

Clinicopathological Significance of Core 2 β 1,6-*N*-Acetylglucosaminyltransferase Messenger RNA Expressed in the Pulmonary Adenocarcinoma Determined by *in Situ* Hybridization¹

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ABSTRACT

Cell surface carbohydrates of epithelial cells play important roles in tumor progression. Previously, we have shown that expression of core 2 branched *O*-glycans in colorectal cancer is closely correlated with the vessel invasion and depth of invasion (K. Shimodaira *et al.*, *Cancer Res.*, 57: 5201–5206, 1997). To test whether this is also the case in human lung cancer, we have examined the expression pattern of core 2 β 1,6-*N*-acetylglucosaminyltransferase (C2GnT) mRNA responsible for the biosynthesis of core 2 branched *O*-glycans in 41 cases of lung cancer. Using *in situ* hybridization, C2GnT mRNA was detected in 73.2% of the lung cancer cells, irrespective of the histopathological type; whereas in normal lung tissues, its expression was restricted to the basal cells of bronchial mucosa. These results indicate that the expression level of C2GnT mRNA was significantly enhanced in association with malignant transformation. Statistical analysis between the C2GnT mRNA expressed in pulmonary adenocarcinoma and clinicopathological variables revealed that the expression of C2GnT was correlated with vessel invasion and lymph node metastasis with significant difference ($P < 0.05$), but expression of sialyl Le^x, which is frequently expressed in the adenocarcinoma, was not significantly correlated with lymph node metastasis. These results indicate that C2GnT mRNA detected by *in situ* hybridization reflects the malignant potentials of pulmonary adenocarcinoma, because lymph node metastasis is the most affecting factor to the patients' prognosis.

INTRODUCTION

Neoplastic transformation of epithelial cells is accompanied by alterations of the cell surface carbohydrates, and many of them can be detected by specific antibodies, thus providing tumor-associated antigens (1–3). Among these carbohydrates, mucin-type glycoproteins carrying sialyl Le^a or sialyl Le^x are particularly interesting because they are enriched on the cell surface of tumor cells such as colorectal cancer and serve not only as a tumor marker but also as preferential ligands for cell adhesion molecules, E- and P-selectins (4–6). During the metastatic process of tumor cells, it is well documented that binding between the tumor and endothelial cells in the remote organs is crucial for the successful colonization in target organs (7). Thus, the malignant cells expressing sialyl Le^a or sialyl Le^x on their cell surface are expected to frequently metastasize to the distal organs when compared with the malignant cells that lack these carbohydrates. In fact, it was shown that the

expression of sialyl Le^a or sialyl Le^x on the cell surface of colorectal cancer or breast cancer cells is positively correlated with poor outcome of the patients (8–11). However, clinicopathological values of sialyl Le^a and sialyl Le^x in the lung cancer have remained controversial. Previously, it was reported that the detection of sialyl Le^a or sialyl Le^x in lung cancer cells was valuable in predicting the recurrence of nonadenocarcinoma cells or adenocarcinoma cells (12). In contrast, it was reported that these carbohydrate antigens may not reflect the prognosis of pulmonary adenocarcinoma (13).

C2GnT³ is a key enzyme to form core 2 branched *O*-glycans (Gal β 1 \rightarrow 3(GlcNAc β 1 \rightarrow 6)GalNAc α \rightarrow Ser/Thr) by catalyzing the transfer of GlcNAc from UDP-GlcNAc with β 1,6-linkage to α -GalNAc of core 1 *O*-glycan (Gal β 1 \rightarrow 3GalNAc α \rightarrow Ser/Thr) (Fig. 1). Sialyl Le^a as well as sialyl Le^x present in nonreducing terminals of *O*-glycans are formed via this particular branch structure (14), and the C2GnT cDNA was isolated from human promyelocytic leukemia HL-60 cells by expression cloning (15). Previously, we have shown that the expression of core 2 branched *O*-glycans is closely correlated with the vessel invasion and depth of invasion of the colorectal cancer cells by analyzing the expression of C2GnT mRNA in fresh colorectal tumor samples (16). However, the same study did not identify a cell type that expresses C2GnT mRNA, because the analysis was conducted by RT-PCR. This point is critical because leukocytes and activated T lymphocytes surrounding cancer cells also express C2GnT (14). To determine whether tumor cells express increased levels of C2GnT and whether its expression level is actually associated with tumor progression of lung cancer, we examined the expression pattern of C2GnT mRNA in lung cancer using *in situ* hybridization and then statistically analyzed the correlation between the expression of C2GnT mRNA in pulmonary adenocarcinoma and clinicopathological variables. We report here that the expression of C2GnT but not sialyl Le^x, which is frequently expressed in the adenocarcinoma, is highly correlated with the lymph node metastasis of the adenocarcinoma.

