Expression of N-Acetylglucosaminyltransferase V Is Associated with Prognosis and Histology in Non-Small Cell Lung Cancers

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ABSTRACT

Purpose: N-Acetylglucosaminyltransferase V (GnT-V), a key enzyme in the formation of branching of asparagine-linked oligosaccharides, is strongly linked to tumor invasion and metastasis of colon and breast cancers. However, GnT-V is expressed in many tissues, including normal lung. GnT-V expression has not been examined previously in human lung cancers. The objective of this study is to examine GnT-V expression in non-small cell lung cancers (NSCLCs) and to determine its relationship to biological and clinicopathological characteristics and prognosis.

Experimental Design: GnT-V expression was studied by immunohistochemistry in 217 surgically resected NSCLCs and analyzed statistically in relation to various characteristics.

Results: High GnT-V expression was found in 113 (52.1%) NSCLCs, and low GnT-V expression was found in 104 (47.9%) NSCLCs. Multivariate logistic regression analysis revealed a significant association between low GnT-V expression and squamous cell carcinomas, as compared with nonsquamous cell carcinomas (P = 0.02). Among biological characteristics of tumors, Ki-67 labeling index was higher in tumors with low GnT-V expression than in those with high GnT-V expression, although this difference was not statistically significant (P = 0.09). Patients with tumors having low GnT-V expression had significantly shorter survival time than patients with tumors having high GnT-V expression in 103 patients with pStage I NSCLCs (5-year survival rates, 49% and 86%, respectively; P = 0.0009), as well as in 59 patients with pStage I non-squamous cell carcinomas (5-year survival rates, 54% and 89%, respectively; P = 0.007).

Low GnT-V expression was a significant unfavorable prognostic factor in pStage I NSCLCs (hazard ratio, 2.86; P = 0.002) and in pStage I nonsquamous cell carcinomas (hazard ratio, 3.02; P = 0.02). Furthermore, β1–6 branching of asparagine-linked oligosaccharides, which are products of GnT-V, were increased highly or moderately in 8 of 10 tumors with high GnT-V expression, as judged by leukoagglutinating phytohemagglutinin staining.

Conclusions: GnT-V expression is associated with histology in NSCLCs. Low GnT-V expression is associated with shorter survival and poor prognosis in pStage I overall NSCLCs and non-squamous cell carcinomas.

INTRODUCTION

Lung cancer is one of the leading causes of cancer death throughout the world. Although the management and treatment of non-small cell lung cancers (NSCLCs) have improved, there is no evidence to suggest that therapeutic advances have resulted in a marked increase of survival rates, and the overall 5-year survival rate remains <15% (1, 2). The clinical observations that patients with NSCLCs in comparable stages may have different clinical courses and may respond differently to similar treatments have yet to be fully understood. A more sophisticated understanding of the pathogenesis and biology of these tumors (3, 4) could provide useful information for predicting clinical outcome, individualizing treatment (5–8), and identifying molecular targets of the treatment (9).

Oligosaccharides on glycoproteins are altered in tumorigenesis, and they often play a role in the regulation of the biological characteristics of tumors (10). Each oligosaccharide is synthesized by a specific glycosyltransferase, the expression of which affects specific functions of glycoproteins through glycosylation in normal and malignant cells (11). Among many glycosyltransferases, N-acetylglucosaminyltransferase V (GnT-V), a key enzyme in the formation of branching of asparagine-linked oligosaccharides, is the most strongly linked to tumor invasion and metastasis in cancers of the colon and breast (12–14). In such organs, normal epithelial cells do not express GnT-V (14) or β1–6 branching asparagine-linked oligosaccharides, which are synthesized by GnT-V (15). On the other hand, GnT-V has been shown to be expressed in many mouse tissues, including normal lung (16). In addition, expression of β1–6 branching asparagine-linked oligosaccharides, which are synthesized by GnT-V, is found in normal human bronchial epithelial cells and alveolar pneumocytes (15). However, GnT-V expression has not been examined previously in human lung cancers.

In the present study, we examined GnT-V expression by immunohistochemistry in surgically resected NSCLCs and an-
alyzed its biological and clinical importance, especially as a potential prognostic factor.

