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Analysis of the Response and Toxicity to Gefitinib of Non-small Cell Lung Cancer

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Abstract. *Background: Gefitinib is an oral agent that inhibits the tyrosine kinase of epidermal growth factor receptor (EGFR), which had antitumor activity in patients with previously treated non-small cell lung cancer (NSCLC). We analyzed the efficacy, toxicity and overall survival time of gefitinib treatment in patients with NSCLC. Patients and Methods: One hundred and twenty-two patients with NSCLC, who received gefitinib between 2002 and 2004 in our institutes, were evaluated retrospectively. Results: The objective response rate was 24.6%. The variables identified as significant in univariate analysis included gender and smoking habit. The median overall survival time was 14.4 months. Significant variables associated with improved survival included good performance status (PS), female, adenocarcinoma and never smoked status, while never smoked status and good PS were independent prognostic factors in multivariate analysis. Four patients (3.3%) developed interstitial pneumonitis associated with gefitinib. Conclusion: Gefitinib showed favorable anti-tumor activity in females, never smokers and adenocarcinoma.*

Lung cancer is one of the most common fatal malignancies, with an increasing worldwide incidence (1). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. The current standard of care for patients with locally advanced or metastatic NSCLC is systemic chemotherapy with a two-drug combination regimen that includes a platinum-based chemotherapy (2). Although platinum-based chemotherapy offers modest efficacy and survival advantage over best supportive care (BSC) alone for chemotherapy-naive patients with advanced NSCLC, the overall 5-year survival rate after diagnosis remains at 10-15% due to frequent recurrence and metastasis of the NSCLC (1-4). Docetaxel is currently approved as the second-line chemotherapy on the basis of randomized trials in patients who failed on previous chemotherapy (5, 6). The objective response rates of docetaxel are only 5-10%, associated with a modest survival improvement. No agent has produced a tumor response in more than 5% of patients in the third-line treatment setting. In addition, elderly patients with poor performance status (PS) or patients with complications can not receive standard chemotherapy due to the toxicity associated with anticancer drugs. Thus, there is need for new therapies with novel mechanisms of action that are well tolerated, effective and convenient.

The epidermal growth factor receptor (EGFR) is a type I family of transmembrane glycoproteins and regulates the growth of both epithelial and non-epithelial cell types. EGFR is highly expressed in NSCLC and the association between EGFR expression and poor patient prognosis has been extensively documented (7, 8). Because activation of EGFR enhances the processes responsible for tumor growth and progression, including the promotion of proliferation,

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angiogenesis, and invasion/metastasis and inhibition of apoptosis, EGFR is a rational molecular target for antitumor therapy and many strategies which are currently being developed (9, 10).

Gefitinib (IressaR, ZD1839; AstraZeneca, Wilmington, DE, USA) is an oral inhibitor of the intracellular tyrosine kinase domain of EGFR, which blocks signal transduction pathways implicated in the proliferation and survival of cancer cells (11, 12). In phase I trials, gefitinib was generally tolerated and confirmed partial responses or survival prolongation were seen in some patients (13, 14). Subsequently, two phase II trials, IDEAL 1 and IDEAL 2, confirmed that response occurred in 12% to 18% in previously treated NSCLC patients receiving 250 mg/day gefitinib, and that gefitinib showed significant antitumor activity (15, 16). However, some patients who received gefitinib developed severe acute interstitial pneumonitis as an adverse event and more detailed investigation is thought to be required (17).

In the present study, we retrospectively analyzed response rates, survival time and toxicity in patients with NSCLC at our institutes who were given 250 mg/day gefitinib. In addition, we compared the relationship between response or survival time and clinical features.

Patients and Methods

Patients and treatment. Between July 2002 and March 2004, 122 patients (66 males and 56 females) with histologically or cytologically confirmed NSCLC received 250 mg/day gefitinib orally at our institutes (Hokkaido University Hospital, National Hospital Organization Hokkaido Cancer Center, Sapporo City General Hospital and Fukushima Medical University Hospital, Japan). Those records were analyzed retrospectively. The histopathological classification was based on WHO criteria for histopathological classification (18) and Tumor-Node-Metastasis (TNM) was determined based on the American Joint Committee on Cancer guidelines (19). Patients, who had recurrent or refractory disease after surgery, radiotherapy or prior systemic chemotherapy, or who could not tolerate standard systemic chemotherapy as judged by a physician, were included. Patients continued treatment until disease progression, no efficacy, intolerable toxicity, or withdrawal of consent. Patients received no systemic anticancer treatment during treatment with gefitinib.

Efficacy. We assessed objective tumor response as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) by repeat radiographic imaging in accordance with the Response Evaluation Criteria in Solid Tumors Group (RECIST) guidelines (20). Disease control was defined as CR, PR, or SD. Patient responses were defined as Unknown if they died of progressive disease or they stopped gefitinib due to adverse events within 10 days. The responses of patients in whom efficacy could not be assessed, due to short duration of observation, were also defined as Unknown. Tumor dimensions were assessed every 4 weeks by radiographic examinations. PR was sustained for 4 weeks or longer and SD was sustained for 8 weeks or longer.

Table I. *Patient characteristics.*

Characteristics	No. of patients	%
Number of patients	122	
Gender		
Male	66	54
Female	56	46
Age, Years		
Median	64	
Range	31-84	
Performance status		
0	15	12.3
1	70	57.4
2	23	18.8
3	8	6.6
4	6	4.9
Stage		
I~II	4	3.3
IIIA	6	4.9
IIIB	15	12.3
IV	76	62.3
Recurrence after surgery	21	17.2
Tumor histology		
Adenocaricinoma[a]	97	79.5
Squamous cell carcinoma	18	14.8
Large cell carcinoma	3	2.5
Other NSCLC	4	3.2
Previous chemotherapy		
0 regimen	20	16.4
1 regimen	40	32.8
2 regimens	39	31.9
More than 3 regimens	23	18.9
Surgery	31	25.4
Radiation	15	12.3
Smoking habit		
Never	48	39.3
Current	23	18.9
Former	51	41.8

[a]One patient with bronchiolar carcinoma was included in this group.

Toxicity. Toxicity was evaluated based on the National Cancer Institute Common Toxicity Criteria, version 2, every 2 weeks or 4 weeks.

Overall survival. Overall survival was defined as the period from the date of start of gefitinib to the date of tumor-related death. Living patients at data cut-off were censored at the last date that they were confirmed to be alive.

Statistical analysis. The associations between response and patient characteristics were statistically analyzed using Fisher's exact test as appropriate using Statview software (version 5.0; SAS Institute, CA, USA). Those variables that were significant in the univariate analysis were included in a multivariable logistic regression analysis. The progression-free survival curves and survival curves were estimated by the Kaplan-Meier method for estimation and the log-rank test for comparing the characteristics of patients. Those variables that were significant in the univariate analysis were

Table II. *Best overall objective responses.*

Response	Number of patients	(%)
Complete response (CR)	0	0
Partial response (PR)	30	24.6
Stable disease (SD)	35	28.7
Progressive disease (PD)	47	38.5
Unknown[a]	10	8.2

[a]No conclusion was achieved about best overall tumor response due to the short duration of observation, death from progressive disease within 10 days, or cessation of gefitinib by adverse events within 10 days.

included in a multivariable Cox proportional hazard model. Values of $p<0.05$ were considered to indicate statistical significance and all tests were two-tailed.

Results

Patients. We assessed 122 patients who received 250 mg/day gefitinib. Table I shows the baseline patient characteristics. The most common tumor histology was adenocarcinoma, including 1 patient with bronchioloalveolar carcinoma. The median PS of all patients was 1 (range 0-4), the median number of chemotherapy regimens was 2 (range, 0-4), and the median duration of treatment for all 122 patients who consented to taking gefitinib was 2.6 months (range, 0-21.7 months).

Efficacy. The best overall tumor responses are shown in Table II. PR was observed in 30 patients (24.6%), SD in 35 patients (28.7%) and PD in 47 patients (38.5%). No CR was achieved. In 10 patients, tumor response could not be determined. Three patients died of progressive diseases within 10 days of gefitinib administration, 2 patients ceased gefitinib due to adverse events (diarrhea and nausea) within 10 days, and in 5 patients efficacy could not be defined due to the short duration of observation. The objective response rate was 24.6% and disease control rate was 53.3%.

Female patients showed a higher response rate (37.5%) than male patients (13.6%) ($p<0.01$) and those who had never smoked showed a higher response rate (41.7%) than current or former smokers (13.5%) ($p<0.01$) (Table III). Although the tumor response in patients with adenocarcinoma tended to be higher than that in patients with other histological types, in the univariate analysis, no statistical difference was observed (27.8% *vs.* 12%, $p=0.1$). Furthermore, no statistical differences were observed between tumor response and

Table III. *Univariate analysis of characteristics associated with response to gefitinib.*

Characteristics	Number of patients[a]	(%)	*P*
Gender			
Male	9/66	13.6	<0.01[b]
Female	21/56	37.5	
Age			
75<	24/102	23.5	
75≥	6/20	30	0.54[c]
Tumor histology			
Adenocarcinoma	27/97	27.8	
Non-adenocarcinoma	3/25	12	0.1[b]
Smoking habit			
Never	20/48	41.7	
Current / Former	10/74	13.5	<0.01[b]
Number of previous chemotherapy regimens			
0	5/20	25.0	
1	12/40	30	
2-4	13/62	21	0.63[b]
Performance status			
0-2	28/108	27.5	
3-4	2/14	14.3	0.34[b]

[a]The number of patients who showed PR/ the number of total patients. [b]Fisher's exact test and [c]*t*-test

gender or smoking habit in the multivariate analysis, although the never smoked group tended to show favorable tumor response (odds ratio of 2.5, 95% CI, 0.89 to 7.21; $p=0.08$) (data not shown). On the other hand, there were 20 patients who had no previous chemotherapy before treatment with gefitinib. PR was observed in 5 patients (25.0%), SD was observed in 7 patients (35.0%), and PD was observed in 6 patients (30.0%) (Table III).

The duration of treatment for patients with PR or SD who consented to take gefitinib was 7.6 months (range, 1 ~ 21.7 months) and 18 patients were treated with gefitinib for more than 10 months (data not shown). Although 5 out of 18 patients with long duration of treatment stopped gefitinib due to progression of disease and one patient stopped gefitinib due to adverse event, the remaining patients still had PR or SD at the data cut-off and treatment with gefitinib was continued.

Survival. In all 122 patients, the median overall survival was 14.4 months. The 1-year survival rate was 50% (Figure 1). Female patients had significantly better survival compared to male patients ($p<0.01$) (Figure 2A). Survival of those who had never smoked was significantly longer than that of current or former smokers ($p<0.01$) (Figure 2B). Patients with adenocarcinoma had significantly better survival than

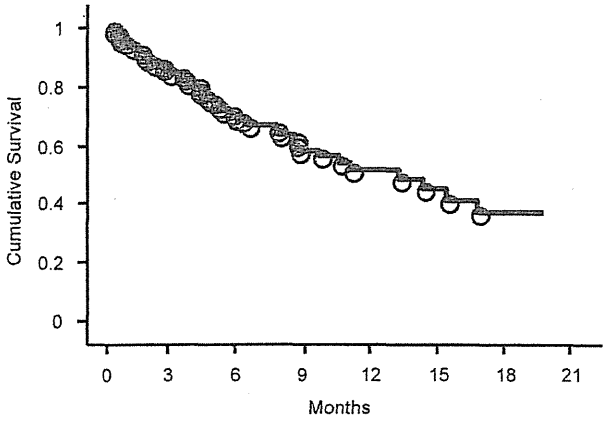

Figure 1. *Kaplan-Meier method of overall survival in all 122 patients. Median survival time was 14.4 months.*

patients with non-adenocarcinomas ($p<0.01$) (Figure 2C). The survival of patients with PS 0 to 2 was prolonged significantly longer compared to that of patients with PS 3 to 4 ($p<0.01$) (data not shown).

A multivariate analysis was carried out using the Cox hazard proportional model with significant prognostic factors identified in the univariate analyses (Table IV). Never smoked ($p<0.05$) and good PS (PS 0 to 2) ($p<0.01$) remained independent significant prognostic factors statistically, and female patients tended to have better survival in the multivariate analysis ($p=0.07$).

Toxicity. Most treated patients were assessable for toxicity (Table V). The main toxicity was Grade 1 or 2 rash, seen in 52.5% patients. Grade 1 or 2 pruritis and diarrhea were seen in 27.9% and 24.6%, respectively. Of the 122, 4.1% of patients and 2.5% of patients developed Grade 3 anorexia and rash, respectively. In addition, 8 patients (6.6%) stopped gefitinib due to diarrhea, nail change, nausea, anorexia, or elevated transaminases.

Four patients developed gefitinib-related interstitial pneumonitis. Two of them were complicated with interstitial pneumonitis, and one with radiation pneumonitis prior to gefitinib, although their pulmonary diseases were clinically stable. Three of the 4 were males. One patient recovered by stopping gefitinib and 2 patients recovered by stopping gefitinib and beginning steroid treatment. However, one patient died from progression of interstitial pneumonitis despite steroid treatment (Table VI).

Discussion

In the present analysis, we retrospectively analyzed the efficacy of 250mg/day gefitinib for patients with NSCLC.

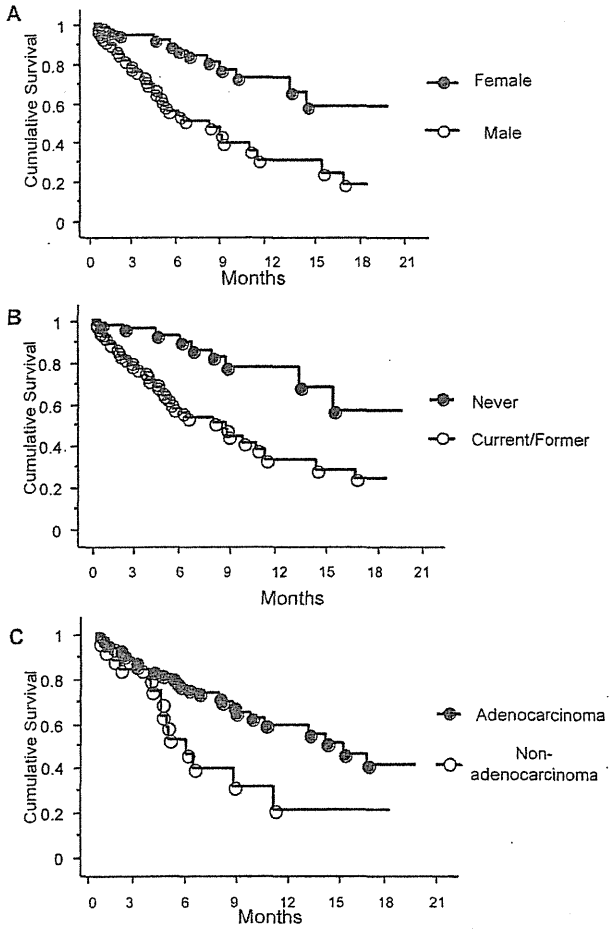

Figure 2. *Kaplan-Meier method of overall survival according to each patient's characteristics. (a) Overall survival is shown separately on the basis of gender. (b) Overall survival is shown separately on the basis of never having smoked and current/former smokers. (c) Overall survival is shown separately on the basis of patients with adenocarcinoma and non-adenocarcinoma. The survival differences in each category are statistically different ($p<0.01$).*

The objective response rate was 24.6% of the total patient population. Two phase II trials of gefitinib in patients with pretreated advanced NSCLC (IDEAL 1 and IDEAL 2) demonstrated antitumor activity (objective response rates, 11.8% to 18.4%) (15, 16), whereas the response rates were higher for Japanese patients (27.5%) than non-Japanese patients (10.4%) (15). The response rate in the present analysis was comparable to that of Japanese patients in IDEAL 1. The median survival was 14.4 months in the present analysis. In IDEAL 1 and IDEAL 2, the median survival was 7.6 months and 7 months, respectively. Subset analysis of data from the Japanese patients in IDEAL 1 showed that median survival was 414 days, which is comparable to the present data (21).

Table IV. *Multivariable analysisa of features associated with improved survival.*

Characteristics	Hazard Ratio	95% CI	*P*
Gender			
Male	0.46	0.20 to 1.08	0.07
Female	1		
Smoking habit			
Never	2.59	1.02 to 6.62	0.046
Current/Former	1		
Histology			
Non-adenocarcinoma	0.70	0.37 to 1.32	0.27
Adenocarcinoma	1		
Performance status			
3-4	0.18	0.08 to 0.37	<0.0001
0-2	1		

[a]Cox hazard analysis

Table V. *Treatment-related toxicity (%).*

	Grade 1/2	Grade 3	Grade 4
Rash	52.5	2.5	0
Pruritus	27.9	0.8	0
Diarrhea	24.6	0.8	0
Anorexia	13.1	4.1	0
Dry skin	14.8	0.8	0
Nail changes	8	0	0
Dehydration	2.5	1.6	0
GOT or GPT[a]	5.7	0.8	0
Intersitial pneumonitis	2.5	0	0.8

[a]Elevated liver transaminase

Table VI. *Characteristics of patients with interstitial pneumonitis.*

No.	Age	Gender	History	Smoking habit	PS	No. of former chemotherapy	Prior pulmonary complications	Length (day)	Adverse effect	Current status
1	74	M	SCC[a]	Yes	1	0	Interstitial pneumonitis	47	Rash Diarrhea	Death[c]
2	74	M	Ad[b]	No	1	2	Interstitial pneumonitis	24	No	Ceased
3	70	M	Ad[b]	Yes	1	2	Radiation pneumonitis	52	Rash Pruritus Nail changes Dry skin	Ceased
4	76	F	Ad[b]	No	1	1	No	71	Rash Pruritus Nail changes Dry skin Diarrhea	Ceased

[a]Squamous cell carcinoma
[b]Adenocarcinoma
[c]Possibly therapy-related death

We also analyzed the association between sensitivity to gefitinib and patient characteristics. In the present analysis, gender and smoking habit were associated with favorable tumor response, and four factors were associated with improved survival in patients receiving gefitinib; females, never smoked, adenocarcinoma and good PS in the univariate analysis. Among them, smoking habit was predictive of tumor response in the multivariate analysis, although no statistical difference was observed ($p=0.08$). In addition, good PS (PS 0 to 2) and never smoked remained independent prognostic factors and female patients tended to have better survival in the multivariate analysis. Previously, multivariate analysis of all patients in IDEAL 1 revealed that PS, gender, histology and prior immuno/hormonal therapy were significant prognostic factors associated with objective tumor response (15). Subset analysis of data from Japanese patients in IDEAL 1 showed that survival was longer in patients with adenocarcinoma, those with symptom improvement, or female patients in the univariate analysis, but not in the multivariate analysis (21). Moreover, Janne *et al.* reported that significant independent prognostic factors associated

with improved survival were females, good PS and adenocarcinoma (22). Our results are almost comparable to these results and confirmed the result of subset analysis of Japanese patients in IDEAL 1.

Miller *et al.* reported that bronchioloalveolar carcinoma was a more significant prognostic factor for gefitinib sensitivity than adenocarcinoma (23). We had only one patient with bronchioloalveolar carcinoma, who showed an objective response. In the present analysis, most of the patients were diagnosed by small specimens of transbronchial lung biopsy, by which it was difficult to definitely diagnose bronchioloalveolar carcinoma, and we were unable to analyze the association between tumor response and bronchioloalveolar carcinoma

The present analysis included 20 patients with no previous chemotherapy. Among them, 5 patients showed a tumor response (25%), which was almost compatible with that in patients with previous chemotherapy (24.5%) in the present analysis. In addition, the median survival of patients with no previous chemotherapy was 8.6 months. There are few reports about the efficacy of gefitinib monotherapy as first-line therapy. Argiris *et al.* reported that 25 patients, who were unfit or refused chemotherapy, received 250mg oral gefitinib daily as first-line therapy for the treatment of NSCLC, and 4 of the 22 evaluable patients (18%) had an objective response (24). The median time to progression was 2.2 months, median survival was 12.6 months and 1-year survival was 52% (24). The present results were compatible with this former analysis and suggested that gefitinib was effective as first-line therapy. Gefitinib monotherapy should undergo further evaluation as first-line therapy in advanced NSCLC.

In the present analysis, the median duration of treatment with gefitinib for patients with PR or SD was 7.6 months and many patients received gefitinib for a long time. There are few precise reports on long duration of treatment with gefitinib. The present results suggested that the efficacy of gefitinib could be sustainable for a long time and long duration of treatment could contribute to the prolongation of survival.

Recently, somatic mutations in the tyrosine kinase domain of the EGFR gene have been shown to be correlated with clinical responsiveness to gefitinib (25, 26). Screening for such mutations in lung cancers may identify patients who will have a response to gefitinib, as does being female, having adenocarcinoma and never having smoked. We have not yet analyzed whether the 30 patients with objective responses have specific mutations in the EGFR gene and investigation for EGFR mutations would definitely be needed in the future study.

Major adverse events were rash, pruritis, diarrhea and anorexia, which is consistent with previous reports. Patients given gefitinib have been documented to develop severe acute interstitial pneumonitis (17). In the present analysis, 4 patients developed interstitial pneumonitis, and one died of it. Three of them had a history of prior pulmonary complications. Although the cause of interstitial pneumonitis by gefitinib is unknown, the frequency of interstitial pneumonitis in Japanese patients tends to be higher than in non-Japanese patients, and may be related to population or environmental differences.

In conclusion, gefitinib showed favorable antitumor activity for patients with NSCLC, particularly females, never smokers, or patients with adenocarcinoma. On the other hand, one patient died from gefitinib-related interstitial pneumonitis and closer investigation is required in patients with prior pulmonary complications.

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Expression of N-Acetylglucosaminyltransferase V in the Development of Human Esophageal Cancers: Immunohistochemical Data from Carcinomas and Nearby Noncancerous Lesions

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Key Words
GnT-V · Carcinogenesis · Esophageal cancer · L4-PHA · Ki-67

Abstract
Objective: N-Acetylglucosaminyltransferase V (GnT-V) is a key enzyme in the formation of branching asparagine-linked oligosaccharides and is linked to tumor invasion and metastasis in colon and breast cancers. In normal esophageal epithelium, β1,6-branched asparagine-linked oligosaccharides synthesized by GnT-V are seen in the basal cell layers but not in the superficial cell layers, and its presence has been shown in invasive esophageal cancers. However, neither GnT-V expression nor its clinical significance has been previously examined in human normal, premalignant and malignant esophageal tissues. ***Methods:*** GnT-V expression was studied by immunohistochemistry using a specific monoclonal antibody in 121 surgically resected specimens of esophageal squamous cell carcinomas (SCCs) and adjacent tissues, and was analyzed statistically in relation to various characteristics. ***Results:*** GnT-V expression was observed in none (0%) of the 19 normal epithelial tissues, 1 (2%) of the 43 hyperplastic tissues, 30 (54%) of the 56 mildly dysplastic tissues, 27 (63%) of the 43 moderately dysplastic tissues, 21 (44%) of the 48 in situ SCCs and 29 (26%) of the 110 invasive SCCs ($p < 0.005$). GnT-V expression was observed significantly more frequently in mildly and moderately dysplastic tissues when compared with normal epithelial and hyperplastic tissues ($p < 0.005$), and its frequency was decreased in in situ and invasive SCCs ($p < 0.005$). GnT-V expression was frequently observed in SCCs of small size and without distant metastasis or lymph node metastasis. ***Conclusions:*** Increased expression of GnT-V is associated with the early event of esophageal tumorigenesis.

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Introduction

The prognosis for esophageal carcinoma remains poor, although advances in surgical techniques and chemoradiotherapy have improved survival to some extent. The overall 5-year survival rate generally remains at less than 50%, despite the use of multimodality therapy [1–5]. A better understanding of the molecular basis of esophageal carcinogenesis and identification of prognostically important biological markers are needed in order to improve survival.

It is known that sugar chains have a variety of functions and play key roles in development, differentiation, cell adhesion and cell growth [6, 7]. An enzyme that specifically catalyzes β1,6 branching of N-acetylglucosamine is UDP-N-acetylglucosamine:mannoside β1,6-N-acetylglucosaminyltransferase (GnT-V). This enzyme transfers N-acetylglucosamine from UDP-N-acetylglucosamine to α-*D*-6 mannoside of asparagine-linked oligosaccharides [8]. This enzyme has been shown to be linked to tumor metastasis [9–14]. High expression levels of the GnT-V gene are correlated with distant or lymph node metastasis and poor prognosis in human colorectal and breast cancer [15–17]. On the other hand, GnT-V expression is an early event in rat and human hepatocarcinogenesis [12, 18], and levels of GnT-V expression do not correlate with postsurgical prognosis in patients with hepatoma [18], thus suggesting that GnT-V is involved in or associated with the early stages of tumorigenesis. In normal esophageal epithelium, β1,6-branched asparagine-linked oligosaccharides synthesized by GnT-V are seen in basal cell layers but not in superficial cell layers [19], and its presence has been observed frequently in invasive esophageal cancers [20]. However, neither GnT-V expression nor its biological and clinical significance has been examined in human normal, premalignant or malignant esophageal tissues.

In the present study, we immunohistochemically studied GnT-V expression in 121 human esophageal squamous cell carcinomas (SCCs) and surrounding tissues in order to elucidate the significance of GnT-V in the development of esophageal cancers. We also investigated the presence of β1,6-branched asparagine-linked oligosaccharides transferred by GnT-V using *Phaseolus vulgaris* leukoagglutinin (L4-PHA) staining, which specifically binds to β1,6 branching [16], in order to examine the relationship between GnT-V expression and β1,6 branch synthesis.

Materials and Methods

Patients and Esophageal Specimens

One hundred and twenty-one patients (105 men and 16 women; mean age 62.3 years) with primary esophageal SCC underwent radical esophagectomy at the Department of Surgical Oncology, Hokkaido University Hospital, and its affiliated hospitals (Teinekeijinkai Hospital and Hokkaido Gastroenterology Hospital) between September 1989 and May 1999. In all cases, informed consent was obtained for the use of resected tumor specimens. No distant metastasis was detected in any patients on preoperative examination. No patients had received anticancer treatment before surgery. The clinical staging of tumors was determined according to the TNM classification system of the International Union Against Cancer [21].

Survival was analyzed for the 70 patients who met the following criteria: (1) they did not die in hospital after surgery; (2) they did not die of causes other than esophageal cancer within 3 years after surgery, and (3) patients who remained alive were followed up for more than 3 years after surgery. Fifty-one patients who did not meet the above criteria were excluded from survival analysis.

All specimens were fixed in 10% formalin and embedded in paraffin wax. One of the deepest sections from each tumor was selected for evaluation, and 4-μm serial sections were examined by immunohistochemistry. For lectin blotting, samples were immediately frozen in liquid nitrogen and stored at –80°C until use.

Immunohistochemistry for GnT-V and Ki-67

Expression of GnT-V and Ki-67 was analyzed by immunohistochemistry. Briefly, the labeled streptavidin biotin method was used with 4-μm serial sections of formalin-fixed, paraffin-embedded tissues after deparaffinization. For immunostaining of Ki-67, deparaffinized tissue sections were autoclaved in 10 m*M* citrate buffer (pH 6.0) for 10 min at 121°C in order to retrieve antigenicity. Sections were then immersed in methanol containing 1.5% hydrogen peroxide for 20 min in order to block endogenous peroxidase activity and were incubated with normal rabbit serum in order to block nonspecific antibody-binding sites. Sections were consecutively reacted with a mouse monoclonal antibody against recombinant human GnT-V [17] at a dilution of 1:1,600, with mouse monoclonal antibody MIB-1 (Immunotech, Marseilles, France) at a dilution of 1:50, or with control mouse isotype-specific immunoglobulin at 4°C overnight. Immunostaining was performed using the biotin-streptavidin immunoperoxidase method with 3,3′-diaminobenzidine as a chromogen (SAB-PO kit; Nichirei, Tokyo, Japan). Methyl green was used as the counterstain.

L4-PHA Histochemistry

L4-PHA lectin binds preferentially to GlcNAc residues on β1,6 branches of tri- and tetra-antennary sugar chains, which are products of GnT-V [22]. For staining with L4-PHA, deparaffinized tissue sections were incubated with 0.3% hydrogen peroxidase in methanol for 20 min at room temperature in order to eliminate endogenous peroxidase activity. Trypsinization [Tris buffer containing 0.1% trypsin (Difco Laboratories, Detroit, Mich., USA) and 0.1% $CaCl_2$, for 10 min at 37°C] was then performed. To remove sialylation of oligosaccharides, sections were treated with neuraminidase from *Vibrio cholerae* (Roche, Tokyo, Japan) at a concentration of 0.1 U/ml in sodium acetate buffer (pH 5.6) containing 0.04 *M* $CaCl_2$ for 1 h at 37°C. Sections were incubated with 5%

skim milk in PBS for 20 min at room temperature in order to block nonspecific staining and were then incubated with biotinylated L4-PHA lectins (EY Laboratories, San Mateo, Calif., USA) at a dilution of 1:500 at 4°C overnight. Staining was performed using the biotin-streptavidin immunoperoxidase method with 3,3'-diaminobenzidine as a chromogen (SAB-PO kit; Nichirei, Tokyo, Japan). Methyl green was used as the counterstain.

Evaluation of Staining

Expression of GnT-V in SCCs was classified as positive or negative, according to the proportion of positively stained cancer cells. Tumors having cytoplasmic staining in at least 10% of cancer cells were judged to be positive for GnT-V expression, while tumors having cytoplasmic staining in less than 10% of cancer cells were judged to be negative. Noncancerous lesions were considered positively stained when any cytoplasmic staining was present in the Golgi localization pattern.

L4-PHA staining was classified as high or low, according to the proportion of positively stained cells (≥30% or <30%, respectively). For GnT-V and L4-PHA staining, sections were examined by two independent observers without prior knowledge of the clinical or clinicopathological status of the specimens.

Ki-67 labeling index (LI) was defined as the percentage of tumor cells displaying nuclear immunoreactivity and was calculated by counting the number of nuclear Ki-67-stained tumor cells among 1,000 tumor cells in each section. A single representative tissue section from each tumor was surveyed for Ki-67 microscopically at ×100 for at least two or three random areas. Cell counts were performed at ×400 in at least seven fields in these areas, using a Videomicrometer (Model VM-30; Olympus, Tokyo, Japan) equipped with a light microscope. Ki-67 LIs were reliably and reproducibly obtained using this Videomicrometer system.

Lectin Blot Analysis

Tissues were homogenized with a glass homogenizer in TNE buffer containing 10 m*M* Tris-HCl (pH 7.8), 1% NP-40, 0.15 *M* NaCl, 1 m*M* EDTA and a mixture of protease inhibitors (Wako, Osaka, Japan). Homogenized tissues were placed on ice for 20 min and were centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant fraction was saved as the postnuclear supernatant and protein concentrations were determined with a BCA kit (Pierce, Rockford, Ill., USA) using bovine serum albumin as a standard. For lectin blot analysis, 20 μg of proteins were electrophoresed on a 10% polyacrylamide gel and then transferred onto a nitrocellulose membrane. After blocking with PBS containing 3% BSA overnight at 4°C, the membrane was incubated with 1 μg/ml of biotinylated L4-PHA (Seikagaku, Tokyo, Japan) for 3 h. Washing and developing were as described previously [23]. Reactive glycoprotein bands were visualized by chemiluminescence using an ECL system (Amersham Pharmacia Biotech UK, Little Chalfont, UK).

Statistical Analysis

Associations between GnT-V expression and categorical variables were analyzed by χ^2 test or Fisher's exact test, as appropriate. Associations between GnT-V expression and age or labeling indices were analyzed by Student's t test. Associations between Ki-67 LIs and categorical variables were analyzed by Student's t test. Differences in GnT-V expression among normal, premalignant and malignant tissues were analyzed by the Kruskal-Wallis test. 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 in order to identify which factors might have a significant influence on survival. The significance level chosen was $p < 0.05$, and all tests were two-sided.

Results

Typical immunostaining patterns for GnT-V in normal, premalignant and malignant esophageal tissues are shown in figure 1. In tumor cells, GnT-V expression was seen in the Golgi localization pattern (fig. 1), as well as in noncancerous dysplastic epithelial cells. GnT-V expression was observed in none (0%) of the 19 normal epithelial tissues, 1 (2%) of the 43 hyperplastic tissues, 30 (54%) of the 56 mildly dysplastic tissues, 27 (63%) of the 43 moderately dysplastic tissues, 21 (44%) of the 48 in situ SCCs and 29 (26%) of the 110 invasive SCCs ($p < 0.005$ by the Kruskal-Wallis test) (fig. 2). GnT-V expression was observed significantly more frequently in mildly and moderately dysplastic tissues when compared with normal epithelial and hyperplastic tissues ($p < 0.005$), and when compared with in situ and invasive SCCs ($p < 0.005$). The relationship between GnT-V expression and various clinical and clinicopathological features of SCCs is summarized in table 1. In SCCs, tumors exhibiting GnT-V expression were smaller than tumors without GnT-V expression ($p = 0.03$). GnT-V expression was observed significantly more frequently in distant metastasis-negative tumors than in positive tumors ($p = 0.045$), and tended to be found more frequently in lymph node metastasis-negative tumors than in positive tumors ($p = 0.09$).

We next analyzed the relationship between GnT-V expression and patient survival (fig. 3) and the importance of GnT-V as a prognostic factor (table 2). GnT-V expression was not associated with survival (3-year survival rates, 36% for patients having tumors with negative GnT-V expression and 45% for patients having tumors with positive GnT-V expression; $p = 0.4$) (fig. 3), and was not a significant prognostic factor in univariate analysis (table 2). Univariate analysis for overall survival using a Cox proportional hazards model identified pT classification, pN classification, pM classification, pStage, surgical margin, and lymphatic invasion as significant prognostic predictors (table 2). Multivariate analysis of the same set of patients showed that pT classification, pN classification and surgical margin had independent prognostic values, with hazards ratios of 2.51 ($p = 0.01$), 2.83 ($p = 0.008$) and 3.25 ($p = 0.008$), respectively (table 2).

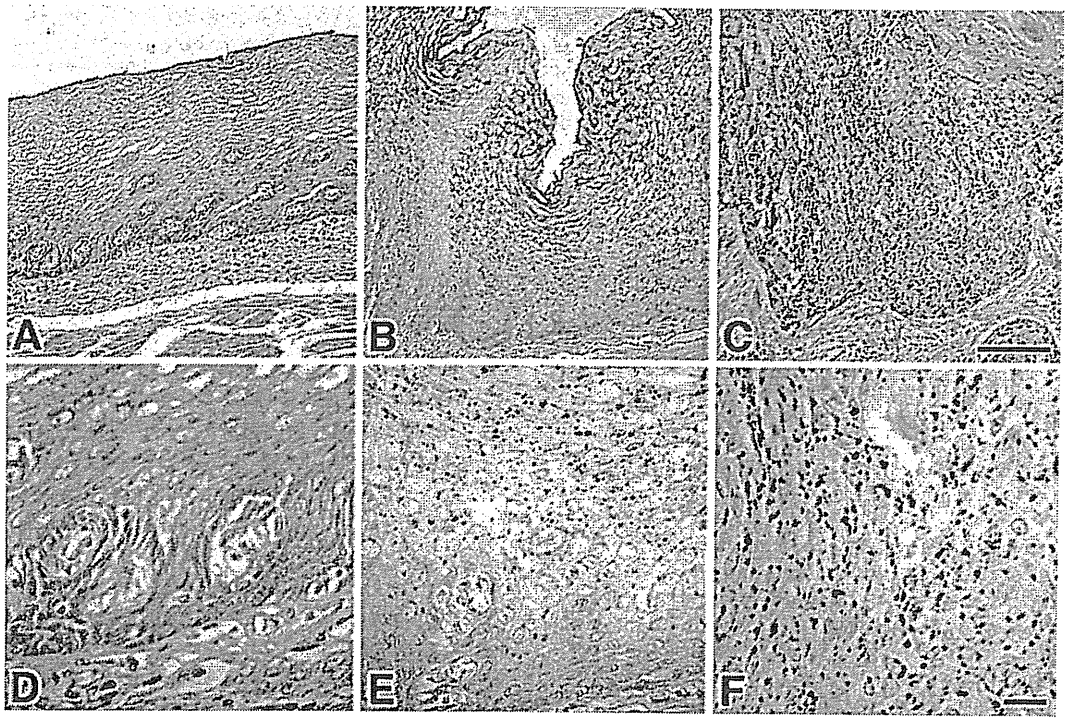

Fig. 1. Immunohistochemical staining patterns for GnT-V in normal epithelium, mild dysplasia and SCCs of the esophagus. GnT-V expression is not observed in normal epithelium (**A**, **D**), but is observed in mild dysplasia (**B**, **E**) and SCCs (**C**, **F**) in the Golgi localization pattern. Scale bar = 100 μm for **A–C** and 20 μm for **D–F**.

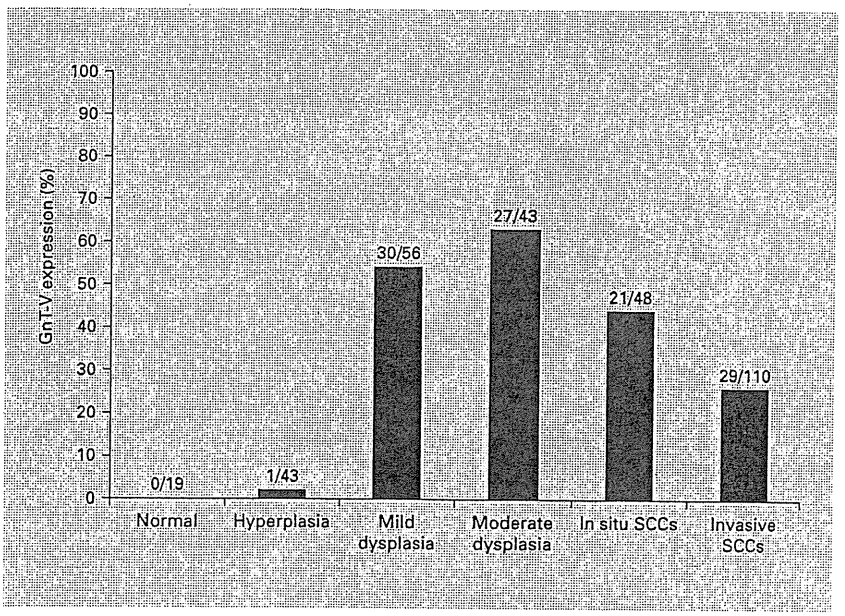

Fig. 2. GnT-V expression in esophageal SCCs and adjacent tissues. GnT-V expression was observed in none (0%) of the 19 normal epithelial tissues, 1 (2%) of the 43 hyperplastic tissues, 30 (54%) of the 56 mildly dysplastic tissues, 27 (63%) of the 43 moderately dysplastic tissues, 21 (44%) of the 48 in situ SCCs and 29 (26%) of the 110 invasive SCCs ($p < 0.005$ by the Kruskal-Wallis test).

Table 1. Relationship between GnT-V expression and clinical and clinicopathological characteristics in surgically resected esophageal SCCs

Characteristics	GnT-V expression		p
	positive (n = 38)	negative (n = 83)	
Age (mean ± SD), years	62.4 ± 8.9	62.3 ± 8.0	0.9
Sex			
Men	31	74	0.3
Women	7	9	
Tumor size (mean ± SD), cm	4.22 ± 2.19	5.31 ± 2.71	0.03
Tumor size[a]			
≥ 4.5 cm	15	48	0.09
<4.5 cm	23	35	
Grade			
G1	9	23	0.8
G2	19	42	
G3	10	18	
pT classification			
T1	19	35	0.5
T2–4	19	48	
pN classification			
N0	23	35	0.09
N1	15	48	
pM classification			
M0	35	63	0.045
M1	3	20	
pStage			
I	14	24	0.2
II	11	23	
III	10	16	
IV	3	20	
Lymphatic invasion			
+	21	46	1.0
–	17	37	
Vascular invasion			
+	13	28	1.0
–	25	55	
Double cancers			
Yes	10	14	0.3
No	28	69	
Ki-67 LI (mean ± SD)	46.7 ± 22.5 (n = 35)	44.9 ± 15.1 (n = 77)	0.6

[a] Tumors were divided into two groups based on the median value (4.5 cm) of tumor size.

Table 2. Cox's proportional hazards model analysis of potential prognostic factors

Characteristics	Hazards ratio (95% CI)	p
Univariate analysis		
Age (≥ 60/<60)	1.42 (0.77–2.64)	0.3
Sex (men/women)	2.43 (0.59–10.06)	0.2
Tumor size (≥ 4.5 cm/<4.5 cm)	1.69 (0.91–3.12)	0.09
Grade (2, 3/1)	1.21 (0.54–2.74)	0.6
pT classification (2, 3, 4/1)	3.42 (1.69–6.99)	0.0007
pN classification (1/0)	3.91 (1.94–7.81)	0.0001
pM classification (1/0)	2.53 (1.33–4.82)	0.005
pStage (II–IV/I)	3.90 (1.53–9.90)	0.004
Lymphatic invasion (+/–)	2.55 (1.33–4.88)	0.005
Vascular invasion (+/–)	1.86 (1.00–3.45)	0.051
Surgical margin (positive/negative)	4.69 (2.02–10.87)	0.0003
Double cancers (yes/no)	0.92 (0.44–1.91)	0.8
Adjuvant therapy (no/yes)	1.01 (0.59–1.93)	0.8
Performance status (1/0)	1.10 (0.46–2.59)	0.8
GnT-V expression (positive/negative)	0.80 (0.42–1.50)	0.5
Ki-67 LI (≥ 45%/<45%) (n = 63)	1.55 (0.83–2.89)	0.2
Multivariate analysis[a]		
Model 1		
pT classification (2, 3, 4/1)	2.51 (1.20–5.24)	0.01
pN classification (1/0)	2.83 (1.31–6.10)	0.008
pM classification (1/0)	1.30 (0.65–2.58)	0.5
Model 2		
pStage (II–IV/I)	2.82 (0.97–8.13)	0.06
Lymphatic invasion (+/–)	1.41 (0.65–3.02)	0.4
Surgical margin (positive/negative)	3.25 (1.37–7.72)	0.008

[a] Significant prognostic factors selected based on the results of univariate analysis.

The presence of β1,6-branched asparagine-linked oligosaccharides usually synthesized by GnT-V was then investigated using L4-PHA histochemistry in 14 randomly selected SCCs with positive GnT-V expression and 35 randomly selected SCCs with negative GnT-V expression (all specimens obtained from the Hokkaido University Hospital). In normal esophageal epithelium, expression of β1,6-branched asparagine-linked oligosaccharides is found in the basal cell layers but not in the superficial cell layers (fig. 4). On the other hand, high L4-PHA staining was observed in all of the 49 in situ and invasive SCCs (obviously more than 30% and at least 70% of tumor cells were stained) (fig. 4). In addition, hyperplastic and dysplastic tissues adjacent to SCCs were also positive for L4-PHA staining (data not shown).

In order to confirm the results of L4-PHA histochemistry, lectin blot analysis was performed. In lectin blotting using L4-PHA, which preferentially recognizes β1,6 branching, increases in β1,6 branching were observed in SCC tissues when compared with normal epithelial tissues in all five pairs of samples analyzed (fig. 5). These

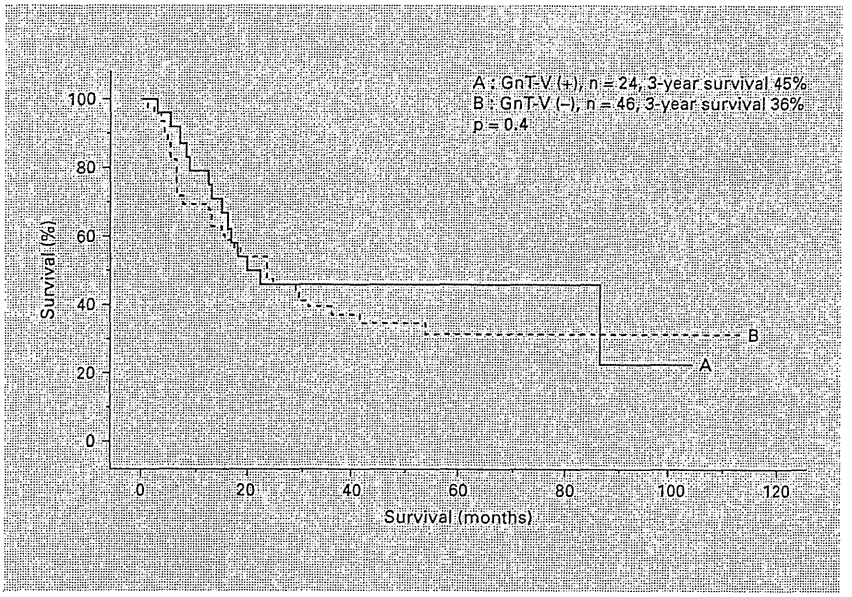

Fig. 3. Kaplan-Meier survival curves for patients with esophageal SCCs. Survival curves are stratified by positive (A) and negative (B) GnT-V expression.

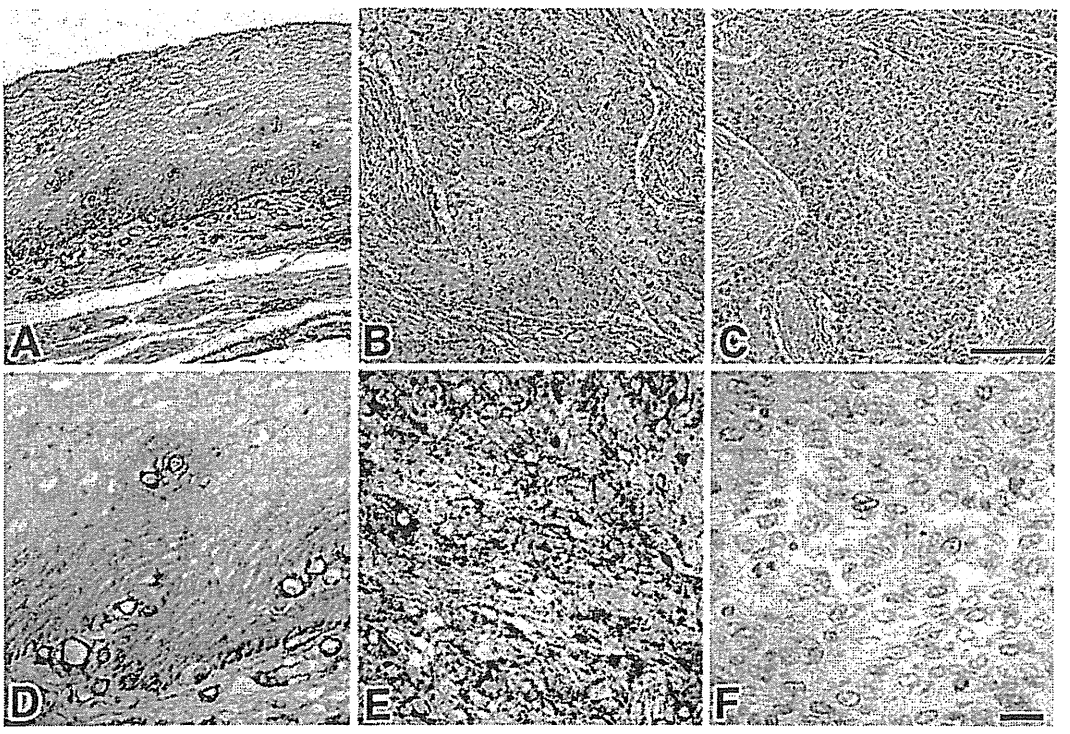

Fig. 4. Histochemical staining patterns for L4-PHA in normal epithelium and in esophageal SCCs. L4-PHA-reactive glycoconjugates are found in the basal cell layer but not in the superficial cell layer of normal epithelium (**A**, **D**). Positive L4-PHA staining (**B**, **E**) is observed diffusely in tumor cells in SCC without GnT-V expression (**C**, **F**). Scale bar = 100 μm for **A–C** and 20 μm for **D–F**.

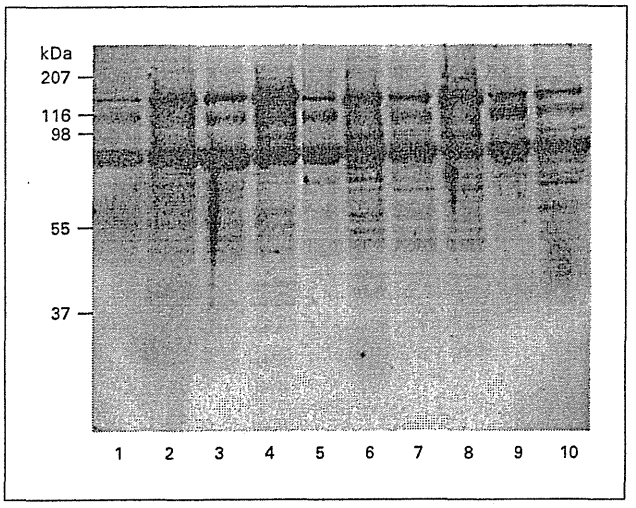

Fig. 5. Lectin blot analysis using L4-PHA on human esophageal SCC tissues. Proteins (20 μg) extracted from SCC tissues (lanes 2, 4, 6, 8 and 10) and surrounding normal esophageal epithelium (lanes 1, 3, 5, 7 and 9) were analyzed by Western blotting using L4-PHA. Increases in β1,6 branching were observed in SCC tissues when compared with normal epithelium. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 were prepared from the same patients.

results were consistent with results of L4-PHA histochemistry, which showed high L4-PHA staining in SCCs.

We then analyzed the relationship between GnT-V expression and tumor cell proliferation, as determined by Ki-67 LI. A monoclonal antibody MIB-1 recognizes a recombinant part of the Ki-67 nuclear antigen expressed during G1, S and G2/M phases, but not G0 phase, of the cell cycle [24]. Thus, the fraction of Ki-67-positive cells, Ki-67 LI, in tumor cells demonstrates tumor proliferative activity [25]. Ki-67 LI was determined in 113 of the 121 SCCs examined. The mean value of Ki-67 LIs was 46.0% (SD: 18.0%; range: 11.0–89.0%). To define an appropriate cutoff level for Ki-67 LIs in the subsequent analyses, we looked at their distribution among SCCs. Ki-67 LIs were considerably skewed and consisted of two populations with a cutoff level of 45%. Accordingly, tumors were divided into two groups having 'low' or 'high' Ki-67 LI: one group with a Ki-67 LI <45%, and the other group with a Ki-67 LI ≥45%. High Ki-67 LI was found in 58 (51%) of the 113 tumors, and low Ki-67 LI was found in 55 (49%). There was no relationship between Ki-67 LI and GnT-V expression. The relationship between Ki-67 LI and various clinical and clinicopathological features of SCCs is summarized in table 3. In SCCs, Ki-67 LI was significantly higher in T2–4 tumors than in T1 tumors ($p = 0.03$), in lymph node metastasis-positive tumors than in negative tumors ($p = 0.002$), and in vascular invasion-positive tumors than in negative tumors ($p = 0.005$). Ki-67 LI tended to be higher in lymphatic invasion-positive tumors than in negative tumors ($p = 0.08$), and in high-

Table 3. Relationship between Ki-67 LI and clinical and clinicopathological characteristics in surgically resected esophageal SCCs

Characteristics	Ki-67 LI (%, mean ± SD)	n	p
Age, years			
≥60	45.3 ± 17.7	71	0.6
<60	47.4 ± 18.5	42	
Sex			
Men	47.1 ± 17.9	98	0.1
Women	38.9 ± 16.9	15	
Tumor size[a]			
≥4.5 cm	48.2 ± 18.0	62	0.2
<4.5 cm	43.5 ± 17.8	51	
Grade			
G1	40.8 ± 18.6	31	0.06
G2, 3	48.0 ± 17.4	82	
pT classification			
T1	41.7 ± 17.5	48	
T2	45.1 ± 16.0	13	
T3	48.2 ± 16.7	40	
T4	57.6 ± 21.5	12	
T1	41.7 ± 17.5	48	0.03
T2–4	49.3 ± 17.7	65	
pN classification			
N0	40.4 ± 17.6	51	0.002
N1	50.7 ± 17.0	62	
pM classification			
M0	45.8 ± 18.4	89	0.8
M1	47.1 ± 16.6	24	
Lymphatic invasion			
+	48.6 ± 18.3	66	0.08
–	42.5 ± 17.1	47	
Vascular invasion			
+	52.6 ± 20.6	38	0.005
–	42.7 ± 15.6	75	
Double cancers			
Yes	38.9 ± 16.8	25	0.02
No	48.1 ± 17.8	88	
GnT-V expression in cancer			
Positive	47.1 ± 22.2	36	0.5
Negative	44.9 ± 15.1	77	

[a] Tumors were divided into two groups based on the median value (4.5 cm) of tumor size.

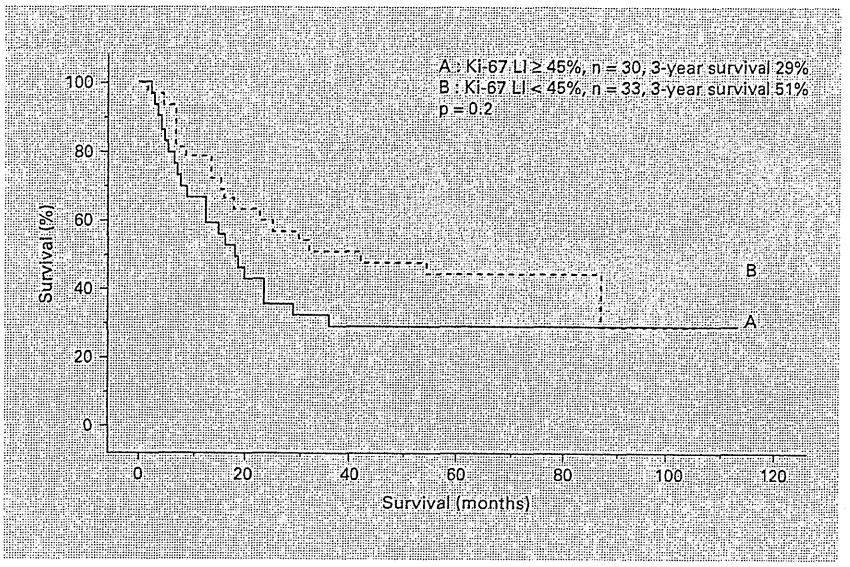

Fig. 6. Kaplan-Meier survival curves for patients with esophageal SCCs. Survival curves are stratified by Ki-67 LI. Ki-67 LI ≥ 45% represents high Ki-67 expression (A) and Ki-67 LI <45% low Ki-67 expression (B).

grade tumors than in moderate- to low-grade tumors ($p = 0.06$). Ki-67 LI was not associated with survival (3-year survival rate, 29% for patients having tumors with a high Ki-67 LI and 51% for patients having tumors with a low Ki-67 LI; $p = 0.2$) (fig. 6), and was not a significant prognostic factor in univariate analysis (table 2).

Discussion

This is the first study that examined GnT-V expression and its clinical significance in human normal, premalignant and malignant esophageal tissues. The present study demonstrates that GnT-V expression is found significantly more frequently in mildly and moderately dysplastic tissues when compared with normal epithelial and hyperplastic tissues, and when compared with in situ and invasive SCCs. Furthermore, GnT-V expression was frequently observed in SCCs of a small size and without distant metastasis or lymph node metastasis. These data suggest that GnT-V expression represents an early event in the development of human esophageal cancers, and that it may not be directly linked to tumor metastasis.

Previous studies demonstrated that high levels of GnT-V expression are correlated with a poor prognosis in human colorectal and breast cancers [15, 16]. On the other hand, expression of GnT-V in the liver is the early event in rat and human hepatocarcinogenesis [12, 18]. Ito et al. [18] reported that positive staining for GnT-V was observed in 60% of surrounding chronic hepatitis tissues, 71% of surrounding liver cirrhosis tissues, 100% of atypical adenomatous hyperplasias, and 75% of hepatocellular carcinomas (HCCs). Furthermore, high levels of GnT-V expression in HCCs were correlated with a low Ki-67 LI, small tumor size, and the absence of portal invasion. Levels of GnT-V expression in HCCs were not correlated with prognosis after surgery. These data in HCCs were similar to the present data in human esophageal SCCs. In addition, we recently found that low GnT-V expression is associated with shorter survival and poor prognosis in pStage I non-small cell lung cancers [26]. The different roles of GnT-V expression among various tumors, including esophageal SCCs, HCCs, non-small cell lung cancers, and colorectal and breast cancers, may be attributable at least partly to differences in the target proteins of GnT-V glycosylation [11, 27–29].

In the present study, the pattern of GnT-V expression did not overlap the staining pattern for β1,6-branched asparagine-linked oligosaccharides, as analyzed by L4-PHA histochemistry. We showed that β1,6-branched asparagine-linked oligosaccharides were found in the basal cell layers but not in the superficial cell layers in normal esophageal epithelium, and L4-PHA staining was observed in all of the 49 in situ and invasive SCCs examined. These results were consistent with previous studies [19, 20]. The discrepancy between L4-PHA staining and GnT-V expression could be attributed to several mechanisms, including expression of other enzymes synthesiz-

ing β1,6 branches and the levels of donor and acceptor substrate. A novel β1,6-N-acetylglucosaminyltransferase cDNA has recently been cloned and was designated GnT-IX by our group [30, 31] or GnT-VB by Pierce's group [32]. Moreover, it has been demonstrated that the in vivo levels of UDP-GlcNAc (donor substrate) are a critical factor for the production of β1,6 GlcNAc branching [33]. The amount of asparagine-linked oligosaccharides (acceptor substrate) is also important. In addition, the action of GnT-III prior to GnT-IV and GnT-V leads to the inhibition of multi-antennary oligosaccharides biosynthesis, because the bisecting N-acetylglucosamine (GlcNAc) moiety, catalyzed by GnT-III, does not serve as an acceptor substrate for α-mannnosidase II, or for GnT-II, GnT-IV, or GnT-V [34]. Thus, the formation of additional branching by GnT-V could be inhibited by the action of GnT-III.

Increased levels of β1,6 GlcNAc branching in human breast and colon carcinoma have previously been correlated with tumor development, invasion and metastasis [15, 16]. Li and Roth [19] reported that differentiated and nonmitotic epithelia in most organs showed strong lectin staining, and the exception was the epithelia of colon and resting mammary gland, which were unreactive to L4-PHA. Thus, an increase in L4-PHA staining intensity from normal epithelium to carcinoma seems to be present only in the colon and breast. The biological importance of this oligosaccharide structure in the regulatory function of normal epithelial cells and in the development, invasion and metastasis of cancer cells would vary in each organ. It is because target proteins of GnT-V could also be different in various cell types from different organs.

In the present study, we also studied Ki-67 LI in human esophageal SCCs. Ki-67 LI has been regarded as a parameter of cell proliferation [35], but a few studies [36, 37] have investigated Ki-67 cell proliferative activity in esophageal SCCs. Kawamura et al. [37] reported that Ki-67 LI was correlated with cancer invasion and lymph node metastasis in carcinomas with submucosal invasion. Our data showed that Ki-67 LI was strongly related to pT classification, pN classification and vascular invasion, and tended to be associated with lymphatic invasion and differentiation in SCCs. The present results were largely consistent with those of previous studies [36, 37].

In conclusion, increased expression of GnT-V was associated with the early event of esophageal tumorigenesis. While the presence of β1,6 GlcNAc branching was observed in SCCs, it was not correlated with GnT-V expression, suggesting that the regulation of β1,6 GlcNAc branching in esophageal SCCs could not be controlled by GnT-V alone.

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E1AF/PEA3 Activates the Rho/Rho-Associated Kinase Pathway to Increase the Malignancy Potential of Non–Small-Cell Lung Cancer Cells

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Abstract

E1AF/PEA3, an Ets family transcription factor, is frequently overexpressed in non–small-cell lung cancers (NSCLCs). Overexpression of E1AF increases motility and invasion of VMRC-LCD and NCI-H226 NSCLC cells, which lack endogenous E1AF expression, and the effect is synergistically increased by hepatocyte growth factor (HGF). The small GTPase Rho/Rho-associated kinase (ROCK) pathway is also involved in motility and invasion. To determine the role of the Rho/ROCK pathway in malignant phenotypes induced by E1AF, we analyzed VMRC-LCD cells transfected with an E1AF expression vector (LCD-E1AF cells) or with empty vector (LCD-vector cells). LCD-E1AF cells had more GTP-bound (active) Rho than LCD-vector cells and Rho activation was synergistically increased by HGF. The Rho activation by E1AF and HGF was also shown in NCI-H226 cells. Phosphorylation of myosin light chain (MLC), a downstream effector of ROCK signaling, was higher in LCD-E1AF cells than in LCD-vector cells, especially under HGF treatment. A specific ROCK inhibitor, Y27632, strongly suppressed MLC phosphorylation, cell motility, and invasion. In nude mice implanted s.c. and intrapulmonarily, LCD-E1AF cells made more local tumors than LCD-vector cells (six of six versus one of seven mice and four of seven versus one of seven mice, respectively). Three of the four mice with lung tumors from LCD-E1AF cells had lymph node metastases whereas the mouse with LCD-vector tumors did not. LCD-E1AF tumors showed higher MLC phosphorylation than LCD-vector tumors. These results suggest that E1AF activates the Rho/ROCK pathway in an HGF-enhanced manner and its activation is important in E1AF-induced motility and invasion as well as tumorigenesis and metastasis in NSCLC cells. (Cancer Res 2005; 65(23): 10776-82)

Introduction

Cell motility and invasion as well as tumorigenesis and metastasis characterize the malignancy potential of cancer. Members of the Ets-related oncoprotein family are transcription factors with a highly conserved ETS domain; they play important roles in the regulation of gene expression during such biological processes as oncogenesis (1). One Ets-related family member, E1AF (2), a human homologue of mouse PEA3 (3), was isolated by its ability to bind adenovirus E1A enhancer elements. E1AF can up-regulate transcription from several subclasses of matrix metalloproteinase (MMP) genes in transient expression assays (2). Expression of E1AF confers an invasive phenotype on MCF7, a human breast cancer cell line (4). E1AF expression is correlated with the transcription of MMPs and the invasive phenotype in oral squamous cell carcinoma cell lines (5). We have previously reported that E1AF is frequently overexpressed in non-small-cell lung cancers (NSCLC) and its expression increases motility and invasion of NSCLC cell lines VMRC-LCD and NCI-H226, which normally lack E1AF expression (6). In addition, recent *in vivo* experiments show that E1AF increases the metastatic activity of fibrosarcoma cells through expression of membrane type 1 MMP (7) and that mammary oncogenesis induced by HER2/neu is strongly inhibited by blockade of E1AF (8). These observations suggest the E1AF has roles in various malignant phenotypes of cancer cells but the mechanisms remain to be discovered.

Rho, a well-known member of the p21 Ras superfamily of small GTPases, exhibits both GDP/GTP binding and GTPase activities. Rho serves as a molecular switch, regulating signal transduction from receptors in the membrane to various cellular events related to cell morphology (9), motility (10), cytoskeletal dynamics (11, 12), and tumor progression (13, 14; for reviews, see refs. 15–17). Rho activates Rho-associated kinase (ROCK), which then increases phosphorylation of myosin light chain (MLC; ref. 18). Phosphorylated MLC induces the contraction of myosin and subsequently organizes the actin stress fibers and focal adhesions. Activation of Rho/ROCK signaling is also known to stimulate the organization of actin stress fibers and to enhance the motility and invasion of rat hepatoma cells (19, 20), human glioma cells (21), and human ovarian cancer cells (22). Recently, studies using clinical specimens showed a relationship between the expression level of RhoC, an isoform of Rho, and tumor aggressiveness in breast cancer (23–25), pancreatic cancer (26), and NSCLCs (27). Furthermore, RhoC also enhances metastasis of melanoma cells (28) and lung cancer cells (29) in mouse model systems.

To determine whether the Rho/ROCK pathway is involved in E1AF-associated malignant phenotypes in NSCLCs, we examined E1AF- and vector-transfected VMRC-LCD NSCLC cells for Rho activity and its relation to motile and invasive activities. To increase the effects of E1AF, we used hepatocyte growth factor (HGF), a strong cell-scattering factor that is frequently overexpressed in various human cancers including NSCLCs. We have shown that HGF acts synergistically with E1AF to increase motile and invasive activities in VMRC-LCD cells (6). We treated the cells with a specific ROCK inhibitor, Y27632 (20), to determine whether activation of the Rho/ROCK pathway is required for

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the enhancement of motile and invasive activities by E1AF. Furthermore, we examined E1AF- and vector-transfected cells for tumorigenic and metastatic activities *in vivo* and their relationship with phosphorylation of MLC, a downstream effector of ROCK.

Materials and Methods

Cell lines and cell culture. VMRC-LCD and A549 NSCLC cell lines were obtained from the Health Science Research Resource Bank of Japan (Osaka, Japan). NCI-H226 and NCI-H520 NSCLC cell lines were kindly provided by Dr. H. Oie (Navy Medical Oncology Branch, National Cancer Institute, Bethesda, MD). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 0.03% glutamine at 37°C in an atmosphere of 5% CO_2. LCD-E1AF cells (VMRC-LCD cells transfected with the E1AF-expression vector pCMV-E1AF and pRSVneo), LCD-vector control cells (VMRC-LCD cells transfected with the empty vector pEV3S and pRSVneo), H226-E1AF cells (NCI-H226 cells transfected with the E1AF-expression vector pCMV-E1AF and pRSVneo), and H226-vector control cells (H226 cells transfected with the empty vector pEV3S and pRSVneo) have been described in our previous article (6). Although not stated in that article, they were pools of G418-resistant cells consisting of at least 100 independent clones, which could minimize possible clonal variations. The established cells had been stocked in aliquots in liquid nitrogen until experiments were started. Only cells in early passages were used for the experiments to avoid molecular changes during prolonged period of culture.

pLNTK-E1AF–infected SiHa cells were cultured in DMEM supplemented with 10% FBS and 0.03% glutamine at 37°C in an atmosphere of 5% CO_2 and used for positive control for Western blot analysis of E1AF protein (30).

Western blot analysis of E1AF. Western blot analyses of E1AF were done as previously described (30). Briefly, purified total protein (30 μg) from isolated subconfluent cells was separated by SDS-PAGE, transferred to nitrocellulose membranes, and reacted with an anti-E1AF monoclonal antibody (a gift from Dr. Chono, Takara Bio, Otsu, Japan). The primary antibody was detected using an antirabbit antibody conjugated with horseradish peroxidase and visualized by the Amersham enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL). pLNTK-E1AF–infected SiHa cells were used for positive control of E1AF protein expression (30). The same lysates were used in Western blotting of actin as an internal control.

***In vitro* motility and invasion assays.** Transwell cell culture chambers (pore size, 8 μm; Costar, Cambridge, MA) were used for the motility and invasion assays. For the motility assay, 1×10^5 LCD-E1AF and LCD-vector cells were suspended in serum-free RPMI 1640 with 0.1% bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) and added to the upper chamber. The reverse side of the upper chamber filter was coated with 10 μg human cellular fibronectin (Biomedical Technologies, Stoughton, MA) as a chemoattractant. Serum-free RPMI 1640 with 0.1% BSA was added to the lower chamber. For the invasion assay, 2×10^5 LCD-E1AF and LCD-vector cells were suspended in serum-free RPMI 1640 with 0.1% BSA and added to the upper chamber. The upper chamber filter was coated with 10 mg mouse Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) and the reverse side of the upper chamber filter was coated with 10 μg human cellular fibronectin as a chemoattractant. Serum-free RPMI 1640 with 0.1% BSA was added to the lower chamber. In both motility and invasion assays, a specific ROCK inhibitor, Y27632 (0, 1, 10, or 100 μmol/L; Calbiochem, Darmstadt, Germany), was added to the upper and lower chambers and HGF (40 ng/mL; Toyobo, Osaka, Japan) was added to the lower chamber as indicated. These cells were incubated at 37°C in an atmosphere of 5% CO_2 for 24 hours for both motility and invasion assays.

Cell growth assay. Cell growth was measured using an assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; non-radioactive proliferation assay, Promega, Madison, WI). Cells (1×10^4) in 0.1 mL of RPMI 1640 with 10% FBS or 0.1% BSA, with or without 100 μmol/L Y27632, were seeded onto each well of 96-well plates and then incubated at 37° C in an atmosphere of 5% CO_2 for 1, 3, or 5 days. To each well was added 15 μL of Dye Solution (Promega) and then cells were incubated at 37° C for 4 hours. After 4 hours, 100 μL of Solubilization/Stop Mix solution (Promega) were added to each well. One hour later, the contents of the wells were mixed to yield a uniformly colored solution. The absorbance at 590 nm was recorded using a 96-well plate reader and the survival fraction was quantified.

Rho pull-down assay. The Rho pull-down assay was done as previously described (31). Briefly, cells (3.5×10^5/mL) were plated and cultured under 10% serum conditions with or without HGF for 24 hours. After incubation, cells were washed twice with PBS and lysed in Laemmli's SDS sample buffer (32). Cell lysates were clarified by centrifugation. For controls, LCD-vector cell lysate or H226-vector cell lysate containing 1.5 mg total protein was incubated at 30°C for 30 minutes with 100 μmol/L GTPγS (Upstate Biotechnology, Lake Placid, NY) for the positive control or with 100 μmol/L GDP (Upstate Biotechnology) for the negative control. Cell lysates containing 1.5 mg total protein were incubated with 30 μg Rhotekin RBD-agarose beads (Upstate Biotechnology) at 4°C for 45 minutes. The beads were washed thrice with PBS. GTP-bound Rho proteins were detected by Western blotting using a polyclonal antibody against Rho (RhoA, RhoB, and RhoC; Upstate Biotechnology) overnight at 4°C. The primary antibodies

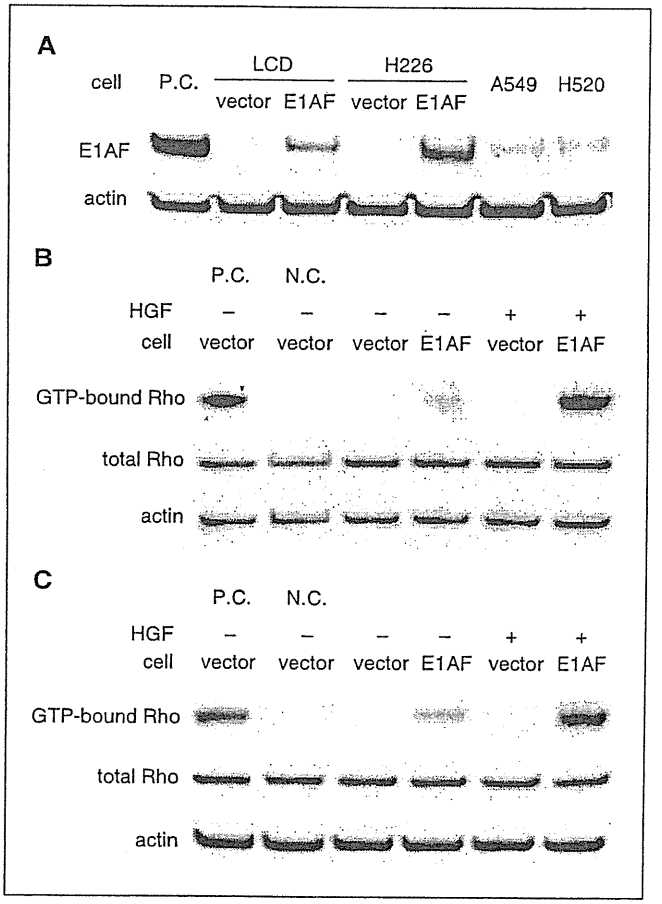

Figure 1. Rho activation by E1AF in NSCLC cells. *A*, Western blot analysis of E1AF protein of LCD-vector and LCD-E1AF cells, H226-vector and H226-E1AF cells, and A549 and NCI-H520 cells. *P.C.*, positive control (pLNTK-E1AF–infected SiHa cells; ref. 30). *B*, Western blot analysis of intracellular levels of GTP-bound Rho and total Rho in LCD-vector and LCD-E1AF cells. For GTP-bound (active) Rho, total protein isolated from cells cultured for 24 hours, with or without HGF (40 ng/mL), was incubated with Rhotekin RBD-agarose beads to bind active Rho. Then GTP-bound Rho was detected by Western blotting using an anti-Rho (RhoA, RhoB, and RhoC) polyclonal antibody. *N.C.*, negative control (see Materials and Methods). The same lysates were used in Western blotting of total Rho and actin as an internal control for the comparison of levels of GTP-bound Rho. *C*, same experiments as in (*B*) were done using H226-vector and H226-E1AF cells.