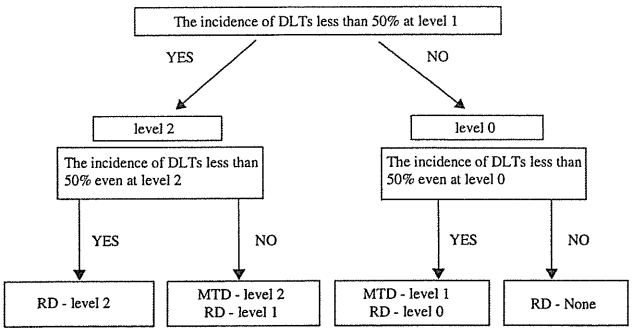

Figure 1. Recommended dosages in each arm. DLT, dose-limiting toxicity; RD, recommended dosage; MTD, maximum tolerated dose.

Toxicity Criteria scale) in 50% or more of patients during the first treatment cycle: grade 4 leukopenia or neutropenia persisting for at least 4 days; grade 3/4 neutropenia associated with a fever ≥38.0°C or infection; thrombocytopenia ($<20 \times 10^9$/l) or need of a platelet transfusion; or grade 3/4 non-hematological toxicities (excluding nausea/vomiting, anorexia, fatigue and hypersensitivity). G-CSFs were administrated for the treatment of grade 4 neutropenia or grade 3 neutropenic fever. A DLT was also reported if any day-8 doses were omitted and dosing requirements were not satisfied until after day 15, or if the second cycle was delayed until after day 29 because the dosing requirements were not satisfied.

The recommended dose for phase II had to be determined from the arm that reached the highest dose level. If at dose level 2 the incidence of DLTs was less than 50%, the recommended dose was defined as dose level 2. The arm that reached the higher dose level reflected the recommended regimen for phase II. If the recommended dose level for the two arms was identical, the recommended regimen would be decided according to the following steps: (i) if frequency of DLTs was 0% in one arm and 33.3% or more in the other arm, the former was selected. If this did not occur, then (ii) if the dose intensity for evaluable patients in one arm was higher by 10% or more than the other arm, the arm with the higher dose intensity was selected. If this did not occur, then (iii) the arm with the fewer day-8 dose omissions in first and second cycles was selected. If the recommended dosage regimen still could not be decided, the sponsor (Aventis Pharma Japan and Eli Lilly Japan K.K.) and the coordinating investigator determined the recommended phase II regimen. If the MTD was dose level 0 in both arms, the study was terminated (Fig. 1).

The sample size for the recommended regimen was determined as follows. The response rate of this regimen and gemcitabine single agent was assumed to be 35 and 20%, respectively, in view of the response rates previously achieved (9,10,17,18). If the sample size of the recommended regimen was set as 40 patients, the probability for the one-sided 90% lower limit of response rate to exceed 20% was 82%. Thus, the target sample size in the recommended regimen including six patients in phase I was set at 40 patients.

The phase II study was conducted with 34 patients. Forty patients who were given the recommended regimen were evaluated for the efficacy and detailed safety profile: these patients consisted of six and 34 patients who entered into the study at phase I and II, respectively.

In this phase I/II study, patients received a minimum of two cycles of gemcitabine–docetaxel and up to four additional cycles.

Dose Modifications

During a cycle, dose modifications were not allowed. If not all of the following requirements were satisfied on either the day of treatment or the previous day, administrations of gemcitabine and docetaxel were delayed until the patient completely recovered. For gemcitabine and docetaxel doses administered on day 1 of Arm 1 or gemcitabine on day 1 of Arm 2, delays occurred for patients with an absolute neutrophil count $<1.5 \times 10^9$/l, a platelet count $<70 \times 10^9$/l, any grade 3/4 non-hematologic toxicities (except PaO_2), or PaO_2 <60 torr. When gemcitabine was given on day 8 of Arm 1, exceptions included leukopenia $<2.0 \times 10^9$/l and an absolute neutrophil count $<1.0 \times 10^9$/l, a platelet count $<70 \times 10^9$/l, any grade 3/4 non-hematological toxicities. When gemcitabine was given on day 8 of Arm 2, exceptions included an absolute neutrophil count $<1.5 \times 10^9$/l, a platelet count $<70 \times 10^9$/l, any grade 3/4 non-hematological toxicities. If a patient developed a DLT, the subsequent doses were cancelled, and in the next cycle the patient could resume the study treatment at the next lower dose level. If a patient developed a DLT at dose level 0, gemcitabine 800 mg/m^2 and docetaxel 40 mg/m^2 were administered in the next cycle.

Baseline and Treatment Assessment

Assessments at baseline included tumor measurements by X-ray and computed tomography (CT) scan within 4 weeks before the day of starting the study treatment. Equally, grading performance status and physical examination were performed within a week; hematology, blood chemistries, urinalysis, arterial blood gas analysis and electrocardiogram were observed within 2 weeks.

After the start of treatment, tumor measurements were obtained every 2 weeks via X-ray and 4 weeks via CT scan. Tumor response was assessed with the World Health Organization (WHO) criteria. Safety assessments, including performance status, hematology, blood chemistries and urinalysis, were obtained weekly. Physical examination, arterial blood gas analysis and electrocardiogram were performed at any time. Adverse events were estimated according to National Cancer Institute–Common Toxicity Criteria version 2.0. All patients were assessed for efficacy and safety. An additional response rate was recorded for patients who received the recommended regimen in phase I and all phase II patients.

RESULTS

Patient Characteristics

Between July 2000 and July 2002, 59 chemonaive patients (43 male, 16 female) with NSCLC were enrolled in phase I and II portions from the five hospitals after approval by the IRB. Twenty-five patients were enrolled in the phase I portion of the study, and 34 patients were enrolled in phase II. Baseline patient characteristics for all patients and patients who received the recommended regimen are summarized in Table 1.

Phase I

Twenty-five patients were enrolled into the phase I portion of the study. The number of patients treated and the DLTs observed in the first cycle at each dose level of gemcitabine and docetaxel are shown in Table 2.

In Arm 1, 50% of patients had DLTs at dose level 1 and dose level 0, therefore Arm 1 could not be the recommended regimen: there were 2/6 and 3/6 patients who achieved partial response (PR) at dose level 1 and 0 in Arm 1, respectively.

Table 1. Baseline characteristics

Patient characteristics	All patients ($n = 59$), n (%)	Patients who received the recommended regimen ($n = 40$), n (%)
Gender		
Male	43 (72.9%)	26 (65.0%)
Female	16 (27.1%)	14 (35.0%)
Age		
Median	62	64
Range	38–74	38–74
ECOG performance status		
0	5 (8.5%)	2 (5.0%)
1	54 (91.5%)	38 (95.0%)
Stage		
IIIB	14 (23.7%)	8 (20.0%)
IV	33 (55.9%)	23 (57.5%)
Postsurgical recurrence	12 (20.3%)	9 (22.5%)
Histological type		
Adenocarcinoma	34 (57.6%)	25 (62.5%)
Squamous cell carcinoma	19 (32.2%)	14 (35.0%)
Large cell carcinoma	5 (8.5%)	1 (2.5%)
Other	1 (1.7%)	0 (0%)
Prior therapy		
None	45 (76.3%)	29 (72.5%)
Surgery	13 (22.0%)	11 (27.5%)
Radiotherapy	0 (0%)	0 (0%)
Radiotherapy and surgery	1 (1.7%)	0 (0%)

ECOG, Eastern Cooperative Oncology Group.

In Arm 2, no DLT was observed at dose level 1: 3/6 patients achieved PR. At dose level 2, one patient discontinued due to progressive disease; therefore, one patient was added. However, another patient discontinued due to grade 3 hypersensitivity (not a DLT). In this regimen, two DLTs had already been observed in five other patients, but the sponsors (Aventis Pharma Japan and Eli Lilly Japan K.K.) and investigators decided not to add one more patient to dose level 2 in Arm 2 in consideration of patients' safety. PRs were observed in 2/7 patients at dose level 2 of Arm 2.

Therefore, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 due to the incidence of DLT.

Dose Administration

In Arm 1, a total of 49 cycles were accomplished. One case delayed the date of administration on day 1 (defined as more than 8 days) as a matter of convenience; seven and four cases delayed their dates of administration on day 8 (defined as more than 1 day) because of adverse events and non-medical reasons, respectively; and four cases could not be treated on day 8 because of adverse events. In Arm 2, including phase I and II portions, a total of 145 cycles were accomplished. Four and five cases delayed their dates of administration on day 1 because of adverse events and non-medical reasons, respectively; 21 and nine cases delayed their dates of administration on day 8 because of adverse events and non-medical reasons, respectively; and two cases could not be treated on day 8 because of

Table 2. Phase I dose-limiting toxicities

Dose level	GEM/DOC (mg/m^2)	Arm 1	Arm 2
0	800/50	3/6 patients: • G3 ALT increased • G1 fever, G3 neutropenia • G2 infection, G3 neutropenia	N/A
1	1000/50	3/6 patients: • G3 infection, G3 neutropenia • G4 neutropenia, G1 fever, G3 infection • G3 neutropenia, G2 infection, G3 arrhythmia, G3 diarrhea	0/6 patients
2	1000/60	N/A	2/5 patients: • G3 ALT increased • G2 fever, G3 neutropenia

GEM, gemcitabine; DOC, docetaxel; G, grade; ALT, alanine aminotransferase; N/A, not applicable.

adverse events. The most common adverse event for a dose delay was neutropenia.

EFFICACY

All 59 patients were involved in the analysis for efficacy, and 19 of 59 patients achieved PR for an overall response rate of 32.2% [95% confidence interval (CI) 20.6–45.6%]. Of the 40 patients who received the recommended regimen in either phase I or phase II, 12 patients achieved PRs for a response rate of 30.0% (95% CI 16.6–46.5%).

The median time to progressive disease in all 59 patients was 111 days (95% CI 71–154 days). Median survival time was 11.9 months (95% CI 7.0–15.0 months), with 1-year survival rate at 47.1% (95% CI 34.0–60.2%).

SAFETY

All 59 patients were evaluable for safety. Grade 3 and 4 drug-related toxicities observed in all 59 patients are shown in Table 3. Grade 3 and 4 drug-related toxicities observed in 40 patients who received the recommended regimen are also shown in Table 4.

In all 59 patients, grade 3 and 4 neutropenia were observed in 19 (32.2%) and 20 (33.9%) patients, respectively. Grade 3 and 4 leukopenia were observed in 24 (40.7%) and four (6.8%) patients, respectively. Grade 3 non-hematological toxicities included infection in four patients (6.8%), anorexia in four patients (6.8%), and nausea, diarrhea, rash and constipation in three patients (5.1%) each. After starting docetaxel administration, grade 3 interstitial pneumonia was reported in three patients (5.1%), all of whom recovered shortly after steroid treatment; grade 4 anaphylaxis was reported in two patients (3.4%). There were no toxic deaths.

DISCUSSION

In this phase I/II study, we examined the activity and tolerability of gemcitabine and docetaxel. In phase I, the recommended regimen was determined as gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8. The response rate of all 59 patients was 32.2% (95% CI 20.6–45.6%). When re-evaluated in the 40 patients who received the recommended regimen, the response rate was 30.0% (95% CI 16.6–46.5%). Although the number of patients was limited, Arm 1 (docetaxel on day 1) had a numerically better response: for the 12 patients in Arm 1, five PRs were recorded for a response rate of 42%. However, Arm 1 had more toxicities than the docetaxel on day-8 schedule.

