of papillary carcinoma, 3/23 and 10/23 cases of follicular carcinoma, 5/13 and 9/13 cases of follicular adenoma, and 11/28 and 6/28 cases of anaplastic carcinoma, respectively. Immunohistochemistry, as well as western blotting, showed that the expression of matriptase paralleled the expression to GnT-V. However, the expression of matriptase mRNA was not correlated with its protein levels, suggesting that the enhancement in matriptase expression could be regulated by a posttranslational modification such as glycosylation through GnT-V-mediated glycosylation. In papillary carcinoma, the levels of expression of both GnT-V and matriptase were significantly higher in tumors 1 cm or less in size (microcarcinoma) and in those without poorly differentiated lesions, and the two proteins were significantly correlated. In contrast,

the prognosis of thyroid carcinoma after surgery was neither correlated with the expression GnT-V nor matriptase, because the levels of their expression were quite low in anaplastic (undifferentiated) carcinomas. These results suggest that prolonged stabilization of matriptase is stabilized by GnT-V-mediated glycosylation *in vivo*, thus extending its halftime and permitting it to play role in the early phases of papillary carcinoma, but not in its later phase progression.

Key words: GnT-V/growth factor/matriptase/thyroid cancer

Introduction

Many types of molecules that regulate malignant transformation of cells, as well as progression and metastasis of carcinoma have been reported. However, the roles of oligosaccharides are understudied in carcinoma progression because of the low gene expression of glycosyltransferases that catalyze the biosynthesis of oligosaccharides and because of the limited success in the identification of target molecules that are modified by specific glycosyltransferase enzymes (Hakomori, 1989). Previously, others and we purified and cloned cDNAs of human UDP-*N*-acetylglucosamine:α-mannoside β-1,6-*N*-acetylglucosaminyltransferase (GnT-V), a key enzyme in the formation of branched asparagine-linked oligosaccharides, which strongly regulates tumor metastasis (Gu *et al.*, 1993; Shoreibah *et al.*, 1993). When GnT-V deficient mice was mated with polyoma middle T antigen transgenic mice, tumor growth and metastasis were dramatically suppressed (Granovsky *et al.*, 2000). However, detailed mechanisms responsible for the regulation of tumor metastasis by GnT-V remain unknown. Our previous studies have shown that an elevated expression of GnT-V is a predictor of a poor prognosis in patients with colonic adenocarcinoma (Murata *et al.*, 2000). In contrast, the expression of GnT-V was reported to be significantly increased in the early phase of hepatocellular carcinoma, as well as in severe liver cirrhosis and in adenomatous hyperplasia (Ito *et al.*, 2001). These findings indicate that, although this enzyme is related to carcinoma progression, its functions may vary, possibly depending on the nature of available target glycoproteins for GnT-V.

Matriptase is a tumor-associated type II transmembrane serine protease, which positively regulates carcinoma metastasis by activating the latent forms of hepatocyte growth factor (HGF) and urokinase-type plasminogen activator (uPA)

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(Lin *et al.*, 1997). Recently, we demonstrated that overexpression of GnT-V in gastric carcinoma cells prolonged the degradation of matriptase and accelerated the peritoneal dissemination of these cancer cells in athymic mice (Ihara *et al.*, 2002). Matriptase purified from GnT-V-transfected gastric cancer cells was resistant to trypsin treatment, and this resistance was dependent on the oligosaccharides linked to the 772 Asn site of matriptase (Ihara *et al.*, 2004). These findings indicate that oligosaccharide modification of matriptase by GnT-V changed the function of the proteases; therefore, matriptase could be considered as target glycoproteins for GnT-V.