MATERIALS AND METHODS

Tissue Specimens. Forty-one surgically dissected tissue specimens of lung cancer were selected from the pathology files of Central Clinical Laboratories, Shinshu University Hospital, Matsumoto, Japan. All patients examined underwent complete resection of the tumor and complete excision of the regional lymph nodes. Histological examinations based on the criteria used for the WHO classification (17) revealed that these tumors consisted of 25 cases of adenocarcinoma, 10 cases of squamous cell carcinoma, 2 cases of large cell carcinoma, and 4 cases of small cell carcinoma. Clinicopathological evaluation including the clinical stages was done according to the histological tumor-node-metastasis classification system. Here, vessel invasion was defined as the presence of tumor cells infiltrating the vessel walls or tumor cells present in vascular lumen by means of elastic staining, and lymphatic invasion was

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³ The abbreviations used are: C2GnT, core 2 β 1,6-*N*-acetylglucosaminyltransferase; PCR, polymerase chain reaction; RT, reverse transcription; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

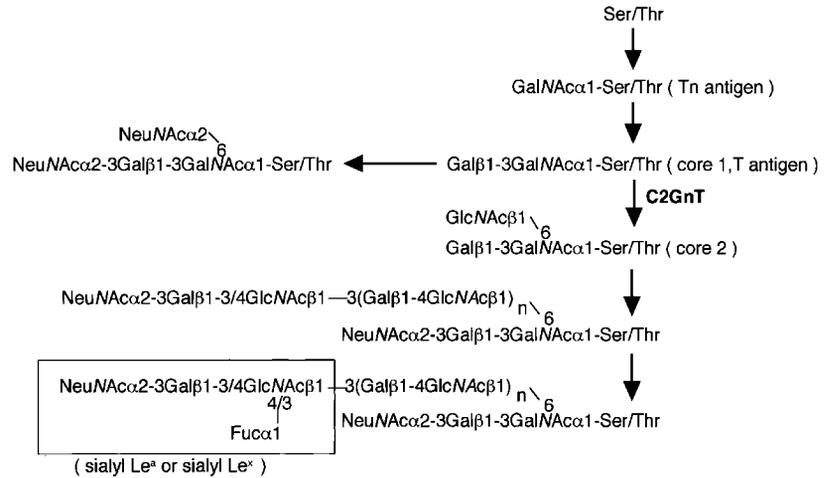


Fig. 1. Synthetic pathway of core 2 branched O-glycans, and sialyl Le^a or sialyl Le^x attached to core 2 branched O-glycans. Core 2 branches are usually galactosylated and sialylated as seen in the second from the bottom at *right*. Sialyl Le^a and sialyl Le^x attached to O-glycans are biosynthesized via the core 2 branched structure formed by C2GnT (based on Refs. 2 and 14–16).

defined as the presence of tumor cells in the endothelium-lined space as described (18). All specimens were fixed for 48 h in 20% phosphate-buffered formalin (pH 7.4) at room temperature, embedded in paraffin, and cut into 7- μ m sections for *in situ* hybridization or into 3- μ m sections for H&E staining, elastic staining, and immunostaining.

Preparation of RNA Probe for Human C2GnT. Using pcDNA1-C2GnT as a template, a C2GnT-specific nucleotide sequence in the catalytic domain (nucleotides +991 to +1143; the first nucleotide of the initiation codon is defined as +1) was amplified by PCR. According to the published sequence (15), the 5'- and 3'-primers were designed to be 5'-GCTCTAGAAGTC-CCGGGCTCACTC-3' and 5'-GGGGTACCGCACACTGAGCGCACATGG-3', respectively. The *Xba*I and *Asp*718 sites are underlined. This amplified DNA fragment was cloned into the *Xba*I and *Asp*718 sites of pGEM-3Zf (+) (Promega), and the resultant vector was used as a template for construction of the RNA probe. A digoxigenin-labeled antisense RNA probe was obtained using an *Xba*I-cut template and T7 RNA polymerase with a DIG RNA Labeling Mixture (Roche Molecular Biochemicals), as described previously (19). Similarly, a sense probe was prepared for negative control experiments by using an *Asp*718-cut template and SP6 RNA polymerase with the DIG RNA Labeling Mixture.

