Expression of \( N\)-Acetylgalactosaminyltransferase V in Colorectal Cancer Correlates with Metastasis and Poor Prognosis

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ABSTRACT

\( N\)-Acetylgalactosaminyltransferase V (GnT-V) is an enzyme that catalyzes \( 1\rightarrow 6 \) branching of \( N\)-acetylgalactosamine on asparagine-linked oligosaccharides of cell proteins. Metastatic potential of various cancer cells has been shown to correlate with increase of GnT-V activity and concomitant \( 1\rightarrow 6 \) branching of \( N\)-acetylgalactosamine. However, protein expression of GnT-V in human cancer tissue and its clinical significance have not yet been demonstrated. To clarify the possible relationship between metastasis and GnT-V in human colorectal cancer, protein expression of GnT-V was studied using surgically resected specimens. We established a monoclonal antibody against GnT-V and performed immunohistochemical analysis of 103 human colorectal cancer cases. Of 103 cases, 26 cases (25.2%) showed specific expression of GnT-V in colorectal cancer tissues. The expression of GnT-V was significantly correlated with distant metastasis \((P < 0.05, \chi^2 \text{ test})\). Overall 5-year survival rate was 52.8% for GnT-V–positive patients and 81.7% for GnT-V–negative patients \((P < 0.01, \text{Log-rank test})\). We showed direct evidence for the relationship between GnT-V and metastasis in human colorectal cancer. Screening of GnT-V expression in colorectal cancer may provide useful information for prognosis of postoperative patients.

INTRODUCTION

Colorectal cancer is now treated mainly by surgery and it is important to make an early diagnosis of metastasis and a prognosis of each patient’s case after initial surgery. It will be beneficial if we can predict each patient’s prognosis, or malignant potential, from the surgical specimen obtained in the initial surgery. Although some of the clinicopathological factors, i.e., lymph node metastasis, are helpful for that purpose, it still needs to be improved.

Modification of cell surface glycoproteins is one of the critical steps for cellular transformation. In particular, branching of asparagine-linked oligosaccharides is shown to regulate metastatic potential in cancer cells (1). Among the several patterns of branching, \( 1\rightarrow 6 \) branching of \( N\)-acetylgalactosamine to \( \alpha\)-d-mannoside enhances metastasis in an experimental cancer model of mice (2). This branching is catalyzed by \( N\)-acetylgalactosaminyltransferase V (GnT-V, 3 EC 4.1.155), whose transcription is shown to be regulated by proto-oncogenes, such as the \( Ets \) family (3), \( Src \), and \( erb B2 \) (1).

The activities of GnT-V in human breast cancer tissues were measured using a synthetic substrate, and increased levels in cancer were reported (4). mRNA levels of GnT-V were elevated during the hepatocarcinogenesis of rats (5, 6), and transforming growth factor \( \beta \) was shown to stabilize mRNA of GnT-V and increase its activity in mouse melanoma cells (7). Increased activity of GnT-V in human hepatocellular carcinoma tissues also was reported recently, and positive correlation to tumor size was observed (8). However, protein expression and localization of GnT-V in human cancer tissues or even in normal tissues have not yet been reported.

Because several biochemical studies suggested that metastatic potential of cancer is in part regulated by GnT-V expression, immunohistochemical study of GnT-V in human cancer tissues should bring about much information regarding to malignant potential. Using human recombinant GnT-V protein as an antigen, we have established a monoclonal antibody against GnT-V and studied protein expression of GnT-V in human colorectal cancer tissues. The comparison of specific GnT-V expression in cancer tissues and conventional clinicopathological analysis raised a possibility of GnT-V expression as a novel, poor prognostic factor for colorectal cancer patients.

MATERIALS AND METHODS

Antibody.

A mouse monoclonal antibody against recombinant human GnT-V was made according to the standard

\[ \text{expression} \]

\[ \text{in vitro} \]

\[ \text{and in vivo} \]

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3 The abbreviations used are: GnT-V, \( N\)-acetylgalactosaminyltransferase V; TNM, tumor, node, metastasis.
Briefly, mice were immunized by recombinant GnT-V, and monoclonal antibodies for GnT-V were screened by the availability for immunohistochemistry. The antibody used in the present study recognized SKNTDFIGKPTILRELTS of the human GnT-V amino acid sequence and gave the best signal for immunohistochemistry. The positive staining was diminished with addition of the excess recombinant GnT-V.

**Tumor Samples and Patient Follow-up.** Surgical specimens of colorectal cancer patients resected at colorectal surgery service in the Department of Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases were used as histological samples. Samples were fixed with 20% formalin in PBS for 72 h and embedded with paraffin. The conventional clinicopathological features and TNM staging were obtained by the pathologists according to the standard. The patients were followed up in outpatient clinic of our hospital for more than 60 months after surgery.