Thyroid carcinoma is one of the most common malignancies originating in the endocrine glands. There are two prominent histological types of thyroid carcinomas originating from follicular cells: papillary carcinoma and follicular carcinoma (the latter is comparatively rare). Follicular carcinoma is thought to arise from preexisting follicular adenoma, although this has not yet been confirmed, whereas the precursor lesion of papillary carcinoma has not been identified (Fagin, 2000). Generally, the malignant potential of these carcinomas is limited. This is particularly true for papillary microcarcinoma, which is papillary carcinoma of 1.0 cm or less in maximum diameter. For such cases, even observation without surgical treatment on diagnosis is acceptable as a therapeutic strategy, if the case does not show clinically apparent lymph node metastasis by ultrasonography, or if the located tumor is adjacent to trachea and/or recurrent nerve. The prognosis of these microcarcinomas is good because most only slowly grow or remain latent (Ito, Uruno, *et al.*, 2003). However, Sakamoto *et al.* (1983) showed that papillary or follicular carcinomas with a solid, trabecular or scirrhous growth pattern showed somewhat worse clinical outcomes than those with pure papillary or follicular structures, and they hypothesized that these cases are in the process of dedifferentiating. They designated them as poorly differentiated carcinomas. Furthermore, when these carcinomas completely dedifferentiate and become anaplastic (undifferentiated), their growing activity increases excessively and patients usually show dire prognosis, even with various therapeutic strategies (Aldinger *et al.*, 1978). These polarized characteristics of thyroid carcinoma have prompted many researchers to study the differences between the biological features of undifferentiated carcinoma and others.

When expression of GnT-V and matriptase was investigated in a variety of cancer cell lines by western blot, their expression levels were highly correlated with each other in gastric cancer, colon cancer, and thyroid cancer cell lines, but not in hepatoma and pancreatic cancer cell lines (manuscript in preparation, Akinaga A, Watanabe Y, Ihara S, Taniguchi N, Miyoshi E). In the present study, immunohistochemical analysis was performed using 132 cases of thyroid cancers to determine the relationship between GnT-V and matriptase expressions. The molecular basis analysis on specifically GnT-V and matriptase expression were investigated in thyroid cancer tissue, and clinical significance of expression was investigated in terms of correlations with histology and biological aggressiveness of thyroid cancers.

Results

Immunohistochemistry

We immunohistochemically investigated GnT-V and matriptase expressions in various thyroid neoplasms, originating from follicular cells. In normal follicular cells, the expression of GnT-V was not observed by the GnT-V antibody used in this study (Fig. 1A) and the expression of matriptase was faint (Fig. 2A).

In all thyroid neoplasms examined, GnT-V and matriptase expressed these proteins in various quantities. We compared the levels of GnT-V and matriptase expressions with histology of thyroid neoplasms. High level of GnT-V expression was observed in five of 13 follicular adenomas (38.5%), two of the 23 follicular carcinomas (8.7%), and 38 of the 68 papillary carcinomas (55.9%) (Figure 1B and C). However, none of the 28 anaplastic carcinomas were classified into the group of high GnT-V expression (Figure 1D, Table I). The incidence of high expression of GnT-V in papillary carcinoma was significantly higher than that in anaplastic carcinoma and follicular carcinoma ($p < 0.0001$). Table II summarizes the relationship between GnT-V expression and clinicopathological parameters of papillary carcinoma. Cases of 1.0 cm or less in maximum diameter, which are classified as microcarcinoma, showed higher GnT-V expression levels than cases of larger size ($p = 0.0171$). Furthermore, the GnT-V levels were significantly higher in cases without poorly differentiated lesion than in those with the lesion ($p = 0.0035$).

High expression for matriptase was observed in two follicular adenomas (15.4%) and 30 papillary carcinomas (44.1%). The intensity of signal for matriptase did not vary between the invasive fronts and central parts of carcinoma nests. None of the follicular carcinomas or anaplastic carcinomas showed high expression of matriptase (Table III).