Overall, the toxicity associated with the gemcitabine–docetaxel regimen was manageable. In Arm 1, five patients (42%) had grade 3/4 neutropenia supervened with infection or fever, while only one patient (9%) had grade 3 neutropenia with infection or fever in Arm 2. This indicated that docetaxel was better tolerated on day 8 than on day 1 in a 21-day cycle. It is speculated that the influence of time to nadir of neutropenia is different in each agent: 14–20 days with gemcitabine and 9 days with docetaxel. The time to recover from nadir is 7–8 days with gemcitabine and 8 days with docetaxel. This could explain why docetaxel on day 8 was better tolerated.

Meta-analysis studies have reported that cisplatin-based regimens produce a significant survival benefit in NSCLC (20–23), improve median survival time by 6–8 weeks and 1-year survival rate from 15% to 25% when compared with the best supportive care (24). But studies with platinum-based combinations have also reported severe toxicities, so the deterioration of patients' quality of life is a major problem to be solved (3).

New effective non-platinum-based therapies have been used in various combinations in recent years, and the combination of gemcitabine and docetaxel has been established as one of the

Table 3. NCI–CTC grade 3/4 toxicities (n = 59)

Toxicities	Grade 3		Grade 4	
	n	%	*n*	%
Hematological toxicities				
Leukopenia	24	40.7	4	6.8
Neutropenia	19	32.2	20	33.9
Lymphopenia	10	16.9	0	0.0
Hemoglobin decreased	4	6.8	0	0.0
Thrombocytopenia	1	1.7	0	0.0
Thrombocytosis	1	1.7	0	0.0
Non-hematological toxicities				
ALT increased	5	8.5	0	0.0
Infection	4	6.8	0	0.0
Anorexia	4	6.8	0	0.0
Nausea	4	6.8	0	0.0
Diarrhea	3	5.1	0	0.0
Interstitial pneumonia	3	5.1	0	0.0
Rash	3	5.1	0	0.0
Constipation	3	5.1	0	0.0
AST increased	2	3.4	0	0.0
Fatigue	2	3.4	0	0.0
Vomiting	2	3.4	0	0.0
Hyperglycemia	1	1.7	0	0.0
Hyponatremia	1	1.7	0	0.0
Allergic reaction	1	1.7	0	0.0
Vasovagal reaction	1	1.7	0	0.0
Body temperature decrease	1	1.7	0	0.0
Weight increase	1	1.7	0	0.0
Hypotension	1	1.7	0	0.0
Pneumonia	1	1.7	0	0.0
Arrhythmia	1	1.7	0	0.0
Edema	1	1.7	0	0.0
Neuropathy peripheral	1	1.7	0	0.0
Anaphylaxis	0	0.0	2	3.4

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Table 4. NCI–CTC grade 3/4 toxicities (n = 40, recommended regimen)

Toxicities	Grade 3		Grade 4	
	n	%	*n*	%
Hematological toxicities				
Leukopenia	13	32.5	2	5.0
Neutropenia	12	30.0	11	27.5
Lymphopenia	5	12.5	0	0.0
Hemoglobin decreased	2	5.0	0	0.0
Thrombocytopenia	1	2.5	0	0.0
Thrombocytosis	1	2.5	0	0.0
Non-hematological toxicities				
ALT increased	2	5.0	0	0.0
Diarrhea	2	5.0	0	0.0
Infection	2	5.0	0	0.0
Interstitial pneumonia	2	5.0	0	0.0
Rash	2	5.0	0	0.0
Fatigue	2	5.0	0	0.0
Nausea	2	5.0	0	0.0
Vomiting	2	5.0	0	0.0
Hyperglycemia	1	2.5	0	0.0
Hyponatremia	1	2.5	0	0.0
AST increased	1	2.5	0	0.0
Allergic reaction	1	2.5	0	0.0
Vasovagal reaction	1	2.5	0	0.0
Anorexia	1	2.5	0	0.0
Body temperature decrease	1	2.5	0	0.0
Weight increase	1	2.5	0	0.0
Hypotension	1	2.5	0	0.0
Pneumonia	1	2.5	0	0.0
Edema	1	2.5	0	0.0
Constipation	1	2.5	0	0.0
Peripheral neuropathy	1	2.5	0	0.0
Anaphylaxis	0	0.0	2	5.0

NCI–CTC, National Cancer Institute–Common Toxicity Criteria version 2.0; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

well-examined regimens. In recent studies using gemcitabine–docetaxel in NSCLC, response rates of 25–50% (19,25–29) and time-to-progression of disease of 106–132 days (31,32) have been reported. Georgoulias et al. (16) reported that the gemcitabine–docetaxel and docetaxel–cisplatin regimens they compared were equivalent in efficacy, but toxicity was severe in the latter. While docetaxel–cisplatin regimens showed severe toxicities of grade 3 anemia (5%), grade 3/4 neutropenia (13%/21%), grade 3 nausea/vomiting (10%) and grade 3 diarrhea (8%), gemcitabine–docetaxel regimens had grade 3/4 anemia (1%/1%), grade 3/4 neutropenia (11%/11%), grade 3 nausea/vomiting (2%) and grade 3/4 diarrhea (2%/1%) in 441 patients. However, the difference of efficacy and safety by the administration schedule and dosage of gemcitabine and docetaxel has not been well documented.

There are some studies that have examined the efficacy and safety of the same schedule as the recommended regimen in our study, namely gemcitabine on days 1 and 8 plus docetaxel on day 1. In these studies dosages were various: gemcitabine was 800–1100 mg/m^2 and docetaxel was 60–100 mg/m^2 (18,19,27–30). Response rates in these studies also varied from 16 to 38%, which indicates that the response rate of the recommended regimen in our study (30.0%) was clinically meaningful because the dosage of docetaxel (50 mg/m^2) in our study is less than that in any other studies. This might have contributed to the relatively mild toxicities of our recommended regimen.

In another study (26), a high response rate (50.0%) was achieved in patients with another administering schedule: gemcitabine 1000 mg/m^2 on days 1 and 10 plus docetaxel 80 mg/m^2 on day 1, administered every 21 days. The most common treatment-related toxicity was myelosuppression. Grade 3/4 leukopoenia and neutropenia occurred in only six (18%) and eight (24%) patients, respectively.

The median survival was 11.9 months in our study, being slightly better than the result from the median survival of the phase III study with gemcitabine and cisplatin, which was 8.7–9.1 months (33,34). This result suggests that the regimen we selected in the phase II portion of this study is comparable in survival with the cisplatin-based regimen.

In conclusion, the combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be better tolerated and has equivalent efficacy to cisplatin-based therapy. These results should be verified by a phase III study in Japanese patients.

CONCLUSION

In this phase I/II study, we studied the activity and tolerability of gemcitabine and docetaxel in Japanese patients. The combination of gemcitabine 1000 mg/m^2 on days 1 and 8 plus docetaxel 50 mg/m^2 on day 8 is suggested to be well tolerated and has equivalent efficacy to cisplatin-based therapy.

Acknowledgments

We thank Dr N. Masuda for his helpful comments with the preparation of the paper; and Drs T. Taguchi, Y. Ariyoshi, N. Hara and M. Kawahara for overseeing the management of the study. This work was supported by Eli Lilly Japan K.K.

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ANTICANCER RESEARCH *26*: 777-782 (2006)

Phase II Study of Weekly Paclitaxel for Relapsed and Refractory Small Cell Lung Cancer

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Abstract. *The purpose of this study was to evaluate the efficacy and toxicity of single-agent paclitaxel given weekly to patients with relapsed and refractory small cell lung cancer (SCLC). Patients were treated with 80 mg/m^2 paclitaxel administered weekly for 1 h for 6 weeks in an 8-week cycle. Twenty-two patients were enrolled, 21 of whom were eligible. The patient characteristics included: 20 males, 1 female; median age 66 years (range 48 - 75); performance status 0/1 in 19 and 2 in 5 patients. Grade 3/4 leukopenia and neutropenia occurred in 47.5% and 64%, respectively. Other grade 3/4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. There were 5 partial responses in 3 out of the 11 sensitive cases and 2 out of the 10 refractory cases, respectively. Paclitaxel, administered as a weekly infusion at a dose of 80 mg/m^2, was effective in treating relapsed and refractory SCLC.*

More than 95% of patients with small cell lung cancer (SCLC), who are initially treated with paclitaxel 80 mg/m^2, present a relapse and their response to a second-line therapy is poor. The responses obtained are usually brief, and the median survival is generally less than 4 months (1). Nevertheless, second-line chemotherapy may provide a significant palliation of symptoms and does result in a prolongation of survival in many patients.

The activity of paclitaxel as a single agent has been investigated in both previously-untreated and -treated SCLC patients. Two phase II trials were conducted to investigate its efficacy as a first-line treatment for SCLC. In a trial conducted by the Eastern Cooperative Oncology Group (ECOG), Ettinger *et al.* administered 250 mg/m^2 paclitaxel as a 24-h infusion to 36 patients (2), among whom 11 partial responses were observed. Kirschling *et al.* obtained a similar response rate, 41%, in a group of 37 patients on an identical paclitaxel dose-schedule (3). The results of a phase II study in previously treated patients were reported by Smit *et al.* (4). All 24 patients in that trial developed progressive disease within 3 months of receiving at least one previous chemotherapy regimen. Seven patients (29%) had a partial response to 175 mg/m^2 paclitaxel as a 3-h infusion. These data show that paclitaxel exhibits single-agent efficacy in SCLC comparable to that of the best agents. The results of Smit *et al.'s* study in patients with refractory SCLC are particularly impressive, since most response rates reported with single-agent or combination regimens in this population have been less than 15%. However, life-threatening toxicity occurred in 4 of these patients, 2 of whom experienced hematological toxicity.

Recent reports of the activity and tolerability of weekly doses of paclitaxel have generated a great deal of clinical interest. Weekly paclitaxel therapy has generally been quite well tolerated, causing minimal toxicity and no apparent cumulative myelosuppression. Substantial evidence from clinical trials indicates that weekly paclitaxel is effective and generally well tolerated as both a first- and second-line treatment for advanced NSCLC. A phase I/II trial by Koumakis *et al.* in a second-line setting tested weekly paclitaxel infused for the first 6 weeks of each 8-week cycle, and demonstrated that a paclitaxel dose escalation from 60 mg/m^2 to 90 mg/m^2 was tolerated (5).

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Key Words: Paclitaxel, small cell lung cancer.

Fennelly *et al.* reported a recommended dose of 80 mg/m^2 administered weekly for 6 weeks of an 8-week cycle in patients with recurrent ovarian cancer (6).

Based on this evidence, a phase II trial of 80 mg/m^2 weekly paclitaxel as a 1-h infusion for 6 consecutive weeks followed by 2 weeks without treatment (8-week cycle) was conducted in patients with relapsed SCLC. The objective of this study was to evaluate the efficacy and safety of weekly paclitaxel in patients with relapsed and refractory SCLC. The primary end-point was the response rate, while the secondary end-points were the toxicity profile and survival rate.

Patients and Methods

Patient selection. Patients who met all of the following criteria were considered eligible: a) histological or cytological proof of SCLC with no response to prior chemotherapy or progression after chemotherapy, b) measurable disease, c) most recent cytotoxic treatment less than 4 weeks before entry, d) ECOG performance status 0-2, e) age ≤75 years, f) adequate bone marrow function (leukocyte count ≥4,000/μl, hemoglobin level ≥9.0 g/dl and platelet count ≥100,000/μl), hepatic function (transaminases ≤2.5 times the upper limit of normal, bilirubin level ≤1.5 mg/dl), and renal function (creatinine ≤1.5 times upper limit of normal) and g) arterial oxygen partial pressure ≥60 torr. Excluded patients were those with any active concomitant malignancy, symptomatic brain metastases, a past history of drug allergy reactions, complication by interstitial pneumonia, treatment with non-steroidal anti-inflammatory drugs or steroids or other serious complications such as uncontrolled angina pectoris, myocardial infarction within 3 months, heart failure, uncontrolled diabetes mellitus or hypertension, massive pleural effusion or ascites or serious active infection. All patients gave written informed consent and our institutional review board for human experimentation approved the protocol.

