1. Introduction

Patients with advanced non-small-cell lung cancer (NSCLC) have a poor prognosis with 1–5% 5-year survival rates [1]. A recent meta-analysis demonstrated that platinum-based combination chemotherapy is currently considered to be the most effective treatment for advanced NSCLC, and these have improved the median survival time (MST) by 2 months and caused a 10% increase in 1-year survival rates [2]. As platinum-based chemotherapy improves survival and quality of life in advanced NSCLC patients, most patients will receive second line chemotherapy. With recurrence or progression, docetaxel has been approved as a second line chemotherapy treatment due to demonstrated survival benefit compared with best supportive care (BSC) or vinorelbine/ifosfamide [3,4]. Currently, there is no proven effective chemotherapy for patients previously treated with platinum-based and docetaxel therapies.

The epidermal growth factor receptor (EGFR) is a promising target for anticancer therapy because many types of cancer cells express or overexpress EGFR (including NSCLC, renal cell carcinoma and breast cancer) [5,6]. EGFR overexpression has been reported as a poor prognostic factor in many types of human solid tumors including NSCLC in several studies [7–9]. Currently, monoclonal antibodies that bind to the extracellular domain of EGFR and intracellular tyrosine kinase inhibitors have been developed [10,11]. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation, angiogenesis, invasion, metastasis and survival of cancer cells [12,13]. Several phase I trials demonstrated safety and tolerability of gefitinib in pretreated patients with solid tumors, in which trials an 11% response rate was seen in 100 patients with heavily pretreated advanced NSCLC [14]. On the other hand, in Japan, a phase I trial demonstrated five responders out of a total of 31 patients who all had adenocarcinoma of the lung [12]. To confirm anti-tumour activity and the safety profile of gefitinib, an international phase II study (IDEAL-1) and United States trial (IDEAL-2) were conducted as a second or third line treatment in patients with advanced NSCLC [15,16]. Patients enrolled in these studies were randomized into two different doses, 250 and 500 mg/day. These trials demonstrated that toxicity was mild and showed an encouraging response rate with an RR of 18.4 and 11.8% of patients in the 250 mg arm, respectively, and an improvement in disease related symptoms and quality of life were observed. The IDEAL-1 study has also confirmed that there were statistically significant differences in efficacy for 'adenocarcinoma' and 'female' using multivariate analysis. Two large randomized phase III studies [17,18], which are standard chemotherapy (cisplatin/gemcitabine or carboplatin/paclitaxel) with or without gefitinib, failed to demonstrate a survival benefit for advanced NSCLC patients as a first line chemotherapy. Although the results of the phase III studies were negative, gefitinib is still considered a promising molecular targeted agent as a new generation treatment in patients with advanced NSCLC. Information on the clinical prognostic factors following a single regimen of gefitinib should be helpful in finding which patients are likely to receive benefit, and in the development of a future treatment. Although the previous phase II trial (IDEAL) showed that several predictive factors were associated with the response to gefitinib, the population was essentially biased towards the young, with good performance status (PS) and conserved, good organ functions.

In this study, to find factors associated with an objective response and survival benefit of gefitinib, we retrospectively analysed patients who received a single regimen of gefitinib at our institute.

2. Methods

All patients with stage IIIB or IV NSCLC, who received a single regimen of gefitinib from August 1998 until July 2003 at the Kinki University School of Medicine, Osaka, were retrospectively reviewed. We evaluated patients who participated in clinical trials (phase I trial, phase II trial; IDEAL-1), or phase II trial for investigating surrogate gene therapy, and in 53 patients who were administered the drug after marketing (including elderly or poor performance status patients). Patients who received gefitinib as part of a compassionate use program were excluded. All patients were checked for age, gender, histology, Eastern Cooperative Oncology Group (ECOG), PS, stage, pre-treatment regimen, number of prior regimen, and smoking status before treatment of gefitinib. Smoking status was evaluated by the Brinkmann index; number of cigarettes per day multiplied by number of years. We analyzed the response, overall survival rate and the adverse effects of gefitinib, and investigated predictive factors associated with response and prognosis. The response was assessed using physical examination, biochemical profile, chest X-ray, chest computed tomography (CT), head CT or magnetic resonance imaging (MRI) scan, abdominal echo-graphic or abdominal CT scan, bone scinti-graph, bronchoscope, and was evaluated according to the response eval-

uation criteria in solid tumor (RECIST) [19]. The severity of all the adverse events (AEs) that related to gefitinib administration was assessed by the NCPCTC (version 2.0) grading system. The predictive factors associated with the response that were analyzed in this study were age, gender, PS, histology, stage, number of prior regimen and smoking status. Variables were tested for any possible relationship with the response to gefitinib, at first by univariate analysis, and subsequently by the application of a multivariate model. Response rates were compared between strata using Fisher's exact test. Logistic regression models were used to explore observed differences and identify baseline factors that may independently predict for response rates. The survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. *P*-values less than 0.05 were considered significant.

3. Results

3.1. Patient profiles

From August 1998 until July 2003 at our institute, a total of 105 patients, who were already cytologically or histologically diagnosed as NSCLC, were treated by a single regimen of gefitinib. Patients received gefitinib until disease progression or intolerable toxicity. Of these, 101 patients were evaluated as suitable for analysis; four patients were excluded from analysis because they received gefitinib as part of a compassionate use program. As shown in Table 1, the 101 patients included: 2 patients who received gefitinib at a once daily dose of 50 mg; single patients who each received 100, 225, 400, 525 and 700 mg, respectively; 89 patients who received 250 mg; and 5 patients who received 500 mg. In the phase I trial, we used an intermittent administration schedule with 14 days continuous dosing followed by 14 days off.

Table 1 Patient characteristics

	Number of patient ($N = 101$)
Phase I	7
50 mg	2
100 mg	1
225 mg	1
400 mg	1
525 mg	1
700 mg	1
Phase II (IDEAL-1)	11
250 mg	6
500 mg	5
Phase II (gene expression) (250 mg)	30
Post marketing (250 mg)	53

Table 2 Patient characteristics ($N = 101$)

	Number of patients
Age (year)	
Median (range)	62 (31–84)
<69	74
≥70	27
Gender	
Male	64
Female	37
Performance status	
0	15
1	62
2	17
3	7
Tumor histology	
Adenocarcinoma	81
Squamous	18
Large-cell	2
Stage	
III	18
IV	83
Previous treatment	
No treatment	5
Failed 1 previous chemotherapy regimens	53
Failed 2 previous chemotherapy regimens	34
Failed 3 previous chemotherapy regimens	9
Smoking (smoker:never-smoker)	55:46
Index[a] 0:1–999:1000	46:32:23

[a] Index: number of cigarettes per day multiplied by number of years.

Patient characteristics are shown in Table 2. The median age was 62 years (ranging from 31–84) and 74 patients (73.3%) were less than 69 years old. 63.4% of the patients were male, 76.2% had performance status (ECOG) 0–1, 80.2% had adenocarcinoma of which 83.2% had stage IV disease. Fifty-three patients had received one prior regimen, 43 had more than two prior regimens and only five had previously been untreated. 54.5% of them were smokers, and the non-smokers were almost all female. This study included patients

Table 3 Overall objective response

	Number	%
Number of patients evaluated	101	
Complete response (CR)	1	1.0
Partial response (PR)	19	18.8
Stable disease (SD)	52	51.5
Progressive disease (PD)	25	24.8
Not evaluable	4	4.0
Response rate % (95% CI)	19.8 (12.0–27.6)	
Disease control rate[a] % (95% CI)	71.3 (62.5–80.1)	

[a] CR + PR + S.D.

who had failed several previous chemotherapy regimens, and patients with an ECOG PS score of 3.

3.2. Response to treatment

Table 3 shows an objective response observed in this study. Twenty responders were evaluated and the overall response rate was 19.8%. One patient achieved a complete response, 19 patients exhibited a partial response and 52 patients had stable disease, resulting in a disease control rate (objective responses plus stable disease) of 71.3%. When evaluated using patient characteristics, we determined the response rate detailed in Fig. 1. All patients that responded had adenocarcinoma of the lung as the histological subtype. In addition, for the factors 'female' and 'never-smoker', there were higher response rates than in 'male' and 'smoker' respectively, while RR was similar for age, stage and pre-treatment. The response rate of 'female' and 'never-smoker' were 37.8 and 32.6%, respectively. Using the Fisher's exact test, the predictive factors which were associated with a response were 'female' (37.8% versus 9.4%; $P = 0.0006$), 'adenocarcinoma' (24.7% versus 0%; $P = 0.0104$), 'good PS' (0–1) (26.0% versus 0%; $P = 0.0028$), and never-smoker (32.6% versus 9.1%; $P = 0.0025$). There were no significant differences for age, stage and pre-treatment (Table 4). A multivariate analysis was performed against the four significant predictive factors in univariate analysis (Table 5). Because the incidence of the female factor is very strongly correlated to the never-smoker factor, the statistical assay was rather unstable if the two factors were analyzed simultaneously. We then investigated two patterns of multivariate analysis. One analysis excluded smoking and the other excluded gender. If smoking status was extracted, then female and good performance status were statistically significant. If gender was extracted, then non-smoking and good performance were statistically significant. The odds of a response were over three times higher for patients with adenocarcinoma than for patients with other histologies, however, this is not considered to be statistically significant because the group in this study was of a small size and included a high percentage of adenocarcinoma.

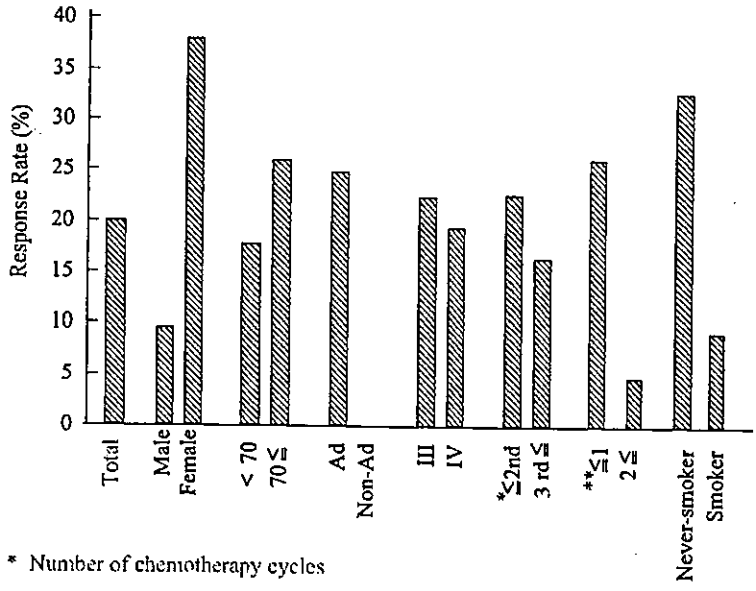

Fig. 1 Tumor response rate of the subgroups.