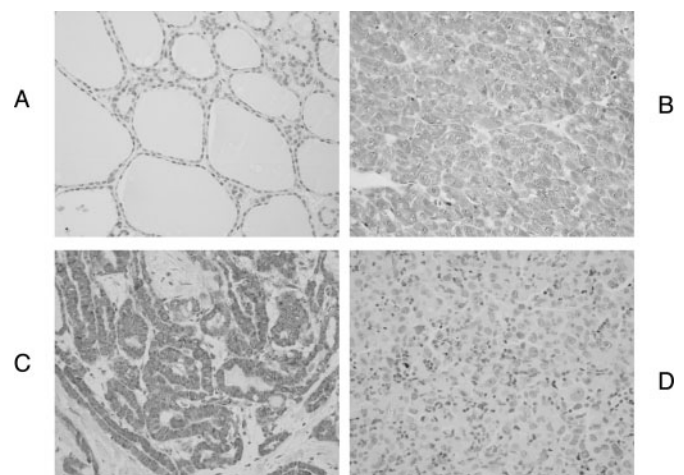


Fig. 1. Immunohistochemistry of GnT-V in thyroid cancer tissues. Immunostaining of GnT-V was performed as described in *Materials and Methods*. Panel A indicates normal thyroid tissue negative for GnT-V, panel B indicates high GnT-V expression in follicular carcinoma, panel C indicates high GnT-V expression in papillary carcinoma, and panel D indicates anaplastic carcinoma negative for GnT-V (original magnifications $\times 450$).

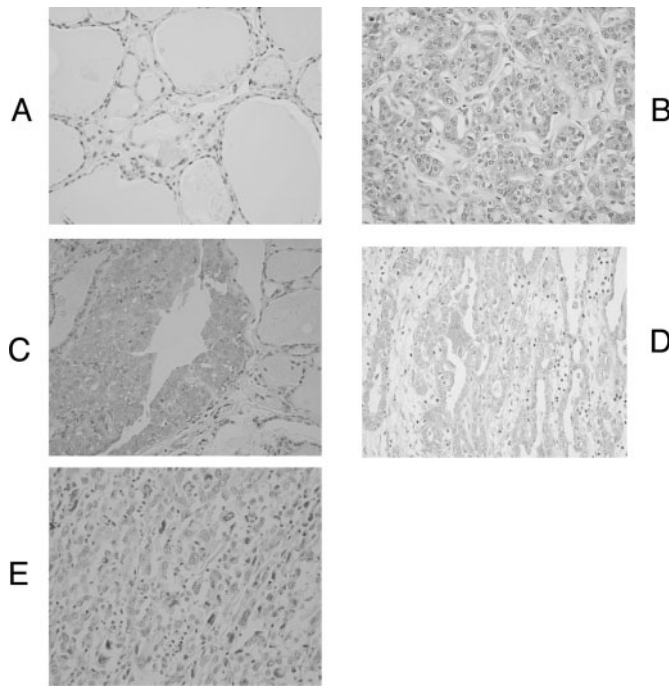


Fig. 2. Immunohistochemistry of matriptase in thyroid cancer tissues. Immunostaining of matriptase was performed as described in *Materials and Methods*. Panel **A** indicates normal thyroid tissue faintly positive for matriptase, panel **B** indicates high matriptase expression in follicular adenoma, panel **C** indicates high matriptase expression in invasive front of papillary carcinoma, panel **D** indicates high matriptase expression in the central part of papillary carcinoma nest, and panel **E** indicates anaplastic carcinoma negative for matriptase (original magnifications $\times 450$).

Table I. Expression of GnT-V in various types of thyroid tumors

Histological type	High (++)	Low		Total
		+	-	
*Anaplastic (undifferentiated) carcinoma	0	11	17	28
*^Papillary carcinoma	38	28	2	68
^Follicular carcinoma	2	8	13	23
Follicular adenoma	5	4	4	13
Total	45	51	36	132

* $p < 0.0001$, ^ $p < 0.0001$.

Similar to GnT-V expression, matriptase was inversely linked to tumor size of papillary carcinoma ($p = 0.0155$), and the presence of poorly differentiated lesions ($p = 0.0077$) (Table IV). We could not find any relationships between the expression levels of GnT-V or matriptase and other parameters such as nodal metastasis, multiple tumor formation, International Union Against Cancer (UICC) stage, extrathyroid invasion (Tables II and IV), and age at diagnosis (data not shown).