In Situ Hybridization of C2GnT Transcripts. Tissue specimens were subjected to *in situ* hybridization to detect C2GnT transcripts with nonradioactive labeling (19). After the tissue sections were deparaffinized in xylene, hydrated slides were immersed in 0.2 M HCl for 20 min and then digested with 100 μ g/ml proteinase K at 37°C for 20 min, followed by postfixation with 4% paraformaldehyde. These slides were rinsed with 2 mg/ml glycine and then acetylated for 10 min in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). The hydrated slides were defatted with chloroform and then air-dried. After prehybridization with 50% deionized formamide/2 \times SSC for 1 h at 45°C, the slides were hybridized with 0.5 mg/ml of the antisense or sense probe in 50% deionized formamide, 2.5 mM EDTA (pH 8.0), 300 mM NaCl, 1 \times Denhardt's solution, 10% dextran sulfate, and 1 mg/ml brewer's yeast tRNA at 45°C for 16 h.

After hybridization, the slides were washed in 50% formamide/2 \times SSC for 1 h at 45°C and digested with 10 mg/ml RNase A at 37°C for 30 min. After washing sequentially with 2 \times SSC/50% formamide at 45°C for 1 h, 1 \times SSC/50% formamide at 45°C for 1 h, and 1 \times SSC/50% formamide at room temperature for 30 min, the sections were subjected to immunohistochemistry for detection of the hybridized probes using an alkaline phosphatase-conjugated antidigoxigenin antibody (Roche Molecular Biochemicals). The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium in the presence of 10% polyvinyl alcohol. A control study using the sense probe showed no specific reactivity.

Immunohistochemistry. Deparaffinized tissue specimens were subjected to immunohistochemical staining for detection of sialyl Le^a or sialyl Le^x. Mouse monoclonal antibodies directed against sialyl Le^a (NS19-9) and sialyl Le^x (CSLEX-1) were purchased from Dako (Glostrup, Denmark) and Becton Dickinson (San Jose, CA), respectively. Immunohistochemical detection was performed by an indirect method as described previously (20). Antimouse

immunoglobulin antibody conjugated with horseradish peroxidase (Dako) was used as the secondary antibody, and peroxidase activity was visualized with diaminobenzidine-H₂O₂ solution. A control experiment was done omitting the primary antibody from the staining procedure, and no specific staining was found. Tissue specimens containing >5% positively stained carcinoma cells were defined as positive, and others were classified as negative according to the criteria of Nakamori *et al.* (8).

Statistics. The relationship between the expression of sialyl Le^a, sialyl Le^x, or C2GnT mRNA in pulmonary adenocarcinoma and clinicopathological variables was statistically evaluated by Fisher's exact test with the use of StatView 4.5 (Abacus Concept, Berkeley, CA), and the significant correlation was established when $P < 0.05$ was obtained. The clinicopathological variables examined in the present study included tumor size, lymph node metastasis, pleural invasion, pulmonary metastasis, vessel invasion, and lymphatic invasion.

RESULTS

Expression of Sialyl Le^a or Sialyl Le^x in Normal and Carcinoma Tissues of Lung. To investigate the expression profile of sialyl Le^a or sialyl Le^x in normal and carcinoma tissues of the lung, immunohistochemistry using NS19-9 or CSLEX-1 antibody was conducted. In the normal lung tissue, sialyl Le^a was expressed in the mucous cells of bronchial glands (Fig. 2A), and occasionally in the apical surface of the ciliated cells of bronchial mucosa. However, other cells including alveolar pneumocytes were negative for sialyl Le^a (Fig. 2B). Similarly, sialyl Le^x was detected in the mucous cells of bronchial glands (Fig. 2C) and sometimes in the apical surface of the ciliated cells of bronchial mucosa. In addition, the lateral surface of the serous cells of bronchial glands and the goblet cells of bronchial mucosa also expressed sialyl Le^x, but alveolar pneumocytes were negative for sialyl Le^x (Fig. 2D). These results on sialyl Le^a and sialyl Le^x expression in the normal lung are consistent with those previously reported by Kasai *et al.* (21).

In lung cancer, on the other hand, expression of sialyl Le^a was limited to 10 of 41 patients (24.4%). When the expression of sialyl Le^a was analyzed with respect to the histological types of the lung cancer, it was expressed only in 36% of the adenocarcinomas and 10% of the squamous cell carcinomas. In addition, sialyl Le^a was not detectable in large cell carcinoma or small cell carcinoma of the lung (Fig. 3 I and M). Sialyl Le^x was, on the other hand, expressed in 29 of 41 patients (70.7%), irrespective of the histological type; *i.e.*, 76% of adenocarcinoma (Fig. 3B), 70% of squamous cell carcinoma, 100% of the large cell carcinoma (Fig. 3J), and 25% of the small cell carcinoma.