MATERIALS AND METHODS

Tumor Specimens and Survival Data. Primary tumor specimens from 217 NSCLCs were consecutively obtained by surgery performed at the Hokkaido University Medical Hospital between 1976 and 1994. The patients with NSCLCs consisted of 145 men and 72 women. The histological classification of the tumor specimens was based on WHO criteria (17), and the specimens included 90 squamous cell carcinomas, 109 adenocarcinomas, 9 large cell carcinomas, and 8 adenosquamous cell carcinomas. For this study, non-squamous cell carcinoma included adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma. The specimens represented 120 Stage I, 18 Stage II, 71 Stage IIIa, 1 Stage IIIb, and 7 Stage IV tumors. The postsurgical pathological tumor-node-metastasis stage (pTNM) was determined according to the guidelines of the American Medical Association. Of the 120 patients with tumors, 71 Stage IIIa, 1 Stage IIIb, and 7 Stage IV tumors. The specimens included adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma. For this study, non-squamous cell carcinoma included adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma. The specimens represented 120 Stage I, 18 Stage II, 71 Stage IIIa, 1 Stage IIIb, and 7 Stage IV tumors. The postsurgical pathological tumor-node-metastasis stage (pTNM) was determined according to the guidelines of the American Medical Association.

A total of 120 patients who met the following criteria: (a) survived for >3 months after surgery; (b) did not die of causes other than lung cancer within 5 years after surgery; and (c) were followed for >3 years after surgery (for patients who remained alive). Seventeen patients did not meet the above criteria (four patients died within 3 months after surgery, six died of causes other than lung cancer within 5 years, and seven had no survival records after surgery) were excluded from the survival analysis. Of the 103 patients for whom survival was analyzed, 54 patients had died of cancer. Of these 54 patients, 27 had squamous cell carcinomas, 22 had adenocarcinomas, 3 had large cell carcinomas, and 2 had adenosquamous cell carcinomas. Karnofsky performance status was 90% or greater in all 103 patients for whom survival was analyzed. This study was approved by the Medical Ethical Committee of Hokkaido University School of Medicine. Because all patients were coded, they could not be individually identified.

Immunohistochemistry for GnT-V. GnT-V expression was analyzed by immunohistochemistry. The labeled streptavidin biotin method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. Briefly, deparaffinized tissue sections were incubated with normal rabbit serum at room temperature to block nonspecific antibody binding sites. The sections were consecutively reacted with a mouse monoclonal antibody against recombinant human GnT-V (1:400 dilution; Ref. 14) or with control mouse isotype-specific immunoglobulin at 4°C overnight. Immunostaining was performed by the biotin-streptavidin immunoperoxidase method with 3,3′-diaminobenzidine as a chromogen (19). The sections were counterstained with hematoxylin.

Immunohistochemistry. Expression of Ki-67, p27KIP1, Cyclin E, and GalNAc-T3. Expression of Ki-67, p27KIP1, cyclin E, and GalNAc-T3 was analyzed by immunohistochemistry. For these proteins, the slides and results that were reported previously (20–21) were used for the present study. The methods for the staining of these proteins in resected tumors have been described previously (20–21). The labeled streptavidin biotin method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. The primary antibodies were mouse monoclonal MB-1 antibody (Immuno-Tech, Marseille, France), a mouse monoclonal anti-human p27KIP1 antibody (clone 1B4; Novocastra, Newcastle, United Kingdom), a mouse monoclonal antihuman cyclin E antibody (HE12; PharMingen, San Diego, CA), and a rabbit polyclonal antibody against a synthesized peptide of human GalNAc-T3 (21).

Leukoagglutinating Phytohemagglutinin (L-PHA) Histochecmy. Expression of β1-6 branching asparagine-linked oligosaccharides was analyzed by L-PHA histochemistry. The labeled streptavidin biotin method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization, as described previously (22). Briefly, trypsinization was done in Tris buffer containing 0.1% trypsin (Difco Laboratories, Detroit, MI) and 0.1% CaCl2 for 10 min at 37°C after blocking endogenous peroxidase activity. To remove sialic acids from the terminal residues of L-PHA-reactive oligosaccharides, the sections were treated with neuraminidase from Vibrio Cholerae (Roche, Tokyo, Japan) at a concentration of 0.1 unit/ml in sodium acetate buffer (pH 5.6) containing 0.04 M CaCl2 for 1 h at 37°C. The sections were incubated with 5% skim milk in PBS for 20 min at room temperature to block nonspecific staining. The sections were incubated with biotinylated L-PHA lectins (E. Y. Laboratories Inc., San Mateo, CA) at a dilution of 1:500 at 4°C overnight. Staining was performed by the biotin-streptavidin peroxidase method with 3,3′-diaminobenzidine as a chromogen (Nichirei). Hematoxylin was used for counterstain.