Treatment schedule. Paclitaxel was infused intravenously (*i.v.*). over a 1-h period at a dose of 80 mg/m^2 each week for 6 consecutive weeks followed by a 2-week break. This 8-week period comprised one treatment cycle. Premedication consisted of 20 mg dexamethasone, 50 mg ranitidine and 50 mg diphenhydramine given *i.v.* 30 min prior to paclitaxel.

If the leukocyte count fell below 2,000/μl or the neutrophil count fell below 1,000/μl, recombinant granulocyte colony-stimulating factor (rhG-CSF) at a daily dose of 2 μg/kg was administered until the leukocyte count recovered to ≥10,000/μl, except on the days of paclitaxel administration. The toxicity assessment was based on the National Cancer Institute – Common Toxicity Criteria version 2.0. If grade 3 leukopenia, grade 4 neutropenia, grade 2 neuropathy or other grade 3 non-hematological toxicities occurred, the dose of paclitaxel in subsequent cycles was reduced by 10 mg/m^2 from the planned dose. Paclitaxel was not administered if the leukocyte count was <2,000/μl, the platelet count was <5,000/μl, or if there was grade 3 nausea/vomiting, infection with a fever of more than 38°C, or other grade 2 non-hematological toxicities except alopecia. The treatment was discontinued if there was disease progression, grade 3 neuropathy, other grade 4 non-hematological toxicities or a 2 consecutive weeks without paclitaxel administration.

Evaluation of response and survival. The tumor response was classified according to the WHO criteria (7). A complete response (CR) was defined as the total disappearance of all measurable and assessable disease for at least 4 weeks. Partial response (PR) was defined as a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors lasting for at least 4 weeks without the appearance of any new lesions. No change (NC) was defined as a decrease of <50% or an increase of <25% in tumor lesions for at least 4 weeks with no new lesions. Progressive disease (PD) was defined as the development of new lesions or an increase of 25% in the sum of the products of the 2 largest perpendicular diameters of all measurable tumors. The overall survival was measured from the time of study entry until death.

Statistical methods. The median probability of survival was estimated by the method of Kaplan and Meier (8). This study was designed as a phase II study, with the response rate as the main end-point. According to the Simons minimax design, with a sample size of 20 our study had a 90% power to accept the hypothesis that the true response rate was greater than 25%, while a 10% significance sufficed for rejection of the hypothesis that the true response rate was less than 5% (9).

Results

Patient characteristics. Between December 1999 and February 2002, a total of 22 patients were enrolled in the study, 1 of whom was deemed ineligible due to age (>75 years), leaving a total of 21 patients assessable for toxicity, response and survival. The main demographic characteristics of the cohort are summarized in Table I. The patient cohort consisted of 1 female and 20 males with a median age of 66 years (range, 48 to 75). Four patients exhibited limited disease and 19 exhibited extensive disease at the start of treatment. The majority of the patients had received no prior surgical treatment, while 67% had received prior radiation therapy. All patients had been treated with some form of cisplatin- or carboplatin-based combination chemotherapy regimen. Eighteen patients had received prior etoposide-containing chemotherapy and 10 prior irinotecan-containing chemotherapy. The median number of previous chemotherapy regimens administered was 1 (range, 1 to 2). Among the 10 patients who proved refractory to chemotherapy, 5 had NC or PD on first- or second-line treatment, 2 had PR but experienced disease progression during treatment and 3 had a relapse within a 90-day treatment-free interval after completing their treatments.

Toxicity. The toxicity of the regimen is summarized in Table II. Neutropenia was the main toxicity, with 6 out of the 21 patients experiencing grade 4 neutropenia during the entire study. Grade 3 anemia was observed in 2 patients. One patient experienced grade 4 anemia, secondary to digestive tract bleeding. Thrombocytopenia remained infrequent throughout the study. No cases of grade 3 or 4 thrombocytopenia were observed and there was no evidence of cumulative hematological toxicity.

Table I. *Baseline characteristics of all patients.*

Baseline characteristics		No. of patients
Sex	Male / Female	20 / 1
Age (years)	Median (Range)	66 (48-75)
ECOG PS	0/1/2	5 /12 /4
Disease extent	LD/ ED	4 / 17
Previous treatment	Chemotherapy only	4
	Chemotherapy + radiotherapy	14
	Chemotherapy + others	3
Previous chemotherapy	Platinum + etopoisde +/- others	18
	Including irinotecan HCl	10
	Others	1
No. of previous chemotherapy regimens	1 / 2 / 3	16 / 4 / 1
Response to prior chemotherapy	CR / PR / NC / PD / NE	2 / 13 / 5 / 0 / 1

No.: number
PS: performance status, LD: limited disease, ED: extensive disease.

Table II. *Toxicity of treatment for all cycles.*

Toxicity	No. of patients with event by grade				
	G0	G1	G2	G3	G4
Nausea	12	7	2	0	0
Vomiting	19	1	1	0	0
Diarrhea	17	3	1	0	0
Constipation	10	5	6	0	0
Mucositis	21	0	0	0	0
Gastric ulcer	20	0	1	0	0
Fever	16	3	2	0	0
Fatigue	13	0	8	0	0
Skin rash	20	0	0	1	0
Infection	18	0	0	3	0
Neuropathy	9	9	1	2	0
Myalgia	16	4	1	0	0
Dyspnea	17	0	1	2	1
Hemoglobin	1	9	9	1	1
WBC count	2	1	8	8	2
Neutrophil count	0	5	2	8	6
Platelet count	16	5	0	0	0
GOT	12	7	2	0	0
GPT	16	4	1	0	0
Total bilirubin	19	1	1	0	0

Other grade 3 and 4 toxicities included infection, skin rash, neuropathy and pulmonary toxicity. Grade 1 or 2 neuropathy was seen in 10 patients, and greater than grade 2 was observed in 2 individuals. No hypersensitivity reactions were encountered. Grade 3 or 4 pulmonary toxicity was reported in 3 patients and was characterized by dyspnea. Life-threatening complications of grade 4 infection and grade 4 dyspnea were encountered in 1 patient, who experienced febrile neutropenia and respiratory failure secondary to pneumonia after the third weekly dose. He was treated with antibiotics and supportive measures, but the respiratory distress worsened and he died on day 41. One of 2 grade 3 pulmonary toxicities was pneumonitis, probably induced by paclitaxel, but was resolved by steroid therapy.

Response to treatment and survival. The responses to therapy are shown in Table III according to whether the patient had primary refractory disease or primary sensitive cancer that subsequently relapsed. Although 1 out of the 21 patients was not assessable for response, having died during the first cycle, a ≥50% decrease in the sum of the products of the 2 largest perpendicular diameters of the tumor was achieved in this patient. Five of the 22 patients had a PR, but no CRs were observed and the overall response rate

Table III. *Response data.*

		No. of patients					Response rate (%)
		CR	PR	NC	PD	NE	
Total	21	0	5	4	11	1	23.8
Sensitive	11	0	3	3	5	0	27.3
Refractory	10	0	2	1	6	1	20.0

CI = confidence interval; CR = complete response; NE = not evaluable; PD = progressive disease; PR = partial response; NC = no change.

was 23.8% (95% confidence interval, 5.59 to 42.03). When only evaluable patients were included in the analysis, however, the response rate improved to 25% (95% confidence interval, 6.02 to 43.98). Two PRs (20%) occurred in refractory cases and 3 PRs (27%) were achieved in sensitive cases. Four patients showed no change, and 1 exhibited disease progression. The survival analysis was performed in January 2003, by which point 10 patients had died and 2 were still alive. The median survival time (MST) was 5.8 months and the 1-year survival rate was 13.4% (Figure 1).

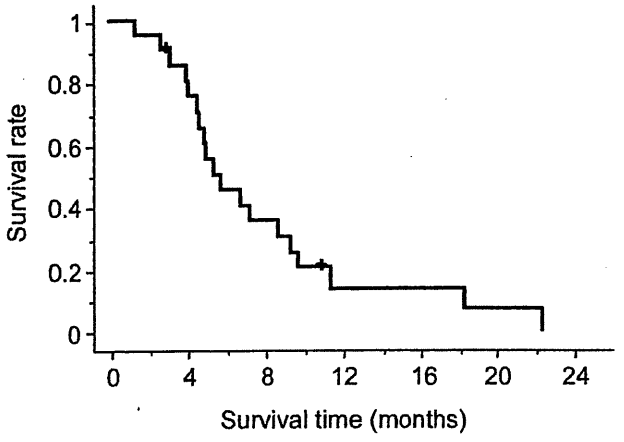

Figure 1. *Overall survival.*

Discussion

Since the outlook for SCLC patients who receive second-line therapy is poor, several new drugs, such as paclitaxel, docetaxel, gemcitabine, vinorelbine, topotecan and irinotecan, are currently under investigation. The new chemotherapy agents that have been most extensively evaluated in SCLC are the topoisomerase I inhibitors, including topotecan and irinotecan. Von Pawel *et al.* conducted a phase III study comparing single-agent topotecan with cyclophosphamide, doxorubicin and vincristine (CAV) in patients with progression at least 60 days after initial therapy and reported response rates of 24.3% for topotecan and 18.3% for CAV with a median survival time (MST) of 25.0 and 24.7 weeks, respectively, and found that topotecan was at least as effective as CAV in the treatment of patients with recurrent SCLC (10). Two studies of irinotecan in patients with refractory SCLC have been reported in Japan and the response rates in both studies were high, *i.e.*, 50% in 16 patients, and 47% in 15 patients, respectively (11, 12). We therefore consider that topoisomerase I inhibitors, such as topotecan and irinotecan, are key drugs in the second-line treatment of SCLC. However, the number of SCLC patients treated with an irinotecan-containing regimen as first-line chemotherapy has increased in Japan since, in a randomized phase III trial in Japan (13), a combination of irinotecan and cisplatin was shown to yield better survival than the standard etoposide and cisplatin regimen in patients with untreated extensive SCLC. Therefore, the search for effective drugs, other than topoisomerase I inhibitors, for previously treated SCLC, especially refractory SCLC, must be continued.

Single-agent paclitaxel, at a dose of 175 mg/m^2 as a 3-h infusion every 3 weeks in patients with previously treated SCLC, produced a response rate of 29% and an MST of 100 days (4). The results of our phase II study demonstrated that weekly paclitaxel at a dose of 80 mg/m^2 yielded a similar response rate of 23.8% and a much better MST of 5.8 months than that of paclitaxel given every 3 weeks. Because the antiproliferative activity of paclitaxel is cell-specific, prolonging patient exposure to a low dose of the drug beyond a threshold concentration is ultimately more efficacious than a short-term exposure to higher drug concentrations, a hypothesis supported by *in vitro* experiments with a variety of cell lines and suggested by the results of clinical studies. As clinical experience with paclitaxel treatment of various types of tumors has progressed, so has the use of weekly regimens at lower doses administered as 1-h infusions, as opposed to standard higher doses delivered once every 3 weeks as 3-h infusions.

A response rate of more than 10% is considered evidence of drug efficacy in previously-treated SCLC patients (14). Before newer drugs, such as topoisomerase I inhibitors, taxane, gemcitabine and vinorelbine were introduced, salvage chemotherapy did not usually prolong survival in SCLC and MSTs after relapse were 2.5 – 3.9 months (1). Single-agent phase II trials of gemcitabine, docetaxel and vinorelbine in patients with relapsed or refractory SCLC have been reported. Smyth *et al.* (15), using a 100 mg/m^2 dose of docetaxel, obtained a response rate of 25% in 28 assessable patients who had received prior chemotherapy. A trial of gemcitabine in 46 previously-treated patients yielded an 11.9% response rate (16) and vinorelbine provided response rates of 12% and 16% in second-line patients with sensitive disease (17,18). Thus, the MST of 5.8 months and response rate of 23.8% in this study compare favorably with those of published single-agent trials in relapsed or refractory SCLC.

The toxicity profile noted in this trial was predictable based on the toxicity profile previously described in weekly paclitaxel trials, neutropenia being the major toxic effect. All side-effects, except fatal neutropenic pneumonia in 1 case, were manageble. Grade 3 or 4 neutropenia occurred in 14 of the patients in our study but was immediately alleviated by treatment with G-CSF. Grade 3 or 4 anemia occurred in 1 patient, but there was no grade 3 or 4 thrombocytopenia in our study. The incidence of grade 3/4 myelosuppression was considered tolerable. There were 3 cases of grade 3 or 4 pulmonary toxicity, 2 of which occurred due to bacterial infection. This regimen required a dose of 20 mg of dexamethasone weekly as premedication. We believe that this occurrence of bacterial pneumonia might be related to the use of steroids.

Testing new drugs in previously-treated patients has the clear advantages of determining the degree of non-cross resistance with other drugs. Its greatest disadvantage is the risk of a considerable dose reduction (especially of myelotoxic drugs) to avoid extensive hematological side-

effects, perhaps resulting in doses that are too low to fairly evaluate the drug. Since a weekly administration of paclitaxel causes only mild myelosuppression and as there may be no cross resistance with platinum, etoposide, irinotecan, or topotecan, which are usually used to treat SCLC, we find this regimen suitable for previously-treated SCLC.

In summary, the weekly paclitaxel regimen is moderately effective in SCLC patients who have received prior chemotherapy. Based on the statistical design of this study, the 5 PR observed suggest that weekly paclitaxel warrants further evaluation in this patient population. Additional investigations will serve to clarify the role of this agent, either alone or in combination with other agents. Combining paclitaxel with other agents with proven non-cross resistance such as irinotecan, topotecan, or gemcitabine or new target-based agents is the next step needed to evaluate second-line situations, especially in patients with resistant disease.

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Received September 20, 2005
Accepted November 10, 2005

Int. J. Cancer: **118,** 812–820 (2006)

Small interfering RNA targeting survivin sensitizes lung cancer cell with mutant p53 to adriamycin

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Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in cancer tissues. p53 is one of the tumor suppressor genes; its induction in response to DNA damage causes apoptosis and correlates with drug sensitivity. To investigate the possible regulation of survivin by p53, we examined the level of survivin expression in lung cancer cell lines in response to adriamycin. Levels of survivin mRNA and protein in cell lines with wild-type p53 decreased dramatically after p53 induction, but no such reduction of survivin was observed in cell lines with mutated or null p53. Inhibition of wild-type p53 in A549 cells by small interfering (si) RNA significantly upregulated the expression of survivin. Survivin inhibition by siRNA in PC9 cells with mutated p53 significantly depressed cell proliferation. To investigate the sensitivity of cancer cells to adriamycin after inhibition of survivin, we depressed survivin expression using siRNA, and then added adriamycin at an IC_{50} dose. After a further 48 hr incubation with adriamycin, proliferation was significantly depressed in the cells treated with siRNA targeting survivin, in comparison with siRNA targeting scramble. Furthermore, both TUNEL and pro-caspase3 expression assay showed a significant increase in apoptosis after combined treatment with adriamycin and siRNA targeting survivin. Our results demonstrate that survivin is downregulated by p53, and that siRNA targeting of survivin increases cell sensitivity to adriamycin and promotes apoptosis. siRNA targeting of survivin could be potentially useful for increasing sensitivity to anticancer drugs, especially in drug-resistant cells with mutated p53.

Key words: Survivin; siRNA; p53; lung cancer; Adriamycin

The success of cancer treatment depends on the response to chemotherapeutic agents. However, malignancies often acquire resistance to drugs if they are used frequently. Inhibition of the apoptosis pathway is one of the factors that may be responsible for such drug resistance.[1] Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is specifically overexpressed in various cancers but not in normal adult tissues.[2] Overexpression of survivin is correlated with poor prognosis in a number of tumor types, including lung cancer,[3] colorectal cancer[4] and gastric cancer.[5] Like other mammalian IAPs (*e.g.*, XIAP, c-IAP-1, c-IAP-2 and livin), survivin binds to caspase-3 and caspase-7.[6] It has been suggested that survivin expression is regulated in a cell cycle-dependent manner.[7] Survivin is maximally expressed in the G2/M phase and physically associates with mitotic spindle microtubules that regulate progression through mitosis. In contrast, survivin is definitively depressed in the G1 phase. p53 is one of the tumor suppressor genes, and it is frequently mutated in cancer tissue/cells.[8] The crucial role of p53 is to maintain genetic stability through its participation in cell cycle checkpoints. After DNA damage induced by various cytotoxic agents, cells with wild-type p53 become preferentially arrested in the G0/G1 phase, after which they choose a path that results in either DNA repair or apoptosis. Apoptosis is closely linked to transcripts that are downregulated by p53. In contrast, mutation or deletion of p53 leads cells away from the apoptosis pathway, causing drug resistance.[9] It is generally accepted that p53 functions as a transcriptional factor and transactivates some genes, resulting in cell growth modulation or death. For example, an elevated level of p21, the first product of p53 transactivation, results in underphosphorylation of the retinoblastoma (Rb) protein, which in turn sequesters the E2F transcription factor; as a result, the cell cycle is blocked in the G1 phase.[10,11] Additionally, some genes, such as stathmin or cdc2, could be negatively regulated by p53.[12,13] Previous reports suggest that p53 also downregulates the expression of survivin in some cell models and cancer cell lines.[14,15] More recent reports have shown that inhibition of survivin by anti-sense oligonucleotide blocks the cell proliferation of myeloid leukemic cells[16] or lung cancer cells,[17] although the mechanism of this transcriptional regulation is not fully understood and requires additional research.

From another viewpoint, inhibition of survivin might play a role in overcoming acquired drug resistance. It has not been clarified how DNA-damaging agents influence survivin expression and cause cell cycle arrest and apoptosis. One report has suggested that anti-sense targeting of survivin sensitizes lung cancer cells to chemotherapy.[17] However, that study employed only 1 lung cancer cell line containing wild-type p53 and did not address the outcome that would be expected with mutated or deleted p53.

RNA interference (RNAi) is a mechanism whereby double-stranded RNA post-transcriptionally silences a specific gene. It has been reported that synthetic, double-stranded small-interfering RNA (siRNA) can effectively silence a gene through the RNAi mechanism.[18] siRNA can be a novel tool for clarifying gene function in mammalian cells and may be applicable to gene-specific therapeutics.[1] In our study, using siRNA, we aimed to sensitize lung cancer cell line to adriamycin. Our results suggest that siRNA targeting of survivin can inhibit cell growth and produce a combined anti-proliferative effect and apoptosis when combined with adriamycin, especially in cell lines containing mutated p53.

Material and methods

Drugs and cell lines

Adriamycin, obtained from Kyowa Hakko Kogyo Co. (Tokyo, Japan), was dissolved in distilled water and stored at −30°C until use. All cell lines used in our study were derived from patients with lung cancer. Lines NCI H226, H292, H358, H460, H522 and H1299 were obtained from the American Type Culture Collection (Manassas, VA). Lines A549, EBC-1, LK-2, Lu99, Lu99B, OBA-LK-1 and Sq-1 were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). SBC3, Lu65 and RERF-LC-KJ were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). Lines PC9 and PC14 were kindly donated by Prof. Hayata, Tokyo Medical University (Tokyo, Japan). SBC3/ADM,[20]

Abbreviations: dH_2O, distilled H_2O; DW, distilled water; FBS, Fetal Bovine Serum; GAPDH, glyceraldehyde-3- phosphate; IAP, inhibitor of apoptosis protein; IC_{50}, 50% inhibitory concentration; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non-small cell lung cancer; si RNA, small interfering RNA; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; SD, standard deviation; SE, standard error, TUNEL, TdT mediated dUTP nick end labeling.

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Received 22 September 2004; Accepted after revision 29 April 2005
DOI 10.1002/ijc.21350
Published online 17 August 2005 in Wiley InterScience (www.interscience.wiley.com).

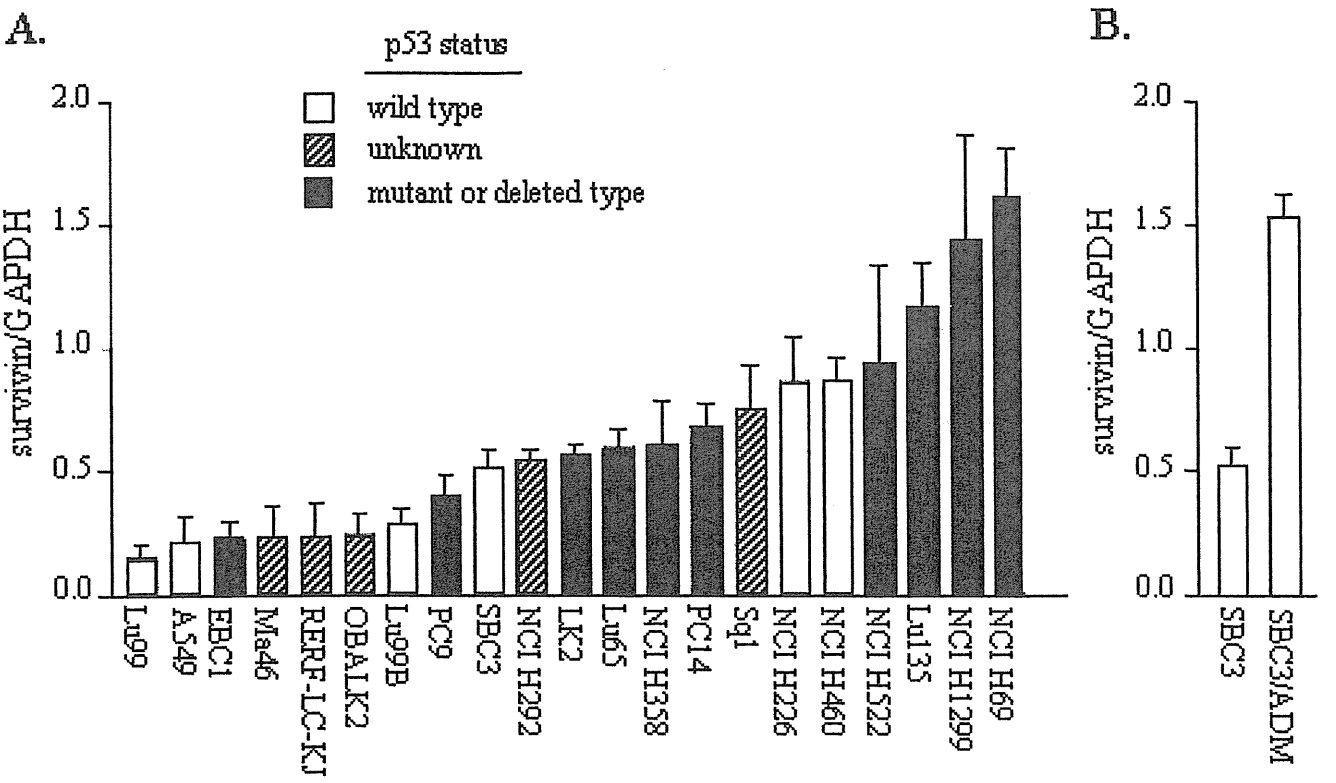

FIGURE 1 – Level of survivin mRNA in 22 lung cancer cell lines. (*a*) Cells were incubated in a 75 cm^2 flask, harvested and analyzed using real-time PCR as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH and are presented as the mean ± SD for at least 3 independent experiments. p53 status is presented. (*b*) Comparison between SBC3 and SBC3/ADM, the adriamycin-resistant subline, is shown.

a subline of SBC3 with approximately 8-fold stronger resistance to the growth-inhibitory effect of adriamycin, as determined by the MTT assay, was provided by Dr. Kiura, Okayama University (Okayama, Japan). Lu135 was provided by Riken Cell Bank (Tokyo, Japan). Ma46 was established in our laboratory from malignant effusion of an NSCLC patient. The cells were cultured in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 and air at 37°C. All cell lines were discarded after 20 generations, and new lines were obtained from frozen stocks. Some cell lines were analyzed for their IC_{50} values using the MTT assay by incubating them with adriamycin for 72 hr.[21] With regard to p53 status, NCI H226, H460, A549, SBC3, SBC3/ADM, Lu99 and Lu99B possess wild-type p53. EBC-1, PC9, LK2, Lu65, NCI H358, H522, H69, PC14, Lu135 and Lu65 possess mutated p53. NCI H1299 has deleted p53.[22–26]

Real-time RT-PCR

Total RNA was extracted from cells treated with adriamycin, siRNA or water using an RNeasy Mini Kit (Qiagen, Inc., Tokyo, Japan). For first-strand cDNA synthesis, 1 μg total RNA from a sample was added to components of the Super Script Preamplification System (Life Technologies, Inc., Gaithersburg, MD), as described in the user's manual. Real-Time PCR was performed using the Gene Amp 5700 Sequence Detection System (Perkin-Elmer), and mRNA expression was quantified. For this purpose, 1 μl cDNA was mixed with commercial reagents (TaqMan PCR Reagent Kit, Perkin-Elmer Biosystems), following the manufacturer's protocol. Survivin cDNA was amplified using a forward primer consisting of 5′-ATGGGTGCCCCGACGT-3′ and a reverse primer consisting of 5′-AATGTAGAGATGCGGTGG-TCCTT-3′ and detected by a Taqman probe consisting of 5′-CCCCTGCCTGGCAGCCCTTTC-3′, each nucleotide corresponding to positions 50–65, 92–114 and 69–89 of the 1,619 bp survivin mRNA (GenBank NM001168). Relative quantification of gene expression was performed as described previously,[27] using the housekeeping gene glyceraldehyde-3-phosphate (GAPDH) as an internal standard.

Western-blotting analysis

Cells treated with adriamycin, siRNA or water were harvested with trypsin/EDTA, and PBS-washed cell pellets were treated with HEPES lysate buffer (30 mM HEPES, 1% Triton X-100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA and 10 mM NaCl). Equal amounts of protein extracts were loaded onto sodium dodecyl sulfate-polyacrylamide gels and ran at 200 V for 45 min followed by transfer to nitrocellulose membranes at 100 V for 30 min. at room temperature. The membranes were probed with the following primary antibodies: affinity-purified rabbit anti-survivin antibody (R&D Systems, Inc., Minneapolis, MN), mouse monoclonal anti-p53 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-actin affinity isolated antibody (Sigma-Aldrich Co., St. Louis, MO) and mouse monoclonal anti-caspase3 antibody (Santa Cruz Biotechnology) at room temperature for 120 min. As secondary antibodies, goat anti-rabbit labeled with horseradish peroxidase (Amersham Biosciences, England) and sheep anti-mouse labeled with horseradish peroxidase (Santa Cruz Biotechnology) were used. Blots were developed using a chemiluminescence detection system (Perkin Elmer Life Sciences, Boston, MA).[28]

Flow cytometry

Cells were treated with adriamycin, harvested, washed with PBS, fixed with 70% methanol, washed with PBS and stained with propidium iodide solution (0.05 mg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA and 0.05 mg/ml RNase A). Approxi-

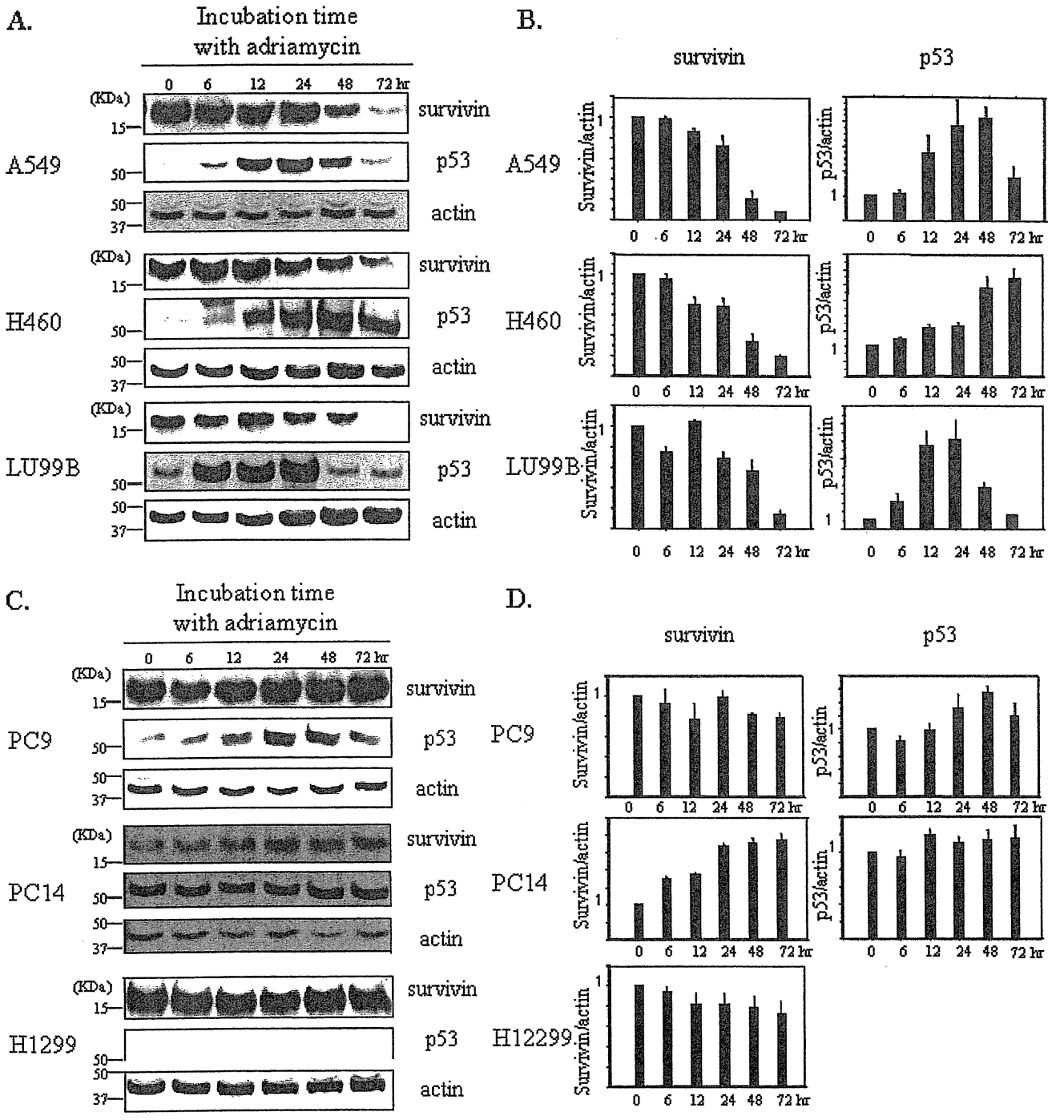

FIGURE 2 – Expression of survivin and p53 protein in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. (*a*) Western-blotting analysis for expression of survivin and p53 in cell lines possessing wild-type p53, including A549, NCI H460 and LU99B. Each of the cell lines was incubated with adriamycin at the IC_{50} dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*c*) Western-blotting analysis for expression of survivin and p53 in PC9 and PC14, possessing mutated p53, and in NCI H1299, possessing deleted p53. Each of the cell lines was incubated with adriamycin at the IC_{50} dose for the indicated time. Actin served as a control. Treatment, harvest and analysis were repeated 3 times. (*b*,*d*) Protein expression levels were presented as the mean±SD.

mately 1×10^5 stained cells were analyzed by flow cytometry in a Becton Dickinson FACS calibur.[28]

siRNA transfection

The siRNA duplexes for survivin and p53 were synthesized by Dharmacon Research, Inc. (Lafayette, CO) using 2′-ACE protection chemistry. The siRNA targeting survivin corresponded to the coding region 206–404 relative to the start codon (GenBank NM001168). The siRNA targeting p53 corresponded to the coding region 775–793. BLAST searches of the human genome database were carried out to ensure the sequences would not target other gene transcripts. Cells in the exponential phase of growth were

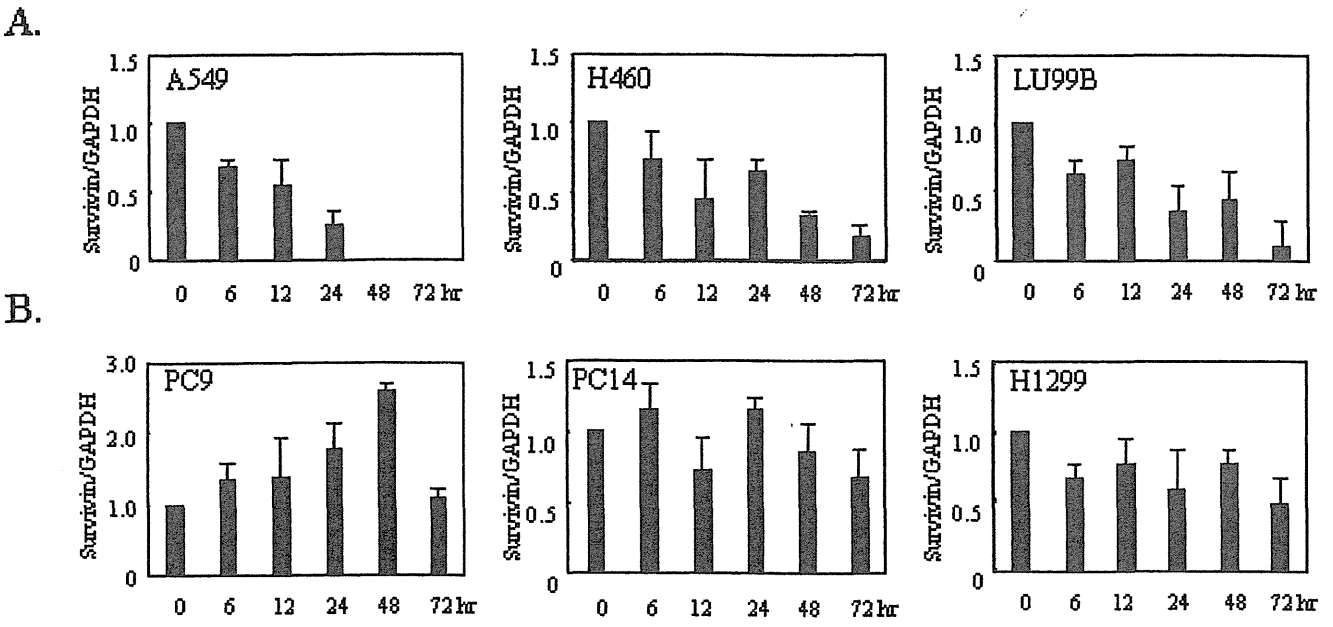

FIGURE 3 – Expression of survivin mRNA in lung cancer cell lines with different p53 phenotypes following exposure to adriamycin for the indicated time. Each of the cell lines with wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for the indicated time and analyzed by real-time PCR, as described in Material and methods. All data were normalized relative to the concentration of mRNA for the housekeeping gene GAPDH, and are presented as the mean ± SD for at least 3 independent experiments.

plated in 12-well tissue culture plate at 4×10^4 cells/well, grown for 24 hr and then transfected with 300 nM siRNA using oligofectamine and OPTI-MEM. Serum media (Invitrogen Life Technologies, Inc., Carlsbad, CA) were reduced according to the manufacturer's protocol. Gene silencing was examined with Western blotting 24–72 hr after transfection. Control cells were treated with siRNA duplex targeting scramble (Dharmacon). These studies were repeated 3 times and the data was presented as mean ± SE.

TUNEL assay

Cells were fixed in 4% paraformaldehyde (pH 7.4) and then stained and analyzed for apoptosis using an In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). Fixed cells were permeabilized using a mixture containing 0.1% sodium citrate and 0.1% TritonX100 and incubated with TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP at 37°C for 60 min. Flow cytometric analysis using a FACS calibur was done to quantitate apoptosis.[29]

Cell viability analysis

Cells treated with adriamycin or transfected with siRNA duplex were washed with medium once and PBS twice, after staining with trypan blue.

Statistical analysis

All data are presented as mean ± SD or mean ± SE, and statistical analysis was done by Student's 2-tailed *t*-test (Stat View, SAS Institute, Inc.). Differences at $p < 0.05$ were considered significant.

Results

Survivin mRNA expression in lung cancer cell lines

The level of expression of survivin mRNA in the 22 human lung cancer cell lines was analyzed by TaqMan real-time PCR (Fig. 1). Normalization was performed using GAPDH as an internal control. Harvest and analysis of each cell line was repeated at least 3 times, and the mean and standard deviation for each cell lines is shown. All lung cancer cell lines expressed survivin mRNA, although the expression level varied. Among the 22 cell lines, the p53 status of 17 has been reported. The mean survivin expression of cells with wild-type p53, except for SBC3/ADM, tended to be less than that of cells with mutated or deleted p53 ($p = 0.0192$). Moreover SBC3/ADM, which is 8 times more adriamycin-resistant than SBC3 in terms of IC_{50}, expressed about 3 times more survivin mRNA than did SBC3.

Decrease of survivin expression after adriamycin exposure is dependent on functional p53 accumulation

To examine the p53 regulation of survivin expression, we monitored the expression of survivin protein in cells treated continuously with adriamycin at the IC_{50} dose by Western blotting (Fig. 2). Harvest, treatment and analysis of each cell line were repeated 3 times. The p53 phenotype of cell lines A549, NCI H460 and Lu99B has been reported previously as wild-type p53; PC9, PC14 and NCI H1299 possess mutant or deleted p53. In the cells with wild-type p53 (A549, H460 and Lu99B), p53 expression was induced 6 hr after adriamycin exposure and reached a peak level by 24 hr or later. Survivin protein expression was repressed for 72 hr after p53 accumulation (Fig. 2*a*). On the other hand, expression of survivin protein in cells with mutated or deleted p53 (PC9, PC14 and H1299) was not significantly decreased, and in fact appeared to be strongly increased in PC14 (Fig. 2*b*). Additionally, we analyzed survivin mRNA modification after adriamycin exposure using real-time PCR (Fig. 3). As was observed for the protein, the level of survivin mRNA showed a temporal decrease in all cell lines (A549, H460 and LU99B) containing wild-type p53. Repression of survivin mRNA in these cell lines started with accumulation of p53 during the first 6 hr (Fig. 3*a*). In contrast, in cell lines with mutated or deleted p53 (PC9, PC14 and H1299), survivin mRNA did not decrease throughout the period of adriamycin exposure. Furthermore, in cell line PC9, the level of survivin mRNA tended to increase (Fig. 3*b*).

Dependence of altered cell cycle distribution on p53 phenotype following exposure to adriamycin

In each of the cell lines treated with adriamycin, the cell cycle distribution was analyzed by flow cytometry (Fig. 4). It was found that the cell cycle distribution varied markedly depending on the p53 phenotype. That is, following exposure to adriamycin cells possessing wild-type p53 tended to show arrest in G1/S phase, whereas cells with mutated or deleted p53 became arrested in G2 phase. In cells containing wild-type p53, the G2/M peak tended to decline along with repression of survivin protein after 24 hr of adriamycin exposure, and the proportion of apoptotic cells (sub-G1) increased. On the other hand, in cells with mutated or deleted p53, the decline in the G2 peak was delayed in comparison with wild cells possessing wild-type p53, and only a small proportion of the cells became apoptotic after 24 hr of expression to adriamycin (Fig. 4).

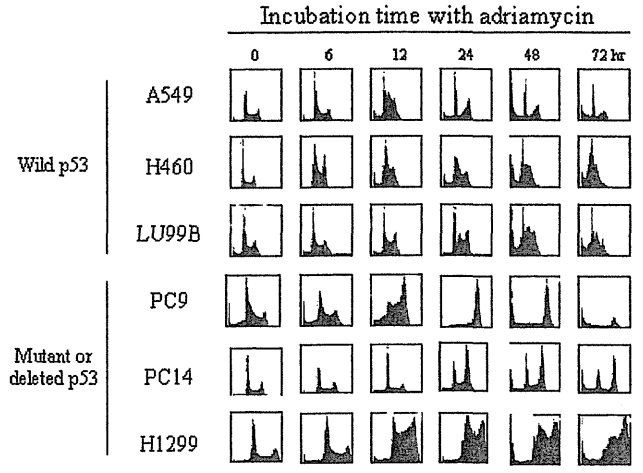

FIGURE 4 – Cell cycle analysis of lung cancer cell lines with different p53 phenotypes after exposure to adriamycin. Each of the cell lines possessing wild-type p53 (A549, NCI H460 and LU99B), mutated p53 (PC9 and PC14) or deleted p53 (NCI H1299) was incubated with adriamycin at the IC_{50} dose for the indicated time and analyzed by flow cytometry as described in Material and methods.

Inhibition of p53 using siRNA duplex, and resulting change in survivin expression

We examined whether wild-type p53 functionally regulates survivin, using the novel siRNA technique, which specifically inhibits p53. The siRNA duplex was designed to target coding region 775–793 after the start codon of p53. A549, a lung cancer cell line possessing wild-type p53, was transfected with siRNA duplex targeting p53, or scramble as a control, and the resulting levels of survivin expression were determined by Western blotting (Fig. 5*a*). All siRNA molecules have some intrinsic effect on treated cells. We compared cells treated with scrambled siRNA and cells treated with distilled water about p53 and survivin expression. In a result, there is not a significant difference between these. The siRNA duplex targeting p53 reduced p53 protein expression to 54% of the control level within 48 hr (Fig. 5*b*), and this was accompanied by an increase of survivin protein by as much as 2 times the control level (Fig. 5*c*).

Inhibition of survivin expression by siRNA duplex inhibits cell proliferation and induces cell death

To evaluate the biological effect of survivin inhibition in lung cancer cell lines, transfection with siRNA duplex was performed. Cell line PC9, with mutated p53, was transfected with siRNA duplex targeting survivin or with that targeting scramble as a con-

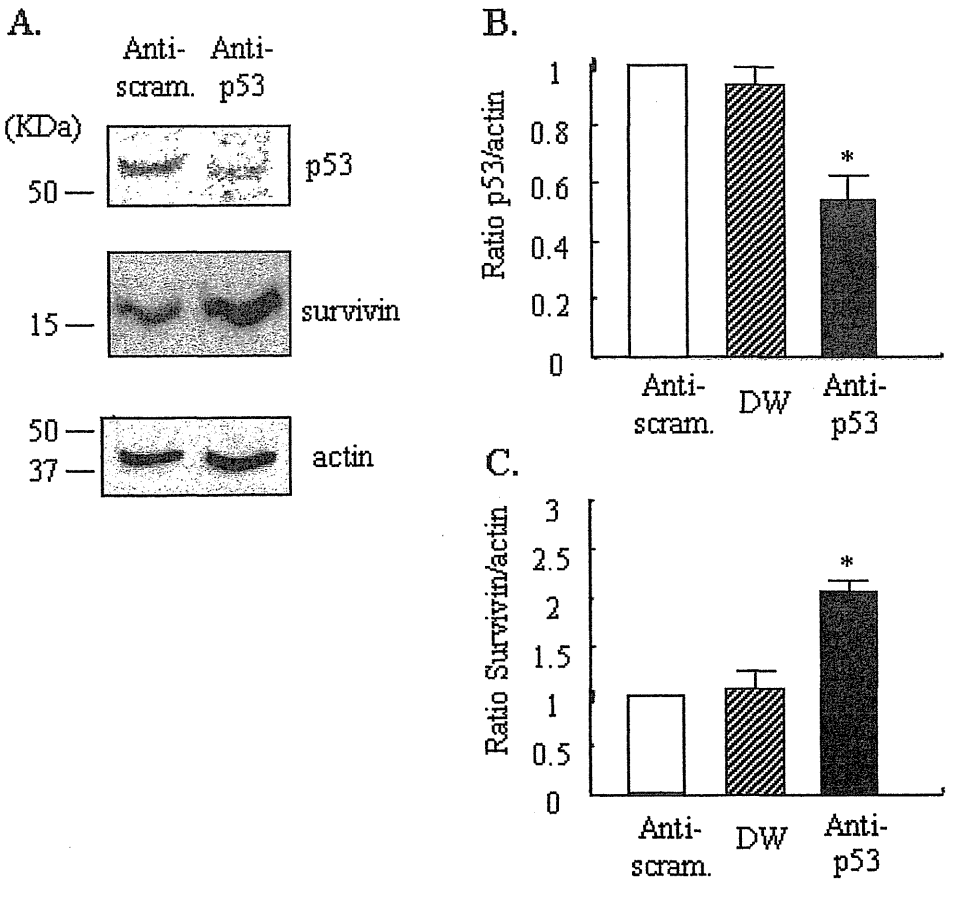

FIGURE 5 – (*a*) Increasing survivin expression in A549 lung cancer cells possessing wild-type p53 as a result of p53 inhibition by siRNA duplex. A549 cells were treated with siRNA duplex targeting p53, scramble or distilled water and then 48 hr later, cell lysates were prepared from the siRNA-treated cells. (*a*) Expressions of p53, survivin and actin were analyzed by Western blotting. (*b*) The expression of p53 protein was analyzed densitometrically using a ChemiImager AlphaImager (ASTEC Co., Japan) and corrected relative to actin. (*c*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager and corrected relative to actin. All data are presented as the mean ± SD for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $*p < 0.05$ *vs.* cells treated with siRNA duplex targeting scramble.

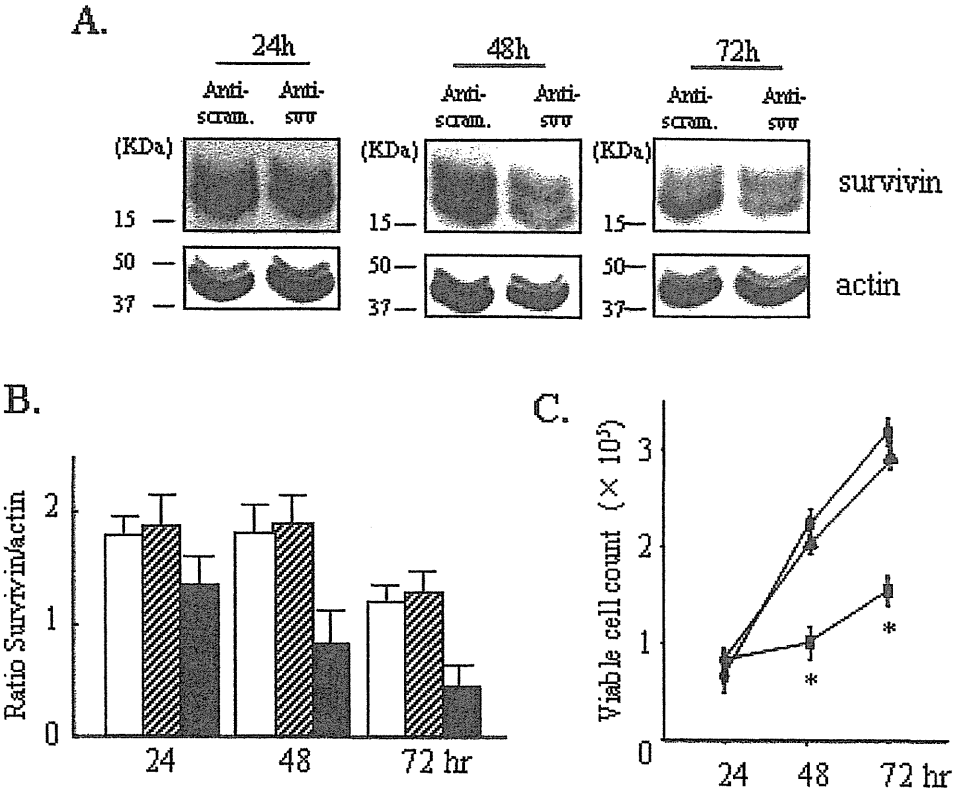

FIGURE 6 – Effects of siRNA targeting survivin on proliferation of PC9 lung cancer cells. PC9 cells were treated with siRNA duplex targeting surviving, scramble or distilled water. At the indicated time, the cells were harvested and assayed using the following procedure. (*a*) Expression of survivin and actin was analyzed by Western blotting, and actin was used as a control. (*b*) The expression of survivin protein was analyzed densitometrically using a ChemiImager AlphaImager, and corrected relative to actin. (*c*) Effect of siRNA targeting survivin (closed square), scramble (closed circle) or distilled water (closed triangle) on proliferation of PC9 cells. Cell proliferation was measured by counting the viable cells using trypan blue staining. All data are presented as the mean±S.E. for at least 3 independent experiments. Statistical analysis was performed by Student's 2-tailed t test. $*p < 0.05$ versus cells treated with siRNA duplex targeting scramble.

trol. Scrambled siRNA did not have unspecific effect on survivin expression compared to distilled water in each point. It was found that expression of survivin protein was significantly repressed after transfection with anti-survivin, compared to the control (Fig. 6*a,b*). The level of survivin protein was reduced to 62% of the control within 48 hr and to 45% within 72 hr. We then counted the number of viable cells after siRNA transfection. As shown in Figure 6*c*, the repression of survivin had a direct effect on cell proliferation. At 48 hr post-siRNA, survivin repression significantly reduced the viable cell count to 45% of the scrambled siRNA treated cells ($p < 0.05$) and 47% of the control level at 72 hr ($p < 0.05$). Viable cell count of the scrambled siRNA treated cells was not different from distilled water treated cells in each point. In addition, apoptosis was induced to a greater extent by survivin repression, which is measured by the TUNEL assay (data not shown).

Sensitization of lung cancer cell lines to adriamycin by siRNA targeting survivin

Based on the fact that cell lines with mutated or deleted p53 stably expressed survivin after exposure to adriamycin, we investigated the impact of survivin inhibition on adriamycin sensitivity in cells with mutated p53. Cell line PC9 possessing mutated p53 was transiently transfected with siRNA duplex targeting survivin, or with that targeting scramble as a control, for 48 hr. After the transfection, which significantly inhibited survivin expression, the medium was replaced and adriamycin at the IC_{50} dose, or water, was added. Adriamycin exposure was continued for 48 hr, and the cells were then harvested separately for Western blotting, viable cell assay, TUNEL assay and procaspase 3 assay. It was found that siRNA inhibited the expression of survivin by 57% at the start of adriamycin exposure and that survivin inhibition was weakened to 20% by 48 hr (data not shown). In terms of cell proliferation, anti-survivin siRNA duplex alone, adriamycin alone or a combination of both was significantly more repressive than anti-scramble siRNA followed by water, as a control ($*p < 0.05$, Fig. 7). That is, 48 hr after exposure to adriamycin or water, anti-survivin siRNA alone inhibited cell growth to 55% of the control, adriamycin alone reduced cell growth to 39%, and a combination of the 2r reduced cell growth to 21% of the control. Within 12 hr after exposure to adriamycin or water, exposure to anti-survivin siRNA or adriamycin alone did not significantly inhibit cell proliferation compared to the control; however the combination of the 2 significantly repressed cell proliferation to 44% of the control ($*p < 0.05$), and we compared anti-scrambled siRNA with distilled water followed by adriamycin or not. As a result, the scrambled siRNA effect on cell proliferation was small.

Induction of apoptosis in lung cancer cells by siRNA targeting survivin, and resulting sensitization to adriamycin

Additionally, we performed a TUNEL assay to evaluate apoptosis (Fig. 8). Cells were transfected with anti-scramble, anti-survivin siRNA duplex or distilled water for 48 hr and harvested for the assay 24 hr after exposure to adriamycin or water. Cells treated with water after anti-scramble were 5.1% TUNEL-positive, whereas cells treated with anti-survivin siRNA alone or adriamycin alone were 24.1% and 18.8% TUNEL-positive, respectively. Anti-survivin siRNA duplex induced significantly more apoptosis than that seen in the control ($*p = 0.0298$). Finally, the combination of anti-survivin siRNA duplex and adriamycin exposure resulted in 51.2% TUNEL-positivity, which was a significantly more potent effect than each of the other treatments ($**p < 0.05$). Intrinsic effect of scrambled siRNA on apoptosis was small, compared to cells treated with scrambled siRNA and cells treated with distilled water.

We additionally assessed procaspase-3 expressed in cells exposed to adriamycin after treatment with anti-scramble, anti-survivin siRNA duplex or distilled water (Fig. 9). It has already been reported that survivin potentially inhibits caspase-3 acti-

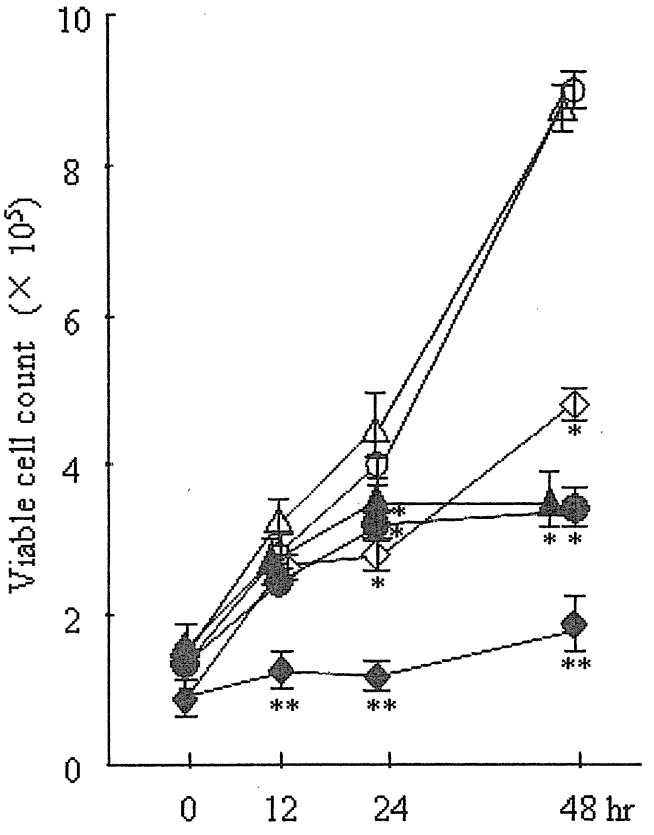

FIGURE 7 – Effects of siRNA duplex targeting of survivin on proliferation of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin or water after 48 hr transfection with siRNA duplex targeting surviving, scramble or distilled water. Open triangle:water after distilled water; open circle: water after transfection with siRNA duplex targeting scramble; open diamond: water after transfection with siRNA duplex targeting survivin; closed triangle: adriamycin after distilled water; closed circle: adriamycin after transfection with siRNA duplex targeting scramble; closed diamond: adriamycin after transfection with siRNA duplex targeting survivin. The data are presented as the mean±S.E. from 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test. $*p < 0.05$ *vs.* cells treated with water after transfection with siRNA duplex targeting scramble. $**p < 0.05$ vs. other treatments.

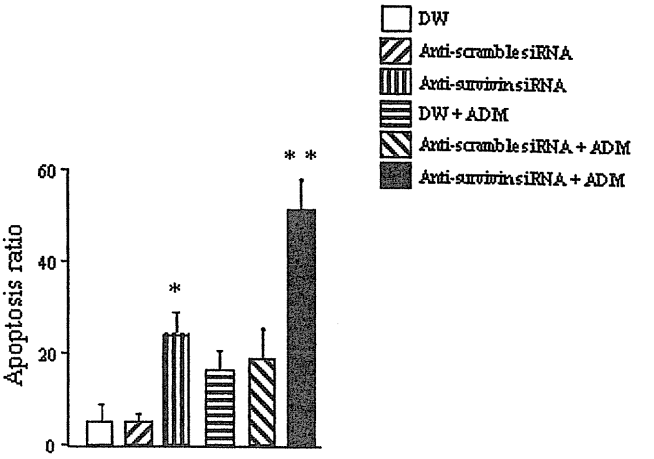

FIGURE 8 – Effects of siRNA targeting survivin on apoptosis of PC9 lung cancer cells treated with adriamycin, evaluated by TUNEL assay. PC9 cells were exposed to adriamycin or water for 24 hr after 48 hr transfection with duplex siRNA targeting surviving, scramble or distilled water. The data are presented as the mean±S.E. for 3 independent experiments. Statistical analysis was performed by Student's 2-tailed *t*-test, $*p<0.05$ *vs.* cells treated with anti-scrambled siRNA. $**p<0.05$ *vs.* cells treated with each of the other treatments.

vation and inhibits apoptosis. The procaspase-3 level in the cells exposed to adriamycin and treated with anti-survivin siRNA decreased to 50% of the level in cells exposed to adriamycin followed by treatment with anti-scramble siRNA duplex. We treated distilled water to replace anti-scramble siRNA, and there is small effect on pro-caspase3 expression in anti-scrambled siRNA.