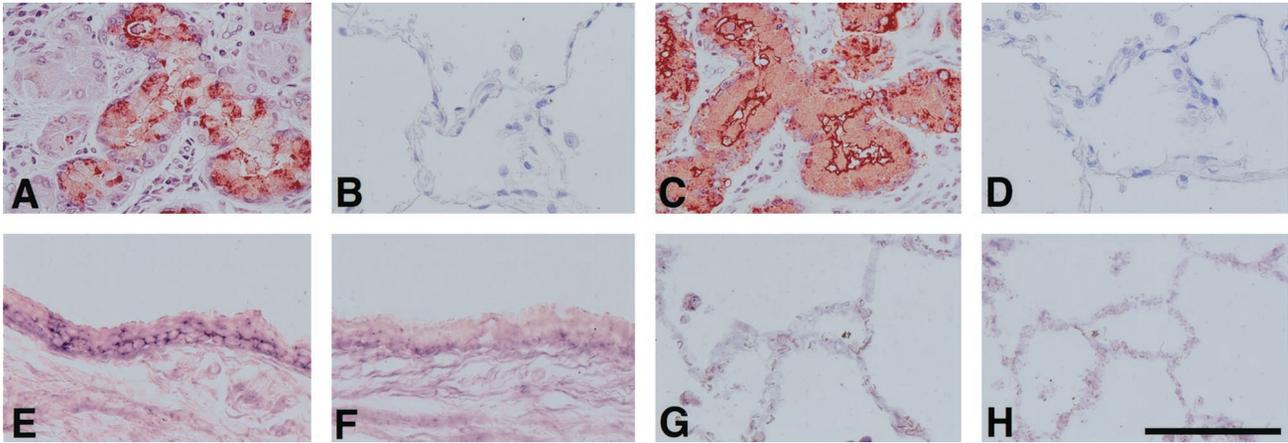


Fig. 2. Expression of sialyl Le^a, sialyl Le^x, and C2GnT mRNA in normal lung tissue. In normal lung, sialyl Le^a is expressed in the bronchial glands (A) but not in the alveolar pneumocytes (B). Similarly, sialyl Le^x is also detected in the bronchial gland (C) but not in the alveolar pneumocytes (D). The transcripts of C2GnT are expressed in the basal cells of the bronchial mucosa (E) but not in the alveolar pneumocytes (G). Immunohistochemistry using NS 19-9 (for sialyl Le^a) (A and B) or CSLEX-1 (for sialyl Le^x) (C and D). *In situ* hybridization for C2GnT using antisense probe (E and G) or sense, control probe (F and H). Bar, 100 μ m.

Enhanced Expression of C2GnT mRNA in Lung Cancer. To determine the expression pattern of transcripts of C2GnT, a key enzyme for the biosynthesis of *O*-glycans (Fig. 1), *in situ* hybridization was performed using a RNA probe specific for C2GnT. In normal lung tissue, the transcripts of C2GnT were exclusively limited to the

basal cells of the bronchial mucosa (Fig. 2, E and F), and other cells including alveolar pneumocytes did not express C2GnT mRNA (Fig. 2, G and H). In lung cancer, on the other hand, a significant signal for the C2GnT transcripts was detected in 30 of 41 patients (73.2%), irrespective of the histopathological types of carcinoma cells; *i.e.*,

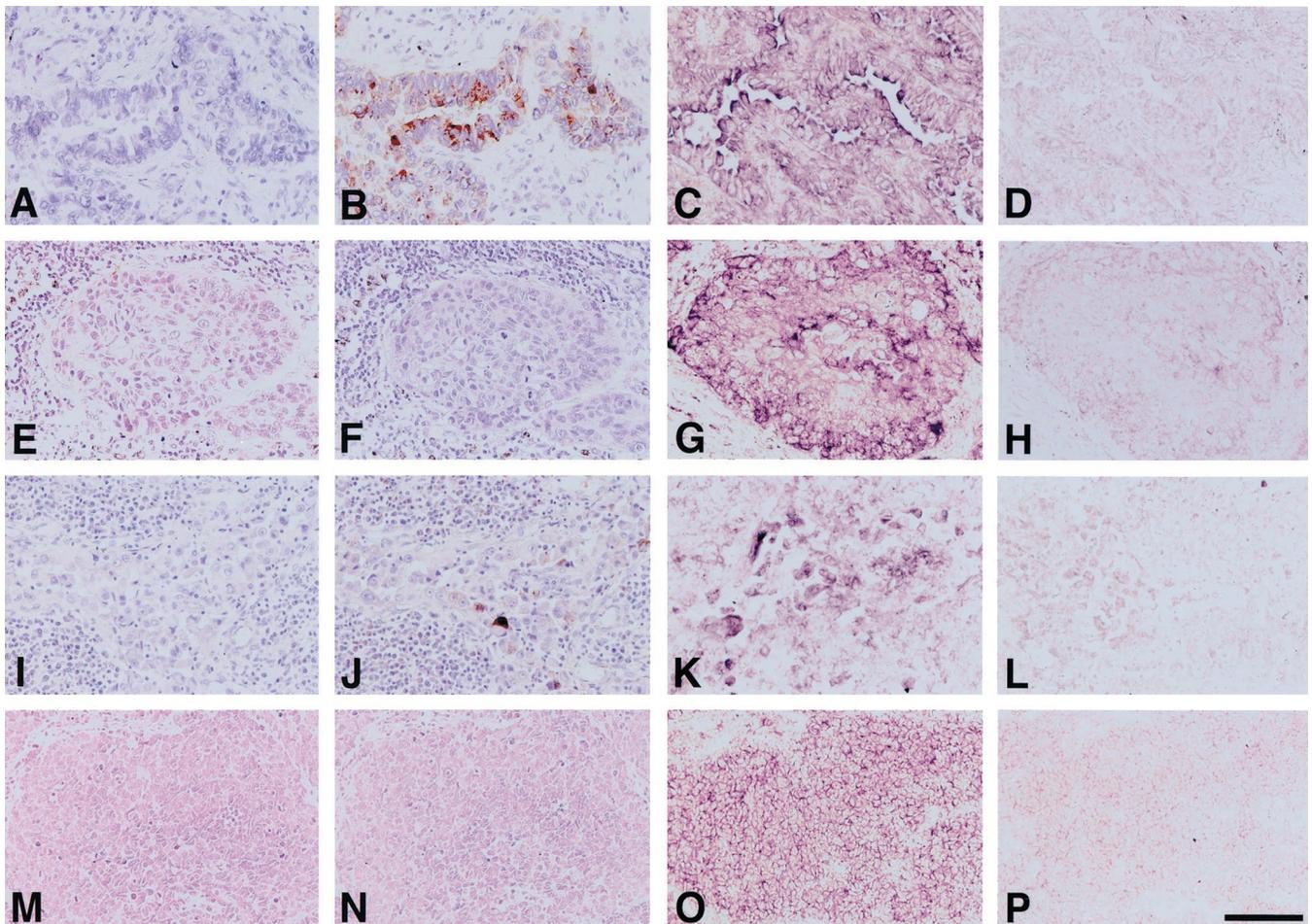


Fig. 3. Expression of sialyl Le^a, sialyl Le^x, and C2GnT mRNA in lung cancer. In lung cancer, sialyl Le^a is rarely detectable in the cancer cells (A, E, I, M), whereas sialyl Le^x is expressed in the adenocarcinoma (B) and large cell carcinoma (J). C2GnT mRNA is detectable in the adenocarcinoma (C), squamous cell carcinoma (G), large cell carcinoma (K), and small cell carcinoma (O). The lung cancer tissues (A–D, adenocarcinoma; E–H, squamous cell carcinoma; I–L, large cell carcinoma; M–P, small cell carcinoma) were prepared from serial sections. Immunohistochemistry using NS 19-9 (for sialyl Le^a) (A, E, I, M) or CSLEX-1 (for sialyl Le^x) (B, F, J, N). *In situ* hybridization for C2GnT using antisense probe (C, G, K, O) or sense, control probe (D, H, L, P). Bar, 100 μ m.

Table 1 Statistical analysis of the relationship between the expression of sialyl Le^a or sialyl Le^x in pulmonary adenocarcinoma cells and clinicopathological variables

	Sialyl Le ^a , positive (n = 9)	Sialyl Le ^a , negative (n = 16)	p ^a	Sialyl Le ^x , positive (n = 19)	Sialyl Le ^x , negative (n = 6)	p ^a
Tumor size						
30 mm \geq	5 (20.0) ^b	9 (36.0)	>0.9999	10 (40.0)	4 (16.0)	0.6609
30 mm<	4 (16.0)	7 (28.0)		9 (36.0)	2 (8.0)	
Pleural invasion						
Negative	3 (12.0)	12 (48.0)	0.0872	10 (40.0)	5 (20.0)	0.3449
Positive	6 (24.0)	4 (16.0)		9 (36.0)	1 (4.0)	
Pulmonary metastasis						
Negative	5 (20.0)	14 (56.0)	0.1425	14 (56.0)	5 (20.0)	>0.9999
Positive	4 (16.0)	2 (8.0)		5 (20.0)	1 (4.0)	
Lymph node metastasis						
Negative	3 (12.0)	13 (52.0)	0.0308 ^c	11 (44.0)	5 (20.0)	0.3644
Positive	6 (24.0)	3 (12.0)		8 (32.0)	1 (4.0)	
Vessel invasion						
Negative	3 (12.0)	11 (44.0)	0.1153	9 (36.0)	5 (20.0)	0.1804
Positive	6 (24.0)	5 (20.0)		10 (40.0)	1 (4.0)	
Lymphatic invasion						
Negative	2 (8.0)	13 (52.0)	0.009 ^c	10 (40.0)	5 (20.0)	0.3449
Positive	7 (28.0)	3 (12.0)		9 (36.0)	1 (4.0)	

^a Analyzed by Fisher's exact probability test.^b Numbers in parentheses, percentage.^c p < 0.05.

60% of adenocarcinoma (Fig. 3, C and D), 90.0% of squamous cell carcinoma (Fig. 3, G and H), 100% of large cell carcinoma (Fig. 3, K and L), and 100% of small cell carcinoma (Fig. 3, O and P). Lymphocytes surrounding the cancer cells also exhibited strong signal for C2GnT mRNA.

Sialyl Le^a and sialyl Le^x in O-glycans are synthesized via core 2 branched oligosaccharide formed by C2GnT (Fig. 1; Refs. 2 and 14); thus, it is possible that sialyl Le^a or sialyl Le^x displayed by the cancer cells expressing C2GnT mRNA is O-glycosylated. In the present study, by combining the results obtained from the immunohistochemistry for sialyl Le^a or sialyl Le^x and those from the *in situ* hybridization for C2GnT, we found that the expression of C2GnT mRNA and the expression of sialyl Le^a or sialyl Le^x were independently regulated in the lung cancer (Fig. 3). For example, in the 10 patients positive for sialyl Le^a in the lung cancer cells, 7 patients (70%) also expressed C2GnT mRNA in the cancer cells, but 3 patients (30%) did not express C2GnT. Similarly, 21 of 29 patients (72.4%) positive for sialyl Le^x in the cancer cells also expressed C2GnT mRNA, whereas 8 patients (27.6%) did not express C2GnT mRNA. Thus, it is possible that sialyl Le^a or sialyl Le^x in the cancer cells expressing C2GnT

mRNA is attached to core 2 branched O-glycan, whereas these carbohydrates present in the cancer cells lacking C2GnT mRNA are attached to N-glycans or belong to glycolipids.

Expression of C2GnT Transcripts in Pulmonary Adenocarcinoma Is Closely Associated with Vessel Invasion and Lymph Node Metastasis. It is well known that biological behavior and clinicopathological features of non-small cell lung cancer are quite distinct from those of small cell lung cancer. In addition, among non-small cell lung cancer, sialyl Le^x is more frequently expressed in adenocarcinoma than other cancers (22). Thus, we have statistically analyzed the relationship between the expression of sialyl Le^a or sialyl Le^x in pulmonary adenocarcinoma and clinicopathological variables. Due to the limited numbers of other patients examined in this study, we have excluded other types of lung cancers from the analysis. As shown in Table 1, significant correlation was found between the expression of sialyl Le^a and lymph node metastasis or lymphatic invasion (P < 0.05), whereas no significant correlation was obtained between the expression of sialyl Le^x and any clinicopathological variables examined.

Next, we statistically analyzed the relationship between the expres-

Table 2 Statistical analysis of the relationship between the expression of C2GnT mRNA and clinicopathological variables in patients expressing sialyl Le^a or sialyl Le^x in the pulmonary adenocarcinoma cells

	No. of patients (%)					
	Sialyl Le ^a -positive group (n = 9)			Sialyl Le ^x -positive group (n = 19)		
	C2GnT, positive (n = 6)	C2GnT, negative (n = 3)	p ^a	C2GnT, positive (n = 12)	C2GnT, negative (n = 7)	p ^a
Tumor size						
30 mm \geq	2 (22.2) ^b	3 (33.3)	0.1667	7 (36.8)	3 (15.8)	0.6499
30 mm<	4 (44.4)	0 (0)		5 (26.3)	4 (21.1)	
Pleural invasion						
Negative	1 (11.1)	2 (22.2)	0.2262	5 (26.3)	5 (26.3)	0.3498
Positive	5 (55.6)	1 (11.1)		7 (36.8)	2 (10.5)	
Pulmonary metastasis						
Negative	2 (22.2)	3 (33.3)	0.1667	8 (42.1)	6 (31.6)	0.6027
Positive	4 (44.4)	0 (0)		4 (21.1)	1 (5.3)	
Lymph node metastasis						
Negative	0 (0)	3 (33.3)	0.0119 ^c	5 (26.3)	6 (31.6)	0.1473
Positive	6 (66.7)	0 (0)		7 (36.8)	1 (5.3)	
Vessel invasion						
Negative	0 (0)	3 (33.3)	0.0119 ^c	2 (10.5)	7 (36.8)	0.0007 ^c
Positive	6 (66.7)	0 (0)		10 (52.6)	0 (0)	
Lymphatic invasion						
Negative	0 (0)	2 (22.2)	0.0833	5 (26.3)	5 (26.3)	0.3498
Positive	6 (66.7)	1 (11.1)		7 (36.8)	2 (10.5)	

^a Analyzed by Fisher's exact probability test.^b Numbers in parentheses, percentage.^c p < 0.05.

Table 3 Statistical analysis of the relationship between the expression of C2GnT mRNA in pulmonary adenocarcinoma cells and clinicopathological variables

	No. of patients (n = 25)		p ^a
	C2GnT, positive (n = 15)	C2GnT, negative (n = 10)	
Tumor size			
30 mm \geq	8 (32.0) ^b	6 (24.0)	>0.9999
30 mm<	7 (28.0)	4 (16.0)	
Pleural invasion			
Negative	7 (28.0)	8 (32.0)	0.2107
Positive	8 (32.0)	2 (8.0)	
Pulmonary metastasis			
Negative	10 (40.0)	9 (36.0)	0.3449
Positive	5 (20.0)	1 (4.0)	
Lymph node metastasis			
Negative	7 (28.0)	9 (36.0)	0.0405 ^c
Positive	8 (32.0)	1 (4.0)	
Vessel invasion			
Negative	4 (16.0)	10 (40.0)	0.0005 ^c
Positive	11 (44.0)	0 (0)	
Lymphatic invasion			
Negative	7 (28.0)	8 (32.0)	0.2107
Positive	8 (32.0)	2 (8.0)	

^a Analyzed by Fisher's exact probability test.

^b Numbers in parentheses, percentage.

^c $p < 0.05$.

sion of sialyl Le^a or sialyl Le^x possibly attached to core 2 branched O-glycans in adenocarcinoma and the clinicopathological variables by combining the results of the immunohistochemistry and *in situ* hybridization, with respect to the patients expressing sialyl Le^a or sialyl Le^x in the cancer cells (Table 2). Among the patients positive for sialyl Le^a in the adenocarcinoma, the cancer cells expressing C2GnT mRNA exhibited more vessel invasion and lymph node metastasis than those lacking C2GnT mRNA ($P < 0.05$). Similarly, among the patients expressing sialyl Le^x, significant correlation between the expression of C2GnT mRNA and vessel invasion was found ($P < 0.05$). These combined results strongly suggest that sialyl Le^a or sialyl Le^x expressed in the cancer cells more accurately reflect the patients' prognosis when the sialyl Le^a and sialyl Le^x are attached to core 2 branched O-glycans.

We have clearly demonstrated that expression of C2GnT mRNA is significantly enhanced in lung cancer cells compared with normal lung (Figs. 2 and 3). Thus, we have finally evaluated the clinicopathological significance of the C2GnT expression in the adenocarcinoma, irrespective of the sialyl Le^a or sialyl Le^x expression. Interestingly, the expression of C2GnT mRNA *per se* was significantly correlated with vessel invasion and lymph node metastasis of the cancer cells ($P < 0.05$; Table 3). These results suggest that core 2 branched O-glycans play an important role in the progression of pulmonary adenocarcinoma.

DISCUSSION

In this study, we have demonstrated that the expression level of C2GnT mRNA was significantly enhanced in association with malignant transformation of the lung, irrespective of the histological type of the lung cancers. Previously, we have shown that transcripts of C2GnT are detectable in colorectal cancer tissues, but not in their normal counterparts, suggesting the presence of C2GnT mRNA in colorectal cancer cells (16). However, the same study could not provide a direct evidence that the cancer cells actually express C2GnT mRNA by themselves, because C2GnT transcripts were analyzed by using RT-PCR of total RNA isolated from the fresh colorectal tumor samples (16). In that case, granulocytes and activated T lymphocytes surrounding tumor cells may have contributed to the C2GnT mRNA level. In fact, the present study showed that the lymphocytes sur-

rounding the cancer cells actually expressed C2GnT mRNA. Thus, the present study extended the previous study and has clearly demonstrated the presence of C2GnT transcripts in lung cancer cells using *in situ* hybridization.

Extensive studies have been carried out to determine the clinicopathological significance of sialyl Le^a or sialyl Le^x expressed in the lung cancer (12, 13, 23, 24). Ogawa *et al.* (23) reported that sialyl Le^a or sialyl Le^x expressed in non-small cell lung cancer in stage I, which is defined by the absence of lymph node or distant metastasis, is positively correlated with the vessel invasion of the tumor cells. Similarly, Satoh *et al.* (24) reported that the high serum level of sialyl Le^x of >38.0 units/ml in patients with non-small cell lung cancer is significantly correlated with mediastinal lymph node metastasis. In contrast, Kawai *et al.* (13) demonstrated that sialyl Le^a or sialyl Le^x expressed in pulmonary adenocarcinoma is not significantly correlated with postoperative survival time. In the present study, we have shown a significant correlation between the expression of sialyl Le^a and lymphatic invasion or lymph node metastasis in the adenocarcinoma, consistent with the results by Ogawa *et al.* (23). On the other hand, we could not establish any significant correlation between sialyl Le^x expressed in the adenocarcinoma and any clinicopathological variables. However, when the same patients were analyzed by immunohistochemistry for sialyl Le^a or sialyl Le^x combined with *in situ* hybridization for C2GnT mRNA, we could demonstrate for the first time that these carbohydrates possibly attached to core 2 branched O-glycans were significantly correlated with the vessel invasion of the pulmonary adenocarcinoma cells. Thus, these results collectively suggest that for the evaluation of the progression of pulmonary adenocarcinoma, it appears much more valuable to detect sialyl Le^a or sialyl Le^x present in O-glycans than just simply detect sialyl Le^a or sialyl Le^x.

It is established that lymph node metastasis is the most affecting factor to the patients' prognosis of lung cancer (18). The present study demonstrated that lymph node metastasis is significantly correlated with the expression of not only C2GnT mRNA *per se* but also sialyl Le^a in pulmonary adenocarcinoma (see Tables 1 and 3). Taking into account that in pulmonary adenocarcinoma, sialyl Le^a was less frequently expressed than C2GnT mRNA, *in situ* hybridization for C2GnT will be helpful to predict the prognosis of adenocarcinoma of the lung. As shown in the present study, C2GnT mRNA was expressed not only in the adenocarcinoma but also in the other types of lung cancers. Future study will be of significance to test whether the expression of C2GnT in lung cancer other than adenocarcinoma is closely correlated with the tumor progression. Recently it was reported that by using *in situ* hybridization for C2GnT, the transcripts are detectable not only in breast cancer cells but also in normal or benign breast tissues, in particular, lactating breast (25). In the same study, however, the relationship between the expression of C2GnT in breast cancer and lymph node metastasis was not evaluated. It is of significance to determine whether breast cancer cells expressing C2GnT more frequently metastasize to lymph nodes compared with those lacking C2GnT.

The selectin-mediated interaction of tumor cells to endothelial cells of the vasculatures plays important roles in tumor progression, in particular, metastasis (2, 3). Although sialyl Le^a and sialyl Le^x are well established ligands for E- and P-selectins, various studies indicate that these carbohydrates attached to core 2 branched O-glycans serve as higher affinity ligands for P-selectin than those attached to N-glycans (14). In the present study, we have clearly demonstrated that pulmonary adenocarcinoma expressing C2GnT mRNA more frequently metastasized to lymph nodes than did the adenocarcinoma lacking C2GnT mRNA, irrespective of the presence of sialyl Le^a or sialyl Le^x in the cancer cells. As exemplified by the presence of

galactose- and GalNAc-specific C-type lectin in macrophages (26) or sialyl Le^x in short N-glycan-specific C-type lectin in NK cells (27), the present results strongly suggest that C-type lectins or selectin-like molecules that preferentially bind to core 2 branched oligosaccharides other than those with sialyl Le^a and sialyl Le^x are present in the lymph nodes. In fact, Wagers *et al.* (28) has recently demonstrated that an sialyl Le^x-deficient variant of HL60 cells apparently binds to E- and P-selectins, even in the absence of sialyl Le^x as well as sialyl Le^a. The same authors have also shown that this binding between the variant HL60 cells and the selectins is significantly reduced after the pretreatment with sialidase and suggested that sialylated and fucosylated carbohydrates other than sialyl Le^x, that have not been identified yet, might function as the ligands for E- and P-selectins (28). Future study is required to determine the carbohydrate moieties in core 2 branched O-glycans other than sialyl Le^a or sialyl Le^x, which apparently facilitates metastasis to lymph nodes (29).

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Clinicopathological Significance of Core 2 β 1,6-*N*-Acetylglucosaminyltransferase Messenger RNA Expressed in the Pulmonary Adenocarcinoma Determined by *in Situ* Hybridization

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