Table 4 Predictive factors associated with an objective response by univariate analysis

Parameter	N	Responder	RR (%)	P-value
Smoking index				
Non-smoker	55	15	32.6	
Smoker	46	5	9.1	0.0025
Gender				
Female	37	14	37.8	
Male	64	6	9.4	0.0006
Histology				
Adenocarcinoma	81	20	24.7	
Others	20	0	0.0	0.0104
PS				
0–1	77	20	26.0	
≥2	24	0	0.0	0.0028
Pre-treatment				
≤2 regimens	58	13	22.4	
≥3 regimens	43	7	16.3	N.S.
Age (years)				
≤70	74	13	17.6	
≥71	27	7	25.9	N.S.
Stage				
IIIB	18	4	22.2	
IV	83	16	19.3	N.S.

Abbreviations: N.S., not significant.

3.3. Toxicity

Drug-related AEs of all patients are shown in (Table 6). A total of 101 patients were evaluated for toxicity. The most frequent drug-related AEs were a rash, dry skin and diarrhea. Most of these AEs were mild (Grade 1 or Grade 2) and were controllable. Of all the drug-related AEs evaluated, Grade 3 or Grade 4 AEs were seen in less than 5%, and Grade 4 drug-related AEs were only pneumonitis. Grade 3 or 4 AEs required a treatment interruption, but recovered after discontinuation of gefitinib, except with pneumonitis. Four patients developed greater than Grade 3 pneumonitis requiring hospitalization. All patients had a fever and severe hypoxemia on admission. As soon as possible, all patients were administered steroid therapy. While two patients recovered with the steroid therapy, two patients died within 40 days after the administration of gefitinib. Hematological toxicities were not observed.

3.4. Survival

The median survival time of the patients who were 'good PS' (0 or 1) and 'poor PS' (2 or 3) was 353 and 97 days, respectively, and this difference was significant ($P = 0.0001$, log-rank test) (Fig. 2A). The MST of females was significantly longer than that of males (596 days versus 178 days, $P = 0.004$) (Fig. 2B). Furthermore, a low smoking index (<900) significantly prolonged survival (MST: 301 days versus 149 days, $P = 0.031$) (Fig. 2C). Age did not influence the survival benefit of the patients treated with gefitinib (Fig. 2D).

4. Discussion

Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways, and is one of the promising molecular targeted drugs used in the treatment of advanced NSCLC [16,17,20]. Although the large scale of the phase II study (IDEAL-1) [15] has already confirmed that there were statistically significant differences in efficacy for 'adenocarcinoma' and 'female' by multivariate analysis, the population was essentially biased towards young people with good performance status who had conserved, good organ functions. To clarify the predictive prognostic fac-

Table 5 Predictive factors associated with an objective response by multivariate analysis

Parameter	Odds ratio	95% CI	P-value
Extraction of smoking			
Gender (female vs. male)	0.163	0.040–0.585	0.0032
Performance status (1 vs. 2)	0.061	0.000–0.415	0.0018
Histology (Adeno[a] vs. others)	3.326	0.435–infinity	N.S.
Extraction of gender			
Non-smoking (non vs. ≥1)	0.297	0.063–0.959	0.0417
Performance status (1 vs. 2)	0.096	0.000–0.628	0.0101
Histology (Adeno vs. others)	4.385	0.588–infinity	N.S.

Abbreviations: N.S., not significant; CI, confidence interval.
[a] Adenocarcinoma.

Table 6 Patients with drug-related adverse events (NCI-CTC)

Adverse event	Number of patients ($N = 101$)				
	Grade 1	Grade 2	Grade 3	Grade 4/5	Total
Rash	33 (32.6%)	21 (20.8%)	3 (3.0%)	0	57 (56.4%)
Dry skin	24 (23.7%)	3 (3.0%)	0	0	27 (26.7%)
Pruritis	9 (9.0%)	7 (7.0%)	0	0	16 (16.0%)
Diarrhea	19 (18.8%)	4 (4.0%)	0	0	23 (22.8%)
Nausea	6 (6.0%)	1 (1.0%)	0	0	7 (7.0%)
Vomiting	3 (3.0%)	0	0	0	3 (3.0%)
Anorexia	7 (7.0%)	0	0	0	7 (7.0%)
ALT increased	5 (5.0%)	2 (2.0%)	5 (5.0%)	0	12(13.0%)
AST increased	8 (8.0%)	2 (2.0%)	3 (3.0%)	0	13 (13.0%)
Pneumonitis	0	0	2 (2.0%)	2[a] (2.0%)	4 (4.0%)

[a] Treatment-related death (Grade 5).

tors in a practical setting, we retrospectively analysed the patients who received a single regimen of gefitinib at our institute. Multivariate analysis demonstrated that the predictive factors which were associated with a response were 'female', 'good PS' and 'never-smoker'. In survival analyses, the factors 'female', 'good PS', and a low smoking index also significantly prolonged survival.

The mechanism by which these factors produced better prognosis has not been clarified.

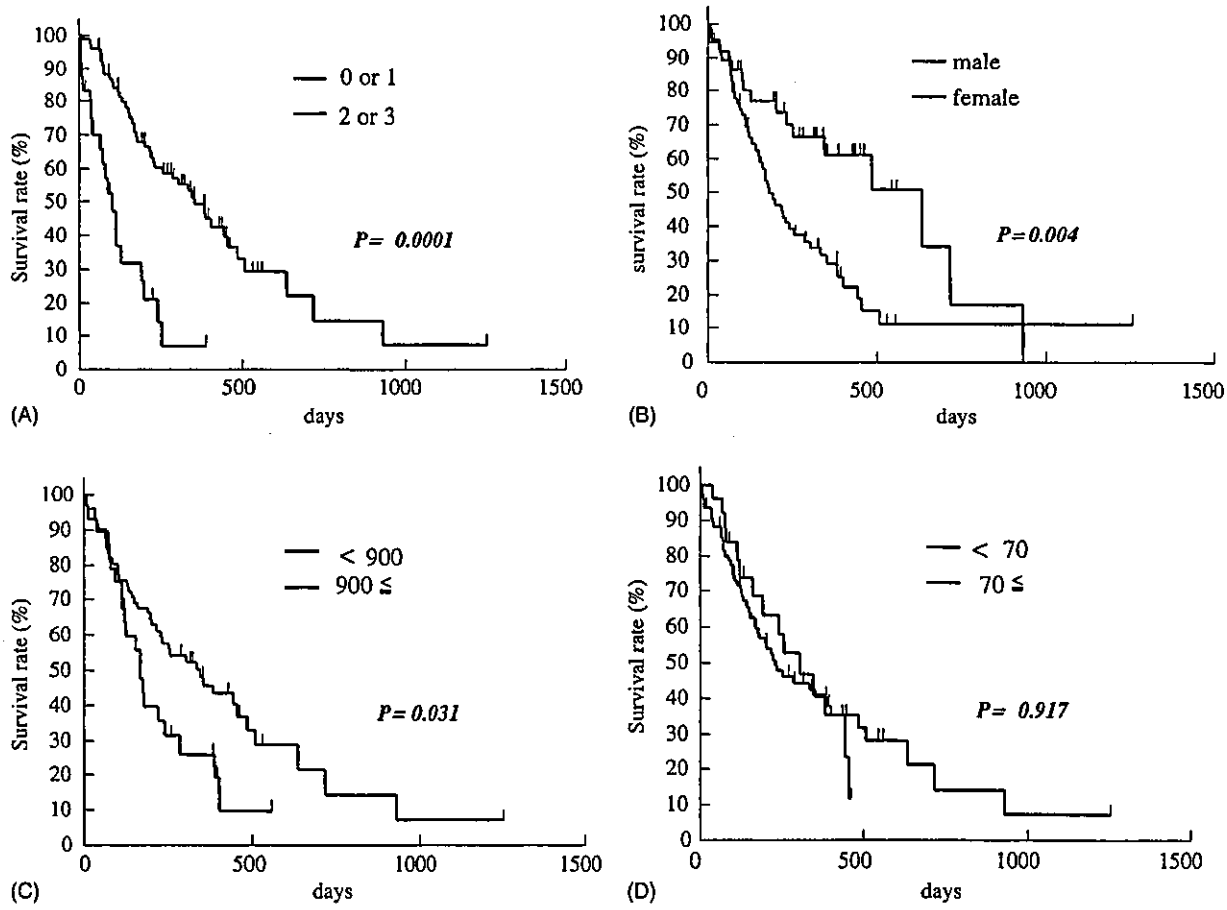

Fig. 2 A comparison of survival of: (A) PS 0, 1 vs. PS 2, 3; (B) gender: male vs. female; (C) smoking index: <900 vs. ≥900; and (D) age: <70 vs. ≥70.

Estrogen and progesterone may up-regulate EGFR in normal tissues [21], and activation of steroid hormones might impact on EGFR function in NSCLC [22]. Another explanation may be that the steroid hormone receptor might interact with EGFR and influence the response of an EGFR inhibitor.

Multivariate analysis in IDEAL-1 showed that PS was not a significant prognostic factor, however, the population of the study was restricted with regards to good PS. Although gefitinib was considered as an effector of symptom improvement in the phase II trial, the indication for patients with poor PS is controversial. Several authors described the case reports about the efficacy of gefitinib in NSCLC patients with poor PS [23,24] or with brain metastases [25]. Although 'good PS' were significant prognostic factor in this trial, gefitinib still might be a candidate drug for patients with poor PS, because of restriction of the use of other anti-cancer drug by their toxicities.

Elderly patients exhibited an equivalent response to young patients in this study. Recent data suggested, gefitinib is safe and well tolerated in elderly pretreated NSCLC patients [26]. A phase II study of gefitinib for elderly patients in NSCLC is needed.

A low smoking index was revealed as a predictive prognostic factor following a single regimen of gefitinib. Erlotinib is also administered orally and is a highly selective EGFR tyrosine kinase inhibitor [27] with a quinazolinamine-based structure similar to that of gefitinib. In the phase II study of erlotinib in NSCLC or bronchial alveolar carcinoma [28], a non-smoking history was also a prognostic factor. Chronic exposure to nicotine increases the expression level and phosphorylation status of EGFR and impairs its function [29]. Moreover, smoking produces overexpression of Her2/neu that binds to EGFR as a hetero-dimer in the tissue of normal bronchus. Expression of EGFR or Her2/neu or both in tissue samples by immunohistochemistry has not correlated in the response of gefitinib [30], however the different type of dimers formed between EGFR families might influence the response to gefitinib.

Four patients (4% of the patients) developed interstitial lung disease (ILD). Continuous smoking disrupted surfactant protein A or D [31,32], and the serum levels of the proteins were increased [33]. As 'smoking history' and 'male' are significant risk factors of ILD and also in treatment with gefitinib [34], a serum level of the surfactant protein A or D might be a predictive marker of ILD. Patients who are female and non-smokers are most likely to receive a high benefit and low risk with gefitinib treatment.

Although more basic biological research is needed to find the mechanism of action, we have found several predictive prognostic factors associated with the practical use of gefitinib. This is necessary clinical information which is important in order to set eligibility criteria for future clinical trials with gefitinib.