Discussion

Survivin mRNA is expressed to various degrees in all of the 22 lung cancer cell lines used in our study. It has been reported that survivin mRNA is detectable in 85.5% of NSCLC tissue samples and that its expression level is correlated with poor prognosis.[3] The mean survivin expression in 6 cell lines with wild-type p53, except for SBC3/ADM, tended to be low in comparison with the mean expression in 10 cell lines possessing mutant p53 ($p = 0.019$). There is no relationship between survivin expression and histology or origin of carcinoma (Table I). It has been reported that survivin expression is associated with accumulation of mutant p53 in gastric cancer and pancreatic carcinoma, assayed by immunohistochemical staining.[30–31] These data suggest that p53 might regulate survivin expression. In addition, after exposure to adriamycin, survivin expression show a transcriptional decrease following accumulation of wild-type p53. Adriamycin is generally classified as a topoisomerase II inhibitor that induces DNA double-strand breaks. The cellular response to DNA damage, which includes nuclear accumulation of p53, has been studied extensively using adriamycin. Thus, we used adriamycin in this study. In our study, p53 inhibition by siRNA duplex resulted in downregulation of survivin expression. The dependence of survivin repression on functional p53 has been investigated previously in a number of different cell models and cancer cell lines.[14,15] Although it is generally accepted that p53 activates a number of genes through direct interaction with their promoter DNA, the mechanism whereby p53 regulates survivin expression is still unclear.[8] One possibility is that p53 might directly bind to the promoter of survivin and repress survivin transcription. In fact, a p53-binding motif is reported to exist within the promoter of survivin.[14,15] In contrast, Mirza *et al.*[15] suggested that a p53-binding motif was not required for transcriptional repression of survivin. They suggested that chromatin deacetylation in the survivin promoter could contribute to p53-dependent repression of survivin gene expression. It is also possible that p53 might increase the level of another transcriptional regulator (*e.g.*, p21) and indirectly downregulate survivin elsewhere downstream.[11] In our study, both survivin and p53 expressions were low in 2 cell lines with wild-type p53 treated with adriamycin for 72 hr (Fig. 2*a*). It may be explained by indirect survivin regulation by another transcriptional factor. Z. Wang *et al.*[32] previously showed that survivin post-translationally increased Mdm2 protein, and subsequently ubiquitination of p53, by blocking caspases that could cleave Mdm2 protein. We showed that p53 functionally repressed survivin expression. In our study, there is a possibility that survivin repression followed by adriamycin exposure might affect p53 accumulation in wild-type p53 cell lines. Survivin expression increased after adriamycin treatment in PC14 possessing mutant p53. Wall NR *et al.*[33] also showed survivin protein increase in MCF7 following adriamycin treatment, and they suggested that survivin was phosphorylated by cdc2 and very little degraded by an ubiquitination-dependent mechanism.

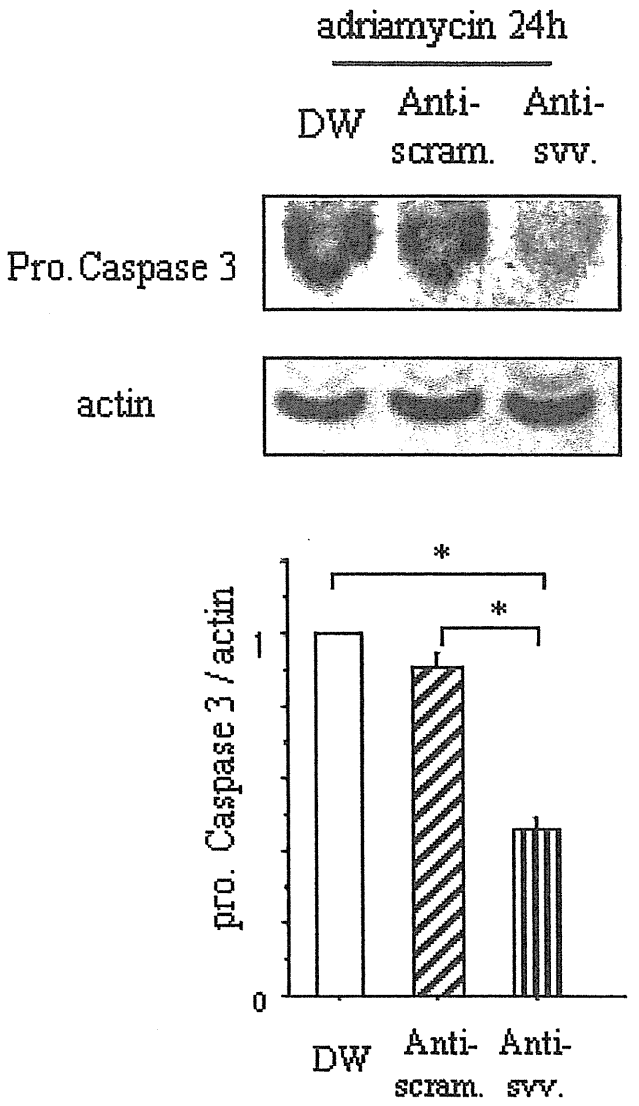

FIGURE 9 – Effects of siRNA targeting survivin on pro-caspase3 expression of PC9 lung cancer cells treated with adriamycin. PC9 cells were exposed to adriamycin for 24 hr after 48 hr transfection with duplex siRNA targeting survivin, scramble or distilled water, and each sample was analyzed by Western blotting. The data are presented as the mean ± S.E. for the 3 independent experiments. A representative blot is shown. Statistical analysis was performed by Student's 2-tailed *t*-test, $*p < 0.05$ *vs.* cells treated with other agents.

TABLE I – HISTOLOGY AND ORIGIN OF EACH CELL LINE[1]

Cell Line	Histology	Origin
LU99	La	Prim.
A549	Ad	Prim.
EBC1	Sq	Prim.
MA-46	Sq	Effu.
RERF-LC-KJ	Ad	Prim.
OBALK1	La	Effu.
Lu99B	La	Effu.
PC9	Ad	Prim.
SBC3	Sm	Prim.
NCI-H292	Muc	Prim.
LK-2	Sq	Prim.
LU65	La	Prim.
NCI-H358	Ad	Prim.
PC14	Ad	Prim.
Sq1	Sq	Prim.
NCI-H226	Metho	Effu.
NCI-H460	La	Effu.
NCI-H522	Ad	Prim.
Lu 135	Sm	Prim.
NCI-H1299	La	Lym.
NCI-H69	Sm	Prim.

[1]Ad: adenocarcinoma, Sq: squamous cell carcinoma, La: large cell carcinoma, Sm: small cell carcinoma, Metho: mesothelioma, Muc.: mucoepidermoid carcinoma, Prim.: primary, Lym.: lymph node, Effu.: effusion.

Investigation of cell cycle distribution after exposure to adriamycin has shown that cells possessing wild-type p53 tend to become arrested in G1 phase. In these cell lines, transcriptional p21 activation generally leads to G1 arrest. Additionally, we found G2/M phase repression and apoptosis progression accompanying repression of survivin protein. It has been reported previously that transfection with survivin anti-sense or dominant negative survivin gene resulted in accumulation of apoptotic cells and concomitant loss of G2/M phase cells.[34,35] Li *et al.*[7] showed that cells transfected with a mutant survivin gene or survivin anti-sense appeared to show increased caspase3 activity when synchronized in G2/M phase but not in G1/S phase. We therefore analyzed the cell cycle distribution of cell lines possessing mutated or deleted p53. In contrast to cells with wild-type p53, these cells became arrested in G2/M phase. Thus, survivin retention in cells possessing mutant p53 might make them able to resist apoptosis at the G2/M checkpoint.

One critical point of our study was to investigate differences in the proliferation of cancer cells following survivin repression, with the expectation that survivin inhibition itself would have a potent anti-proliferation effect. In cells possessing mutated or deleted p53, survivin was stably expressed even after adriamycin exposure and cell cycle arrest at the G2/M phase, indicating an anti-apoptotic effect. Survivin inhibition by siRNA downstream of p53 induced cell apoptosis and enhanced the anti-proliferative effect. Survivin associates with microtubules of the mitotic spindle at the beginning of mitosis, and disruption of survivin-microtubule interactions increases caspase-3 activity.[7] In order to inhibit survivin specifically, we used siRNA. This efficiently repressed survivin expression and inhibited cell proliferation in the absence of any cytotoxic stimulus. It has been reported that antisense targeting of survivin induces apoptosis in lung cancer cells. Using TUNEL assay, we also confirmed that anti-survivin siRNA duplex induced apoptosis.

Finally, survivin inhibition was found to sensitize PC9 to an anti-cancer agent. Exposure to Adriamycin after repression of survivin by siRNA significantly inhibited cell proliferation compared to cells exposed to either adriamycin alone or anti-survivin siRNA alone. Data obtained by the TUNEL assay confirmed that the difference in cell proliferation was based on apoptosis. *In vitro* binding experiments have indicated that survivin specifically binds to caspase-3 and -7, but not to caspase-8.[6] We also identified repression of procaspase-3 (which means activation of caspase-3) in cells exposed to adriamycin after treatment with anti-survivin siRNA. Activation of caspase-3 by inhibition of survivin may thus promote sensitivity to adriamycin. In our study, the expression of survivin mRNA in SBC3/ADM cells was greater than that in the parental SBC cells (Fig. 1*b*), indicating that survivin expression is related to cell resistance to adriamycin. We identified survivin inhibition by siRNA in cells with mutated p53 sensitized to adriamycin. Combining transfection with a mutant survivin gene with exposure to adriamycin did not enhance apoptosis in HeLa cells and MCF-7 cells, which have wild-type p53, compared to a mutant survivin gene transfection alone or adriamycin alone.[36] The combined effect of the two against apoptosis may be dependent on the character of each cell type, including p53 status or the compound targeting survivin. Additional studies will be needed to

determine the combined effect of survivin inhibition and other drugs on other cell lines.

In conclusion, siRNA targeting survivin could be of potential value for increasing the sensitivity of cancer cells to anti-cancer drugs, especially drug-resistant cells that possess mutated p53.

Acknowledgements

We thank E. Hatashita, Y. Yamada, T. Wada and M. Nagasaka for experimental assistance. This investigation was selected for a Scholar-in-Training Award at the 95th Annual Meeting of the AACR.

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

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ABSTRACT

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

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

Results: **High GnT-V expression was found in 113 (52.1%) NSCLCs, and low GnT-V expression was found in 104 (47.9%) NSCLCs. Multivariate logistic regression analysis revealed a significant association between low GnT-V expression and squamous cell carcinomas, as compared with nonsquamous cell carcinomas ($P = 0.02$). Among biological characteristics of tumors, Ki-67 labeling index was higher in tumors with low GnT-V expression than in those with high GnT-V expression, although this difference was not statistically significant ($P = 0.09$). Patients with tumors having low GnT-V expression had significantly shorter survival time than patients with tumors having high GnT-V expression in 103 patients with pStage I NSCLCs (5-year survival rates, 49% and 86%, respectively; $P = 0.0009$), as well as in 59 patients with pStage I non-squamous cell carcinomas (5-year survival rates, 54% and 89%, respectively; $P = 0.007$). Low GnT-V expression was a significant unfavorable prognostic factor in pStage I NSCLCs (hazard ratio, 2.86; $P = 0.002$) and in pStage I nonsquamous cell carcinomas (hazard ratio, 3.02; $P = 0.02$). Furthermore, β1–6 branching of asparagine-linked oligosaccharides, which are products of GnT-V, were increased highly or moderately in 8 of 10 tumors with high GnT-V expression, as judged by leukoagglutinating phytohemagglutinin staining.**

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

INTRODUCTION

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

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

In the present study, we examined GnT-V expression by immunohistochemistry in surgically resected NSCLCs and an-

Received 7/15/03; revised 11/20/03; accepted 12/3/03.
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