We investigated the relationship between GnT-V and matriptase expression in thyroid neoplasms. As shown in Table V, the expression of these proteins was significantly

Table II. Relationship between GnT-V expression and clinical features of 68 cases of papillary carcinoma

Clinical features	High	Low	Total
Tumor size			
≤ 1.0 cm	15	4	19
> 1.0 cm	23	26	49
	$p = 0.0171$		
Lymph node metastasis			
Absent	18	18	36
Present	18	12	30
	NS (two cases unknown)		
Multiple tumor formation			
Absent	15	10	25
Present	23	20	43
	NS		
UICC stage			
I	20	8	28
II	9	11	20
III or IV	9	11	20
	NS		
Extrathyroidal invasion			
Absent	31	19	50
Present	7	11	18
	NS		
Poorly differentiated lesion			
Absent	32	15	47
Present	6	15	21
	$p = 0.0035$		
Total	38	30	68

NS, not significant.

Table III. Expression of matriptase in various types of thyroid tumor

Histological type	High (++)	Low		Total
		+	+/-	
*Anaplastic (undifferentiated) carcinoma	0	6	22	28
*^Papillary carcinoma	30	22	16	68
^Follicular carcinoma	0	3	20	23
Follicular adenoma	2	3	8	13
Total	32	34	66	132

* $p < 0.0001$, ^ $p < 0.0001$.

correlated with each other ($p < 0.0001$). This result was consistent with that of western blot analysis, using 50 cases of thyroid cancer tissues (data not shown).

RT-PCR and western blot analyses in thyroid cancer tissues

A high expression of matriptase mRNA was observed in certain cases of thyroid cancer tissues but not in normal

Table IV. Relationship between matriptase expression and clinical features of 68 cases of papillary carcinoma

Clinical features	High	Low	Total
Tumor size			
≤1.0 cm	13	6	19
>1.0 cm	17	32	49
	$p = 0.0155$		
Lymph node metastasis			
Absent	15	21	36
Present	14	16	30
	NS (two cases unknown)		
Multiple tumor formation			
Absent	12	13	25
Present	18	25	43
	NS		
UICC stage			
I	15	13	28
II	7	13	20
III or IV	8	12	20
	NS		
Extrathyroidal invasion			
Absent	24	26	50
Present	6	12	18
	NS		
Poorly differentiated lesion			
Absent	26	21	47
Present	4	17	21
	$p = 0.0077$		
Total	30	38	68

NS, not significant.

Table V. Relationship between the expressions of GnT-V and matriptase in thyroid neoplasms

		GnT-V expression			Total
		++	+	-	
Matriptase expression	++	27	5	0	32
	+	6	22	6	34
	+/-	12	24	30	66
	Total	45	51	36	132

Data were analyzed by chi-square test.
 $p < 0.0001$.

surrounding thyroid tissues (Figure 3A). Only one case of normal thyroid tissue showed high level of matriptase mRNA (Figure 3A, lane 1). In this case, cancer cells were found to infiltrate in normal thyroid tissues under microscopic observation. Figure 3B shows a representative data for western blotting of matriptase and GnT-V. Densitometry analysis using 14 samples of papillary carcinomas