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References

[1] Mountain CF. Revisions in the International System for Staging Lung Cancer. Chest 1997;111:1710–7.
[2] Non-Small-Cell Lung Cancer Collaborative Group. Chemotherapy in non-small-cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Br Med J 311 (1995) 899–909.
[3] Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. J Clin Oncol 2000;18:2095–103.
[4] Fossella FV, DeVore R, Kerr RN, et al. Randomized phase II trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum containing chemotherapy regimens. J Clin Oncol 2000;18:2354–62.
[5] Salomon D, Brandt R, Ciardiello F, et al. Epidermal growth factor-related peptides and their receptors in human malignancies. Crit Rev Oncol Hematol 1995;19:183–232.
[6] Rusch V, Baselga J, Cordon-Cardo C, et al. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small-cell lung cancers and adjacent benign lung. Cancer Res 1993;53:2379–85.
[7] Fujino S, Enokibori T, Tezuka N, et al. A comparison of epidermal growth factor receptor levels and other prognostic parameters in non-small-cell lung cancer. Eur J Cancer 1996;32A:2070–4.
[8] Pavelic K, Banjac Z, Pavelic J, et al. Evidence for a role of EGF receptor in the progression of human lung carcinoma. Anticancer Res 1993;13:1133–7.
[9] Volm M, Rittgen W, Drings P. Prognostic value of ERBB-1, VEGF, cyclin A, FOS, JUN and MYC in patients with squamous cell lung carcinomas. Br J Cancer 1998;77:663–9.
[10] Baselga J, Pfister D, Cooper M, et al. Phase I study of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. J Clin Oncol 2000;18:904–14.
[11] Baselga J, Averbuch S. ZD1839 ('Iressa') as an anticancer agent. Drugs 2000;60(Suppl l):33–40.
[12] Nakagawa K, Tamura T, Negoro S, et al. Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Japanese patients with solid malignant tumors. Ann Oncol 2003;14:922–30.

[13] Ranson M, Hammond L, Ferry D, et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of phase I trial. J Clin Oncol 2002;20:2240–50.
[14] Herbst R, Maddox AM, Rothenberg M, et al. Selective oral epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is generally well-tolerated and has activity in non-small-cell lung cancer and other solid tumors: results of a phase I trial. J Clin Oncol 2002;120:3815–25.
[15] Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
[16] Kris M, Natale R, Herbst R, et al. A phase II trial of ZD 1839 ('Iressa') in advanced non-small-cell lung cancer (NSCLC) patients who had failed platinum- and docetaxel-based regimen (IDEAL 2). Proc Am Soc Clin Oncol 2002;21:292a.
[17] Giaccone G, Johnson DH, Manegold C, et al. A phase III clinical trial of ZD 1839 ('Iressa') in combination with gemcitabine and cisplatin in chemotherapy-naive patients with advanced non-small-cell lung cancer (INTACT 1). Ann Oncol 2002;113(Suppl 5):A-4.
[18] Johnson DH, Herbst R, Giaccone G, et al. ZD1839 ('Iressa') in combination with paclitaxel and carboplatin in chemotherapy naive patients with advanced non-small-cell lung cancer (NSCLC): results from a phase III clinical trial (INTACT 2). Ann Oncol 2002;13(Suppl 5):A-468.
[19] Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.
[20] Santoro A, Cavina R, Latteri F, et al. Activity of a specific inhibitor, gefitinib (Iressa™, ZD1839), of epidermal growth factor receptor in refractory non-small-cell lung cancer. Ann Oncol 2004;15:33–7.
[21] Shimomura Y, Matsuo H, Samoto T, Maruo T. Up-regulation by progesterone of proliferating cell nuclear antigen and epidermal growth factor expression in human uterine leiomyoma. J Clin Endocrinol Metab 1998;83:2192–8.
[22] Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001;2:127–37.
[23] Fujiwara K, Kiura K, Ueoka H, et al. Dramatic effect of ZD 1839 ('Iressa') in a patient with advanced non-small-cell lung cancer and poor performance status. Lung Cancer 2003;40:73–6.
[24] Ranson M, Thatcher N. Commentary on ZD1839 (Iressa) in non small cell lung cancer. Lung Cancer 2003;40:77–8.
[25] Cappuzzo F, Ardizzoni A, Soto-Parra H, et al. Epidermal growth factor receptor targeted therapy by ZD1839 (Iressa) in patients with brain metastases from non-small-cell lung cancer (NSCLC). Lung Cancer 2003;41:227–31.
[26] Cappuzzo F, Bartolini S, Ceresoli GL, et al. Efficacy and tolerability of gefitinib in pretreated elderly patients with advanced non-small-cell lung cancer (NSCLC). Br J Cancer 2004;90:82–6.
[27] Hidalgo M, Siu LL, Nemunaitis J, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. J Clin Oncol 2001;19:3267–79.
[28] Miller VA, Patel J, Shah N, et al. The epidermal growth factor receptor tyrosine kinase inhibitor Erlotinib (OSI-774), shows promising activity in patients with bronchioalveolar cell carcinoma (BAC): Preliminary results of a phase II trial. Proc Am Soc Clin Oncol 2003;22:A-2491.
[29] Wang SL, Milles M, Wu-Wang CY, et al. Effect of cigarette smoking on salivary epidermal growth factor (EGF) and EGF receptor in human buccal mucosa. Toxicology 1992;75:145–57.
[30] Cappuzzo F, Gregorc V, Rossi E, et al. Gefitinib in pretreated non-small-cell lung cancer (NSCLC): analysis of efficacy and correlation with HER2 and epidermal growth factor receptor expression in locally advanced or metastatic NSCLC. J Clin Oncol 2003;21:2658–63.
[31] Subramaniam S, Whitsett JA, Hull W, Gairola CG. Alteration of pulmonary surfactant proteins in rats chronically exposed to cigarette smoke. Toxicol Appl Pharmacol 1996;140:274–80.
[32] Foster DJ, Yan X, Bellotto DJ, et al. Expression of epidermal growth factor and surfactant proteins during postnatal and compensatory lung growth. Am J Physiol Lung Cell Mol Physiol 2002;283:981–90.
[33] Zhang F, Pao W, Umphress SM, Jakowlew SB, et al. Serum levels of surfactant protein D are increased in mice with lung tumors. Cancer Res 2003;63:5889–94.
[34] Tamura K, Yamamoto N, Takeda K, et al. An epidemiological survey for interstitial lung disease induced by gefitinib in patients with advanced non-small-cell lung cancer. West Japan Thoracic Oncology Group (WJTOG). Proc Eur Cancer Soc 2003;1(Suppl 5):A-56.

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 statiscally 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-

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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 Joint Committee on Cancer (18). Of the 120 patients with pStage I tumors resected with curative intent, survival was analyzed for the 103 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 (SAB-PO kit; Nichirei, Tokyo, Japan). Methyl green was used for counterstain. GnT-V expression found in normal bronchial epithelial cells and alveolar pneumocytes served as internal positive controls.

GnT-V expression in tumors was classified as high or low, based on the proportion of positively stained cancer cells (≥50% of cancer cells stained or <50% of cancer cells stained) and the staining intensity (classified as retained or decreased and negative, as compared with the staining of normal bronchial epithelial cells). Tumors that retained staining in at least 50% of cancer cells were judged as having high GnT-V expression (retaining expression of GnT-V). Tumors that retained staining in <50% of cancer cells were judged as having low GnT-V expression (losing expression of GnT-V).

Immunohistochemistry for Ki-67, $p27^{KIP1}$, Cyclin E, and GalNAc-T3. Expression of Ki-67, $p27^{KIP1}$, cyclin E, and GalNAc-T3 was analyzed by immunohistochemistry. For these proteins, the slides and results that were reported previously (19–21) were used for the present study. The methods for the staining of these proteins in resected tumors have been described previously (19–21). The labeled streptavidin biotin method was used on 4-μm sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. The primary antibodies used were a mouse monoclonal MIB-1 antibody (Immunotech, Marseilles, France), a mouse monoclonal antihuman $p27^{KIP1}$ 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) Histochemistry. 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% $CaCl_2$ 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 $CaCl_2$ 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 χ^2 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 generalized 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*s < 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 χ^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 ($P = 0.02$; 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 ($P = 0.09$; 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; $P = 0.001$; Fig. 2*A*). 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

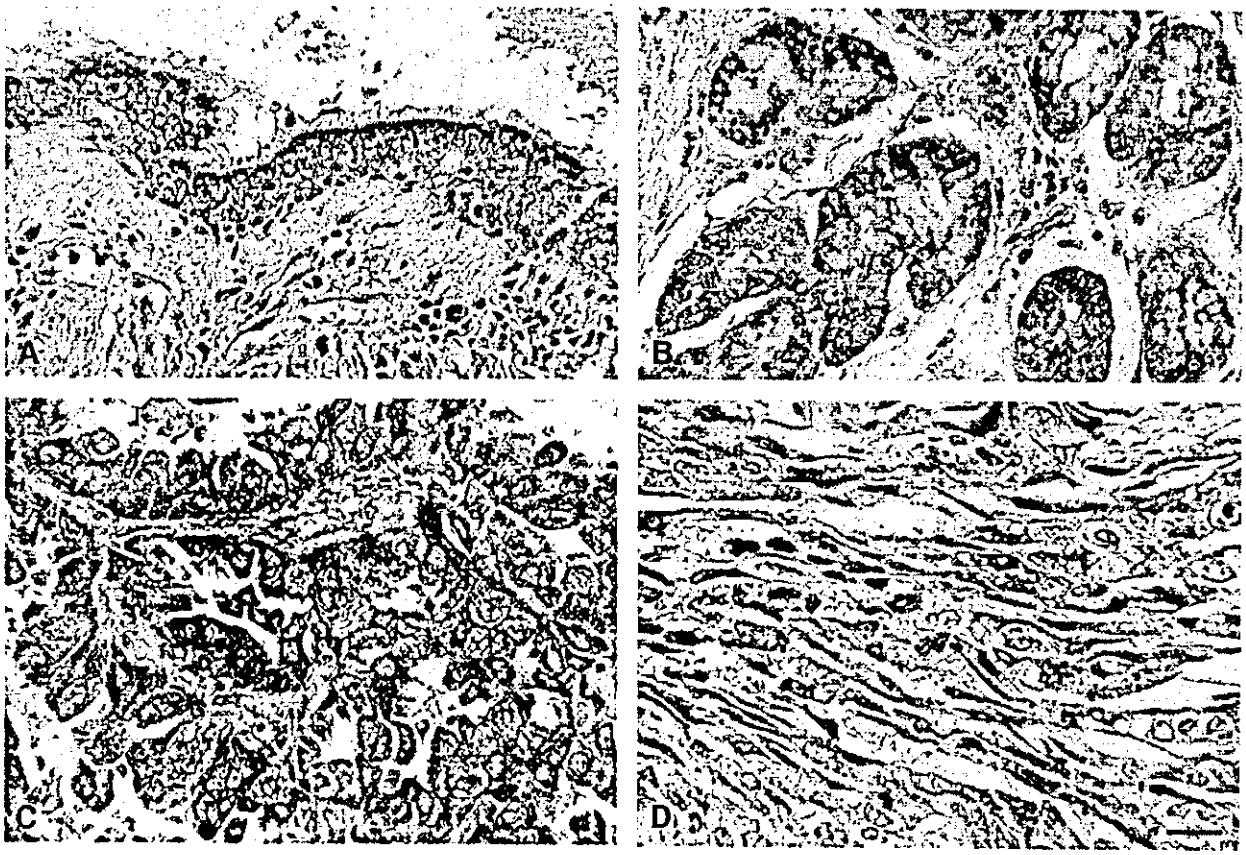

Fig. 1 Immunohistochemical staining patterns for *N*-acetylglucosaminyltransferase V (GnT-V) in normal bronchial tissue and non-small cell lung cancers. Normal bronchial epithelial cells (*A*) and bronchial gland cells (*B*) show GnT-V expression. An adenocarcinoma tumor shows high GnT-V expression diffusely in the cytoplasm (*C*), and a squamous cell carcinoma tumor shows low GnT-V expression (*D*). *Scale bar* = 20 μm.