**Immunohistochemistry and Pathology Review.** Immunohistochemical analysis of cancer tissue and adjacent noncancerous mucosa was performed with a monoclonal antibody against GnT-V. Incubation with the primary antibody was followed by incubation with biotinylated goat anti-mouse IgG antibody and avidin-horseradish peroxidase. 3,3′-Diaminobenzidine was used as a chromagen with hematoxylin counterstain. The sections were examined by two independent observers without prior knowledge of the clinical status of the patients.

**Statistical Analysis.** Statistical analysis was performed using χ² for independence test, Fisher’s exact probability test, or Student’s t test. For survival analysis, the Kaplan-Meier method was applied, and statistical significance was calculated using the Log-rank test. Differences were judged statistically significant if P was <0.01.

**RESULTS**

**Immunohistochemical Staining of GnT-V in Colorectal Cancer Tissues.** One hundred three patients were analyzed in this study. Figure 1A shows a typical case with positive GnT-V staining in moderately differentiated adenocarcinoma of colon and negative staining in adjacent noncancerous mucosa. There were 26 cases in which GnT-V was expressed in cancer tissues, and they were defined as “positive.” Among the 26 cases, one case had GnT-V expression both in cancer and in noncancerous mucosa. The rest of the cases (77 cases) that did not express GnT-V in cancer tissues were defined as “negative.” Among the negative cases, there was a case with GnT-V expression only in noncancerous mucosa. Golgi localization in noncancerous mucosa of that case is shown in (D).

![Fig. 1](image)

Immunohistochemical staining of GnT-V in colorectal cancer and adjacent noncancerous mucosa. Staining was performed on formalin-fixed tissues using a monoclonal antibody against human GnT-V (see Materials and Methods) detected with peroxidase-3,3′-diaminobenzidine (brownish pigment). (A) Low-power view (original magnification, 100×). (B–D) High-power views (original magnification, 400×). A typical example of “positive” expression of GnT-V, which has expression of GnT-V only in cancer tissue (right side) but not in normal adjacent mucosa (left side) (A). An example of GnT-V expression in moderately differentiated adenocarcinoma (B). An example of GnT-V expression in well-differentiated adenocarcinoma (C). GnT-V was expressed in Golgi apparatus (arrows). In contrast, GnT-V was not expressed in noncancerous mucosa (arrowheads). Of 26 positive cases, there was a case with expression of GnT-V in both cancer and noncancerous mucosa. Golgi localization in noncancerous mucosa of that case is shown in (D).
GnT-V is a member of Golgi enzymes that modulate branching of oligosaccharides in cells. In fact, an immunofluorescent study of GnT-V in B16 mouse melanoma cells showed its localization in Golgi apparatus (9). Our study also showed Golgi localization of GnT-V in human colon cancer tissues, although the structure of Golgi apparatus in cancer tissues usually is deformed because of the tumorigenesis of normal mucosa (Fig. 1, B and C). One case with expression of GnT-V both in cancer and noncancer clearly showed Golgi localization of GnT-V in noncancer mucosa (Fig. 1D).

**The Clinicopathological Features of the Patients.** Twenty-six patients had positive staining of GnT-V in cancer tissues, and clinicopathological features by GnT-V expression were analyzed (Table 1). No association was observed between GnT-V expression and patients’ gender, age, or tumor size (maximal diameter). There was also no significant association between GnT-V and tumor location or histological grade. According to the standard of TNM classification revised in 1997, lymph vessel invasion and venous invasion were assessed. No significant relationship was observed between GnT-V and these invasions.

**TNM Staging and GnT-V Expression.** The TNM classification was performed for the cases examined (Table 2). There was not a significant correlation between GnT-V and T factor (tumor depth) or N factor (lymph node metastasis). However, GnT-V expression strongly correlated with M factor (distant metastasis). Of 26 patients with positively stained GnT-V, 8 patients (31%) had distant metastasis. GnT-V has been shown to be involved in distant metastasis in an experimental animal model (2), but this is the first report to show the significant relationship between GnT-V expression and distant metastasis in cancer patients. Sites of distant metastasis in GnT-V–positive patients are shown in Table 3. There was not a significant difference in terms of sites of metastasis between GnT-V–negative (not shown) and–positive patients.

**Survival Rate and GnT-V Expression.** Five-year overall survival rate for GnT-V–negative patients was 81.7%. On the other hand, the 5-year overall survival rate for GnT-V–positive patients was 52.8%, significantly lower than that for the GnT-V–negative (P < 0.01, Log-rank test) (Fig. 2). The site(s) of metastasis, survival period, outcome, and cause of death in stages II, III, and IV patients with GnT-V–positive expression are shown in Table 3. For stage 0 and I cases, all of the patients including GnT-V–negative cases survived for more than 5 years. However, for stage II GnT-V–positive patients, 3 of 5 died within 5 years, whereas only 1 of 25 GnT-V–negative patients died within 5 years (Table 3). There was a significant difference in survival rate between GnT-V–positive and –negative in stage II (P < 0.01, Log-rank test) (Fig. 3). For those cases in stage III, the survival rate was not significantly different by GnT-V expression. In stage IV cases, 7 of 8 GnT-V–positive patients died, and 7 of 9 GnT-V–negative patients died within 5 years (not significant). These data implicate that the poor survival rate in all GnT-V–positive patients is mainly attributable to the poor survival rate in stage II patients and partly to the larger number of patients in stage IV.
Importance of GnT-V Expression in Stage II Patients.

Because there was a significant difference between GnT-V–negative (not shown) and –positive patients in stage II (P = 0.0022, Log-rank test).

Another important point in Table 4 is that GnT-V expression significantly correlated with venous invasion in stage II cases (P < 0.05, Fisher’s exact probability test). All of the patients with GnT-V–positive expression had venous invasion. Although a larger number is required to make a conclusive statement, venous invasion in GnT-V–positive patients in stage II could account for their poor prognosis.


discussion

An enzyme that specifically catalyzes β 1–6 branching of N-acetylgalactosamine is GnT-V. This enzyme transfers N-acetylgalactosamine from UDP-N-acetylgalactosamine to α-D-6 mannose of asparagine-linked oligosaccharides. The specific activity was measured using a synthetic substrate (10). Although biochemical purification (11–13) and molecular cloning (14,15) of GnT-V were achieved, immunohistochemical study and its prognostic value have not been reported thus far. This was probably because of the lack of a specific antibody against GnT-V that facilitates immunohistochemical study in formalin-fixed, paraffin-embedded specimens. The monoclonal antibody that we used in this study was screened for its titer in immunohistochemistry, and the specificity was confirmed by the competition experiments using an excess amount of recombinant GnT-V (unpublished data). This antibody prompted us to first report the expression of GnT-V in human colorectal cancer tissues. In addition, this report clearly showed the significant relationship between GnT-V expression and metastasis. As predicted by the in vitro or animal model study, GnT-V was related to the malignant potential of the colorectal cancer specimen.

Elevated levels of β 1–6 branching of N-acetylgalactosamine, transferred by GnT-V, are shown to be correlated with metastatic potential and tumor invasiveness in several reports. In most cases, phaseolus vulgaris leukoagglutinin binding was used to assess β 1–6 branching in situ. Dennis demonstrated that increased β 1–6 branching is not associated with transformation or tumorigenicity but with metastatic potential (2). An increased level of β 1–6 branching in human breast and
Table 4  Expression of GnT-V and clinicopathological features in state II colorectal cancer

<table>
<thead>
<tr>
<th></th>
<th>GnT-V Negative</th>
<th>Positive</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>21 (100%)</td>
<td>5 (100%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M/F</td>
<td>14/7</td>
<td>4/1</td>
<td>0.502</td>
</tr>
<tr>
<td>Age (yr)b</td>
<td>58 ± 13</td>
<td>62 ± 8</td>
<td>0.602</td>
</tr>
<tr>
<td>Size (mm)b</td>
<td>51.4 ± 20.0</td>
<td>29.6 ± 14.3</td>
<td>&lt;0.05 (0.023)</td>
</tr>
<tr>
<td>Locationa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>8 (38)</td>
<td>0 (0)</td>
<td>0.129</td>
</tr>
<tr>
<td>Left colon</td>
<td>7 (33)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>6 (29)</td>
<td>1 (20)</td>
<td></td>
</tr>
<tr>
<td>Histologya</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>15 (71)</td>
<td>2 (40)</td>
<td>0.277</td>
</tr>
<tr>
<td>Moderately</td>
<td>5 (24)</td>
<td>3 (60)</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>1 (5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Tc</td>
<td>12 (57)</td>
<td>3 (60)</td>
<td>0.654</td>
</tr>
<tr>
<td>Lymph vessel invasionb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>9 (43)</td>
<td>1 (20)</td>
<td>0.343</td>
</tr>
<tr>
<td>Positive</td>
<td>12 (57)</td>
<td>4 (80)</td>
<td></td>
</tr>
<tr>
<td>Venous invasiond</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>11 (52)</td>
<td>0 (0)</td>
<td>&lt;0.05 (0.046)</td>
</tr>
<tr>
<td>Positive</td>
<td>10 (48)</td>
<td>5 (100)</td>
<td></td>
</tr>
</tbody>
</table>

a Fisher’s exact P test.
b Values are means ± SD.
c Student’s t test.
d Values in parentheses are percentages.
e Well, well-differentiated; Moderately, moderately differentiated; Poorly, poorly differentiated.

GnT-V expression also is shown using phaseolus vulgaris leukoagglutinin staining that specifically binds to β 1–6 branching (16). They reported higher levels of β 1–6 branching in Dukes C cases, which have lymph node metastasis. Another report showed increase of β 1–6 branching in human esophageal carcinomas (17). They showed disposition of β 1–6 branching predominantly in the outer surface of the tumor adjacent to normal tissues, suggesting that tumor invasiveness is regulated by β 1–6 branching.

The relationship between GnT-V and malignancy also has been reported. Transcription of GnT-V is induced by src (18). GnT-V also is shown to be induced by proto-Ha-ras oncogenes in NIH3T3 cells (19). There are in vitro studies to demonstrate the invasiveness and metastatic potential induced by GnT-V (reviewed in Ref. 1). The mobility of Mv1Lu, mint lung epithelial cells, was elevated by the transfection of the GnT-V gene (20). GnT-V expression causes decreased adhesion to fibronectin and collagen type IV. Transfection of GnT-V into mouse mammary cancer cells resulted in increased lung metastasis (21). The oncogene her-2/neu induces GnT-V expression and increases its oligosaccharide products (22). These data supported the positive correlation in our report between metastasis and GnT-V expression in colorectal cancer patients.

The mechanisms underlying this relationship can be speculated by recent advances in glycoprotein biology. Beta 1–6 branching structure, a product of GnT-V, is a good substrate for attachment of poly-N-acetyllactosamine, whose synthesis is controlled by complemental branch specificity of i-extension enzyme and β 1–4 galactosyltransferase I (23). This structure is preferentially fucosylated to form sialyl Lewis X, a ligand for selectin in vascular endothelial cells. The level of poly N-acetyllactosamine is increased in highly metastatic colon cancer cells (24) and the level of sialyl Lewis X expression is correlated with poor survival in human colon cancer patients (25, 26). From these points, GnT-V may induce tumor metastasis through the formation of sialyl Lewis X on tumor cell surface.

Recently, in pancreatic carcinoma, increased activity of GnT-V is shown (27). Yao et al. (8) also reported that increased activity of GnT-V was observed in hepatocellular carcinoma. They showed the relationship of GnT-V activity and tumor size assessed by the TNM classification and suggested that GnT-V activity correlated with progression of hepatocellular carcinoma. Our report of colorectal cancer did not show the relationship between tumor size and GnT-V expression. In stage II, we observed even a negative correlation between size (maximal diameter) and GnT-V expression. This is partly because of difference in tumor progression between hepatocellular and colon carcinoma and partly because of the discrepancy between the activity and enzyme expression of GnT-V.

The survival rate of GnT-V–positive patients was significantly less than that of GnT-V–negative patients. This is mainly attributable to the poor prognosis of GnT-V patients in stage II. Colorectal cancer patients in stage II by TNM staging are defined as those negative for lymph node metastasis and free from distant metastasis. Causes of death in stage II are mostly distant metastasis, probably via venous invasion of cancer cells at original sites (28). The relationship between venous invasion and GnT-V expression and concomitant poor prognosis in stage II patients may provide insights for molecular mechanisms involved in venous invasion. Because overall survival rate is 80% or more in stage II colorectal cancer patients, it is a critical issue whether the adjuvant chemotherapy is necessary or not for those patients (29–31). The standard to select patients with poor prognosis in stage II is not currently established. The expression of GnT-V in resected specimen will not only help such selection but also give us information about patients’ prognosis so that intensive follow-up may be done.

Clinical trials are ongoing to test the possibility of swainsone, an inhibitor for expression of β 1–6 branched oligosaccharides, as an anticancer drug (32, 33). The effects of GnT-V transfection are blocked by swainsone (20). Swainsone inhibits basement membrane invasion of metastatic tumor cells with β 1–6 branching (34). Swainsone (35) also has been shown to reduce tumor metastasis, enhance cellular immune responses, and reduce solid tumor growth in mice. Screening of GnT-V expression in resected cancer tissues may be able to identify postoperative patients eligible for such an inhibitor.

All of the patients in this study were examined for distant metastasis preoperatively and diagnosed for M factor. However, information about GnT-V expression in resected specimens would encourage physicians to detect distant metastasis after surgery because most cases of the distant metastasis after surgery are considered to exist before surgery.

In conclusion, the data we showed raise the possibility of GnT-V expression as a novel prognostic factor for colorectal cancer. GnT-V expression may help physicians to predict metastatic recurrence in stage II, postoperative colorectal cancer patients.
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REFERENCES


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