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

Yasuhiro Ito, Ayumi Akinaga, Kanako Yamanaka, Takatoshi Nakagawa, Akihiro Kondo, Robert B. Dickson, Chen-Yong Lin, Akira Miyachi, Naoyuki Taniguchi, and Eiji Miyoshi

2Department of Surgery, Kuma Hospital, 8-2-35 Shimoyamate-dori, Chuo-ku, Kobe 650-0011, Japan; 3Department of Biochemistry, Osaka University Graduate School of Medicine, 2-2 Yamadaoka Suta, Osaka 565-0871, Japan; 4CREST, JST, 4-1-8 Honcho Kawaguchi, Saitama, 332-0012, Japan; 5Department of Glyco-therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka Suta, Osaka 565-0871, Japan; 6Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, DC 20057-1421

Received on July 5, 2005; revised on January 23, 2006; accepted on January 27, 2006

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 are 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 of 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 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 than 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)
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.

(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 ×450).

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).
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, extrathyroidal 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 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 I.** Expression of GnT-V in various types of thyroid tumors

<table>
<thead>
<tr>
<th>Histological type</th>
<th>High (++)</th>
<th>Low</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaplastic (undifferentiated) carcinoma</em></td>
<td>0</td>
<td>11</td>
<td>17</td>
</tr>
<tr>
<td><strong>Papillary carcinoma</strong></td>
<td>38</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td><em>Follicular carcinoma</em></td>
<td>2</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>51</td>
<td>36</td>
</tr>
</tbody>
</table>

\({*p < 0.0001, ^p < 0.0001.}\)

---

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

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>High</th>
<th>Low</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size (\leq 1.0 \text{ cm})</td>
<td>15</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>(&gt; 1.0 \text{ cm})</td>
<td>23</td>
<td>26</td>
<td>49</td>
</tr>
<tr>
<td>(p = 0.0171)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>18</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Present</td>
<td>18</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>NS (two cases unknown)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple tumor formation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>15</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Present</td>
<td>23</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UICC stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>20</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>II</td>
<td>9</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>III or IV</td>
<td>9</td>
<td>11</td>
<td>20</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extrathyroidal invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>31</td>
<td>19</td>
<td>50</td>
</tr>
<tr>
<td>Present</td>
<td>7</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated lesion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>32</td>
<td>15</td>
<td>47</td>
</tr>
<tr>
<td>Present</td>
<td>6</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>(p = 0.0035)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>30</td>
<td>68</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Histological type</th>
<th>High (++)</th>
<th>Low</th>
<th>+/– Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anaplastic (undifferentiated) carcinoma</em></td>
<td>0</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td><strong>Papillary carcinoma</strong></td>
<td>30</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>+Follicular carcinoma</td>
<td>0</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>Follicular adenoma</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td>34</td>
<td>66</td>
</tr>
</tbody>
</table>

\(*p < 0.0001, ^p < 0.0001.\)

---

**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 ×450).
Matriptase and GnT-V in thyroid cancer

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

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

NS, not significant.

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

<table>
<thead>
<tr>
<th></th>
<th>GnT-V expression</th>
<th>+/−</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matriptase expression</td>
<td>++</td>
<td>27</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>45</td>
<td>66</td>
</tr>
</tbody>
</table>

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 (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.
Addition of protein for GnT-V, which has been well characterized at the cellular level, and the tissue level. Matriptase is a target proteolytic enzyme that can be modified by glycosyltransferases, and their addition to proteins, target glycoproteins for glycosylation, and possibly influence the metastatic potential of gastric cancer cells (Ihara et al., 2004), and this is proposed to modulate the metastatic potential of gastric cancer cells (Ihara et al., 2004). 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 immuohistochemical 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 immunochemical 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, S5; 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′-GTCAAGGCAACACAGCAACAA; downstream: 5′-AGGCACGGTAGGGTGTGGTTT), and for GAPDH (upstream: 5′-AAGCAGGAGTCTTGTCAAT; downstream: 5′-GCCAGTAGCTTCCGTTCA). 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 SYBR 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′-CGCGGGACTCAAGTACAACCT-3′ and antisense 5′-GAGCTCTTGGTACCTGTTAGAG-3′; for GnT-V sense 5′-TCACTCGTTGAAAGTCTCT-3′ and antisense 5′-TGAGTTTCTGCTTGGATGGT-3′. The relative mRNA levels of each molecule were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and were amplified using the primers, sense 5′-ATTGCCTCAAACGACCCACTT-3′ and antisense 5′-AGTCCACCACACCCTGTTGCT-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°C/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

**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 benzamidine). 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 carcino-
ma tissues were investigated in this study. Twenty micro-
grams of the proteins were electrophoresed on a 10%
polyacrylamide gel and then transferred onto a polyvinylidi-
dine 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.

**Acknowledgments**

This study is supported by Grant-in-Aid for Cancer Research and
Scientific Research of Priority Areas No. 16023237 from the
Ministry of Education, Science, Culture, Sports, the
21st Century COE program and Technology, and Japan
Science and Technology Agency (JST). This work was also
supported by NIH R01CA096851 (R.B.D and C.Y.L.).

**Conflict of Interest Statement**

None declared.

**Abbreviations**

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

**References**

plastic carcinoma of the thyroid: a review of 84 cases of spindle and

tion by acid guanidinium thiocyanate-phenol-chloroform extraction.


Falvo, L., Catania, A., D’Andrea, V., Marzullo, A., Giustini, M.C.,
and De Antoni, E. (2005) Prognostic importance of histological vascular

Granovsky, M., Fata, J., Pawling, J., Muller, W.J., Khokha, R., and

Gu, J., Nishikawa, A., Tsuruoka, N., Ohno, M., Yamaguchi, N.,
Kawagawa, K., and Taniguchi, N. (1993) Purification and characteriza-
tion of UDP-N-acetylgalcosamine:alpha-6-D-mannose beta 1-6-
acetylgalcosaminyltransferase (N-acetylgalcosaminyltransferase V)

Hakomori, S. (1989) Aberrant glycosylation in tumors and tumor-

Ihara, S., Miyoshi, E., Ko, J.H., Murata, K., Nakahara, S., Honke, K.,
of N-acetylgalcosaminyltransferase V is due to modification and
stabilization of active matriptase by adding beta-1-6 GlcNAc branch-

Ihara, S., Miyoshi, E., Nakahara, S., Sakiyama, H., Ihara, H., Akinaga, A.,
of beta-1, 6 GlcNAc branching to the oligosaccharide attached to
Asn 772 in the serine protease domain of matriptase plays a pivotal role
in its stability and resistance against trypsin. *Glycobiology*, 14, 139–146.

Ito, Y., Miyauchi, A., Yoshida, H., Urino, T., Nakano, K., Takamura, Y.,
Miya, A., Kobayashi, K., Yokozawa, T., Matsuzuka, F., and others
(2003) Expression of alpha 1, 6 fucosyltransferase (FUT8) in papillary
carcinoma of the thyroid: its linkage to biological aggressiveness and

Ito, Y., Miyoshi, E., Sakon, M., Takeda, T., Noda, K., Tsujimoto, M.,
Ito, S., Honda, H., Takemura, F., Wakasa, K., and others (2001) The
 Elevated expression of UDP-N-acetylgalcosamine: alpha mannoside
beta 1, 6-N-acetylgalcosaminyltransferase (GnT-V) is an early event in

Ito, Y., Urano, R., Nakano, K., Takamura, Y., Miya, A., Kobayashi, K.,
Yokozawa, T., Matsuzuka, F., Kuma, S., Kuma, K., and others
(2003) An observation trial without surgical treatment in patients with
papillary microcarcinoma of the thyroid. *Thyroid*, 13, 381–388.

Kakudo, K., Tan, W., Ito, Y., Mori, I., Nakamura, Y., and Miyauchi, A.
(2004) Papillary carcinoma of the thyroid in Japan: subclassification of
common type and identification of low risk type. *J. Clin. Pathol.*, 57,
1041–1046.

(1997) Characterization of a novel, membrane-bound, 80-kDa matrix-
degradating protease from human breast cancer cells. *J. Biol. Chem.*, 272,
9147–9152.

data using real-time quantitative PCR and the 2(T) (-Delta Delta C)

Encapsulated papillary neoplasm of the thyroid: retrospective clinico-

Murata, K., Miyoshi, E., Kameyama, M., Ishikawa, O., Kabuto, T., Sasaki,
Y., Hiratsuka, M., Ohigashi, H., Ishiguro, S., Ito, S., and others
(2000) Expression of N-acetylgalcosaminyltransferase V in colorectal cancer corre-
lates with metastasis and poor prognosis. *Clin. Cancer Res.*, 6,
1772–1777.

Oberst, M., Anders, J., Snow, D., Xie, B., Singh, B., Ossandon, M.,
HAI-1 are expressed by normal and malignant epithelial cells in vitro

Oberst, M.D., Singh, B., Ozdemirli, M., Dickson, R.B., Johnson, M.D.,

carcinoma of the thyroid. A clinicopathological entity for a high-risk

Shoreibah, M., Perng, G.S., Adler, B., Weinstein, J., Basu, R., Cupples, R.,
Wen, D., Browne, J.K., Buckhaults, P., Freqjen, N., and others (1993)
Isolation, characterization, and expression of a cDNA encoding N-