Table 1 Relationship between GnT-V[a] expression and clinical and clinicopathological characteristics in 217 surgically resected NSCLCs

Characteristics	GnT-V expression		*P*
	Low	High	
Age (mean ± SD) (yrs)	64.3 ± 8.9	62.3 ± 9.5	0.1
Sex			
Male	79	66	0.009
Female	25	47	
Smoking			
Nonsmoker	21	38	0.04
Smoker	76	68	
Smoking (pack-years)			
$0 \leq x < 20$	23	43	0.01
≥ 20	74	61	
Histology[b]			
Squamous	53	37	0.003
Adenocarcinoma	39	70	
Other	11	6	
Differentiation			
Well	18	33	0.2
Moderate	33	46	
Poor	26	21	
pT classification			
1	27	34	0.6
2–4	76	79	
pN classification			
0	67	66	0.4
1–3	36	47	
pM classification			
0	101	108	0.5
1	2	5	
pStage			
1	56	64	0.6
2	9	9	
3a	37	34	
3b	0	1	
4	2	5	

[a] GnT-V, *N*-acetylglucosaminyltransferase V; NSCLC, non-small cell lung cancer.
[b] Squamous, squamous cell carcinoma; Other, large cell carcinoma and adenosquamous cell carcinoma.

Table 2 Multivariate logistic regression analysis for the correlation between GnT-V[a] expression and clinical and clinicopathological characteristics

Characteristics[b]	Odds ratio	*P*
Gender (male/female)	1.58	0.4
Smoking (smoker/nonsmoker)	1.21	0.7
Histology (squamous/nonsquamous[c])	2.39	0.02
Differentiation (moderate & poor/well)	0.99	1.0

[a] GnT-V, *N*-acetylglucosaminyltransferase V.
[b] Selected from Table 1.
[c] Including adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma.

tumors having low GnT-V expression survived a significantly shorter time than patients with tumors having high GnT-V expression (5-year survival rates, 54% and 89%, respectively; $P = 0.007$; Fig. 2*B*). Low GnT-V expression was the only significant unfavorable prognostic factor (hazard ratio, 3.02; $P = 0.02$) in pStage I non-squamous cell carcinomas that we found from our analysis (Table 4B). In 44 patients with pStage I squamous cell carcinomas, GnT-V expression was not associated with survival (5-year survival rates, 47% for low GnT-V expression and 75% for high GnT-V expression; $P = 0.2$) and was not a prognostic factor ($P = 0.1$).

We examined the expression of β1–6 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 β1–6 branching oligosaccharides (Fig. 3, Table 5). L-PHA binds with high specificity and affinity to complex-type tri- and tetra-antennary oligosaccharides, which contain β1–6 branches

Table 3 Relationship between GnT-V[a] expression and cell biological characteristics

Characteristics	GnT-V expression		*P*
	Low	High	
Ki-67 (mean ± SD)	40.4 ± 28.2	34.0 ± 26.1	0.09
$p27^{KIP1}$ (mean ± SD)	29.4 ± 23.8	27.9 ± 23.7	0.6
Cyclin E (mean ± SD)	38.7 ± 27.9	37.2 ± 29.9	0.7
GalNAc-T3			
Low	64	29	0.0001
High	39	81	

[a] GnT-V, *N*-acetylglucosaminyltransferase V.

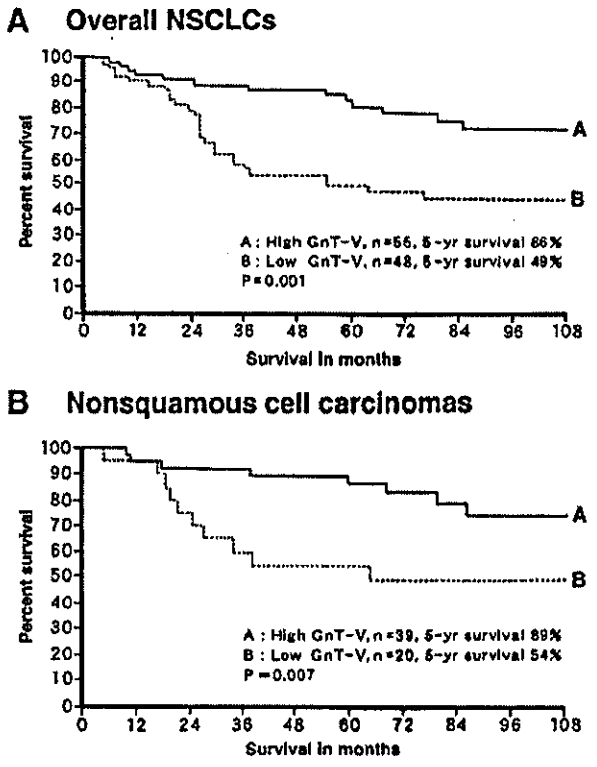

Fig. 2 Kaplan-Meier survival curves for patients with non-small cell lung cancers. Survival curves for patients with pStage I tumors are stratified by low and high *N*-acetylglucosaminyltransferase V expression for overall non-small cell lung cancers ($n = 103$; *A*) and for non-squamous cell carcinomas ($n = 59$; *B*).

Table 4 Cox's proportional hazards model analysis of prognostic factors in patients with pStage I NSCLCs[a]

A. Overall NSCLCs			
Characteristics	Hazard ratio	95% CI	*P*
Sex (male/female)	0.63	0.35–1.16	0.1
Age (≥65 yrs/<65 yrs)	0.75	0.43–1.31	0.3
Chemotherapy	1.24	0.72–2.15	0.4
Histology (non-squamous[b]/squamous)	1.34	0.77–2.35	0.3
Differentiation (moderate, poor/well)	0.89	0.44–1.80	0.8
pT classification (pT_2/pT_1)	1.15	0.66–2.00	0.6
GnT-V expression (high/low)	2.86	1.45–5.56	0.002
B. Non-squamous cell carcinomas[b]			
Characteristics	Hazard ratio	95% CI	*P*
Sex (male/female)	0.72	0.33–1.55	0.4
Age (≥65 yrs/<65 yrs)	0.70	0.32–1.51	0.8
Chemotherapy	1.42	0.63–3.20	0.4
Differentiation (moderate, poor/well)	0.96	0.38–2.43	0.9
pT classification (pT_2/pT_1)	1.33	0.61–2.87	0.5
GnT-V expression (high/low)	3.02	1.19–7.69	0.02

[a] NSCLC, non-small cell lung cancer; CI, confidence interval; GnT-V, *N*-acetylglucosaminyltransferase V.

[b] Including adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma.

(24). Hence, L-PHA has been used as a reliable reagent to detect β1–6 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 β1–6 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 β1–6 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 β1–6 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 β1–6 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 β1–6 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 β1–6 branching oligosaccharides. Increased GnT-V activity and β1–6 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 matriptase (40), 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 β1–6 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

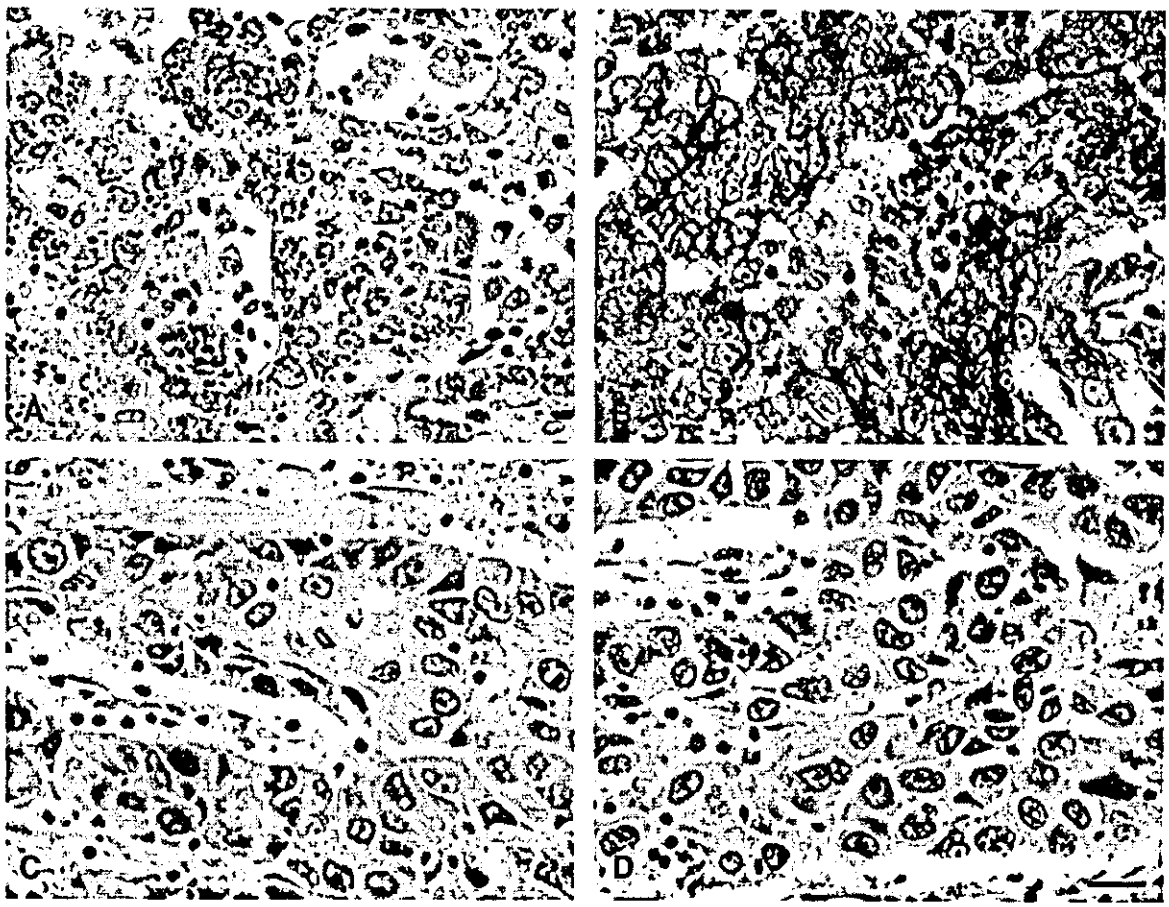

Fig. 3 Immunohistochemical staining patterns for *N*-acetylglucosaminyltransferase V (GnT-V; *A* and *C*) and staining patterns for leukoagglutinating phytohemagglutinin (L-PHA; *B* and *D*) in non-small cell lung cancers. The staining pattern of a tumor with high GnT-V expression and high L-PHA staining 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.