L-PHA binding reactivity was classified as high, moderate, or low, according to the proportion of positively stained cancer cells (≥30%, between 10% and 30%, or <10%, respectively).

Statistical Analysis. The associations between GnT-V expression and categorical variables were analyzed by the χ2 test or Fisher’s exact test, as appropriate. The associations between GnT-V expression and age or Ki-67 labeling index (LI) were analyzed by Student’s t test. To simultaneously examine the effect of more than one factor on GnT-V expression, multivariate logistic regression analysis was used (23). The survival curves were estimated using the Kaplan-Meier method, and differences in survival distributions were evaluated by the general Wilcoxon test. Cox’s proportional hazards modeling of factors potentially related to survival was performed to identify which factors might have a significant influence on survival. P < 0.05 were considered statistically significant. All tests were two-sided.
RESULTS

Typical immunostaining patterns for GnT-V in normal bronchial tissue and NSCLCs are shown in Fig. 1. Normal bronchial epithelial cells, bronchial gland cells, and alveolar pneumocytes (data not shown) showed GnT-V expression, consistent with previous findings that GnT-V is expressed in normal mouse lung (16) and that β1–6 branching oligosaccharides synthesized by GnT-V are found in normal bronchial epithelial cells and alveolar pneumocytes (15). In cancer cells, GnT-V expression was found diffusely in the cytoplasm or localized in the Golgi apparatus, as reported previously for colon cancers (14).

High GnT-V expression was found in 113 (52.1%) NSCLCs, and low GnT-V expression was found in 104 (47.9%) NSCLCs (Table 1). Low GnT-V expression was significantly more prevalent in tumors from men than in those from women \((P = 0.009)\), in tumors from smokers compared with nonsmokers \((P = 0.04)\), and in squamous cell carcinomas compared with non-squamous cell carcinomas \((P = 0.003)\) by the \(\chi^2\) test (Table 1). GnT-V expression was not associated with pTNM classifications or pStage. Multivariate logistic regression analysis for the correlation between GnT-V expression and various characteristics showed a significant association between low GnT-V expression and squamous cell carcinomas \((P = 0.02; \text{Table 2})\).

Among biological characteristics of tumors studied previously in this cohort of NSCLCs (19–21), Ki-67 LI was higher in tumors with low GnT-V expression than in those with high GnT-V expression, although this difference was not statistically significant \((P = 0.09; \text{Table 3})\). Low GalNAc-T3 expression was significantly more prevalent in tumors with low GnT-V expression than in those with high GnT-V expression \((P = 0.0001)\). There were no differences in p27 KIP1 LI and cyclin E LI between tumors with low GnT-V expression and those with high GnT-V expression.

We next analyzed the relationship between GnT-V expression and patient survival (Fig. 2) and the importance of GnT-V as a prognostic factor (Table 4) in pStage I disease. In 103 patients with pStage I NSCLCs, patients with tumors having low GnT-V expression survived a significantly shorter time than patients with tumors having high GnT-V expression (5-year survival rates, 49% and 86%, respectively; \(P = 0.001; \text{Fig. 2A}\)). Low GnT-V expression was the only significant unfavorable prognostic factor (hazard ratio, 2.86; \(P = 0.002\)) found in our analysis (Table 4A). Squamous cell carcinomas and non-squamous cell carcinomas were analyzed separately because histology was significantly correlated with GnT-V expression in the multivariate logistic regression analysis (Table 2). In 59 patients with pStage I non-squamous cell carcinomas, patients with

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Fig. 1 Immunohistochemical staining patterns for N-acetylglucosaminyltransferase V (GnT-V) in normal bronchial tissue and non-small cell lung cancers. Normal bronchial epithelial cells (A) and bronchial gland cells (B) show GnT-V expression. An adenocarcinoma tumor shows high GnT-V expression diffusely in the cytoplasm (C), and a squamous cell carcinoma tumor shows low GnT-V expression (D). Scale bar = 20 µm.
tumors having low GnT-V expression survived a significantly shorter time than patients with tumors having high GnT-V expression (5-year survival rates, 47% for low GnT-V expression and 75% for high GnT-V expression; \( P = 0.02 \)) and was not a prognostic factor (\( P = 0.1 \)). We examined the expression of \( \beta 1-6 \) branching asparagine-linked oligosaccharides by L-PHA histochemistry in 10 randomly selected NSCLCs with high GnT-V expression and 10 randomly selected NSCLCs with low GnT-V expression to determine whether GnT-V expression resulted in the synthesis of \( \beta 1-6 \) branches.
Table 4  Cox’s proportional hazards model analysis of prognostic factors in patients with pStage I NSCLCs

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Overall NSCLCs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>0.63</td>
<td>0.35–1.16</td>
<td>0.1</td>
</tr>
<tr>
<td>Age (≥65 yrs/&lt;65 yrs)</td>
<td>0.75</td>
<td>0.43–1.31</td>
<td>0.3</td>
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<tr>
<td>Chemotherapy</td>
<td>1.24</td>
<td>0.72–2.15</td>
<td>0.4</td>
</tr>
<tr>
<td>Histology (non-squamous(\textsuperscript{c})/ squamous)</td>
<td>1.34</td>
<td>0.77–2.35</td>
<td>0.3</td>
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<tr>
<td>Differentiation (moderate, poor/well)</td>
<td>0.89</td>
<td>0.44–1.80</td>
<td>0.8</td>
</tr>
<tr>
<td>pT classification (pT(\textsubscript{1}/\textsubscript{2}))</td>
<td>1.15</td>
<td>0.66–2.00</td>
<td>0.6</td>
</tr>
<tr>
<td>GnT-V expression (high/low)</td>
<td>2.86</td>
<td>1.43–5.56</td>
<td>0.002</td>
</tr>
<tr>
<td>B. Non-squamous cell carcinomas(\textsuperscript{d})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>0.72</td>
<td>0.33–1.55</td>
<td>0.4</td>
</tr>
<tr>
<td>Age (≥65 yrs/&lt;65 yrs)</td>
<td>0.70</td>
<td>0.32–1.51</td>
<td>0.8</td>
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<tr>
<td>Chemotherapy</td>
<td>1.42</td>
<td>0.63–3.20</td>
<td>0.4</td>
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<tr>
<td>Differentiation (moderate, poor/well)</td>
<td>0.96</td>
<td>0.38–2.43</td>
<td>0.9</td>
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<tr>
<td>pT classification (pT(\textsubscript{1}/\textsubscript{2}))</td>
<td>1.33</td>
<td>0.61–2.87</td>
<td>0.5</td>
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<tr>
<td>GnT-V expression (high/low)</td>
<td>3.02</td>
<td>1.19–7.69</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(\textsuperscript{a}\) NSCLC, non-small cell lung cancer; CI, confidence interval; GnT-V, N-acetylglucosaminyltransferase V.

\(\textsuperscript{b}\) Including adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma.

(24). Hence, L-PHA has been used as a reliable reagent to detect B1–6 branching oligosaccharides by histochemistry (13, 25). Among the 10 tumors with high GnT-V expression, 6 tumors had high L-PHA staining, and 2 tumors had moderate L-PHA staining. Among the 10 tumors with low GnT-V expression, 6 tumors had low L-PHA staining, and 2 tumors had moderate L-PHA staining.

DISCUSSION

In the present study, we demonstrate that GnT-V expression is decreased or lost in about half of NSCLCs, although GnT-V is expressed in bronchial epithelial cells, bronchial gland cells, and alveolar pneumocytes. Histology was significantly correlated with GnT-V expression; low GnT-V expression was more frequently found in squamous cell carcinomas than in non-squamous cell carcinomas. Furthermore, low GnT-V expression was associated with a shorter survival period and was an unfavorable prognostic factor in pStage I resected non-squamous cell carcinomas.

GnT-V expression is not equal to the expression of B1–6 branching asparagine-linked oligosaccharides analyzed by L-PHA histochemistry, because (a) GnT-V has been shown to have a function as an inducer of angiogenesis (26) that is a completely different function from the original function of glycosyltransferase, and (b) GnT-V expression does not necessarily result in the synthesis of B1–6 branching oligosaccharides, depending on the cell and tissue types (data not shown). Therefore, we analyzed the relationship between GnT-V expression and L-PHA staining in selected specimens of NSCLCs. As a result, in 8 of 10 tumors with high GnT-V expression, there was high or moderate L-PHA staining, indicating the synthesis of B1–6 branching oligosaccharides.

Interestingly, in this study, only 1 of 8 goblet cell-type adenocarcinomas (27) had high GnT-V expression (data not shown), although 70 of 109 overall adenocarcinomas had high GnT-V expression (Table 1). Goblet cell-type adenocarcinoma is supposed to be an independent subtype that is distinct from other cell types of adenocarcinoma with respect to molecular biological and immunohistochemical features (28, 29). Normal bronchial goblet cells are negative for B1–6 branching oligosaccharides synthesized by GnT-V (15). Collectively, these findings suggest cell type-specific and developmentally regulated modes of GnT-V expression. When the eight goblet cell-type adenocarcinomas were excluded from the analysis, Ki-67 LI was significantly lower in tumors with high GnT-V expression than in tumors with low GnT-V expression (mean ± SD, 34.0 ± 26.1 and 41.7 ± 28.4, respectively; \(P = 0.04\)). This finding of low Ki-67 LI in tumors with high GnT-V expression is consistent with that in hepatoma (30).

Li et al. (15) reported expression of B1–6 branching, asparagine-linked oligosaccharides, which are products of GnT-V, in almost all postmitotic, fully differentiated epithelial cell types of normal human and rat tissues, including bronchial epithelial cells and alveolar pneumocytes. Exceptions were the epithelia of the colon, esophagus, and resting mammary gland, which showed no expression of B1–6 branching oligosaccharides. Increased GnT-V activity and B1–6 branching oligosaccharides were found in human colon and breast cancers, as compared with the respective normal epithelium (12, 31). In cancers derived from these epithelia and experimental tumors, GnT-V expression has been shown to be linked to malignant transformation, invasion, and metastatic potential (25, 31–38), as well as unfavorable prognosis of patients bearing tumors (14, 27). In these tumors, glycoproteins, such as integrins (34, 39), lysosomal-associated membrane protein 2 (34, 36), and matrix metalloproteinase, have been shown to be target glycoproteins that are glycosylated by GnT-V.

However, in NSCLCs, which derive from bronchial and alveolar epithelia that normally express GnT-V, GnT-V expression was associated with favorable prognosis in this study. The biological importance of GnT-V expression for maintaining physiological function, as well as for the development and progression of cancer, may be different in each organ and tissue, depending on the biological function of target substrate glycoproteins, which can vary among organs and tissues. GnT-V expression is regulated in a tissue-specific manner (16), and certain cancer-associated loss or gain in glycosylation by GnT-V may contribute directly to cellular transformation (34). Decreased (low) expression of GnT-V may contribute to altered biological properties of NSCLCs by decreased synthesis of B1–6 branching oligosaccharides of certain target glycoproteins, resulting in a shorter survival of patients having tumors with low GnT-V expression compared with those having tumors with high GnT-V expression. The target glycoproteins of GnT-V in the lung and bronchus remain to be determined.

In conclusion, decreased GnT-V expression is found in about half of NSCLCs in association with the histology and is associated with an unfavorable clinical outcome in pStage I.
overall NSCLCs and non-squamous cell carcinomas. GnT-V expression may have great value in stratification of patients with pStage I tumors into groups at high and low risks of recurrence in NSCLCs (especially in non-squamous cell carcinomas) and thus in selecting patients who will benefit from adjuvant therapy.

Fig. 3  Immunohistochemical staining patterns for N-acetylglucosaminyltransferase V (GnT-V; A and C) and staining patterns for leukoagglutinating phytohemagglutinin (L-PHA; B and D) in non-small cell lung cancers. The staining pattern of a tumor with high GnT-V expression and high L-PHA staining is shown in A and B. Staining for a tumor with low GnT-V expression and low L-PHA staining is shown in C and D. GnT-V expression is found as Golgi localization in the cytoplasm of tumor cells (A). L-PHA-reactive glycoconjugates are found diffusely in the tumor cells (B). GnT-V expression is not found in most of the tumor cells (C), and L-PHA-reactive glycoconjugates are not found in most of the tumor cells (D). Scale bar = 20 μm.

Table 5  Relationship between GnT-V$^a$ expression and L-PHA staining in NSCLCs

<table>
<thead>
<tr>
<th>GnT-V expression</th>
<th>L-PHA staining</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>High</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$GnT-V, N-acetylglucosaminyltransferase V; NSCLC, non-small cell lung cancer; L-PHA, leukoagglutinating phytohemagglutinin.

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