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. *Ps* < 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

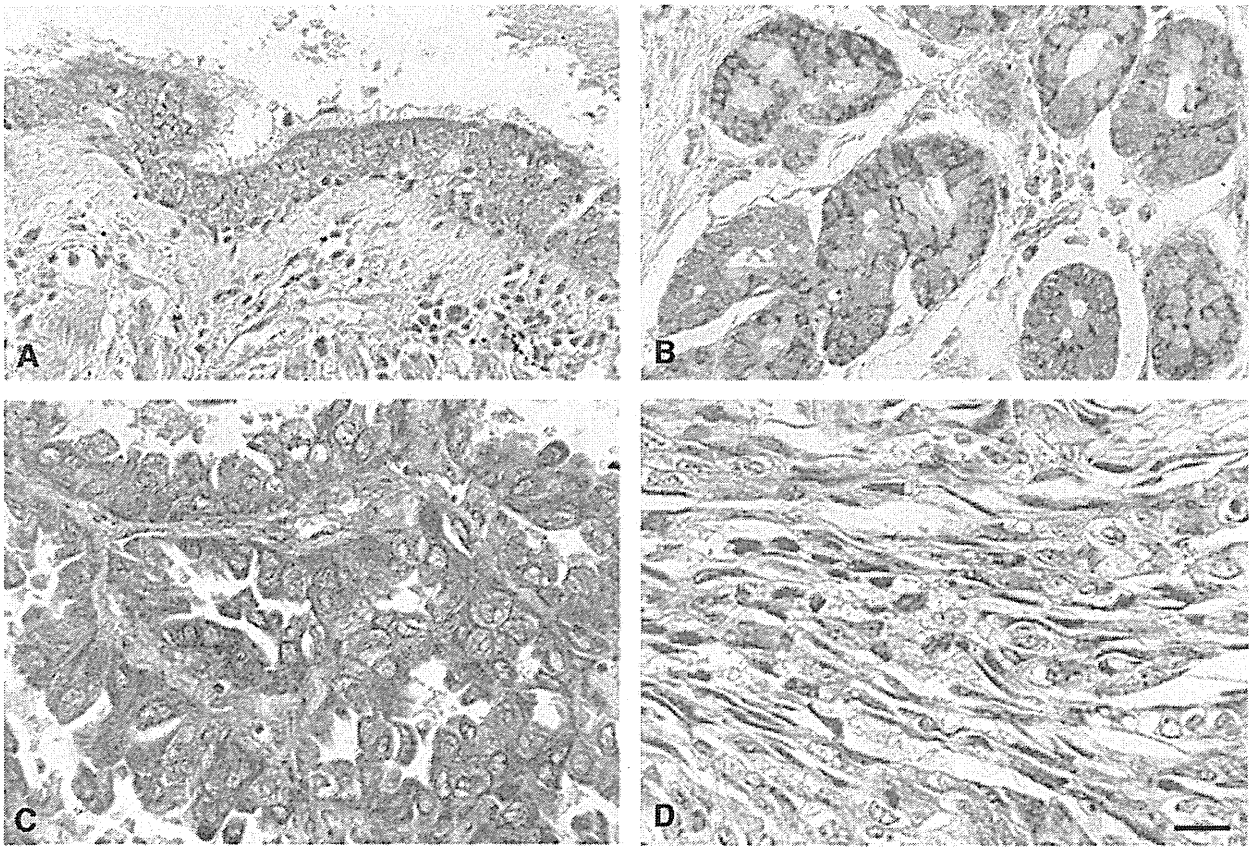

Fig. 1 Immunohistochemical staining patterns for *N*-acetylglucosaminyltransferase V (GnT-V) in normal bronchial tissue and non-small cell lung cancers. Normal bronchial epithelial cells (*A*) and bronchial gland cells (*B*) show GnT-V expression. An adenocarcinoma tumor shows high GnT-V expression diffusely 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.

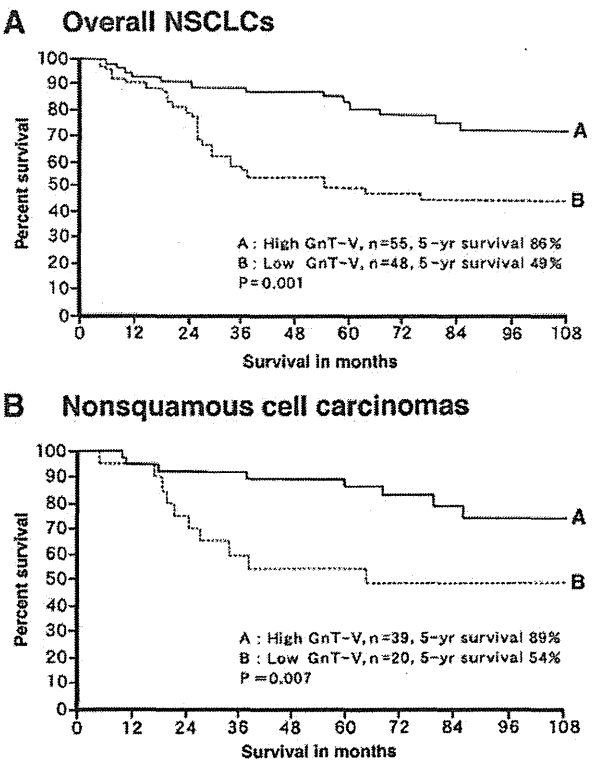

Fig. 2 Kaplan-Meier survival curves for patients with non-small cell lung cancers. Survival curves for patients with pStage I tumors are stratified by low and high *N*-acetylglucosaminyltransferase V expression for overall non-small cell lung cancers ($n = 103$; *A*) and for non-squamous cell carcinomas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

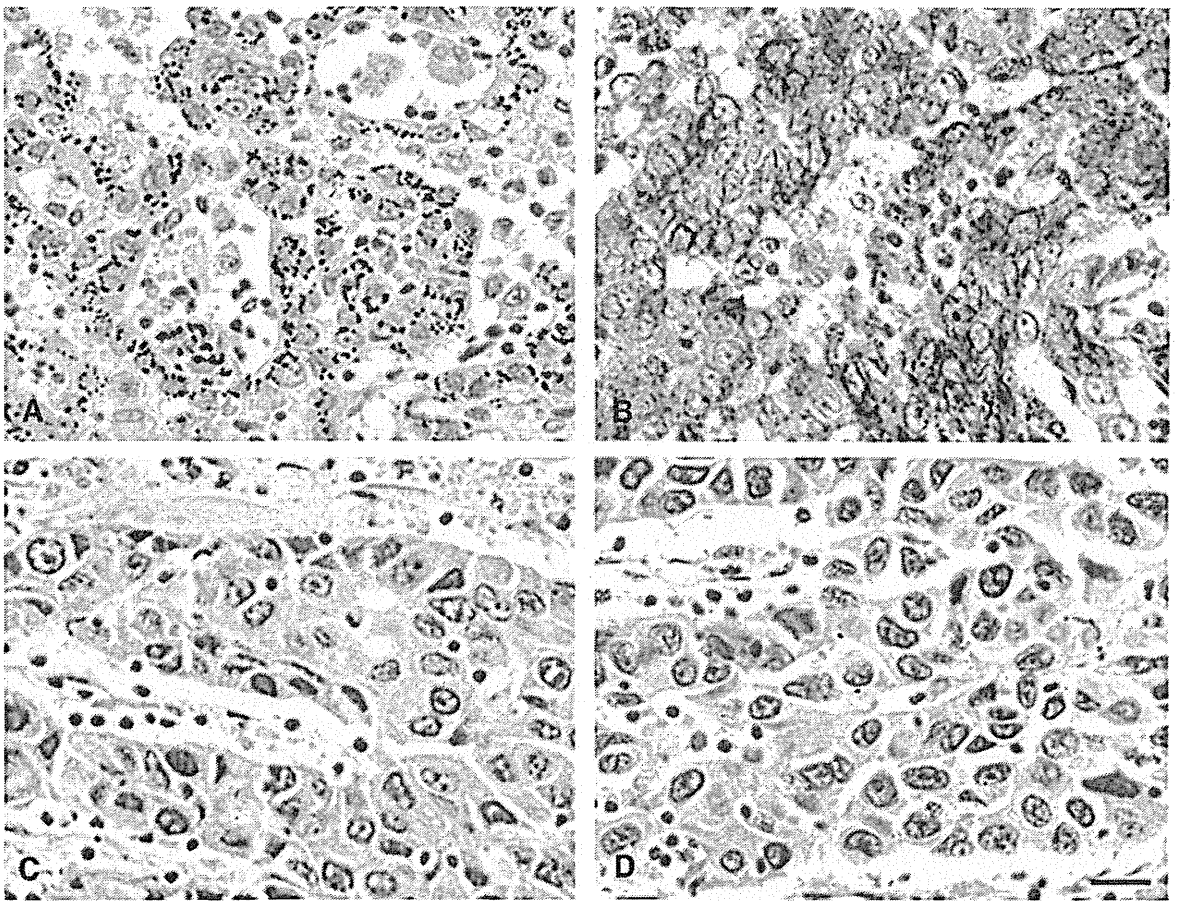

Fig. 3 Immunohistochemical staining patterns for *N*-acetylglucosaminyltransferase V (GnT-V; *A* and *C*) and staining patterns for leukoagglutinating phytohemagglutinin (L-PHA; *B* and *D*) in non-small cell lung cancers. The staining pattern of a tumor with high GnT-V expression and high L-PHA staining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

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Phase I Trial of Carboplatin and Weekly Paclitaxel in Patients With Advanced Non-small-cell Lung Cancer

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Objective: This study was designed to determine the maximum tolerated dose of paclitaxel administered weekly in combination with carboplatin and to assess its dose limiting toxicity and preliminary activity in patients with previously untreated, advanced non-small-cell lung cancer.

Methods: Carboplatin was administered at a fixed dose that maintained an area under the curve of 6. Paclitaxel was given over 1 h once a week for 3 weeks starting at 60 mg/m^2 and escalated in 10 mg/m^2 increments.

Results: Twenty-one patients were treated with six dose levels (60, 70, 80, 90, 100, 110 mg/m^2) of paclitaxel. The dose limiting toxicity was infection and the maximum tolerated dose was 110 mg/m^2. Nine of 21 (42.9%) patients demonstrated a therapeutic response.

Conclusion: Weekly paclitaxel and carboplatin were well tolerated. Based on our results, 100 mg/m^2 of paclitaxel for 3 weeks of a 4-week cycle, in combination with carboplatin, was recommended for a phase II study.

Key words: carboplatin – non-small-cell lung cancer – paclitaxel – phase I study

INTRODUCTION

Non-small-cell lung cancer (NSCLC) is the leading cause of death from cancer in the world. Unfortunately, when NSCLC is diagnosed most patients have locally advanced or disseminated cancers. The median survival of patients with stage IIIB and IV NSCLC ranges from 6 to 8 months and only 20–30% survive for 1 year.

Paclitaxel (Taxol; Bristol-Myers Squibb) is a clinically active anticancer drug that inhibits cell division by promoting the assembly of microtubules and stabilizing the tubulin polymers in the G_2/M phase (1). Consequently, paclitaxel causes the formation of abnormal bundles of microtubules during the cell cycle, and it has antiangiogenic activity (2). Carboplatin (Paraplatin; Bristol-Myers Squibb) is a less toxic analog of cisplatin, which is thought to inhibit DNA synthesis by forming interstrand and intrastrand cross-linking of DNA molecules. Carboplatin is as efficacious as cisplatin in treating NSCLC.

A recent Eastern Cooperative Oncology Group (ECOG) study compared third-generation chemotherapy regimens, which included cisplatin with paclitaxel, cisplatin with gemcitabine, cisplatin with docetaxel and carboplatin with paclitaxel. The results showed that there were no differences in survival, and carboplatin with paclitaxel had the lowest degree of toxicity. Therefore, ECOG selected carboplatin with paclitaxel as its reference regimen (3). In addition, the combination of carboplatin [area under the curve (AUC) = 6] and paclitaxel (225 mg/m^2) administered every 3 weeks is the most commonly used regimen in the USA. The response rate with this regimen ranges from 17% to 25%, with median survival times averaging 8 months (3–5). While the regimen is well tolerated, it is associated with a 10% to 17% incidence of grade 3 neuropathy (3–5).

Weekly regimens of paclitaxel and carboplatin were developed in an effort to increase efficacy and reduce toxicity. Belani et al. (6) studied various regimens and found that paclitaxel (paclitaxel 100 mg/m^2 weekly for three of 4 weeks) plus carboplatin (AUC = 6 on day 1) was the most effective and least toxic. For example, this regimen had a response rate of 32%, a median survival time of 49 weeks, and a 1-year survival rate of 47%. Comparison with the previous studies using the standard every-3-week schedule of paclitaxel and carboplatin indicated that the weekly regimens achieved favorable efficacy with a highly tolerable toxicity profile.

In Japan, a phase I trial of carboplatin plus weekly paclitaxel was conducted in advanced NSCLC, and the recommended dose level of paclitaxel was 70 mg/m^2 on days 1, 8 and 15 in

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combination with carboplatin (AUC = 6) on day 1 of a 4-week cycle (7). The dose level of paclitaxel was much lower than in Belani's study. In order to reconfirm the dose level of paclitaxel, we conducted a phase I trial of weekly paclitaxel (on days 1, 8 and 15) with carboplatin (on day 1) of a 4-week cycle for advanced NSCLC.

SUBJECTS AND METHODS

Patient Eligibility

This phase I trial was designed to determine the maximum tolerated dose (MTD) and toxicity of paclitaxel administered on a weekly schedule to patients with advanced NSCLC. A secondary objective was the determination of efficacy. The ethics committee at Hokkaido University School of Medicine approved this study. Inclusion criteria were: (i) histological or cytological evidence of NSCLC with no prior chemotherapy; (ii) stage IIIB or IV disease that was not curable with chemoradiation as the first choice; (iii) measurable or assessable disease; (iv) ECOG performance status (PS) ≤1; (v) between 20 and 75 years of age with white blood cell (WBC) counts $>4 \times 10^9$/l, hemoglobin (Hb) >9.5 g/dl, platelet (PLT) counts $>100 \times 10^9$/l, bilirubin <1.5 mg/dl, GOT and GPT less than twice upper limits of normal, and creatinine <1.5 mg/dl, P_aO_2 >70 torr; (vi) anticipated survival at least 3 months and (vii) provided written informed consent. Exclusion criteria were: (i) serious concomitant systemic disorders; (ii) severe heart failure within 3 months, uncontrollable angina or hypertension; (iii) diabetes mellitus; (iv) interstitial pneumonia; (v) active infection, ulcer or second primary malignancy; (vi) history of severe hypersensitivity or hypersensitivity to the study drug or polioxyetilene and (vii) pregnancy. We also measured plasma paclitaxel concentrations during treatment to compare with those established for every-3-week regimens.

Treatment Schedule

The trial was designed as a dose-escalation study of paclitaxel and carboplatin used in combination therapy scheduled every 4 weeks. The dose of carboplatin was fixed to target AUC 6 on day 1. The starting dose of paclitaxel was 60 mg/m^2 given in 1-h i.v. infusions on days 1, 8 and 15 every 4 weeks. If treatment was well tolerated, then successive dose levels were increased in intervals of 10 mg/m^2 in groups of three patients to 70, 80, 90 mg/m^2, if there was no dose-limiting toxicity (DLT). DLT was defined as: (i) persistent (>4 days) leucopenia (<1000/μl); (ii) active infection or fever (>38°C) with grade 3/4 neutropenia; (iii) a PLT count <20 000/μl; (iv) any grade 3/4 non-hematological toxicities, except appetite loss, nausea and vomiting; (v) a delay of second cycle within 6 weeks. If no one encountered dose limiting toxicity, then subsequent patients entered the study at the next greater dose level. If one of the three patients encountered DLT, then subsequent patients entered at the same level, to a total of six patients. If more than one of three or more than two of six patients had DLT at a specific dose level, then that dose levels was defined as the maximum tolerated dose. Anaphylactic premedication included diphenhydramine (10 mg i.v.) and ranitidine (50 mg i.v.) and dexamethasone (20 mg i.v.) 1 h before paclitaxel infusion.

The National Cancer Institute (NCI) common toxicity scale (8) was used to grade side effects. The treatment plan was put on hold for any of the following reasons: (i) white blood cell (WBC) count <3000/μl or PLT count 75 000/μl within 24 h of the day of treatment; (ii) fever >38°C; (iii) PS 3 or (iv) grade 3/4 non-hematological toxicities. Treatment was discontinued when disease progressed, patient died, patient withdrew or experienced septic shock or grade 4 non-hematological toxicity, or decision of clinician. The dose modifications were made after the first 4 weeks of therapy as necessary. Repeated cycles were delivered as their assigned first-dose level unless modified for toxicity. If a patient developed hematologic DLT, each drug was reduced by 20% of previous cycle. The response was evaluated according to WHO criteria (9).

Phamacokinetic Analysis

The disposition of paclitaxel was determined in three patients who received 100 mg/m^2. Blood samples were collected at 0, 2.5, 3.0, 6.0, 10, 24 and 48 h after infusion in tubes that contained potassium edetic acid. Plasma was immediately separated by centrifugation at 3000 r.p.m. for 3 min and stored at −20°C until analysis. Plasma paclitaxel concentrations were determined by high-performance liquid chromatography (HPLC) at SBS, Inc. (Sagamihara, Japan). The pharmacokinetic parameters were calculated using MOMENT (EXCEL), which was developed using Microsoft Excel.

RESULTS

Twenty-one patients with NSCLC entered this trial through six dose levels (Table 1). There were 14 men and seven women with median age of 65 years (range, 44–75). Seven patients had stage IIIB disease and 14 patients had stage IV disease. Adenocarcinoma was the most common histology (n = 17) followed by squamous cell carcinoma (n = 4). Two patients were treated at relapse after surgical resection and 19 were treated during their initial presentation.

Three patients entered at 60, 70, 80, 90 and 110 mg/m^2 (Table 2). Six patients entered at 100 mg/m^2, because one of the first three patients had a delayed second cycle of 6 weeks because of decrease in WBC counts. This patient developed asymptomatic neutropenia. Paclitaxel on day 15 was postponed to day 30. Since five of six patients completed the first cycle at the 100-mg/m^2 dose level, the dose of paclitaxel was advanced to the next greater level. Two patients at the 110-mg/m^2 dose level developed cases of pneumonia that were associated with grade 3/4 neutropenia that cleared with antibiotics. Thus, criteria of DLT were met in two of three patients treated with 110 mg/m^2 (Table 2).

Table 1. Patient characteristics

Characteristic	No. of patients
Age, years	
Median	65
Range	44–75
Sex	
Male	14
Female	7
Performance status	
0	9
1	12
Histological type	
Adenocarcinoma	17
Squamous cell carcinoma	4
Stage	
IIIB	7
IV	14
Prior therapy	
Surgery	2
Radiotherapy	0
No therapy	19

Table 2. Dose level, emergence of DLT and response

Level	CBDCA (AUC)	Paclitaxel (mg/m^2)	No. of patient	DLT	Resonse
1	6	60	3	0	1 PR
2	6	70	3	0	2 PR
3	6	80	3	0	1 CR + 1 PR
4	6	90	3	0	1 PR
5	6	100	6	1*	1 PR
6	6	110	3	2†	2 PR

*Delay of second cycle within 6 weeks.
†Infection with grade 3/4 neutropenia. CBDCA, carboplatin; AUC, area under the curve (mg/ml/min); DLT, dose limiting toxicity; CR, complete response; PR, partial response.

Seven patients received one cycle of therapy, three completed two cycles, six completed three cycles, three completed four cycles, one completed five cycles and one completed seven cycles. Progression of disease was the most common reason for discontinuation. More than 148 doses of paclitaxel were administered to these 21 patients. The average cumulative dose of paclitaxel was 569 mg/m^2, and the maximum dose was 1278 mg/m^2. The longest duration of therapy was 13 months.

Toxicities of Therapy

Twenty-one patients and six dose levels were eligible for evaluation. The toxicities associated with this schedule were generally mild (Table 3). No significant (grade 3/4) red blood cell and PLT toxicities were noted. Although neuropathy is a complication of paclitaxel therapy, only one patient had grade 1 peripheral neuropathy. There was no anaphylaxis or hypersensitivity. Although no formal analysis of long-term toxicity was performed, no obvious cumulative hematologic, pulmonary or neurologic toxicity was noted.

Table 3. Toxicity by dose level in the first cycle (grade 3/4)

Toxicity*	Level					
	1	2	3	4	5	6
Hematological toxicity						
Leucopenia	1/0	0/0	0/0	0/0	0/0	1/0
Granulocytopenia	0/1	1/0	0/0	1/0	2/1	2/1
Anemia	0/0	0/0	0/0	0/0	0/0	0/0
Thrombocytopenia	0/0	0/0	0/0	0/0	0/0	0/0
Non-hematological toxicity						
Anorexia	0/0	0/0	0/0	0/0	1/0	1/0
Fatigue	0/0	0/0	0/0	0/0	1/0	0/0
Infection with grade 3/4 neutropenia	0/0	0/0	0/0	0/0	0/0	2/0

*NCI common toxicity scale

Response to Therapy

Of 21 assessable patients, one complete response (CR) and eight partial responses (PR) (42.9%) were observed. One occurred at level 1, two at level 2, two at level 3, one at level 4, one at level 5 and two at level 6 (Table 2). One patient attained a CR and he remained disease-free for 13 months. A minor response was also seen in five (23.8%) patients. Seven (33.3%) patients showed progression of their disease.

Pharmacokinetics

Pharmacokinetic studies were performed on three patients at 100 mg/m^2. Their mean peak concentration was 5.3 ± 0.72 (±SD) μmol/l. Their average interval peak plasma paclitaxel concentrations are depicted graphically in Figure 1. The mean 48-h concentration was 0.02 ± 0.00 μmol/l. Plasma paclitaxel concentrations at 48-h were >0.01 μmol/l, which is the minimum for a therapeutic response. Plasma paclitaxel levels remained >0.01 μmol/l for more than 144 h and >0.05 μmol/l for 27.9 ± 4.11 h. Key pharmacokinetic characteristics are shown in Table 4.

DISCUSSION

We conducted a phase I trial of weekly paclitaxel with carboplatin in advanced NSCLC. The recommended dose of paclitaxel in a phase II study was 100 mg/m^2, and the DLT was infection with grade 3/4 neutropenia. While the 100 mg/m^2 dose of paclitaxel was greater than doses previously reported in Japan (7), it was equivalent to the dose reported in Belani's

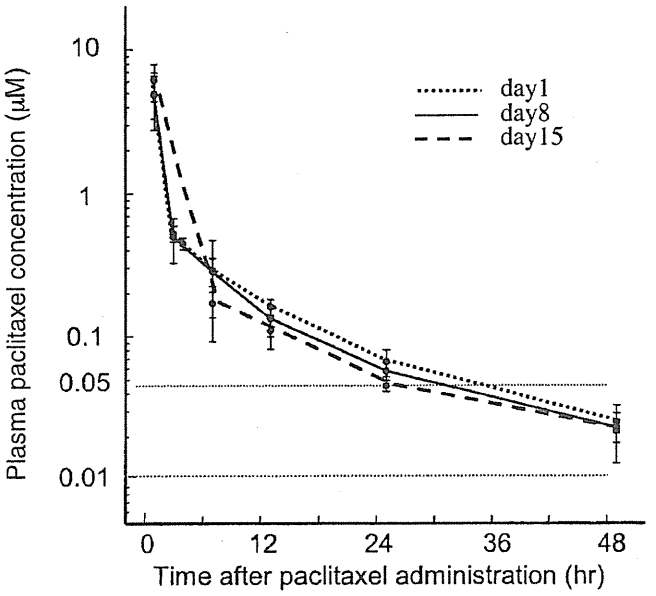

Figure 1. Time versus concentration curves of paclitaxel. Straight line, dotted line, and dashed line show mean ± SD concentration of three patients treated with 100 mg/m^2 on days 1, 8 and 15, respectively.

Table 4. Key pharmacokinetic parameters of paclitaxel

	Cmax (μM)	$t_{1/2}$ (h)	AUC (μM·h)	Time above 0.05 μM (h)
Day 1	6.13 ± 1.75	13.7	15.56	31.8
Day 8	4.93 ± 1.62	14.9	13.26	28.3
Day 15	4.84 ± 2.07	14.6	14.83	23.6

Cmax, maximum plasma concentration; $t_{1/2}$, half-life; AUC, area under the plasma concentration time curve.

study (6). Differences in the ratio of patients with PS 2 between another Japanese trial and the present may be a reason.

Since paclitaxel is a phase-specific agent, frequent or continuous schedules offer the greatest theoretical benefit (10). Depending on the duration of exposure, cellular cytotoxicity can be achieved at relatively low concentrations of this drug that are around 0.01 μmol/l (11,12). On the other hand, myelosuppression was related to the duration of exposure to plasma paclitaxel concentrations >0.05 μmol/l (13). In our study, the time that plasma paclitaxel concentrations remained >0.01 μmol/l was more than 144 h and the time it remained >0.05 μmol/l was 27.9 ± 4.11 h. Our results are similar to the findings in patients with metastatic breast cancer who received similar 1-h infusions of 100 mg/m^2 paclitaxel (14). Thus, a weekly schedule of paclitaxel extended the duration >0.01 μmol/l and >0.05 μmol/l of plasma paclitaxel concentration, as compared to an every-3-week regimen. The pharmacokinetic data of paclitaxel might explain the favorable response rate of a weekly schedule despite weakened intensity of carboplatin every 4 weeks in comparison with an every-3-week regimen. In addition to exposure duration issues, cellular cytokinetic considerations imply that frequent exposure to cytotoxic agents with brief intervals between exposures affords less opportunity for the emergence and regrowth of drug-resistant cell clones (15). On the other hand, severe myelosuppression was not seen in this study, which was not compatible with pharmacokinetic data.

Weekly administration of paclitaxel is dose-intense, but it also has a favorable toxicity profile (16,17). Non-hematologic toxicity was less common with weekly paclitaxel regimens. In our study, no patients developed grade 3 or 4 peripheral neuropathy. A peripheral neuropathy may begin as soon as 24–72 h after treatment with higher doses (>250 mg/m^2) but usually occurs only after multiple courses at conventional doses. Clinically, peripheral neurotoxicity occurs at cumulative doses of approximately 1500 mg/m^2 given at weekly doses of >110 mg/m^2 (18,19). Thus, weekly paclitaxel combination with carboplatin was a favorable regimen in the view of neuropathy, compared to a standard every-3-week regimen.

In conclusion, weekly paclitaxel and carboplatin were well tolerated and 100 mg/m^2 of paclitaxel for 3 weeks of a 4-week cycle in combination with carboplatin was recommended for a phase II study. A multi-institutional phase II study of this treatment is currently underway.

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Overexpression of the Aldo-Keto Reductase Family Protein AKR1B10 Is Highly Correlated with Smokers' Non–Small Cell Lung Carcinomas

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ABSTRACT

Purpose: **Squamous cell carcinoma (SCC) and adenocarcinoma of the lung are currently subject to similar treatment regimens despite distinct differences in histology and epidemiology. The aim of this study is to identify a molecular target with diagnostic and therapeutic values for SCC.**

Experimental Design: **Genes specifically up-regulated in SCC were explored through microarray analysis of 5 SCCs, 5 adenocarcinomas, 10 small cell lung carcinomas, 27 normal tissues, and 40 cancer cell lines. Clinical usefulness of these genes was subsequently examined mainly by immunohistochemical analysis.**

Results: **Seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*), were identified as SCC-specific genes. AKR1B10 was further examined by immunohistochemical analysis of 101 non–small cell lung carcinomas (NSCLC) and its overexpression was observed in 27 of 32 (84.4%) SCCs and 19 of 65 (29.2%) adenocarcinomas. Multiple regression analysis showed that smoking was an independent variable responsible for AKR1B10 overexpression in NSCLCs ($P < 0.01$) and adenocarcinomas ($P < 0.01$). AKR1B10 staining was occasionally observed even in squamous metaplasia, a precancerous lesion of SCC.**

Conclusion: **AKR1B10 was overexpressed in most cases with SCC, which is closely associated with smoking, and many adenocarcinoma cases of smokers. These results suggest that AKR1B10 is a potential diagnostic marker specific to smokers' NSCLCs and might be involved in tobacco-related carcinogenesis.**

INTRODUCTION

Lung cancer is the leading cause of cancer death among all types of cancers and continues to increase in frequency worldwide (1). There are two major types of lung cancer, small cell lung carcinoma (SCLC) and non–small cell lung carcinoma (NSCLC), which account for 20% and 80% of all cases (2), respectively. NSCLC is further classified into squamous cell lung carcinoma (SCC) and lung adenocarcinoma. Despite distinct differences in histologic and epidemiologic features, adenocarcinoma and SCC are similarly treated in clinical practice (3) partly because underlying molecular mechanisms are largely unknown. Even the most recent therapeutic innovations for NSCLC have yielded little improvement to prognosis with overall 5-year survival rates still <15% (4).

We reported previously the clinical relevance of expression of G_1-S transition regulatory molecules in prognosis, such as p53, retinoblastoma protein, $p16^{INK4A}$, and p27 in NSCLCs (5–8). We further showed that Ki-67-positive, high-level cyclin E, low-level *N*-acetylgalactosaminyl transferase-3 (GalNAcT3) and low-level *N*-acetylglucosaminyltransferase (GnT-V) are associated with shorter survival in NSCLCs (8–12). However, we did not observe any differences between SCC and adenocarcinoma.

SCC accumulates a series of genetic alterations in the progression from a normal bronchial epithelium, metaplasia, dysplasia, and carcinoma *in situ* to invasive carcinoma (13). Because most SCC develops in smokers and tobacco smoking reversibly induces metaplasia, smoking has been regarded as a major cause of SCCs (14). As diagnostic markers for SCC, SCC antigen and cytokeratin 19 fragment (CYFRA 21.1) have been widely used (15). Despite their usefulness in distinguishing between SCC and adenocarcinoma, these two molecules are hardly adequate for early detection of cancer (15). Moreover, their expression in normal squamous cell suggests that these two

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Note: This study was carried out as a part of the Technology Development for Analysis of Protein Expression and Interaction in Bioconsortia on R&D of New Industrial Science and Technology Frontiers that was overseen by the Industrial Science, Technology and Environmental Policy Bureau, Ministry of Economy, Trade & Industry, and delegated to New Energy Development Organization.
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molecules are not involved in carcinogenesis and inappropriate as therapeutic targets. Thus, search for genes specific to SCC alone will lead to identification of a novel molecular target of SCC, which may help developing both early detection of SCC and personalized therapeutics of SCC.

Microarray analysis has been applied to several aspects of cancer research, including classification, mechanistic elucidation, discovery of therapeutic targets, and development of tumor makers (16–21). For example, we recently explored potential diagnostic or therapeutic markers of hepatocellular carcinoma using microarray analysis and showed that soluble glypican-3 is a novel serologic marker essential for early detection of hepatocellular carcinoma (19). Recent reports on microarray analysis of lung cancer have shown that SCC and adenocarcinoma have different gene expression signatures, suggesting involvement of distinct pathways in carcinogenesis (22, 23). In the present study, we searched for genes specifically overexpressed in SCC through microarray analysis and identified seven genes, including aldo-keto reductase family 1, member B10 (*AKR1B10*). We investigated potential relevance of AKR1B10 in NSCLCs with a newly generated monoclonal antibody and found that it is overexpressed in smokers' NSCLCs, including most cases with SCC.

MATERIALS AND METHODS

Tissue Samples and Cell Lines. Forty-five primary lung cancers (15 SCCs, 20 adenocarcinomas, and 10 SCLCs) were obtained with informed consent from patients who underwent lobectomy at Jichi Medical School Hospital (Tochigi, Japan), Cancer Institute Hospital, Japanese Foundation for Cancer Research (Tokyo, Japan), and Hokkaido University Medical Hospital (Hokkaido, Japan). All samples were immediately frozen after resection and stored at −80°C until RNA or protein was extracted. Adenocarcinoma cell lines A549, H23, H522, H1648, and H2347 were purchased from the American Type Culture Collection (Manassas, VA). SCLC cell line Lu130 and SCC cell line H157 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan).

RNA Extraction and Microarray Analysis. Tissues or cells were directly lysed in Isogen reagent (Nippon Gene, Osaka, Japan) and homogenized. Total RNA was extracted according to manufacturer's instructions. Surgically resected lung tissues and lung cancers, including 5 SCCs, 10 SCLCs, a pooled sample made up of 12 adenocarcinomas, other 5 adenocarcinomas, and 1 normal lung, were analyzed on GeneChip HG U133 oligonucleotide arrays (Affymetrix, Santa Clara, CA) containing probes for ~40,000 human genes. Further information on the source of other RNA from normal tissues analyzed here is provided on request or is available at http://www.lsbm.org/db/index.html. Microarray analysis was done essentially as described previously (24). For global normalization, the average signal in an array was made equal to 100.

Systematic Selection of SCC-Specific Genes Based on Microarray Analysis. We systematically explored SCC-specific genes that were defined as follows: its expression level is (*a*) up-regulated in SCC but minimal in (*b*) normal lung and bronchial epithelia, (*c*) adenocarcinoma and SCLC, and (*d*) normal squamous epithelia, such as skin. Briefly, genes with a median signal score across 5 SCCs of >150 and >10 times that of normal lung were first selected. Among the 136 genes selected, genes with signal score of >150 in skin and small airway epithelial cell were omitted. We subsequently eliminated genes with signal >150 in SCLCs, adenocarcinomas, and most other normal tissues and various primary culture cells. Among 12 genes selected thus far, we additionally eliminated 5 genes that showed low expression throughout all of 40 cancer cell lines, suggesting expression by surrounding stromal cells but not by cancer cells (Table 1).

Quantitative Real-time Reverse Transcription-PCR. After digesting genomic DNA using DNase I (Invitrogen, Carlsbad, CA) cDNA was synthesized from 1 μg total RNA using SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) in 24 μL volume and diluted up to 80 μL. Quantitative real-time PCR for *AKR1B10* were done using an iCycler iQ Detection System (Bio-Rad, Hercules, CA). Reaction mixtures contained SYBR Green I nucleic acid gel stain (BMA, Rockland, ME) and primers 5′-CCCAAAGATGATAAAGGTAATGCCATCGGT-3′ and 5′-CGATCTGGAAGTGGCTGAAATTGGAGA-3′ for *AKR1B10* or 5′-AGAAGGAGATCACTGCCCTGGCACC-3′ and 5′-CCTGCTTGCTGATCCACATCTGCTG-3′ for *β-actin*. PCR condition was 1 cycle of 94°C for 3 minutes followed by 40 cycles at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. All the samples were run in triplicate, and the results were averaged. Specific amplification of *AKR1B10* was confirmed by the gel electrophoresis and melting curve analysis after PCR. The expression level of *AKR1B10* was indicated as a relative ratio of its signal to that of *β-actin* to normalize the starting amount of template cDNA. We also did semiquantitative PCR using the same condition for six pair-samples of SCC and corresponding noncancerous lung tissues.

Generation of Anti-AKR1B10 Monoclonal Antibodies. Monoclonal antibodies against AKR1B10 were generated as described previously (25). Briefly, glutathione *S*-transferase–fused

Table 1 Genes with SCC-specific overexpression

Symbol	Gene name	UniGene no.	Functional classification
AKR1B10	Aldo-keto reductase family 1, member B10	Hs.116724	Retinal reductase
ELAFIN	Elafin	Hs.112341	Protease inhibitor
AKR1C1	Aldo-keto reductase family 1, member C1	Hs.431175	Detoxification
SPRR3	Small proline-rich protein 3	Hs.139322	Structural component
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	Hs.575	Detoxification
NQO1	NAD(P)H dehydrogenase, quinone 1	Hs.406515	Activation of carcinogens
UGT1A9	UDP glycosyltransferase 1 family, polypeptide A9	Hs.375197	Detoxification

NOTE. Final seven genes selected through microarray analysis are listed. Representative function of each gene is summarized from the literature or National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

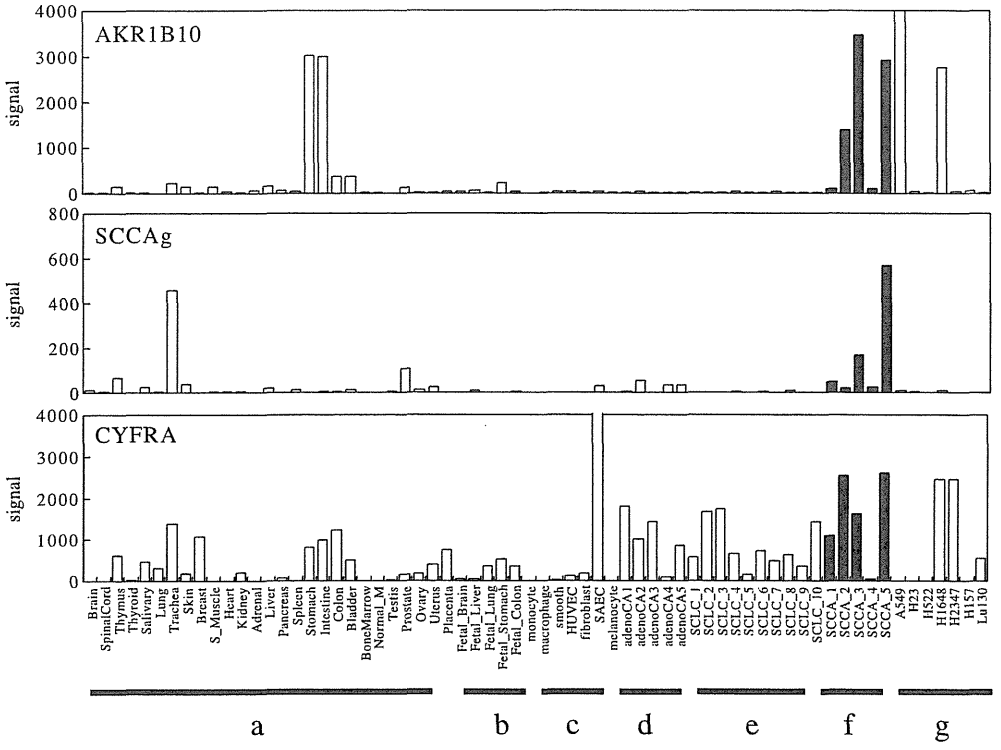

Fig. 1 Expression profiles of AKR1B10. A tissue-wide expression of AKR1B10 was displayed with CYFRA and SCCAg as references. Signal denotes gene expression level obtained from microarray analysis: (*a*) 27 normal tissues, (*b*) 5 fetal tissues, (*c*) 7 cultured normal cells, (*d*) 5 adenocarcinomas, (*e*) 10 SCLCs, (*f*) 5 SCCs, and (*g*) 7 lung cancer cell lines. *Filled columns,* SCC.

full-length AKR1B10 produced in *Escherichia coli* was immunized to female BALB/c mice. Nine clones of monoclonal hybridomas were selected by immunoblotting against recombinant AKR1B10 transiently expressed in COS-7 cells. We selected H4025 as a specific antibody in this study because a single band at around M_r 36,000 was observed only in *AKR1B10*-expressing cell lines as revealed by microarray analysis of 37 cell lines.

Immunoblot Analysis. Immunoblot analysis was done as described previously (25). Briefly, cells or tissues were lysed by 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, 0.1% SDS with protease inhibitor cocktail (Sigma, St. Louis, MO) at 4°C. H4025 (5 μg/mL) or anti-*β-actin* antibody (0.3 μg/mL, Sigma) was used as primary antibodies.

Immunocytochemistry and Confocal Microscopy Analysis. Immunostaining of culture cells were done after fixation in 4% paraformaldehyde and permeabilization in 0.2% Triton X-100 followed by incubation with 2% nonfat milk in TBS. An antibody H4025 (50 μg/mL) was applied as a primary antibody and incubated in a moist chamber at room temperature for 1 hour. The secondary staining was done with FITC-labeled anti-mouse IgG antibody (Sigma) as secondary antibody at room temperature for 1 hour. Dual-color detection by confocal laser scan microscopy (TCS SP2 system, Leica, Bensheim, Germany) was done after treatment with a 0.5 μmol/L solution of the mitochondrial stain MitoTracker Red CMXRos (Invitrogen) or the intercalator of double-strand nucleic acid stain propidium iodide (Invitrogen).

Immunostaining Analysis. Immunohistochemical analysis for AKR1B10 was done with the formalin-fixed, paraffin-embedded tissue archive at the University of Tokyo. The sections were deparaffinized in xylene, washed in ethanol, and rehydrated in TBS. Antigen retrieval was done in 10 mmol/L citrate buffer (pH 6.0) at 120°C for 10 minutes following incubation with TBS with 2% nonfat dried milk. Then, H4025 (50 μg/mL) or cytokeratin 5/6 (1:500, DAKO Ltd., Cambridge, United Kingdom) was applied for 1 hour followed by the secondary staining with DAKO Envision+ reagent. All sections were counterstained with Mayer's hematoxylin. We defined AKR1B10 positive if >10% of tumor cells displayed immunoreactivity.

We first examined archival samples of the University of Tokyo to compare expression of AKR1B10 and that of keratin 5/6 in NSCLCs, squamous epithelia of skin and esophagus, alveolar epithelium, and bronchus. We have analyzed previously 217 primary NSCLC specimens for expression of cyclin E, Ki-67, Bcl-2, p53, retinoblastoma protein, p27, GalNAcT3, and GnT-V (8, 9, 11, 12). Among these, we next examined 101 NSCLCs, which were classified into 32 SCCs, 65 adenocarcinomas, and 4 adenosquamous cell carcinomas according to WHO criteria (26). Clinicopathologic features are summarized in Table 3. The postsurgical pathologic tumor-node-metastasis stage was determined according to the guidelines of the American Joint Committee on Cancer (27). The Medical Ethical Committee of Hokkaido University School of Medicine approved this immunohistochemical study.

Statistical Analysis. We analyzed the statistical significance of the relationship between the expression of AKR1B10 and clinicopathologic variables by χ^2 test, Yates χ^2 test, or Fisher's exact test as appropriate. We also analyzed the associations between AKR1B10 expressions and the cyclin E or Ki-67 labeling index (%; refs. 8, 9) by Student's *t* test.

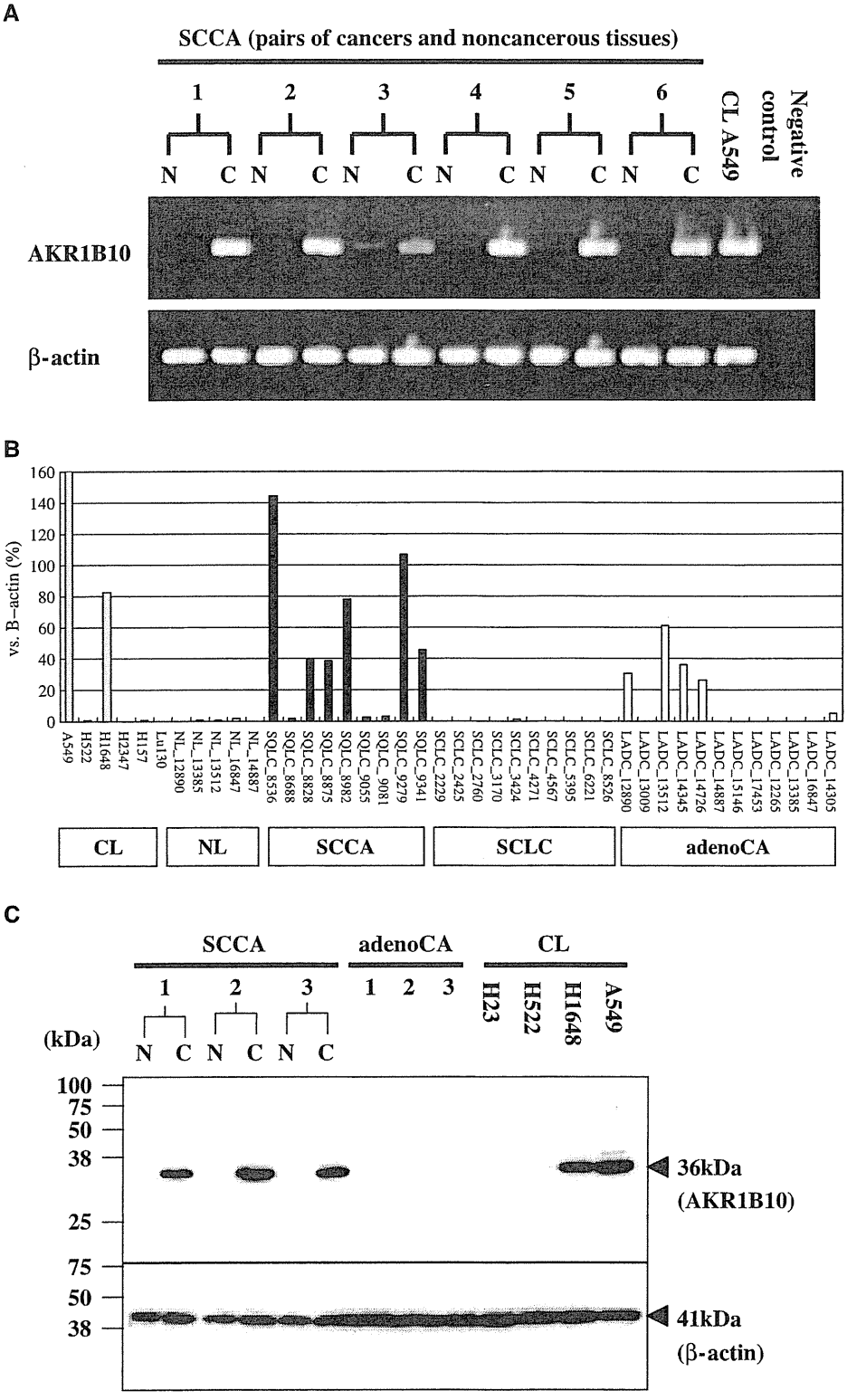

Fig. 2 Overexpression of AKR1B10 in SCC. *A,* semiquantitative PCR using six pairs of SCC and noncancerous lung tissues. Note that *AKR1B10* was up-regulated in all paired samples. *B,* quantitative real-time PCR. Examined samples were 9 SCCs, 12 adenocarcinomas, 10 SCLCs, 6 lung cancer cell lines, and 5 normal lung tissues. Note expression level of *AKR1B10* was remarkably high in 6 SCCs compared with 4 adenocarcinomas. *C,* immunoblot analysis of AKR1B10. A 36-kDa protein was detected in SCCs and AKR1B10-expressing cell lines H1648 and A549. *N,* normal lung tissues; *C,* cancer tissues; *CL,* cell line.

We additionally used multiple regression analysis to extract factors responsible for AKR1B10 expression in NSCLCs and adenocarcinomas alone. Sex, age, smoking history, differentiation, pT classification, pN classification, survival time, histology, cyclin E, Ki-67, GalNAcT3, and GnT-V were used as independent variables and AKR1B10 expression as a dependent variable. Differences were considered significant at $P < 0.05$. We simultaneously examined the correlation coefficient and the partial correlation coefficient between AKR1B10 expression and smoking or sex.

RESULTS

Microarray Analysis Identifies Seven Genes Specifically Up-Regulated in SCC. We selected seven potential SCC-specific genes (see Materials and Methods) using microarray analysis (Table 1). Tissue-wide expression profiles of these genes showed their high specificity compared with two widely used diagnostic markers of SCC, SCC antigen and CYFRA 21.1, suggesting robustness of our selection for SCC-specific genes (Fig. 1; Supplementary Fig. 1). Among these seven genes, *AKR1C1*, *ELAFIN*, *NQO1*, and *UGT1A9* were reported previously as potential target genes for detection or therapy against lung cancer (28–31); *SPRR3* is overexpressed in epidermal SCC (32); and *ALDH3A1* was reported to be involved in metabolism of tobacco carcinogens (33). Overexpression of *AKR1B10* has not been reported previously in lung cancer; then, we investigated whether it represents a good molecular target of SCC.

Overexpression of AKR1B10 in SCC. To confirm array data, we first did semiquantitative PCR. Overexpression of *AKR1B10* in SCC was observed in six pair-samples analysis (Fig. 2*A*). We subsequently investigated expression level of *AKR1B10* across lung cancer tissues by quantitative reverse transcription-PCR. Overexpression of *AKR1B10* was observed in 6 of 9 (67%) SCCs but not in SCLCs or normal lung tissues (Fig. 2*B*). Expression of *AKR1B10* was also observed in 4 of 12 (33%) adenocarcinomas, although its level was not so high as in SCC (Fig. 2*B*).

Next, we investigated expression of AKR1B10 protein by immunoblot analysis with a newly generated monoclonal anti-AKR1B10 antibody, H4025. As for three pair-samples of SCC used in semiquantitative PCR above, AKR1B10 was observed only in cancerous tissues, whereas AKR1B10 was negative in 3 adenocarcinomas (Fig. 2*C*).

Comparison of AKR1B10 with Pan-Squamous Cell Marker Keratin 5/6. As a SCC marker, keratin 5/6 is widely used based on its specificity to squamous cells. Unique feature of AKR1B10 as we identified in our selection is that it is not a merely squamous cell–specific marker unlike keratin 5/6 but a SCC-specific marker. To highlight the difference in "specificity" of these two molecules, we compared their expression in NSCLCs and normal tissues, including squamous epithelia of skin and esophagus, alveolar epithelium, and columnar epithelia of bronchus (Table 2). Keratin 5/6 staining was observed in normal squamous epithelia, columnar epithelia, and 83% of SCCs but not in adenocarcinoma. In contrast, AKR1B10 staining was observed in 64% of SCC and 30% of adenocarcinoma but not in normal epithelia (Table 2).

Distinct Localization of AKR1B10 in SCC and Adenocarcinoma. As we described above, analysis in larger number of samples revealed that AKR1B10 was expressed not only in most cases of SCC but also in a subset of adenocarcinoma. To investigate relevance of AKR1B10 in NSCLCs, we subsequently did immunohistochemical analysis in 101 primary NSCLCs, including 65 adenocarcinoma (Table 3). AKR1B10 staining was observed in 27 of 32 (84.4%) SCCs but also in 19 of 65 (29.2%) adenocarcinomas (Table 3). In adenosquamous cell carcinomas, AKR1B10 staining was observed in 2 of 4 cases and restricted to SCC components of these 2 cases (data not shown). AKR1B10 was preferentially observed in cancer cells with obvious squamous differentiation in SCC (Fig. 3*A-E*), whereas with lower differentiation grade in adenocarcinoma (Fig. 3*G*). Interestingly, we occasionally observed AKR1B10 staining in lesions with metaplasia: squamous metaplasia (Fig. 3*I*) and transitional cell metaplasia (Fig. 3*J*) in noncancerous areas (Fig. 3*K*) of one smoker's SCC specimen. We seldom detected positive staining in noncancerous portion of lung tissue, except two cases in normal bronchial epithelia of smokers (Supplementary Fig. 2A and B).

Table 2 Expression of AKR1B10 and keratin 5/6 in NSCLCs and normal epithelia

		AKR1B10	Keratin 5/6
NSCLCs	SCC (n = 23)	Positive (61%)	Positive (83%)
	Adenocarcinoma (n = 24)	Positive (33%)	Negative (0%)
Normal epithelia	Pulmonary alveoli (n = 3)	Negative	Negative
	Bronchial epithelia (n = 3)	Negative	Positive
	Squamous epithelia		
	Skin (n = 3)	Negative	Positive
	Esophagus (n = 3)	Negative	Positive

AKR1B10 staining was mainly observed in cytoplasm of cancer cells but also in nucleus in a subset of cells (Fig. 3*E* and *F*). Notably, two cases had apparent AKR1B10 staining mainly in nuclei (Fig. 3*M*). These results were essentially confirmed in confocal microscopy analysis of A549 cells. AKR1B10 was generally localized in cytoplasm, neither in nucleus nor in mitochondria in most cells. However, a subset of cells had additional staining in nucleus in 70% confluency (Fig. 3*L* and *M*) but not in full confluency (Fig. 3*N*).

Correlation between AKR1B10 Overexpression and Smoking History in NSCLC and Adenocarcinoma. To clarify the factors that correlate with AKR1B10 immunostaining, we carried out a statistical analysis that examined a variety of clinicopathologic variables and the expression of molecules that we reported previously (refs. 8, 9, 11, 12; Table 3). We observed positive correlations between AKR1B10 overexpression and SCCs (χ^2 test, $P < 0.0001$) and smoking (χ^2 test, $P < 0.0001$) in NSCLCs. AKR1B10 overexpression was observed in 40 of 61 (65.6%) smokers' NSCLCs. The correlation coefficient between AKR1B10 overexpression and smoking was 0.47 in NSCLCs. Partial correlation coefficient was 0.41 even after removing the effect of positive correlation between AKR1B10 overexpression and male ($P < 0.05$). These results indicate the significant correlation between AKR1B10 overexpression and smoking.

Univariate analysis in NSCLCs also showed that AKR1B10 was overexpressed in tumors with high pT classification ($P < 0.05$). Additionally, AKR1B10-positive cases had a higher Ki-67 expression ($P < 0.001$), higher cyclin E expression ($P < 0.01$), lower GalNAcT3 expression ($P < 0.01$), and lower GnT-V expression ($P < 0.05$) than negative cases in NSCLCs. Student's t test revealed that there was a significant difference between AKR1B10 expression and Ki-67 expression ($P < 0.005$) and cyclin E expression ($P < 0.05$) in NSCLCs.

Multiple regression analysis showed that smoking ($P < 0.01$), SCC ($P < 0.01$), and lower GalNAcT3 ($P < 0.05$) were important independent variables responsible for AKR1B10 overexpression in NSCLCs (Table 4). We subsequently analyzed only adenocarcinomas ($n = 65$) because most SCCs were AKR1B10 positive (84.4%) and smokers (96.9%). Interestingly, there was still a remarkable correlation (χ^2 test, $P < 0.01$) between AKR1B10 overexpression and smoking in adenocarcinomas (Table 3). Moreover, it was also shown that smoking was the only important independent variable responsible for AKR1B10 expression in adenocarcinomas ($P < 0.01$; Table 4).

DISCUSSION

Aldo-keto reductases are NAD(P)H-dependent oxidoreductases that catalyze the reduction of a variety of carbonyl compounds (34). AKR1B10 is a member of this superfamily and reduces aromatic and aliphatic aldehyde substrates (34). Reportedly, AKR1B10 mRNA shows expression in adrenal gland, small intestine, and colon, consistent with its putative physiologic roles in steroid metabolism or detoxification of reactive aldehydes in the digested food in intestinal tract (34–36).

Initial goal of our study was to identify SCC-specific molecules, distinct from currently used SCC markers that are specific to squamous cell in general. We eliminated these squamous cell marker genes through our selection and identified *AKR1B10* as a gene highly specific to SCC but not to squamous cells in general. AKR1B10 was expressed in as many as 90% of SCC of the lung but not in normal bronchial epithelium and squamous epithelium from skin and esophagus. This unique feature of AKR1B10 is highlighted when we compared the results of immunohistochemical analysis using AKR1B10 and keratin 5/6 (Table 2). AKR1B10 was highly specific to SCC when SCC and normal epithelia were analyzed by immunohistochemistry, although its specificity and sensitivity for SCC among NSCLCs were lower than those of keratin 5/6.

In the present study, we showed that AKR1B10 is overexpressed in SCC, which is closely associated with smoking. Additionally, we found AKR1B10 expression even in metaplasia, which is also associated with smoking and regarded as precancerous lesions of SCC (37, 38). Unexpectedly, nearly one third of the cases of adenocarcinomas expressed AKR1B10, but it was revealed by multiple regression analysis that smoking was the most important determinant of AKR1B10 expression in adenocarcinomas. Adenocarcinomas can be clustered into several subclasses based on reported expression profiling (22, 23). Together with recent reports that ~40% of adenocarcinomas occur in

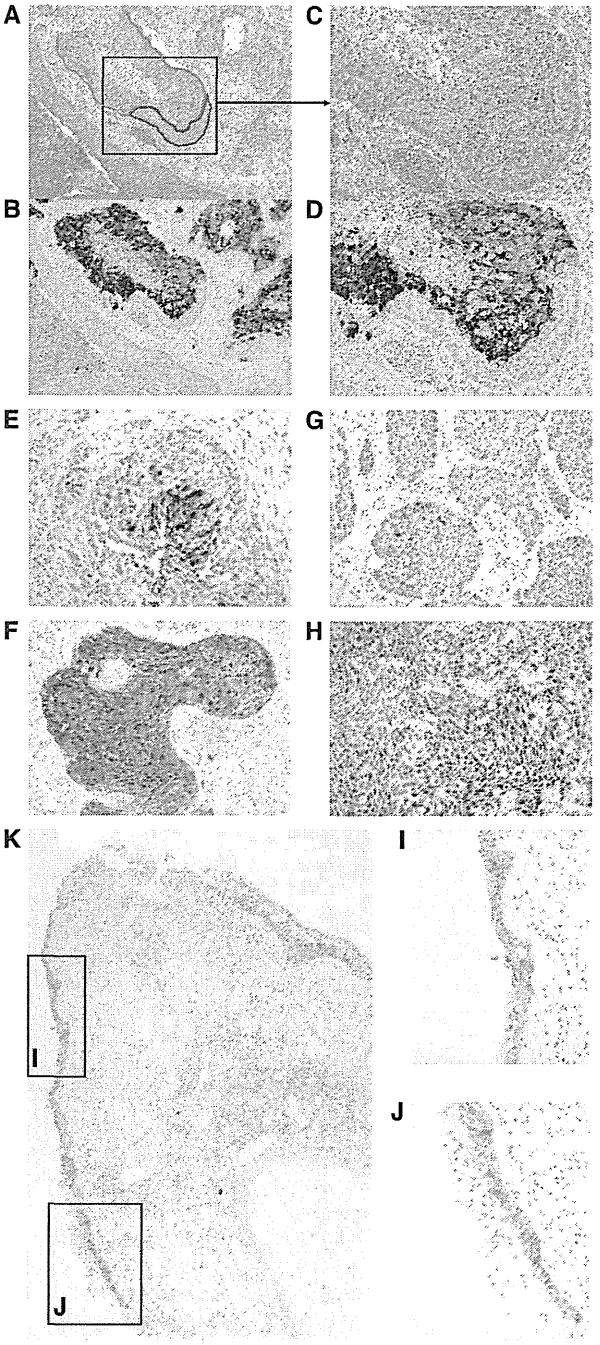

Fig. 3 Immunohistochemical analysis of AKR1B10. *A-E,* two representative cases in SCC. H&E staining, (*A*) ×20 and (*C*) ×100. Cancerous regions with obvious (*red line*) and no (*blue line*) squamous differentiation. Corresponding staining of the same sample (*B* and *D*) and another sample (*E*) by H4025. Note that AKR1B10 is stained in regions with squamous differentiation. *F* and *G,* two representative cases in adenocarcinoma. Homogenous staining was observed in some cases (*F*), whereas preferential staining in regions with lower differentiation was observed in most cases (*G*). *H,* typical case with nuclear staining in SCC (×100). *I-K,* AKR1B10 staining in metaplasia of a smoker. *I,* squamous metaplasia: (*left*) ×20 and (*right*) ×100. *J,* transitional cell metaplasia: (*left*) ×20 and (*right*) ×100. Note that these metaplastic regions are observed successively in noncancerous regions of a case with SCC (*K*).

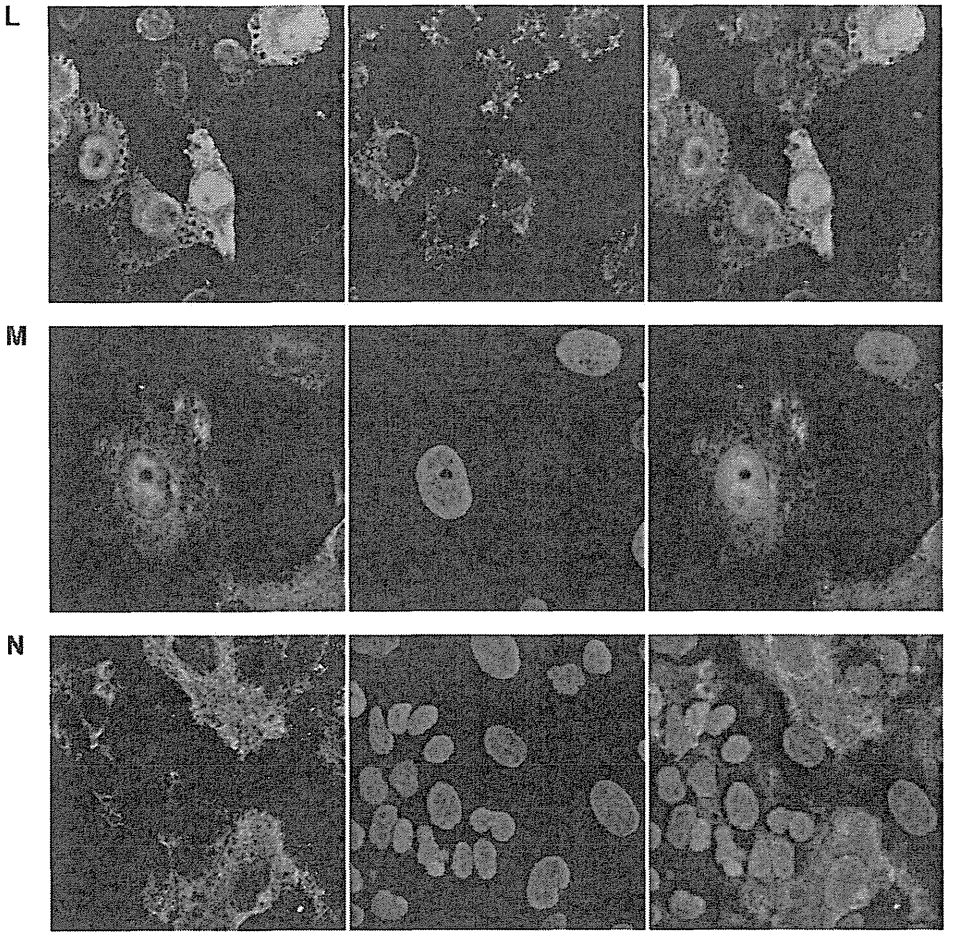

Fig. 3 Continued L-N, subcellular localization of endogenous AKR1B10 in A549 cells in 70% (*L* and *M*) and 100% (*N*) confluency. *Left,* AKR1B10; *middle,* MitoTracker (*L*) or propidium iodide (*M* and *N*); *right,* merged image. Note nuclear staining (*L* and *M*) has disappeared in 100% confluency (*N*).

smokers (39), there is a possibility that AKR1B10 could characterize a subset of adenocarcinoma associated with smoking. Based on our results, AKR1B10 immunostaining could be applied to the early detection of cancer cells or atypical cells in sputum, especially in heavy smokers.

Then, what could be potential roles of AKR1B10 in multistep carcinogenesis of SCCs? There are two possibilities as follows: one is that AKR1B10 may be related to cell proliferation. There was a positive correlation between AKR1B10 expression and putative poor prognosis factors, such as high Ki-67, high cyclin E, low GalNAcT3, and low GnT-V in NSCLCs (8, 9, 11, 12). Moreover, AKR1B10 was localized in nucleus in a fraction of cancer cells in subconfluent culture conditions, which disappeared under confluent culture, suggesting that AKR1B10 translocates during cell cycle and is involved in the regulation of cell cycle in a fashion yet identified.

Another possibility is that AKR1B10 promotes carcinogenesis of SCC through its enzymatic activity that counteracts the conversion of β-carotene to retinoic acid (40). Retinoic acid induces potent differentiation and growth-suppressive effects in diverse premalignant and malignant cells (41). In lung, deficiencies of retinoids are reported to cause hyperplasia and squamous metaplasia of airway epithelium (42) that can be suppressed by retinoic acid (43). Through the analysis of many cancer samples, we noticed positive staining of AKR1B10 even in some cases with metaplasia, precancerous lesion of SCC. Because the number of samples that contained metaplasia was small in the present study, this result was further investigated by another study focusing on idiopathic pulmonary fibrosis, which showed that squamous metaplasia was positive for AKR1B10 in 23 cases of 56 squamous metaplasia lesions.[10] These results strongly suggest that AKR1B10 expression is positive in precancerous lesions and may down-regulate retinoic acid, which could lead to carcinogenesis of SCC. Considering that AKR1B10 is an enzyme related to detoxification and that some smokers' bronchial epithelia without metaplasia were positive for AKR1B10 staining, AKR1B10 may be directly induced by some chemical compounds in tobacco, which should be further investigated. Interestingly, we also observed frequent overexpression of AKR1B10 in SCC of the laryngopharynx and esophagus that is closely associated with smoking and occasional overexpression of esophageal dysplasia and

[10] Fukayama et al., in preparation.

hyperplasia.[10] Remarkably high frequency of its up-regulation specific to SCC warrants further investigation of AKR1B10 in carcinogenesis of SCC.

Various retinoids, including β-carotene, have been shown previously effective for the treatment and prevention of several cancers, including carcinoma of the breast, skin, and kidney (44–49). However, clinical chemoprevention trials of lung cancer by β-carotene have failed to show its effectiveness. Moreover, administration of β-carotene unexpectedly promoted tumorigenesis in smokers (50, 51). Molecular mechanism underlying these adverse effects is currently unknown, but up-regulation of AKR1B10 in precancerous lesions in the bronchial epithelium of smokers may partly explain ineffectiveness of β-carotene observed in the lung.

AKR1B10 was also overexpressed in adenocarcinoma of smokers. Its staining was observed in undifferentiated region in contrast to SCC with staining in differentiated region. Together with its overexpression in hepatocellular carcinomas (34, 36), AKR1B10 may be related to another carcinogenic pathway distinct from that of SCC.

Table 3 Clinicopathologic features correlated to AKR1B10 overexpression

		AKR1B10				AKR1B10		
Characteristics	NSCLC	Positive	Negative	*P*, χ^2 test	Adenocarcinoma	Positive	Negative	*P*, χ^2 test or Fisher test
Age (y)								
Median (range)	63 (31-85)							
<65	58	28	30	NS	37	9	28	NS
≥65	43	20	23		28	10	18	
Sex								
Male	63	36	27	<0.05	30	10	20	NS
Female	38	12	26		35	9	26	
Histology								
Squamous	32	27	5	<0.0001				
Adenocarcinoma	65	19	46					
Adenosquamous	4	2	2					
Differentiation (SCC)								
Poor	14	9	5	<0.05	10	2	8	NS
Moderate/well	15	15	0		49	15	34	
Smoking								
Smoker	61	40	21	<0.0001	30	14	16	<0.01
Nonsmoker	36	6	30		35	5	30	
pT classification								
T_1	33	11	22	<0.05	21	4	17	NS
T_2-T_3	68	37	31		44	15	29	
pN classification								
N_0	56	28	28	NS	36	13	23	NS
N_1-N_3	45	20	25		29	6	23	
Ki-67								
High labeling index*	51	33	18	<0.001	22	11	11	<0.05
Low labeling index†	48	14	34		41	7	34	
Cyclin E								
Positive	76	42	34	<0.01	41	13	28	NS
Negative	24	5	19		23	5	18	
Bcl-2								
Positive	14	8	6	NS	6	2	4	NS
Negative	29	9	20		22	3	19	
p27								
Positive	87	42	45	NS	53	14	39	NS
Negative	9	4	5		8	3	5	
p53								
Positive	28	13	15	NS	16	4	12	NS
Negative	17	5	12		13	1	12	
Retinoblastoma protein								
Positive	31	15	16	NS	18	5	13	NS
Negative	12	2	10		11	1	10	
GalNAcT3								
Positive	63	21	42	<0.01	49	11	38	NS
Negative	34	24	10		14	7	7	
GnT-V								
High‡	47	17	30	<0.05	39	10	29	NS
Low§	52	31	21		24	9	15	

NOTE. NS, not significant.
*≥30% of cancer cells stained.
†<30% of cancer cells stained.
‡50% of cancer cells stained.
§<50% of cancer cells stained.

Table 4 Multiple regression analysis for AKR1B10

	NSCLCs			Adenocarcinomas		
Characteristics	Regression coefficient	*P*	95% Confidence interval	Regression coefficient	*P*	95% Confidence interval
Age	0	0.432	−0.15 to 0.006	−0.01	0.34	−0.02 to 0.007
Sex	−0.19	0.191	−0.474 to 0.096	−0.23	0.13	−0.534 to 0.071
Histology	−0.31	0.004	−0.518 to −0.1			
Differentiation	−0.05	0.694	−0.286 to 0.191	−0.01	0.959	−0.285 to 0.271
Smoking	0.425	0.004	0.143 to 0.707	0.387	0.01	0.0962 to 0.677
pT classification	−0.02	0.763	−0.167 to 0.123	−0.15	0.095	−0.322 to 0.026
pN classification	0.022	0.727	−0.101 to 0.144	0.145	0.088	−0.022 to 0.313
Ki-67	2E−04	0.929	−0.004 to 0.004	−0.01	0.077	−0.011 to 6E−04
Cyclin E	0	0.652	−0.004 to 0.003	0.002	0.424	−0.003 to 0.006
GalNAcT3	−0.23	0.032	−0.483 to −0.02	−0.111	0.507	−0.423 to 0.212
GnT-V	−0.03	0.483	−0.111 to 0.053	0.007	0.889	−0.095 to 0.11
Survival time	2E−06	0.948	−7E−05 to 8E−05	4E−05	0.383	−6E−05 to 1E−04

In summary, we showed that AKR1B10 is overexpressed in most SCCs and in adenocarcinomas that developed in the lung of smokers. Considering its involvement in retinoic acid metabolic pathway, AKR1B10 could be not a mere surrogate marker but a molecule relevant in smoking-related NSCLCs. Elucidation of its roles in carcinogenesis will be required to evaluate AKR1B10 as a therapeutic target in addition to a potential marker of SCC for diagnosis as shown in this study.

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