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.

Table 5 Relationship between GnT-V[a] expression and L-PHA staining in NSCLCs

GnT-V expression	L-PHA staining			*P*
	Low	Moderate	High	
Low	6	2	2	0.1
High	2	2	6	

[a] GnT-V, *N*-acetylglucosaminyltransferase V; NSCLC, non-small cell lung cancer; L-PHA, leukoagglutinating phytohemagglutinin.

REFERENCES

1. Ginsberg, R. J., Vokes, E. E., and Rosenzweig, K. Non-small cell lung cancer. *In*: V. T. DeVita, S. Hellma, and S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology, 6th ed., pp. 925–982. Philadelphia: Lippincott-Raven Publishers, 2001.

2. Carney, D. N. Lung cancer: time to move on from chemotherapy. N. Engl. J. Med., *346:* 126–128, 2002.

3. Sekido, Y., Fong, K. M., and Minna, J. D. Molecular genetics of lung cancer. *In*: C. T. Caskey, C. Austin, and J. Hoxie (eds.), Annual Review of Medicine, Vol. 54, pp. 73–87. Palo Alto: Annual Reviews, 2003.

4. Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57–70, 2000.

5. Strauss, G. M., Kwiatkowski, D. J., Harpole, D. H., Lynch, T. J., Skarin, A. T., and Sugarbaker, D. J. Molecular and pathologic markers in stage I non-small cell carcinoma of the lung. J. Clin. Oncol., *13:* 1265–1279, 1995.

6. Harpole, D. H., Jr., Herndon, J. E., II, Wolfe, W. G., Iglehart, J. D., and Marks, J. R. A prognostic model of recurrence and death in stage I non-small cell lung cancer utilizing presentation, histopathology, and oncoprotein expression. Cancer Res., *55:* 51–56, 1995.

7. Kwiatkowski, D. J., Harpole, D. H., Jr., Goleski, J., Herndon, J. E., II, Dar-Bin, S., Richards, W., Blanco, R., Xu, H., Strauss, G. M., and Sugarbaker, D. J. Molecular pathologic substaging in 244 stage I non-small-cell lung cancer patients: clinical implications. J. Clin. Oncol., *16:* 2468–2477, 1998.

8. Dosaka-Akita, H., Hommura, F., Mishina, T., Ogura, S., Shimizu, M., Katoh, H., and Kawakami, Y. A risk-stratification model of non-small cell lung cancers using cyclin E, Ki-67, and ras p21: different roles of G_1 cyclins in cell proliferation and prognosis. Cancer Res., *61:* 2500–2504, 2001.

9. Gibbs, J. B. Mechanism-based target identification and drug discovery in cancer research. Science (Wash. DC), *287:* 1969–1973, 2000.

10. Hakomori, S. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. Adv. Cancer Res., *52:* 257–331, 1989.

11. Varki, A. Biological roles of oligosaccharides: all of the theories are correct. Glycobiology, *3:* 97–130, 1993.

12. Fernandes, B., Sagman, U., Auger, M., Demetriou, M., and Dennis, J. W. β1–6 Branched oligosaccharides as a marker of tumor progression in human breast and colon neoplasia. Cancer Res., *51:* 718–723, 1991.

13. Seelentag, W. K., Li, W. P., Schmitz, S. F., Metzger, U., Aeberhard, P., Heitz, P. U., and Roth, J. Prognostic value of β1,6-branched oligosaccharides in human colorectal carcinoma. Cancer Res., *58:* 5559–5564, 1998.

14. Murata, K., Miyoshi, E., Kameyama, M., Ishikawa, O., Kabuto, T., Sasaki, Y., Hiratsuka, M., Ohigashi, H., Ishiguro, S., Ito, S., Honda, H., Takemura, F., Taniguchi, N., and Imaoka, S. Expression of *N*-acetylglucosaminyltransferase V in colorectal cancer correlates with metastasis and poor prognosis. Clin. Cancer Res., *6:* 1772–1777, 2000.

15. Li, W. P., and Roth, J. Expression of β1,6 branched asparagine-linked oligosaccharides in non-mitotic and non-migratory cells of normal human and rat tissues. Int. J. Cancer, *71:* 483–490, 1997.

16. Pemg, G. S., Shoreibah, M., Margitich, I., Pierce, M., and Fregien, N. Expression of *N*-acetylglucosaminyltransferase V mRNA in mammalian tissues and cell lines. Glycobiology, *4:* 867–871, 1994.

17. WHO. Histological typing of lung tumors, 2nd ed. Am. J. Clin. Pathol., *77:* 123–136, 1982.

18. American Joint Committee on Cancer. Lung. *In*: O. H. Beahrs, D. E. Henson, R. V. P. Hutter, and B. J. Kennedy (eds.), Manual for Staging of Cancer, 4th ed., pp. 15–122. Philadelphia: J. B. Lippincott Co., 1992.

19. Hommura, F., Dosaka-Akita, H., Mishina, T., Nishi, M., Kojima, T., Hiroumi, H., Ogura, S., Shimizu, M., Katoh, H., and Kawakami, Y. Prognostic significance of $p27^{KIP1}$ protein and Ki-67 growth fraction in non-small cell lung cancers. Clin. Cancer Res., *6:* 4073–4081, 2000.

20. Mishina, T., Dosaka-Akita, H., Hommura, F., Nishi, M., Kojima, T., Ogura, S., Shimizu, M., Katoh, H., and Kawakami, Y. Cyclin E expression, a potential prognostic marker for non-small cell lung cancers. Clin. Cancer Res., *6:* 11–16, 2000.

21. Dosaka-Akita, H., Kinoshita, I., Yamazaki, K., Izumi, H., Itoh, T., Katoh, H., Nishimura, M., Matsuo, K., Yamada, Y., and Kohno, K. *N*-Acetylgalactosaminyl transferase-3 is a potential new marker for non-small cell lung cancers. Br. J. Cancer, *87:* 751–755, 2002.

22. Suzuki, O., Nozawa, Y., Kawaguchi, T., and Abe, M. *Phaseolus vulgaris* leukoagglutinating lectin-binding reactivity in human diffuse large B cell lymphoma and its relevance to the patient's clinical outcome: lectin histochemistry and lectin blot analysis. Pathol. Int., *49:* 874–880, 1999.

23. Cox, D., and Snell, E. Analysis of Binary Data, 2nd ed. London: Chapman and Hall, 1989.

24. Cummings, R. D., and Kornfeld, S. Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leukoagglutinating and erythroagglutinating lectins. J. Biol. Chem., *257:* 11230–11234, 1982.

25. Li, W. P., Zuber, C., Heitz, P. U., and Roth, J. Cytochemical staining for β1,6 branching of asparagine-linked oligosaccharides in variants of metastatic human colon carcinoma cells. Am. J. Pathol., *145:* 470–480, 1994.

26. Saito, T., Miyoshi, E., Sasai, K., Nakano, N., Eguchi, H., Honke, K., and Taniguchi, N. A secreted type of β1,6-*N*-acetylglucosaminyltransferase V (GnT-V) induces tumor angiogenesis without mediation of glycosylation: a novel function of GnT-V distinct from the original glycosyltransferase activity. J. Biol. Chem., *277:* 17002–17008, 2002.

27. Shimosato, Y., Kodama, T., and Kameya, T. Morphogenesis of peripheral type adenocarcinoma of the lung. *In*: Y. Shimosato, M. R. Melamed, and P. Nettesheim (eds.), Morphogenesis of Lung Cancer, Vol. 1, pp. 65–89. Boca Raton, FL: CRC Press, 1982.

28. Konishi, T., Lin, Z., Fujino, S., Kato, H., and Mori, A. Association of p53 protein expression in stage I lung adenocarcinoma with reference to cytological subtypes. Hum. Pathol., *28:* 544–548, 1997.

29. Maeshima, A., Miyagi, A., Hirai, T., and Nakajima, T. Mucin-producing adenocarcinoma of the lung, with special reference to goblet cell type adenocarcinoma: immunohistochemical observation and Ki-ras gene mutation. Pathol. Int., *47:* 454–460, 1997.

30. Ito, Y., Miyoshi, E., Sakon, M., Takeda, T., Noda, K., Tsujimoto, M., Ito, S., Honda, H., Takemura, F., Wakasa, K., Monden, M., Matsuura, N., and Taniguchi, N. Elevated expression of UDP-*N*-acetylglucosamine:α-mannoside β1,6 *N*-acetylglucosaminyltransferase is an early event in hepatocarcinogenesis. Int. J. Cancer, *91:* 631–637, 2001.

31. Dennis, J. W., and Laferte, S. Oncodevelopmental expression of –GlcNAc β1–6Man α1–6Man β1-branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. Cancer Res., *49:* 945–950, 1989.

32. Buckhaults, P., Chen, L., Fregien, N., and Pierce, M. Transcriptional regulation of *N*-acetylglucosaminyltransferase V by the src oncogene. J. Biol. Chem., *272:* 19575–19581, 1997.

33. Chen, L., Zhang, W., Fregien, N., and Pierce, M. The her-2/neu oncogene stimulates the transcription of *N*-acetylglucosaminyltransferase V and expression of its cell surface oligosaccharide products. Oncogene, *17:* 2087–2093, 1998.

34. Demetriou, M., Nabi, I. R., Coppolino, M., Dedhar, S., and Dennis, J. W. Reduced contact-inhibition and substratum adhesion in epithelial cells expressing GlcNAc-transferase V. J. Cell Biol., *130:* 383–392, 1995.

35. Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L., and Kerbel, R. S. β1–6 branching of Asn-linked oligosaccharides is directly associated with metastasis. Science (Wash. DC), *236:* 582–585, 1987.

36. Saitoh, O., Wang, W. C., Lotan, R., and Fukuda, M. Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. J. Biol. Chem., *267:* 5700–5711, 1992.

37. Takano, R., Nose, M., Nishihira, T., and Kyogoku, M. Increase of β1–6-branched oligosaccharides in human esophageal carcinomas invasive against surrounding tissue *in vivo* and *in vitro*. Am. J. Pathol., *137:* 1007–1011, 1990.

38. Granovsky, M., Fata, J., Pawling, J., Muller, W. J., Khokha, R., and Dennis, J. W. Suppression of tumor growth and metastasis in Mgat5-deficient mice. Nat. Med., *6:* 306–312, 2000.

39. Guo, H. B., Lee, I., Kamar, M., Akiyama, S. K., and Pierce, M. Aberrant *N*-glycosylation of β_1 integrin causes reduced $\alpha_5\beta_1$ integrin clustering and stimulates cell migration. Cancer Res., *62:* 6837–6845, 2002.

40. Ihara, S., Miyoshi, E., Ko, J. H., Murata, K., Nakahara, S., Honke, K., Dickson, R. B., Lin, C. Y., and Taniguchi, N. Prometastatic effect of *N*-acetylglucosaminyltransferase V is due to modification and stabilization of active matriptase by adding β1–6 GlcNAc branching. J. Biol. Chem., *277:* 16960–16967, 2002.