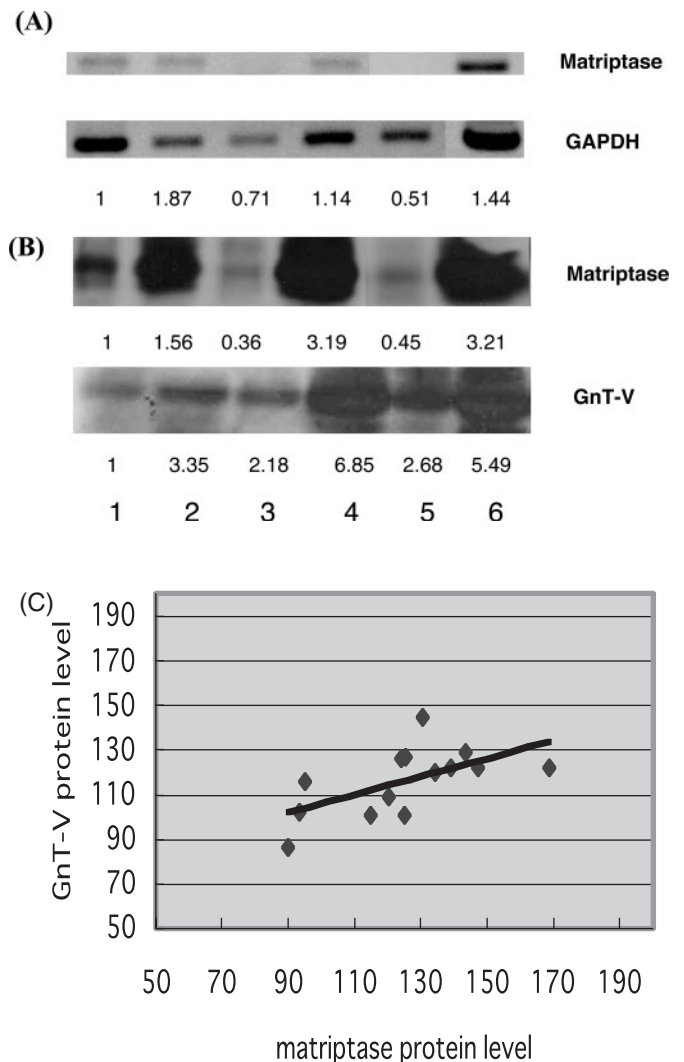


Fig. 3. RT-PCR analysis of matriptase mRNA and western blotting of matriptase on papillary carcinoma and their surrounding tissues. Panel A, One microgram of total RNA extracted from thyroid cancer tissues was used for cDNA synthesis. Matriptase and GAPDH mRNAs were amplified by PCR as described in *Materials and Methods*. Each number indicates relative intensity of matriptase mRNA/GAPDH mRNA. Panel B, Western blotting of matriptase and GnT-V in papillary carcinoma and their surrounding tissues. Each number indicates relative protein levels of GnT-V and matriptase, respectively. Lanes 2, 4, and 6 indicate cancer tissues, and lanes 1, 3, and 5 indicate their surrounding tissues. Detailed procedures are described under *Materials and Methods*. Panel C, Comparison of matriptase and GnT-V protein expression. Levels of matriptase and GnT-V proteins in 14 cases of papillary carcinomas were measured by western blot and densitometry analysis.

(Figure 3C) found that the expression of matriptase was highly correlated with the expression of GnT-V ($r = 0.595$; $p < 0.03$). To quantify mRNA levels of matriptase precisely, real-time PCR was performed, using an additional 11 cases of thyroid carcinoma tissues, and then protein levels and mRNA levels of matriptase were compared (Figure 4). Protein and mRNA levels of matriptase were not correlated, particularly in the cases of cancers which expressed high levels of matriptase proteins.

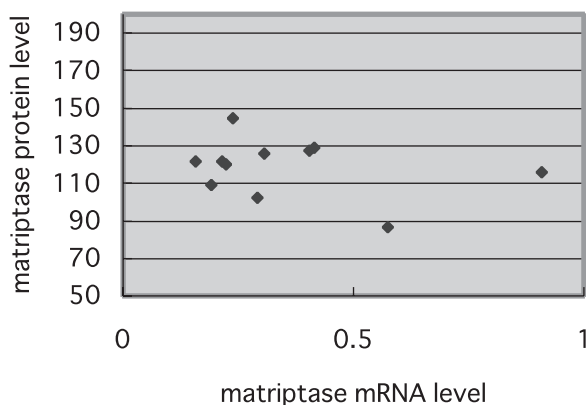


Fig. 4. Comparison of matriptase mRNAs and proteins. Expression of matriptase mRNAs was measured by real-time PCR. Relative levels of matriptase mRNA detected by real-time PCR and relative levels of its protein measured by western blot were compared. Eleven cases of cancer samples were used in this analysis. Details of the procedure are described under *Materials and Methods*.

Discussion

To determine the biological significance of oligosaccharide additions to proteins, target glycoproteins for glycosyltransferases must be investigated at the protein level, the cellular level, and the tissue level. Matriptase is a target protein for GnT-V, which has been well characterized *in vitro*. Addition of β -1-6 GlcNAc branching on matriptase leads to resistance of this protease-trypsin treatment (Ihara *et al.*, 2004), and this is proposed to modulate the metastatic potential of gastric cancer cells (Ihara *et al.*, 2002). These data prompted us to perform immunohistochemical study for matriptase and GnT-V in cancer tissues. We observed expression of matriptase to be highly correlated with GnT-V levels (Table V). Until recently, no common transcriptional factors that regulate gene expression of matriptase and GnT-V have been identified. Overexpression of GnT-V on KAK-1 cells induced delayed degradation of matriptase, suggesting that aberrant glycosylation of matriptase by GnT-V could be involved in the high correlation of the two gene products in thyroid cancer tissues (data not shown).

In cases of thyroid carcinomas, which expressed high levels of matriptase mRNA, matriptase protein level was also increased. However, although expression of matriptase mRNA was only moderate, some cases showed high levels of matriptase protein expression. These cases also showed high expression of GnT-V (seven in 50 cases). Twelve cases, which expressed high level of GnT-V protein but little of matriptase mRNA, showed no expression of matriptase protein. When expression of matriptase and GnT-V was investigated by western blot analysis, they were highly correlated with each other ($p < 0.001$) (Figure 3C). However, there was no significant correlation between matriptase protein and its mRNA expression (Figure 4). These data suggested that expression of matriptase may be regulated by both transcriptional controls and posttranslational modification by GnT-V, with the latter apparently more important in thyroid cancer tissues.

The significance of GnT-V and matriptase expressions in thyroid neoplasm seems unique. In papillary carcinoma,

the incidences of increased expression of GnT-V and matriptase were high and 55.9% and 44.1% belonged to the high group. We then compared their expression with various clinicopathological parameters of papillary carcinoma. To date, several markers reflecting the biological aggressiveness of papillary carcinoma have been proposed. Among them, it is undoubted that tumor size is a prominent factor, because, as described in *Introduction*, most papillary carcinoma measuring 1 cm or less remain latent, and even observation without surgical treatment is acceptable as a therapeutic strategy (Sakamoto *et al.*, 1983). Interestingly, of the 19 cases of microcarcinoma, 15 (78.9%) were positive for GnT-V and 13 (68.4%) for matriptase. Therefore, it is not likely that the prominent significance of matriptase expression is to promote invasion and metastasis of papillary carcinoma. Rather, GnT-V-matriptase cascade is required in early phase of papillary carcinoma progression, possibly including the process of malignant transformation itself. This result was in sharp contrast to that of FUT8 expression previously reported by us (Ito, Miyauchi, *et al.*, 2003). For follicular carcinomas, the incidence of high GnT-V or matriptase expression was significantly lower than that for papillary carcinoma, which reflects the different entity of these two types of thyroid carcinomas with the same origin, normal follicular cells. Furthermore, we could not establish any significant difference between follicular adenoma and carcinoma, suggesting that these enzymes contribute to the progression of follicular tumor to some extent, although their roles are less important, compared with papillary carcinoma.

Another interesting finding is the relationship between expression levels of GnT-V/matriptase and carcinoma dedifferentiation. As undifferentiated thyroid carcinomas display even the most rapid progressive character of all human malignancies (Sakamoto *et al.*, 1983), dedifferentiation is definitely the most important prognostic factor. However, interestingly, in the cases of poorly differentiated and undifferentiated (anaplastic) carcinomas, which show local invasion and distant metastasis much more frequently and massively than in those before the progression, GnT-V and matriptase expressions significantly decreased. This finding was also observed for another glycosyltransferase, FUT8 (Ito, Miyauchi, *et al.*, 2003). Such findings may reflect the unusual biological characteristics of anaplastic thyroid carcinoma, such as loss of epithelial differentiation; matriptase expression has been previously observed only in epithelial tissues and carcinomas (Oberst *et al.*, 2001, 2003). In thyroid cancer cell lines, matriptase protein was not observed in thyroid cancers which did not express GnT-V, even though they expressed matriptase mRNA (data not shown). These data suggest that regulation of matriptase expression was observed in both *in vivo* and *in vitro* systems.

Recent studies showed that some morphological findings reflect the aggressive characteristics and even worse prognosis of papillary carcinoma. For example, Moreno *et al.* (1996) demonstrated that encapsulated papillary carcinoma, a special histological type of papillary carcinoma, shows an excellent prognosis. Kakudo *et al.* (2004) showed that loss of cellular polarity as well as the invasive pattern can be markers of risk of recurrence. Furthermore, according to Falvo's reports, histological vascular invasion may

be considered as a predictor of hematogenic invasion and metastasis (Falvo *et al.*, 2005). Further studies comparing the expression level and localization of matriptase as well as GnT-V and these markers may be important to study the functions of these enzymes in papillary thyroid carcinoma.

In summary, we demonstrated for the first time a direct relationship between matriptase and GnT-V in human cancer tissues, which could result from prolonged stability of matriptase because of aberrant glycosylation by GnT-V.

Materials and Methods

Tissue specimens

Tissue specimens of thyroid neoplasms were obtained from 132 patients who underwent surgery at Kuma Hospital. They consisted of 28 anaplastic carcinomas, 68 papillary carcinomas, 23 follicular carcinomas, and 13 follicular adenomas. These samples were used in subsequent immunohistochemical studies. The other 14 papillary thyroid carcinoma samples, were used for real time PCR and Western blot analyses. For immunohistochemical study, tissues were fixed with 10% formalin, followed by making paraffin-embedded blocks. For RT-PCR and western blotting, tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C until used. The present project was approved by the Ethics Committees of the two hospitals.

Antibodies

A mouse monoclonal antibody, 22G12 against human GnT-V was used in the immunohistochemical study (Murata *et al.*, 2000) and a mouse monoclonal antibody, 24D11 against human GnT-V (Ihara *et al.*, 2002) was used for Western blotting. A rat monoclonal antibody, 21-9 against human matriptase was used for Western blotting and a mouse monoclonal antibody, S5 against human matriptase was used in the immunohistochemical study (Lin *et al.*, 1997).

Immunohistochemistry

Tissue sections 4 μm thick were dewaxed and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min. After rinsing in phosphate-buffered saline (PBS) (pH 7.2), 10% bovine serum (Wako, Osaka, Japan) was applied for 20 min to block nonspecific reactions. Sections were then incubated with a primary antibody (GnT-V [2.2 mg/mL] at a dilution of 1:400, and matriptase mAb 55 [1 mg/mL] at a dilution of 1:200) at 4°C overnight. After rinsing in PBS, sections were treated with peroxidase-labeled anti-mouse and anti-rabbit immunoglobulins (Nichirei, Tokyo, Japan) for 30 min. The peroxidase reaction was visualized by incubating the sections with 0.02% 3,3'-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl (pH 7.5) buffer with 0.01% hydrogen peroxide (Nichirei). The sections were counterstained with hematoxylin. Sections for the negative control were prepared using mouse immunoglobulins instead of the primary antibody.

Immunohistochemical evaluation

We classified the results of GnT-V and matriptase expressions into three groups: (++), more than 50% of neoplastic

cells showed cytoplasmic staining as a signal for GnT-V or matriptase; (+), 10–50% of the cells were positive; and (+/-), positive carcinoma cells were less than 10% or only obscure staining was observed. We regarded (++) cases as high groups and (+) or (+/-) cases as low groups.

Statistical analyses

The chi-square test and the Fisher's exact test were adopted for analyses comparing GnT-V expression with matriptase expression, histology of thyroid neoplasms, and clinicopathological features of papillary carcinomas. A *p*-value less than 0.05 was considered to be significant.

RT-PCR

Total RNA was prepared from thyroid carcinoma and their surrounding tissues, according to the method reported by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Five cases of thyroid carcinomas were investigated by RT-PCR, and three-paired cases are indicated in Figure 3. One microgram of total RNA was reverse transcribed using Reverse Transcription System (Promega, WI) and amplified by using primers specific for matriptase (upstream: 5'-GTCACCAGCAACAGCAACAA; downstream: 5'-AGGCAGCGGTAGGTGTGTTT), and for GAPDH (upstream: 5'-AACGGGAAGCTTGTTCATCAAT; downstream: 5'-GCAGTGAGCTTCCCGTTCA). Polymerase chain reaction (PCR) was performed for 40 cycles (denaturation at 94°C for 1 min, with annealing at 52°C for 1 min, elongation at 1 min, and with a final extension cycle at 72°C for 5 min). The expected amplification products of matriptase (512 bp) and GAPDH (501 bp) were electrophoresed on 0.9% agarose gel containing ethidium bromide.

Quantitative real-time PCR

Total RNA was extracted from frozen thyroid neoplasms from 11 patients (11 follicular carcinomas) using Mixer Mill MW300 (Retsch, Haan, Germany) and EZ1 RNA Universal Tissue Kits (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. RNA concentrations were measured using ND-1000 (Nanodrop, Wilmington, DE). The cDNAs were synthesized using an SYBY RT-PCR Kit (Perfect Real Time, Takara-bio Inc., Shiga, Japan), Reverse Transcription Reagent (Takara-bio Inc.) according to the manufacturer's manual. A random hexamer was used for cDNA synthesis. Real-time PCR was performed using the SYBR RT-PCR Kit (Takara) and was analyzed on Smart Cycler II System (Cepheid, Sunnyvale, CA). The following primer was used: for matriptase sense 5'-CGCGG GACTCAAGTACAACCTC-3' and antisense 5'-GACGTT GTTGACTGGCAGGA-3'; for GnT-V sense 5'-TCACTC CGTGGAAAGTTGTCCT-3' and antisense 5'-TGAGTTC GCTGCTGGATGGT-3'. The relative mRNA levels of each molecule were normalized to the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and were amplified using the primers, sense 5'-ATTGCCCTCAAC GACCACTT-3' and antisense 5'-AGGTCCACCACCCT GTTGCT-3'. The condition for the SYBR RT-PCR was as follows: 95°C for 10 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 20 sec, followed by melt curve step from 60 to 95°C at $0.2^{\circ}\text{C}/\text{sec}$. The endpoint used in the real-time

PCR quantification, Ct, was defined as the PCR cycle number that crossed an arbitrarily selected signal threshold. The levels of gene expression were determined using a Delta Delta Ct method (Livak K.J. et al., 2001.).

Western blot analysis

Thyroid tissue was homogenized in ice-cold NP-40 buffer (10 mM Tris-HCl, pH 7.8, 1% Nonidet P-40, 0.15 M NaCl, 1 µg/mL aprotinin, and 10 mM benzamide). After standing 30 min on ice, the samples were centrifuged for 15 min at 15,000 rpm at 4°C. Protein concentration of the supernatant was measured by a BCA kit (Pierce, IL), using bovine serum albumin as a standard. Fifty cases of thyroid carcinoma tissues were investigated in this study. Twenty micrograms of the proteins were electrophoresed on a 10% polyacrylamide gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, MA). After blocking with PBS containing 3% skim milk, overnight at room temperature, the filter was incubated with 1:1000-diluted anti-GnT-V or matriptase antibodies for 2 h. The filter was washed thrice with Tris-buffered saline (TBS) (pH 7.2), containing 0.05% Tween 20, for 10 min each, and then incubated with TBS containing 1:2500-diluted peroxidase-conjugated goat antibody to mouse IgG (Promega) for 1 h. After washing the membrane thrice with TBS-containing 0.05% Tween 20 for 10 min each, it was developed by an enhanced chemiluminescence system (ECL; Amersham, Buckinghamshire, UK), according to the manufacturer's protocol. Detected bands were measured by densitometry, using NIH image software.

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Conflict of Interest Statement

None declared.

Abbreviations

GnT-V, UDP-N-acetylglucosamine:α-mannoside β-1,6-N-acetylglucosaminyltransferase; RT-PCR, reverse transcriptase polymerase chain reaction.

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