B7-H1 Expression on Non-Small Cell Lung Cancer Cells and Its Relationship with Tumor-Infiltrating Lymphocytes and Their PD-1 Expression

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ABSTRACT

Purpose: **B7-H1/PD-L1 (B7-H1) and B7-DC/PD-L2 (B7-DC) are ligands for the receptor PD-1, which is known to negatively regulate T-cell activation. In the present study, we investigated the expression of B7-H1 and B7-DC in tumor specimens of non-small cell lung cancer and their relationships with clinicopathological variables and postoperative survival. Furthermore, we examined the correlation between B7-H1 expression on tumor cells and the number of tumor-infiltrating lymphocytes (TILs) or PD-1 expression on TILs.**

Experimental Design: **The expression of B7-H1 and B7-DC in 52 surgically resected specimens of non-small cell lung cancer was evaluated immunohistochemically.**

Results: **Expression of B7-H1 and B7-DC was focally observed in all non-small cell lung cancer tumor specimens. No relationship was found between the expression of B7-H1 or B7-DC and clinicopathological variables or postoperative survival. However, in the same sections evaluated, significantly fewer TILs were identified in B7-H1-positive tumor regions than in B7-H1-negative tumor regions in a subset of five patients ($P = 0.01$). Moreover, the percentage of TILs expressing PD-1 was significantly lower in B7-H1-positive tumor regions than in B7-H1-negative tumor regions ($P = 0.02$).**

Conclusions: **The expression of B7-H1 on tumor cells in local areas reciprocally correlated with the number of TILs, and this may contribute to negative regulation in antitumor immune responses in non-small cell lung cancer.**

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INTRODUCTION

Lung cancer is one of the most common fatal malignancies, and the incidence of this type of cancer is increasing worldwide. Platinum-based chemotherapy and radiation have low efficacy against lung cancer due to frequent recurrence and metastasis of the neoplasm; the overall 5-year survival rate after diagnosis remains at 10–15% (1). To improve the poor prognosis of lung cancer patients, studies of new therapeutic strategies including immunotherapy are in progress.

Effective protective immunity against cancer depends on the concordant activity of CTLs (2). T-cell activation is the result of a balance between positive and negative signals. CD28 and ICOS are positive costimulatory receptors interacting with the ligands of the B7 family on professional antigen-presenting cells and are essential for activation and proliferation of antigen-specific T cells (3). In contrast, negative signals through cell surface molecules such as CTLA-4, CD95, CD5, CD31, LAIR, Ly49A, and NKG2A inhibit T-cell activation or induce apoptosis (4).

B7-H1/PD-L1 (B7-H1) and B7-DC/PD-L2 (B7-DC) are members of the B7 superfamily (5, 6). B7-H1 and B7-DC share 40% amino acid homology and are more homologous to each other than to other ligands of the B7 family (7). These B7 family members have been shown to down-regulate T-cell activation through receptor PD-1 (6, 8, 9). Cross-linking of PD-1 by B7-H1 or B7-DC results in decreased interferon γ, interleukin (IL)-10, IL-4, and IL-2 secretion (6, 8). Thus, on T-cell receptor activation, B7-H1 or B7-DC leads to diminished immune responses, and the two molecules may have overlapping functions.

PD-1, which has been identified as a receptor for B7-H1 and B7-DC, belongs to the CD28/CTLA-4 subfamily of the immunoglobulin superfamily and contains tyrosines in ITIM-like motifs that may recruit phosphatases, similar to other negative regulators (7, 10). PD-$1^{-/-}$ mice display a variety of autoimmune diseases, demonstrating the role of PD-1 as a negative regulator of the immune response (10, 11).

B7-H1 and B7-DC are more broadly expressed than the other B7 superfamily members. Initial studies documented the expression of B7-H1 and B7-DC in mRNA in nonlymphoid organs as well as lymphoid organs (5, 6, 8). Recent studies at the protein level have revealed that B7-H1 is expressed on the endothelium in the thymus, heart, and placenta in both humans and mice (12–14) in addition to lymphoid cells, such as activated T cells, B cells, macrophages, and dendritic cells. B7-DC protein is also expressed in the thymus, placenta and heart in humans and in the thymus in mice (12, 14). However, B7-DC expression is more restricted on professional antigen-presenting cells, such as activated monocytes and dendritic cells (5, 6, 8). On the other hand, PD-1 protein is expressed on double-positive

and -negative thymocytes, activated T and B cells, and myeloid cells (9, 10, 14, 15).

B7-H1 is also abundant on tumor cell lines and tumor tissues, including lung carcinomas, ovarian carcinomas, breast carcinomas, glioblastoma, and squamous cell carcinoma of the head and neck (12, 13, 16, 17). Although B7-DC expression has been noted in several murine tumor cell lines (6), little is known about human B7-DC expression in tumor tissue. Cancer cells expressing B7-H1 have been shown to increase apoptosis of antigen-specific human T-cell clones (13) and to inhibit $CD4^+$ and $CD8^+$ T-cell activation *in vitro* (16). In addition, mice succumb to tumors transfected with B7-H1 even after adoptive T-cell immunotherapy, whereas blockade of PD-1/B7-H1 inhibits tumorigenesis *in vivo* (17, 18). However, the functional roles of tumor-related B7-H1 and association of PD-1 with B7-H1 have not been analyzed previously in human tumor tissue.

In the present study, using immunohistochemistry, we investigated the extent of B7-H1 and B7-DC expression in tumor specimens of non-small cell lung cancer, and we analyzed the relationship between their expression and clinicopathological variables or postoperative survival. Furthermore, we also examined the association between B7-H1 expression on tumor cells and PD-1 expression on tumor-infiltrating lymphocytes (TILs).

PATIENTS AND METHODS

Tumor Specimens and Survival Data. Primary tumor specimens were obtained by surgery from 52 non-small cell lung cancer patients (35 men and 17 women; mean age at diagnosis, 66.4 years) at Hokkaido University Medical Hospital, Japan, between 1997 and 2003. Surgically resected specimens were fixed in formalin and embedded in paraffin for routine histopathological diagnosis and embedded in OCT compound (Miles Laboratories, Elkhart, IN) and snap frozen in liquid nitrogen for immunohistochemical analysis. The surgically resected specimens included 31 adenocarcinomas and 21 squamous cell carcinomas, based on World Health Organization criteria of histopathological classification (19). The tumors were classified as stage I (n = 35), stage II (n = 11), stage III (n = 5), and stage IV (n = 1) tumors based on the American Joint Committee on Cancer guidelines for postsurgical tumor-node-metastasis (20). No patient underwent radiation or chemotherapy before surgery. Survival of the 52 non-small cell lung cancer patients was analyzed for patients who met the following criteria: (*a*) survived for >3 months after surgery; and (*b*) did not die of any cause other than lung cancer after surgery. Two patients who did not meet the above-mentioned criteria were excluded from the survival analysis; one died within 3 months after surgery, and the other patient died due to a cause other than lung cancer.

Immunohistochemistry. First, 4–5-μm sections of the specimens were air-dried for 10 min and then fixed in acetone for 10 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxidase in PBS for 30 min. Sections were then washed three times in PBS. After blocking nonspecific binding with serum (Vectastain ABC kits; Vector Laboratories, Burlingame, CA) for 20 min, sections were incubated with the primary antibodies in a humid chamber at 4°C overnight. Anti-B7-H1 (MIH1), anti-B7-DC (MIH14), and anti-PD-1 (MIH4) antibodies (diluted 1:200; Ref. 21) and anti-CD45 antibody (UCHL-1; DAKO, Carpinteria, CA; diluted 1:100) were used as

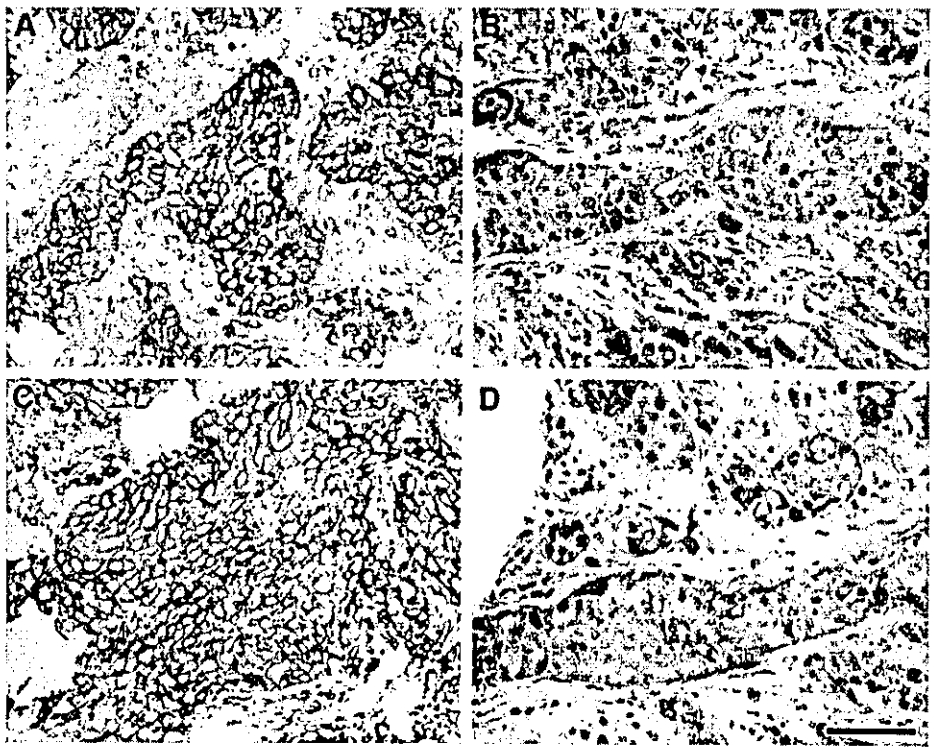

Fig. 1 Representative immunohistochemical staining in non-small cell lung cancer. B7-H1-positive case of non-small cell lung cancer (*A* and *C*) and B7-DC-positive case of non-small cell lung cancer (*B* and *D*) in a focal pattern (*A* and *B*) and a scattered pattern (*C* and *D*). *Scale bar*, 100 μm.

the primary antibodies. After three washes with PBS, sections were incubated with biotinylated secondary antibodies for 30 min, washed three times in PBS, and incubated with streptavidin-conjugated peroxidase for 30 min. After three additional washes in PBS, 3,3'-diaminobenzidine tetrahydrochloride was applied, and sections were then counterstained with hematoxylin. The entire procedure, with the exception of incubation with primary antibodies, proceeded at room temperature. Nonimmunized mouse IgG for B7-H1, B7-DC, and PD-1 was substituted for the primary antibody in the negative controls.

Cell Counting. B7-H1 and B7-DC expression was defined as the percentage of tumor cells displaying immunoreactivity in the cytoplasm or on the membrane and calculated by counting the number of B7-H1- and B7-DC-stained tumor cells among 1000 tumor cells in each section. One or two representative tissue sections were taken from each tumor, and whole areas were surveyed microscopically at ×100 magnification. Cell counts were performed at ×400 in at least five fields in randomly selected tumor areas.

To examine whether B7-H1 expression was associated with infiltration of TILs and PD-1 expression of TILs, we quantified the infiltration of $CD45^+$ cells and PD-1 expression of these cells in PD-L1-positive and -negative non-small cell lung cancer tumor regions, as described previously (22). First, B7-H1-positive and B7-H1-negative areas were located on a B7-H1-stained tumor section. Consecutive slides from the same tumor, stained for either CD45 or PD-1, were superimposed on the B7-H1-stained slide. Using histological landmarks, the corresponding B7-H1-positive and B7-H1-negative areas were located on these slides. The B7-H1-stained section was removed, and a second investigator, who had no prior knowledge of the local status of B7-H1, counted the number of $CD45^+$ cells per 1000 total nuclei or the number of PD-1^+ $CD45^+$ cells per 500 total $CD45^+$ cells. All of the counting was done in a blinded fashion; the observers (J. K. and K. Y.) were not informed of the outcome of the patients or the results of other observers. For analysis of relationships with clinicopathological variables or survival time, patients were divided into two groups using the median number of B7-H1-positive cells or B7-DC-positive cells as the distinguishing factor.

Statistical Analysis. The association between the number of immunoreactive cells and clinicopathological variables was analyzed statistically using Student's paired t test, Wilcoxon's rank-sum test, or the χ^2 test, as appropriate, using Statview software version 4.5 (SAS Institute). The correlation between the percentage of B7-H1 and B7-DC was analyzed statistically using Spearman's rank correlation. The survival curves were estimated by the Kaplan-Meier method. Values of $P < 0.05$ were considered to indicate statistical significance, and all tests were two-tailed.

RESULTS

B7-H1 and B7-DC Expression on Tumor Cells. Among all 52 surgically resected specimens of non-small cell lung cancer without preoperative therapy, the expression of B7-H1 and B7-DC was demonstrated in the cell membrane, cytoplasm, or both, in a focal or scattered pattern (Fig. 1). The percentage of B7-H1-positive cells in non-small cell lung cancer was 27.2 ± 4.7% (mean ± SD; median, 11.2%), whereas the percentage of B7-DC-positive cells was 11.4 ± 2.6% (mean ± SD; median, 7.9%). The percentage of B7-H1-positive cells in adenocarcinoma was larger (mean ± SD, 27.6 ± 33.2%; median, 12.1%) than that in squamous cell carcinoma (mean ± SD, 26.5 ± 35.2%; median, 7.9%). On the other hand, the percentage of B7-DC in squamous cell carcinoma was higher (mean ± SD, 13.1 ± 19.4%; median, 10.5%) than that in adenocarcinoma (mean ± SD, 10.6 ± 18.5%; median, 7.5%). There was a statistically significant correlation between the percentage of B7-H1-positive cells and that of B7-DC-positive cells in both adenocarcinoma ($R^2 = 0.23$; $y = 0.266x + 3.267$; $P < 0.01$; Fig. 2*A*) and squamous cell carcinoma ($R^2 = 0.53$; $y = 0.397x + 2.01$; $P < 0.01$; Fig. 2*B*). However, concomitant expression of B7-H1 and B7-DC was rarely observed on the same tumor cells (Fig. 3).

Next, we divided the 52 non-small cell lung cancer patients who had resected tumors into two groups to analyze correlation between B7-H1 or B7-DC expression and clinicopathological variables, using the median number of the percentages of B7-H1-positive cells and B7-DC-positive cells as the distinguishing factor. Tables 1 and 2 show the relationships between expression of either protein and clinicopathological variables. No correlation was observed between B7-H1 or B7-DC expression and either age, sex, smoking habit, histology, differentiation, pT

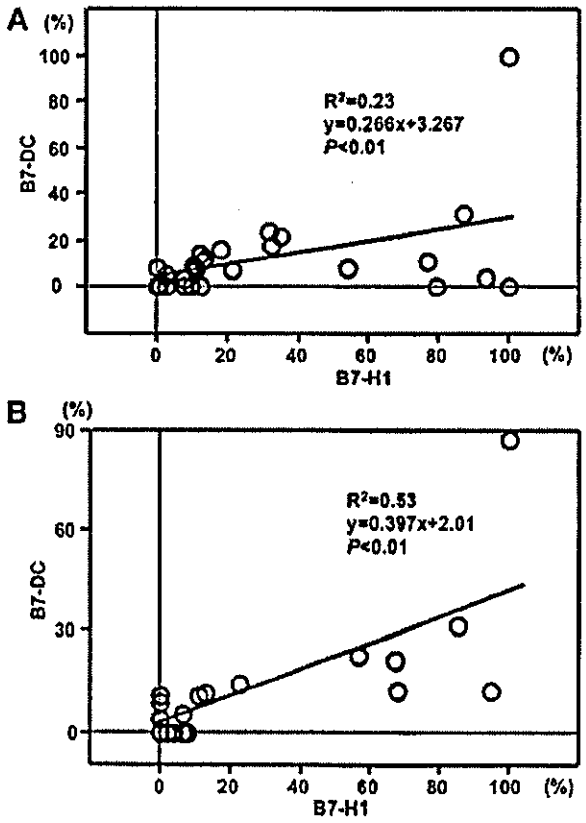

Fig. 2 Relationship between B7-H1 and B7-DC in adenocarcinoma (*A*) and squamous cell carcinoma (*B*).

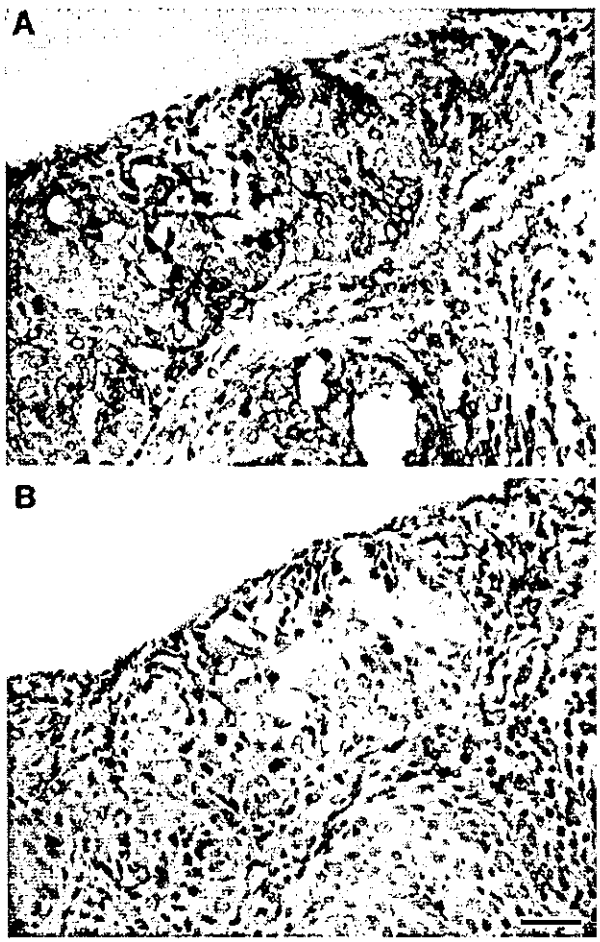

Fig. 3 Representative immunohistochemical staining of B7-H1 (*A*) and B7-DC (*B*) on the same non-small cell lung cancer sections. *Scale bar*, 100 μm.

classification, pN classification, or pStage classification. Among the 50 non-small cell lung cancer patients with potentially resected tumors, no relationship was found between B7-H1 or B7-DC expression and patient survival [5-year survival rates: 59% in those with B7-H1-positive non-small cell lung cancer and 48% in those with B7-H1-negative non-small cell lung cancer ($P = 0.89$); 53% in those with B7-DC-positive non-small cell lung cancer and 50% in those with B7-DC-negative non-small cell lung cancer ($P = 0.43$)]. Although the survival of 10 patients with higher expression of B7-H1 or B7-DC was compared with 10 corresponding patients with no expression of either relevant molecule, no significant differences in either analysis were observed.

Decreased Infiltration of TILs with Decreased PD-1 Expression in B7-H1-Expressing Tumor Regions. All non-small cell lung cancers contained infiltrates of cells that were immunohistochemically positive for CD45 (leukocyte common antigen) and almost exclusively of lymphoid morphology, indicating that they were TILs (Fig. 4). To evaluate whether B7-H1 or B7-DC expression on non-small cell lung cancers limited immune effector cell infiltration within tumors, we counted and compared the TILs in B7-H1- or B7-DC-positive and -negative tumor regions on the same sections. Of all non-small cell lung cancers examined, only five contained separate B7-H1-positive and -negative tumor regions in the same sections. Because the other samples had B7-H1-positive and -negative tumor cells mixed together in the same tumor nests throughout the sections and B7-H1-positive and -negative tumor regions were not separated, no comparison was possible (Fig. 1*C*). On the other hand, all non-small cell lung cancer samples had B7-DC-positive and -negative tumor cells mixed together in the same tumor nests throughout the sections, and the association between B7-DC expression on non-small cell lung cancers and immune effector cell infiltration within tumors also could not be analyzed (Fig. 1*D*). In the five sections that were suitable for analysis, the amount of TIL infiltration was significantly reduced in B7-H1-expressing tumor regions (Fig. 4). B7-H1 expression was associated with a statistically significant (on average, 2.5× reduction) in TILs in B7-H1-positive tumor regions compared with B7-H1-negative tumor regions within the same sections (22.6 ± 13% and 51.5 ± 14.1%, respectively; $P = 0.01$; Table 3). On the other hand, there were no histological or clinicopathological differences compared with the rest of tissues. The percentage of B7-DC-positive cells in these five

Table 1 Correlation between B7-H1 expression on tumor cells and the clinicopathological characteristics of the 52 non-small cell lung cancers

	B7-H1$^+$ expression on tumor cells		
	Low (<11%)	High (≥11%)	*P*
Patients	26	26	
Age (mean ± SD) (yrs)	64.9 ± 1.7	68 ± 2.1	0.28*
Sex			
Male	20	15	0.14†
Female	6	11	
Smoking habits			
Smoker	18	19	NS†
Nonsmoker	8	7	
Histology			
SCC	14	17	0.4†
Adenocarcinoma	12	9	
Differentiation			
Well	10	5	0.13†
Moderate/poor	16	21	
pT classifications			
T_1	8	6	0.54†
T_{2-4}	18	20	
pN classifications			
N_0	16	15	0.78†
N_{1-3}	10	11	
pStage classifications			
I	13	12	0.78†
II–IV	13	14	
CD45$^+$ cells‡	47.8 ± 3.2	42.8 ± 3.1	0.27*

Abbreviations: NS, nonsignificant; SCC, squamous cell carcinoma.
* Student's paired t test.
† χ^2 test.
‡ Percentage of CD45-positive cells per 1000 total nuclei.

Table 2 Correlation between B7-DC expression on tumor cells and the clinicopathological characteristics of the 52 non-small cell lung cancers

	B7-DC$^+$ expression on tumor cells		
	Low (<8%)	High (≥8%)	*P*
Patients	26	26	
Age (mean ± SD) (yrs)	66.0 ± 1.6	66.9 ± 2.3	0.73*
Sex			
Male	18	17	0.77†
Female	8	9	
Smoking habits			
Smoker	16	22	0.06†
Nonsmoker	10	4	
Histology			
SCC	9	12	0.06†
Adenocarcinoma	17	14	
Differentiation			
Well	9	6	0.36†
Moderate/poor	17	20	
pT classifications			
T_1	7	7	NS†
T_{2-4}	19	19	
pN classifications			
N_0	17	14	0.4†
N_{1-3}	9	12	
pStage classifications			
I	14	11	0.4†
II–IV	12	15	
CD45$^+$ cells‡	41.8 ± 3.2	48.8 ± 3.0	0.11*

Abbreviations: SCC, squamous cell carcinoma; NS, nonsignificant.
* Student's paired *t* test.
† χ^2 test.
‡ Percentage of CD45-positive cells per 1000 total nuclei.

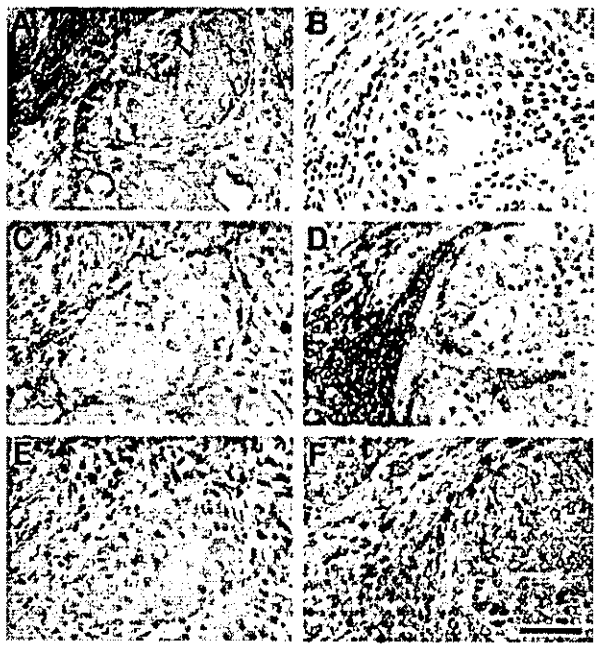

Fig. 4 Representative immunohistochemical staining in B7-H1-positive tumor regions (*A*) and B7-H1-negative tumor regions (*B*) on the same non-small cell lung cancer sections. On consecutive tumor sections, TILs were identified by CD45 staining (*C* and *D*), and PD-1 expression was identified immunohistochemically (*E* and *F*). A low proportion of TILs in B7-H1-positive tumor regions is shown in *C*. A high proportion of TILs in B7-H1-negative tumor regions is shown in *D*. Expression of PD-1 is lower on TILs in B7-H1-positive tumor regions (*E*) compared with that on TILs in B7-H1-negative tumor regions (*F*). *Scale bar*, 100 μm.

sections was 15 ± 3.5% (mean, 12.5%), and there was no difference compared with that in the rest of tissues ($P = 0.65$).

Moreover, we analyzed the expression of PD-1, one of the receptors of B7-H1, on TILs in B7-H1-positive tumor regions and in B7-H1-negative regions. A statistically significant (on average, 3-fold) decrease in PD-1-positive CD45-positive TILs was observed in B7-H1-positive tumor regions compared with B7-H1-negative tumor regions (7.1 ± 4.5% and 20.2 ± 9.0%, respectively; $P = 0.02$; Table 3).

DISCUSSION

We have demonstrated the expression of B7-H1 and B7-DC in surgically resected specimens of non-small cell lung cancer, and we have demonstrated that the expression of these molecules is found in both the plasma membrane and cytoplasm of cancer cells. The expression patterns of these molecules were consistent with previous reports, which examined their expression in human tumor tissue (12, 13, 16, 17). The present study

Table 3 The percentage of CD45$^+$ cells and the expression of PD-1 on CD45$^+$ cells in B7-H1-positive and B7-H1-negative tumor regions

Tumor specimen	Age (yrs)	Sex	Histology (differentiation)	CD45$^+$ cells*		PD-1$^+$/CD45$^+$ cells†	
				B7-H1-positive region	B7-H1-negative region	B7-H1-positive region	B7-H1-negative region
1	73	F	Ad(W)	23.4	43.3	11	28
2	53	F	Ad(M)	32.2	52.9	7.2	9.8
3	60	M	Ad(M)	11.2	42.8	2.2	16
4	79	F	Sq(W)	38.2	42.9	12	15
5	57	M	Ad(W)	8.1	75.5	2.8	31
Mean ± SD				22.6 ± 13	51.5 ± 14.1	7.1 ± 4.5	20.2 ± 9.0
				$P = 0.01$‡		$P = 0.02$‡	

Abbreviations: Ad, adenocarcinoma; Sq, squamous cell carcinoma; W, well differentiated; M, moderately differentiated.
* Percentage of CD45$^+$ cells per 1000 total nuclei counted within a B7-H1-positive tumor region/B7-H1 tumor-negative region.
† Percentage of PD-1$^+$ cells per CD45$^+$ cells counted within a B7-H1-positive tumor region/B7-H1-negative tumor region.
‡ Wilcoxon's rank-sum test (B7-H1-positive tumor region *versus* B7-H1-negative tumor region).

also examined the relationship between the expression of B7-H1 or B7-DC and clinicopathological variables or prognosis of patients, but no statistically significant relationship was found. We also examined expression of B7h, which is a ligand for ICOS, but no obvious expression in surgically resected specimens of non-small cell lung cancer was noted (data not shown). Expression of two other B7 family members, B7.1 and B7.2, is undetectable or low in most tumor cells (23–25). Thus, expression of B7 family members differs among tumor tissues.

Tumor cells evade host immune surveillance by several strategies. These include down-regulation of cell surface major histocompatibility complex class I molecules (24, 26), secretion of immunosuppressive factors [*e.g.*, transforming growth factor β and IL-10 (27, 28)], lack of T-cell costimulation (24, 25, 29), and expression of death ligands or negative ligands (30, 31). Recently, B7-H1 has been shown to be involved in negative regulation of immune responses through the PD-1 receptor on activated T and B cells (8, 9) and has been thought to be a candidate strategy by which cancer cells evade host immune surveillance. The present study is the first to demonstrate in human tumor tissue that the number of TILs in B7-H1-positive tumor regions is significantly lower than that in B7-H1-negative tumor regions and, more importantly, that the number of PD-1-positive TILs in B7-H1-positive regions is significantly lower than that in B7-H1-negative regions. These findings suggest that B7-H1 on tumor cells might contribute to negative regulation against TILs in non-small cell lung cancer. B7-H1 expression on tumor cells may inhibit infiltration of PD-1-expressing TILs or cause down-regulation and apoptosis of infiltrated PD-1-expressing TILs.

The mechanisms regulating B7-H1 expression on tumor cells are not known. Inflammatory mediators are implicated by up-regulation of B7-H1 expression on the surface of several tumor cell lines after exposure to interferon γ (13, 16). Moreover, B7-H1 expression is more frequent in freshly isolated cancer tissue specimens than in cultured tumor cell lines (13), and the expression of B7-H1 on tumor-related dendritic cells may be up-regulated by tumor environmental factors (IL-10 or vascular endothelial growth factor) of ovarian cancer (32). These observations suggest that the cytokine microenvironment induces the expression of B7-H1 on tumor cells. On the other hand, T cells or natural killer cells that infiltrate tumor tissue secrete many cytokines, including interferon γ. Therefore, one possible scenario is that TILs secrete interferon γ in the beginning, followed by up-regulation of B7-H1 on tumor cells; thereafter, the up-regulated B7-H1 on tumor cells induces T-cell apoptosis via PD-1. Although the duration of B7-H1 expression on tumor cells after B7-H1 up-regulation is unclear, immunohistochemical staining might elucidate one step in these sequential events.

On the other hand, B7-H1 expression was absent in several cases in the present study, although TILs existed in most of these cases. This absence is most likely due to original tumor features. This finding is supported by a previous study, in which B7-H1 expression in several tumor cells was not detected even after exposure to interferon γ (17).

In contrast to the negative regulatory functions of B7-H1 and B7-DC against T-cell activation, positive costimulatory functions of B7-H1 and B7-DC in T-cell proliferation and cytokine production have been reported recently, and the functions of these molecules remain controversial. Dong *et al.* (5) reported that B7-H1 costimulated proliferative responses of T cells and induced IL-10 production on polyclonal T-cell stimuli and allogeneic antigens via IL-2-dependent process. Tseng *et al.* (33) reported that B7-DC costimulated T-cell proliferative responses and interferon γ production to greater levels than B7.1. Unlike B7-H1-expressing tumor cells, in one study, B7-DC-transfected tumor cells increased the number of antigen-specific T cells and were rapidly rejected *in vivo* by a PD-1-independent mechanism (34). Additional studies are needed to resolve the precise mechanisms by which B7-H1 and B7-DC affect tumor immunity. Moreover, it has been reported recently (13, 16) that tumor-associated B7-H1 negatively regulates T-cell activation through receptors that remain unidentified, with the exception of PD-1. Additional studies related to tumor-associated B7-H1 and its receptor in non-small cell lung cancer are also required.

In conclusion, we have demonstrated the expression of B7-H1 and B7-DC in surgically resected specimens of non-small cell lung cancer. Moreover, the number of TILs and PD-1 expression on TILs in B7-H1-positive tumor regions were significantly lower than that in negative regions. These results suggest that the expression of B7-H1 on tumor cells might contribute to negative regulatory immune responses against TILs in non-small cell lung cancer. Recently, it has been reported that B7-H1 blockade improves antitumor immunity and represents one approach for cancer immunotherapy (13, 17, 18, 32). The blockade of B7-H1 in non-small cell lung cancer might be one strategy to pursue for future immunotherapy in non-small cell lung cancer.

REFERENCES

1. Ginsberg RJ, Vokes EE, Raben A. Non-small cell lung cancer. In: De Vita VT, Hellmann S, Rosenberg SA, editors. Cancer: principles and practice of oncology, 6th ed. Philadelphia: Lippincott-Raven Publishers; 2001. p. 858–910.
2. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17:138–46.
3. Sperling AI, Bluestone JA. The complexities of T-cell co-stimulation: CD28 and beyond. Immunol Rev 1996;153:155–82.
4. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science (Wash DC) 2000;290:84–9.
5. Dong H, Zhu G, Tamada K, Chen L. B7–H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin 10 secretion. Nat Med 1999;5:1365–9.
6. Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat Immunol 2001;2:261–8.
7. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 2002;20:29–53.
8. Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192: 1027–34.
9. Carter LL, Fouser LA, Jussif J, et al. PD-1: PD-L inhibitory pathway affects both $CD4^+$ and $CD8^+$ T cells and is overcome by IL-2. Eur J Immunol 2002;32:3376–85.
10. Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol 2001;22:265–8.
11. Nishimura H, Nose M, Hirai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene