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Dose Escalation Study of Paclitaxel in Combination with Fixed-Dose Irinotecan in Patients with Advanced Non-Small Cell Lung Cancer (JCOG 9807)

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Key Words
Paclitaxel · Irinotecan · Non-small cell lung cancer · G-CSF

Abstract
Background: Both irinotecan (CPT) and paclitaxel (Pac) are effective against non-small cell lung cancer (NSCLC), and besides, preclinical studies have demonstrated an additive or synergistic interaction between camptothecin and taxane. ***Methods:*** We conducted a phase I/II study of combination chemotherapy consisting of Pac and CPT to determine qualitative and quantitative toxicities and efficacy of the combination against advanced NSCLC. We fixed the dose of CPT at 60 mg/m^2 and escalated the Pac dose in 10 or 20 mg/m^2 increments from a starting dose of 80 mg/m^2, and repeated the cycle every 2 weeks. Prophylactic G-CSF was also administered. ***Results:*** Between February 1999 and April 2001, 24 patients were registered in the study. None of the patients had a history of prior chemotherapy, but surgical resection had been performed in 3 of them. None of the patients experienced dose-limiting toxicity (DLT) up to and including level 6. At dose level 7 of Pac, 180 mg/m^2, 2 patients experienced DLT, that is grades 2 and 3 dyspnea due to pneumonitis. Another patient experienced grade 1 dyspnea due to pneumonitis. Neutropenia, diarrhea, and other toxicities were mild; however, we concluded that dose level 7 of Pac was the maximum-tolerated dose. An objective response was observed in 58.3%. The median survival time was 370 days, and the 1-year survival rate was 54.2%. ***Conclusion:*** Pneumonitis was the DLT in this study, and Pac 160 mg/m^2 and CPT 60 mg/m^2 every 2 weeks are recommended for the phase II study. This combination shows appreciable activity against NSCLC.

Introduction

Current chemotherapy regimens for metastatic non-small cell lung cancer (NSCLC) are not particularly effective, and the disease cannot be cured even with the most effective cisplatin-based combination chemotherapy. New agents and new combination chemotherapies have been investigated for metastatic NSCLC, and in the past decade, a number of new anticancer agents, including vinorelbine, gemcitabine, docetaxel, and paclitaxel (Pac), have been approved for the treatment of advanced NSCLC. Regimens based on combinations of these drugs with platinum compounds have presented interesting new possibilities for treating patients with NSCLC, and randomized studies comparing platinum-based combinations with single-agent treatment have demonstrated a

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small but significant survival benefit for combined treatment [1, 2]. A trial of nonplatinum combination chemotherapy has recently been instituted [3].

The camptothecin derivative irinotecan (CPT) is a topoisomerase I inhibitor and effective against NSCLC [4]. Pac preferentially binds to microtubles and impairs microtubular disassembly, and it is also effective against NSCLC [5]. The combination of a camptothecin and a taxane is attractive, because both have been shown to have a broad spectrum of clinical activity that is dependent on the schedule of administration, and the mechanisms of action and the nonhematologic toxicity profiles of the camptothecins and taxanes are different. Preclinical studies that have evaluated combinations of a camptothecin with a taxane have yielded promising results, and several studies have demonstrated an additive or synergistic interaction between camptothecin and taxane [6–10].

Accordingly, we expected the combination of Pac and CPT to display high activity against NSCLC. Pac was initially infused every 3–4 weeks; however, weekly infusions were shown to produce a higher dose intensity and to have promising activity [11]. CPT has been used in various schedules, including weekly, every 2 weeks, and every 3 weeks. Weekly or 2-week cycles are considerably better than 3-week cycles to increase the dose intensity of both drugs. An important criterion for the feasibility of the combination of Pac and CPT is a reduced incidence of neutropenia, which is dose-limiting toxicity (DLT) of both drugs, and prophylactic granulocyte colony-stimulating factor (G-CSF) has been used to prevent neutropenia. We previously reported that prophylactic administration of G-CSF when monocytopenia is first detected can lessen neutropenia caused by chemotherapy for lung cancer without increasing the total G-CSF dose beyond the standard dose [12]. Administration of G-CSF was started on day 6–8 in this study. A 2-week cycle is reasonable for combinations of Pac and CPT when G-CSF was prophylactically used after the detection of monocytopenia.

We designed a dose escalation study to determine the maximum dose of Pac that could be given with CPT when both drugs were delivered according to a 2-weekly schedule. We fixed the dose of CPT at 60 mg/m^2, which is the dose used in combination with cisplatin [13], and escalated the Pac dose form 80 mg/m^2 in 10 or 20 mg/^{2} increments. The aims of this study were: (1) to determine the qualitative and quantitative toxicities of the combination chemotherapy; (2) to determine the recommended dose of Pac with CPT, and (3) to determine the efficacy of combination chemotherapy against advanced NSCLC by evaluating the objective response rate and survival rate.

Table 1. Patient characteristics

	Patients
Total	24
Age, years	
Median	59
Range	44–69
Gender	
Male	18
Female	6
PS (ECOG)	
0	4
1	20
Clinical stage	
IIIA/B	5
IV	16
Postoperative recurrence	3
Histology	
Adenocarcinoma	16
Squamous cell carcinoma	7
Large cell carcinoma	1
Prior treatment (surgery)	3

Patients and Methods

Patients

Patients with histologically or cytologically proven unresectable NSCLC were registered for Pac and CPT combination chemotherapy. Eligibility criteria were an expected survival of at least 6 weeks, age <70 years, Eastern Cooperative Oncology Group performance score (PS) ≤1, leukocyte count ≥4,000/μl, hemoglobin ≥10 g/dl, platelet count ≥100,000/μl, total serum bilirubin ≤1.5 mg/dl, aspartate aminotransferase and alanine aminotransferase ≤90 IU/l, serum creatinine ≤1.5 mg/dl, and creatinine clearance ≥50 ml/min. Patients who had experienced postoperative recurrence were eligible for this study, but a period of rest of 4 or more weeks was required after surgery. Patients who had received chemotherapy or radiotherapy were excluded from this study. Written informed consent was obtained from every patient.

Chemotherapy

All patients without disease progression were treated every 2 weeks for a total of 4 courses of chemotherapy. CPT was dissolved in 250 ml of 5% glucose and administered as a 90-min infusion at a fixed dose of 60 mg/m^2 on day 1. Pac was dissolved in 500 ml of 5% glucose and administered as a 3-hour infusion at a starting dose of 80 mg/m^2 on day 1 and escalated in 10 or 20 mg/m^2 increments per dose level (table 1). Premedication consisted of a 20-mg dexamethasone infusion (14 and 7 h before Pac), and a 50-mg ranitidine and 50-mg oral dose of diphenhydramine (30 min before Pac). After completion of the 90-min CPT infusion, a 30-min ranitidine infusion was administered, and a 3-hour Pac infusion was then administered. G-CSF, 50 or 2 μg/kg/day, was administered subcutaneously when the leukocyte, neutrophil, or monocyte count fell below 2,000, 1,000 or 150/μl, respectively. G-CSF was stopped if the leukocyte or neutrophil count rose above 10,000 or 5,000/μl, respectively. Patients were

given 5-HT_3 antagonist intravenously before administration of CPT on day 1. Subsequent courses of chemotherapy were started when the patients satisfied the organ function criteria: leukocyte count ≥3,000/μl, neutrophil ≥1,500/μl, platelet count ≥75,000/μl, less than grade 1 nonhematologic toxicities, except alopecia. Grade 3 nausea and vomiting did not preclude subsequent courses of chemotherapy. If the DLT was reached the dose of Pac and CPT in the subsequent course was reduced by 10 mg/m². Chemotherapy was repeated for a maximum of 6 courses unless the disease progressed, but it was stopped if the tumor response was judged to be no change (NC) after 4 courses. Physical examination, a complete blood cell count, biochemical tests, and chest radiographs were obtained weekly. Tumor response was evaluated according to World Heath Organization criteria [14]. Complete response was defined as the complete disappearance of all evidence of tumor for at least 4 weeks. PR was defined as at least a 50% reduction in the sum of the product of the two greatest perpendicular diameters of all indicator lesions or a reduction of more than 50% in evaluable disease for at least 4 weeks with no appearance of new lesions or progression of any existing lesions. PD was defined as at least a 25% increase in the tumor area or the appearance of new lesions. All other outcomes were classified as NC. Toxicities were evaluated according to the JCOG criteria [15].

DLT was defined as toxicity during the first 4 courses consisting of grade 4 neutropenia lasting 4 days or more, or grade 4 neutropenia with a fever of 38°C or higher, grade 4 thrombocytopenia, ≥grade 2 depression of PaO_2, grade 2 dyspnea, grade 3 or 4 other nonhematologic toxicity, except alopecia, nausea, and vomiting, or failure to complete the 4 courses within 9 weeks because of toxicity. Patient refusal was also defined as a DLT.

At least 3 patients assessable for toxicity were treated at each dose level. If none of the first 3 patients experienced a DLT, then escalation to the next dose level proceeded. If 1 patient developed a DLT, the cohort was expanded to 6 patients. We planned a 20 mg/m² increase by dose level after level 3 when none of the patients at levels 1 and 2 experienced DLT. The maximum tolerated dose (MTD) was defined as the dose level at which at least 2 of 3 patients or 3 of 6 patients experienced a DLT. The recommended dose of Pac for the phase II study was defined as the most dose-intensive level below the MTD. If the MTD was not defined by the Pac dose level at 210 mg/m², which is the recommended dose for Pac alone in Japan, the most dose-intensive level including 210 mg/m² of Pac would be recommended as the dose for phase II study. The Committee of JCOG and the Institutional Review Board of the Kanagawa Cancer Center reviewed and approved the protocol prior to commencement.

Results

Patient Characteristics

Between February 1999 and April 2001, 24 patients were registered in the study. The patients' characteristics are summarized in table 1. Eighteen patients were male, and 6 were female; their median age was 59 years (range 44–69 years). Four patients had a PS of 0, and the other 20 patients had a PS of 1. Sixteen patients had adenocarcinoma, 7 had squamous cell carcinoma, and 1 had large cell carcinoma. None of the patients had a prior history of chemotherapy, but surgical resection had been performed in 3 of them.

Table 2. Planned doses and administered doses of Pac and CPT

Dose level	Pac mg/m²	CPT mg/m²	Number of patients	Number of cycles
1	80	60	3	12
2	90	60	3	16
3	100	60	3	12
4	120	60	3	9
5	140	60	3	14
6	160	60	3	13
7	180	60	6	26

Dose Escalation and Determination of MTD

The numbers of patients and cycles at each dose level are listed in table 2. All patients were assessable for toxicity. Three patients each were registered at dose levels 1 and 2. None of the patients experienced DLT during 4 courses. At dose levels 3 and 4, 2 and 1 of the 3 patients registered developed PD during the third cycle and the first cycle, respectively, but none of them experienced DLT. None of the 3 patients registered at level 5 experienced DLT. One of the 3 patients registered at dose level 6 developed PD during the first cycle, but none of them experienced DLT. At dose level 7, 1 of the first 3 patients experienced DLT, grade 2 dyspnea, because of pneumonitis during the third cycle, and another 3 patients were registered. One of the additional 3 patients experienced a DLT, grade 3 dyspnea, because of pneumonitis in the fourth cycle, and another additional patient experienced grade 1 dyspnea because of pneumonitis in the fifth cycle. Two patients experienced DLTs at level 7, but these were both cases of pneumonitis; since another patient experienced pneumonitis, which is a very serious toxicity, we concluded that the dose level 7 of Pac, 180 mg/m², was the MTD. As a result, Pac 160 mg/m² and CPT 60 mg/m² every 2 weeks were recommended for the phase II study.

Treatment Administration and Toxicities during All Cycles of Treatment

The 24 patients received a total of 102 cycles of treatment, and the mean number of cycles was 4 (range 1–6). There were no treatment-related deaths. Five patients were excluded from treatment, 4 because of disease progression and 1 because of an adverse event, that is pneumonitis. Tables 3 and 4 list the overall incidence of hematologic and nonhematologic adverse events among all

Table 3. Hematologic toxicities by number of cycles

Dose level	Number of patients	Number of cycles	Incidence of adverse events (JCOG grade)											
			neutrophils				infection	hemoglobin			platelets			
			1	2	3	4		1	2	3	1	2	3	4
1	3	12	0	0	0	0	0	0	2	0	0	0	0	0
2	3	16	1	1	0	0	0	2	1	0	0	0	0	0
3	3	12	1	0	1	0	0	0	1	0	0	0	0	0
4	3	9	1	1	0	0	3	2	0	0	0	0	0	0
5	3	14	1	1	1	0	1	1	2	0	0	0	0	0
6	3	13	0	1	1	0	0	2	0	0	0	0	0	0
7	6	26	1	2	2	0	1	4	1	0	0	0	0	0

JCOG = Japan Clinical Oncology.

patients in this study. G-CSF was used for a mean of 5.2 days in 97 of the 102 courses in the phase I study, depending on the presence of monocytopenia. One third of the patients experienced grade 3 neutropenia at dose levels 3, 5, 6 and 7, but there was no grade 4 neutropenia. Infectious episodes were observed in 3, 1 and 1 patients at dose levels 4, 5 and 7, respectively, but all of the episodes were mild and improved with response to antibiotics. Anemia was mild, and there was no thrombocytopenia. None of the patients received transfusions. All acute nonhematologic adverse events, i.e. liver, renal, gastrointestinal, cardiac, and circulatory toxicities, were grade 1 or 2, and the patients recovered before the next cycle. Diarrhea was observed in 50% of the patients and nausea or vomiting in 62.5%, both high incidences, but they were mild and improved with medication. The incidences of hypotension and arrhythmia were low, and they were mild. Mild myalgia or arthralgia was observed at all dose levels. All events were observed during the 1st week of administration; the patients recovered before the next cycle. Neuropathy was observed in two thirds of the patients at dose levels 5–7 and persisted for several months. Pneumonitis developed in half of the patients at level 7, even though none of the patients below level 7 experienced it. The episodes were manifested by a high fever between chemotherapy cycles 3–5. All of the patients were treated with methylpredonisolone, and their dyspnea resolved. Every course could be performed in 2-week cycles except 1 course of the second cycle at level 5.

Efficacy

Although patients were treated at different starting dose levels, it was possible to assess the response rate and other efficacy variables for the 24 patients assessable for response. The outcome of chemotherapy in the 24 patients is shown in table 5. There was a complete response in 1, a PR in 13 patients, NC in 4 patients, and PD in 6 patients, and the overall response rate was 58.3%. Median time to progression was 177 days (range 79–413 days). Six patients were alive and the other 18 patients died during the follow-up period. The median survival time was 370 days, and the 1-year survival rate was 54.2%.

Discussion

Combined analysis of the two randomized phase III studies demonstrated that CPT combined with cisplatin significantly improved survival compared to vindesine and cisplatin in patients with advanced NSCLC [13, 16, 17]. CPT is believed to be a key drug against NSCLC in Japan. Over the past decade, a number of new anticancer agents including CPT, vinorelbine, gemcitabine, docetaxel, and Pac have been approved for the treatment of metastatic NSCLC. The combination of one or more of these agents with a platinum compound has resulted in high response rates and prolonged survival [1, 2]. However, a randomized study of a comparison of new anticancer-containing chemotherapy regimens such as cisplatin and Pac, cisplatin and gemcitabine, cisplatin and docetaxel, or carboplatin and paclitaxel conducted in the Eastern Cooperative Oncology Group did not show the differences among them [18]. Therefore, more active new combination chemotherapy is required to improve the treatment of NSCLC, and we conducted the combination chemotherapy with nonplatinum agents.

Table 4. Nonhematologic toxicity

Dose level	Number of patients	Number of cycles	Incidence of adverse events (JCOG grade)																	
			bilirubin		GOT		GPT		creatinine		diarrhea		nausea/ vomitting		hypotension		arrhythmias		skin rash	
			G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4
1	3	12	0	0	0	0	0	0	0	0	3	0	3	0	0	0	0	0	0	0
2	3	16	0	0	1	0	1	0	0	0	2	0	2	0	1	0	0	0	0	0
3	3	12	0	0	2	0	2	0	0	0	2	0	2	0	1	0	1	0	0	0
4	3	9	0	0	0	0	2	0	0	0	2	0	2	0	0	0	0	0	1	0
5	3	14	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0
6	3	13	0	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0
7	6	26	2	0	3	0	3	0	0	0	2	0	4	0	1	0	0	0	0	0

JCOG = Japan Clinical Oncology.
[1] Grade 1 dyspnea was observed in an additional cycle. [2] Not mentioned in JCOG criteria.

The objective response of 58.3% and the survival rate of 54.2% at 1 year in our nonplatinum Pac and CPT regimen have been somewhat better compared to a large phase III trial of four platinum-based chemotherapy regimens which showed response rates of 17–22% and survival rates of 31–34% at 1 year. Good survival and a high response rate in our study are somewhat better than what normally would be expected. This antitumor activity of Pac and CPT combination chemotherapy is thought to be attributable to a synergistic action between these two drugs. A possible mechanism of the synergy is a drug-drug interaction such as shown in a pharmakokinetic study that demonstrated an elevation of the AUC of CPT and SN-38 by Pac infusion [19]. Drug-drug interactions have been demonstrated when Pac has been used in combination regimens in other clinical trials. Toxicity increases when cisplatin is delivered before Pac, in part because of reduced renal clearance of Pac with this sequence [20]. Infusion of Pac before either cyclophosphamide or doxorubicin also results in increased toxicity [21, 22]. When Pac was combined with gemcitabin, Pac caused a decrease in the clearance of gemcitabine [23]. Plasma elimination of CPT is also affected by other chemotherapeutic agents. Saltz et al. [24] have demonstrated modest changes in the conversion of CPT to SN-38 that were dependent on the sequence of drug administration in a regimen that included 5-FU, leukovorin, and CPT. When combined with cisplatin, a modest increase in the dose of CPT has been shown to result in an increase in the AUC SN-38 [25]. Therefore, it is possible that Pac and CPT affect each other's pharmacokinetics, and exert a high activity against NSCLC. Another possible mechanism of this high activity of the Pac-CPT combination is thought to be related to influx and efflux in the cell system. The combination of Pac and SN-38 downregulated the level of multidrug resistance-associated protein, which may be an efflux pump for cisplatin in ovarian cancer cell lines, suggesting that this combination will overcome drug resistance [26]. Unfortunately, since we did not perform a pharmacokinetic study, we could not explain the correlation of response and/or toxic effect of Pac and/or CPT to AUC.

Pac and CPT combination chemotherapy proved to be well tolerated and devoid of significantly hematologic or gastrointestinal toxicities when CPT and Pac are used at the maximum single-agent doses. In particular none of the patients in our study experienced thrombocytopenia compared to a large trial showing grade 3 or 4 thrombocytopenia in 3–50% [18]. This less thrombocytopenic regimen may also be helpful in the treatment of elderly patients. A phase I study of Pac and CPT combination chemotherapy showed that neutropenia was dose-limiting, and Pac 75 mg/m^2 and CPT 50 mg/m^2 were recommended [27]. The difference in the recommended dose between that study and ours can be attributed to the fact that we used prophylactic G-CSF when monocytopenia developed after the start of chemotherapy. The prophylactic use of G-CSF made it easier to repeat the combination chemotherapy at 2-week intervals. The mean duration of G-CSF administration during the 14 days between cycles in this study was 5.2 days. It was not very long and was reasonable compared to other dose-intensive regimens. The results of our study show that prophylactic administration of G-CSF is reasonable when monocytopenia develops.

neurologic		dyspnea[1]				alopecia		stomatitis		myalgia[2]	arthralgia[2]
G1/2	G3/4	G1	G2	G3	G4	G1/2	G3/4	G1/2	G3/4		
0	0	0	0	0	0	3	0	1	0	2	2
0	0	0	0	0	0	3	0	0	0	1	1
0	0	0	0	0	0	3	0	0	0	1	1
0	0	0	0	0	0	2	0	0	0	1	1
2	0	0	0	0	0	3	0	1	0	0	1
2	0	0	0	0	0	2	0	0	0	1	1
4	0	1	1	1	0	3	0	0	0	4	2

Table 5. Antitumor activity by dose level

Dose level	Number of patients	Complete response	Partial response	Stable disease	Progressive disease
1	3	0	0	2	1
2	3	0	2	1	0
3	3	0	1	0	2
4	3	0	2	0	1
5	3	0	2	0	1
6	3	0	2	0	1
7	6	1	4	1	0
Total	24	1	13	4	6

Moreover, if pneumonitis had not occurred as a DLT, a higher dose of Pac might have been possible in this combination.

Both dose-dependent and dose-independent nonhematologic toxicities were observed. The dose-independent toxicities, hypotension, arrhythmia, liver damage, diarrhea, nausea, and vomiting, were mild. The delayed toxicities, pneumonitis and neuropathy, were considered the DLT in this study. Two thirds of the patients at PAC levels 5–7 experienced neuropathy, but none of the patients at levels 1–4 did. Some of them refused to continue chemotherapy despite being responders, and some did not recover from the neuropathy before death. Thus, neuropathy was considered to be a cumulative toxicity. Pneumonitis causing a high fever and depressed PaO_2 was observed in 3 patients only at PAC level 7. No abnormal shadows were detected on plain chest X-P films, but a CT scan showed a reticular shadow in each of the patients. They were all treated with methylpredonisolone, 1 g per day for 3 days, and recovered from respiratory failure. The pneumonitis occurred during the latter half of chemotherapy. The analysis of the relationship between the total doses of Pac and occurrence of pneumonitis revealed a total PAC dose of 540, 720 and 900 mg/m^2, respectively, in the 3 patients who developed pneumonitis. The total dose in the patients who did not experience pneumonitis at levels 6 and 7 was 720 mg/^{2} in 2 patients, 960 mg/m^2 in 2 patients, and 1,080 mg/m^2 in 1 patient. Based on these data, the risk of pneumonitis is unlikely to be dependent on the total dose of Pac during all courses. The pneumonitis is thought to be attributable to a booster effect of an allergic reaction when 180 mg/m^2 or higher of Pac was combined with CPT, and thus it is not expected to occur at doses of PAC below 180 mg/m^2.

In conclusion, this phase I study demonstrated that the DLT of Pac and CPT combination chemotherapy is pneumonitis, but this regimen is feasible for 2-week infusion cycles and has a high activity against NSCLC. A large phase II study will be required to confirm the feasibility and activity of this combination as a treatment for NSCLC.

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High Expression of Integrin β1 and p53 is a Greater Poor Prognostic Factor Than Clinical Stage in Small-Cell Lung Cancer

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Abstract: The purpose of this study was to investigate the possible association between expression of integrin β1 and p53 and survival in patients with small-cell lung cancer (SCLC). One hundred four patients with SCLC who had received an initial course of a full-dose of combination chemotherapy between February 1989 and July 1999 were entered in the study. Transbronchial biopsy specimens of tumors obtained before chemotherapy were subjected to immunostaining for integrin β1 and p53. Twenty-eight and 58 patients could not be evaluated for integrin β1 and p53 immunostaining, respectively, because the tissue samples had been crushed during the biopsy. Fifty-four patients had tumors with less than or equal to 25% integrin β1-positive cells and 22 patients had tumors with more than 25% integrin β1-positive cells, whereas 19 and 27 patients had tumors with less than or equal to 50% and more than 50% p53-positive cells, respectively. By comparison, the overall survival of patients with high expression of integrin β1 and p53 were significantly worse than those of individuals whose tumors had low expression (log-rank test, $p = 0.046$ and $p = 0.0067$, respectively). Moreover, the overall survival of patients with high expression of either integrin β1 or p53 (n = 42) was significantly worse than that of other patients without high expression of integrin β1 and p53 (n = 38; log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$). The association between survival and prognostic factors, including gender, age, performance status, clinical stage, and integrin β1/p53 expression was examined by the Cox proportional hazards model; only integrin β1/p53 expression was found to be a significant independent factor (hazard ratio = 0.394, $p = 0.0005$). In conclusion, the high expression of integrin β1 and p53 in tumor cells is a greater poor prognostic factor than clinical stage in patients with SCLC.

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Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumors, and treatment with combination chemotherapy is known to improve survival. Recently, the Japanese Clinical Oncology Group (JCOG) showed that cisplatin plus irinotecan improved the survival of patients with extensive disease (ED)-SCLC when compared with cisplatin plus etoposide (PE) in a phase III study.[1] However, despite the high response rates and prolonged survival, relapse occurred in the majority of these patients. New therapeutic strategies for SCLC are therefore urgently needed, and these will most likely result from a better understanding of the cell biology of SCLC.

Some forms of chemotherapy exert their cytotoxic effects by inducing apoptosis.[2,3] The regulation of apoptosis in tumor cells is poorly understood; however, the level of protein tyrosine kinase (PTK) activity may determine whether SCLC cells survive and proliferate or die as a result of apoptosis.[4] A recent study showed that SCLC was surrounded by an extensive stroma of extracellular matrix (ECM) at both primary and metastatic sites.[5] Adhesion of SCLC cells to the ECM confers resistance to chemotherapeutic agents as a result of integrin β1-stimulated tyrosine kinase activation, which suppresses chemotherapy-induced apoptosis.[5]

Mutations of p53 in tumors are also suspected to induce resistance to cancer chemotherapy.[6] One response to genotoxic stress involves the p53 tumor suppressor gene product.[7,8] This p53 accumulates after DNA damage and controls cellular proliferation predominantly through its activity as a transcription factor. The expression of downstream genes contributes to tumor suppression either by activating cell arrest, possibly to give the cell time to repair the damage and avoid genetic instability. Mutations in p53 have been shown

to confer sensitivity to drugs whose toxicity is modulated by nucleotide excision repair, such as nitrogen mustard and cisplatin.[9,10]

From these data, chemosensitivity-related genes such as integrin β1 and p53 are suggested to relate to cancer prognosis. Most SCLC respond to chemotherapy, to determine the relationship between integrin β1 and p53 expression in SCLC and clinical resistance to treatment, the expression of these genes in tumors and patients' survival were investigated.

PATIENTS AND METHODS

Patients

Between January 1989 and June 1999, patients with histologically proven SCLC, who had received a full dose of chemotherapy, were entered in this study. Patients who had not received a full dose of chemotherapy because of poor performance status (PS) on the Eastern Cooperative Oncology Group (ECOG) scale were excluded. The tumor responses were evaluated according to the World Health Organization criteria.[11]

Immunohistochemistry

Transbronchial biopsy specimens of tumors obtained before chemotherapy were subjected to immunostaining for integrin β1 and p53. Formalin-fixed, paraffin-embedded, 5-μm-thick tumor sections were mounted on charged glass slides, deparaffinized, and rehydrated in a graded alcohol series. Immunohistochemical staining was performed using an automated processor. The slides were immersed in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity and the sections were then immersed in 10 mmol/l citrate buffer (pH 6.0) for 1 hour at 94°C, and nonspecific staining was blocked with immunoglobulins from normal rabbit serum for 10 minutes at room temperature. Excess serum was removed and the sections were incubated for 1 hour at 4°C with a mouse antiintegrin β1 monoclonal antibody (Oncogene Research Products, U.S.A.) or mouse anti-p53 monoclonal antibody (Novocastra, UK) diluted 1:100 with phosphate-buffered saline (PBS), followed by incubation for 10 min at room temperature with the secondary antibody, biotinylated antimouse immunoglobulin (Nichirei Corp., Japan), for 10 min at room temperature. The slides were again washed in PBS and incubated with streptavidin peroxidase complex (Nichirei Corp., Japan) for 10 min at room temperature. The slides were then incubated with 3,3-diaminobenzidine as the substrate for 5 min to visualize positively immunostained cells. Finally, the slides were counterstained with hematoxylin and coverslips were applied. The identical reaction times used permitted consistent reproducibility, thus allowing accurate comparison of all samples.

Scoring of integrin β1 and p53 immunostaining

Two pathologists examined the staining patterns of integrin β1 and p53 independently, and recorded the percentage of positive cells in each specimen. At least 20 high-power fields were chosen randomly and 2,000 cells were counted. The ratio of integrin β1- and p53-positive cells was calculated by dividing the number of positive cells by the total number of cells, and was expressed as a percentage. The integrin β1 immunostaining levels were classified as high (>25% of the cells were stained) or low (≤25% of the cells were stained), and p53 immunostaining levels were classified as high (>50% of the cells were stained) or low (≤50% of the cells were stained).

Statistical Analysis

Kaplan-Meier survival curves were constructed and analyzed for statistical significance by means of the log-rank and generalized Wilcoxon tests. The influence of each variable on survival was examined by the Cox proportional hazards model in multivariate regression analyses. Differences at $p < 0.05$ were considered to be statistically significant.

RESULTS

From February 1989 to July 1999, 104 patients received an initial course of chemotherapy for SCLC. There were 91 males and 13 females with a median age of 65 years (range 40–85 years). The ECOG PS was 0 in 7 patients, 1 in 69, and 2 in 28. The clinical stages of the tumors were limited disease (LD) in 43 patients and ED in 61.

Transbronchial biopsy specimens were subjected to integrin β1 and p53 immunostaining. Twenty-eight and 58 patients could not be evaluated for integrin β1 and p53, respectively, because the tissue samples had been crushed during the biopsy procedure. Fifty-four and 22 patients had tumors with less than or equal to 25% and more than 25% integrin β1-positive cells, respectively, whereas 19 and 27 patients had tumors with less than or equal to 50% and more than 50% p53-positive cells, respectively.

Each patient received a full dose of combination chemotherapy after confirmation of SCLC. Fourteen of 45 patients treated with PE and 1 of 12 patients treated with carboplatin plus etoposide received concurrent thoracic radiotherapy. Among 80 patients with biopsy specimens evaluated for integrin β1 or p53, 11 patients achieved a complete response and 56 patients achieved a partial response. In three patients there was disease progression, and the remaining 10 patients showed no change. The overall response rate to chemotherapy was 84%. When the relationship between gene expression and tumor response to chemotherapy was considered, 17 of 22 patients with high expression of integrin β1, and 48 of 54 patients with low expression of integrin β1

showed tumor response (77% and 89%, respectively). Only 46 patients could be evaluated for p53 immunostaining. The response rate to chemotherapy in patients with high expression of p53 was similar to that in patients with low expression of p53 (82% vs. 84%, respectively).

When the survival of 80 patients evaluable for integrin β1 or p53 was compared after stratification according to clinical stage, the overall survival of patients with high expression of integrin β1 (n = 22) was significantly worse than that of individuals whose tumors had low expression of integrin β1 (n = 54; log-rank test, $p = 0.046$; Wilcoxon test, $p = 0.043$). The overall survival of patients with high expression of p53 (n = 27) was also significantly worse than that of patients with low expression of p53 (n = 19; log-rank test, $p = 0.0067$; Wilcoxon test, $p = 0.033$). When other prognostic factors were considered, no significant difference in survival was observed according to gender, age, or PS except for clinical stage (Table 1).

Survival curves were constructed for group B of patients with high expression of either integrin β1 or p53 (n = 42) and group A of other patients without high expression of integrin β1 and p53 (n = 38; Fig. 1). The overall survival of group B was significantly worse than that of group A (log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$). When patients were stratified according to clinical stage (LD and ED), the survival of group B was also significantly worse (log-rank test, $p = 0.0007$; Wilcoxon test, $p = 0.0034$). The association between survival and prognostic factors, including gender, age, PS, clinical stage, and integrin β1/p53 expression was examined by the Cox proportional hazards model (Table 2). Only integrin β1/p53 expression was found to be a significant independent factor (hazard ratio = 0.394, $p = 0.0005$).

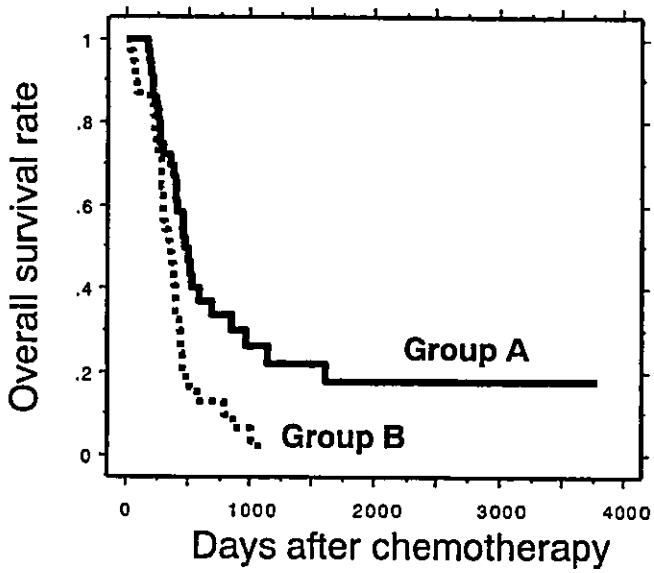

FIGURE 1. Kaplan-Meier survival curves according to integrin β1 and p53 immunostaining. Survival after chemotherapy of patients with high expression of either integrin β1 or p53 (n = 42; group B) was worse than that of other patients without high expression of integrin β1 and p53 (n = 38; group A; log-rank test, $p = 0.0003$; Wilcoxon test, $p = 0.0026$).

TABLE 1. Characteristics of Patients and Overall Survival

		P Value	
	No. of Patients	**Log-Rank**	**Wilcoxon**
Gender			
Male	74	0.36	0.27
Female	6		
Age			
≦65	42	0.7	0.9
>65	38		
PS (ECOG)			
0	6	0.36	0.27
1	52		
2	22		
Clinical stage			
LD	30	0.012	0.028
ED	50		
Integrin β1			
High	22	0.046	0.043
Low	54		
p53			
High	27	0.0067	0.033
Low	19		

ECOG, Eastern Cooperative Oncology Group; ED, extensive disease; LD, limited disease; PS, performance status.

Analysis was performed by stratification of clinical stage between LD and ED except for analysis of clinical stage.

DISCUSSION

Overexpression of the multidrug resistance gene *MDR1* is not common in SCLC, indicating that, unlike in other tumors, this is not an important mechanism for drug resistance in these tumors.[12] During the past few years, it has become clear that integrins are involved not only in cell adhesion but also in signal transduction in both normal cells and tumor cells. Integrins can directly activate many intracellular signaling events after stimulation by ECM proteins or by antibodies that bind to specific sites of integrins.[13] Both receptor clustering and ligand occupancy are critical for the activation of intracellular integrin-mediated responses.[14]

Many mammalian cell types are dependent on adhesion to the ECM for their continued survival. A variety of normal cell types undergo apoptosis when they lose attachment to an appropriate ECM.[15] In vitro and in vivo data provide prelim-

TABLE 2. Multivariate Regression Analysis of Variables in Predicting Overall Survival

Variable	Assigned Score	Hazards Ratio	95% Confidence Interval	*P* Value
Gender		0.622	0.251–1.541	0.305
Male	0			
Female	1			
Age (y)		0.869	0.527–1.431	0.58
≦65	0			
>65	1			
PS (ECOG)		0.710	0.397–1.268	0.247
0 or 1	0			
2	1			
Clinical stage		0.590	0.346–1.006	0.0524
LD	0			
ED	1			
Integrin β1/p53*		0.394	0.233–0.666	0.0005
Negative	0			
Positive	1			

*Positive was defined as patients with tumor specimens of high expression of either integrin β1 or p53, and negative as others. ECOG, Eastern Cooperative Oncology Group; PS, performance status.

inary evidence that adhesion to ECM proteins is essential in SCLC cell resistance to chemotherapy.[5] Cancer cells bound to the ECM may escape chemotherapy-induced cell death and then, with subsequent genetic damage, drug-resistant clones are selected. This is a model to explain not only SCLC behavior in vivo but also why a partial response and local recurrence of SCLC are often seen after chemotherapy. One report has indicated that resistance to chemotherapy induced by integrin β1-mediated adhesion to ECM is due to an increase in the level of PTK activity.[5] However, it is not known how integrin-stimulated PTK activation suppresses the early phase of apoptosis in SCLC cells. R-Ras and insulinlike growth factor-1, which activate phosphatidylinositol-3 kinase (PI3 K), cooperatively inhibit caspase-3 activation, preventing apoptosis of BaF3 cells.[16] Activation of PI3 K by integrins protects epithelial cells from detachment-induced apoptosis.[17] Thus, integrin-stimulated PI3 K activation may impinge on the nuclear response to DNA-damaging agents.

Activation of intracellular signals includes tyrosine phosphorylation of focal adhesion kinase (FAK), which binds to the integrin β1 cytoplasmic domain and is one of the molecules that coclusters with β1 integrins aggregated by noninhibitory antiintegrin antibodies.[18–20] FAK is also reported to suppress a p53-dependent pathway activated by protein kinase C and cytosolic phospholipase A2, and it inhibits apoptosis under serum-starved conditions.[21] From these data, integrin β1 and p53 are considered to be part of the same signal pathway that induces cells in apoptosis. In support of a role for p53 in the cytotoxic mechanism of cisplatin, several studies of ovarian carcinoma cell lines have demonstrated that disruption of p53 function conferred drug resistance.[22–24] Our data from this study show that the high expression of p53 in SCLC was associated with a poor prognosis. We did not confirm whether the high expression of p53 was wild or mutant type, although most are considered to be mutant type, since p53 protein accumulation in tumor cells correlates well with mutations of the p53 gene due to a prolonged half-life of mutated p53 protein[25,26] and high expression of mutated p53 is considered to reflect resistance to chemotherapy.

SCLC is not a candidate for surgical resection, and only a small specimen obtained by transbronchial biopsy was available to investigate the genetic characteristics. It is questionable whether the small tissue sample available truly reflects the genetic characteristics of the total tumor in heterogeneous tumor tissues, although because SCLC is relatively homogeneous we feel justified in using small specimens obtained by transbronchial biopsy to analyze the genetic characteristics of the tumor. High p53 expression in SCLC cells is associated with a poor prognosis, although in this study p53 expression was determined in only 50% of the biopsy specimens because the nucleus of SCLC cells was easily crushed during the biopsy procedure, whereas the ECM was less badly damaged. Therefore, if we investigate the genetic characteristics in small transbronchial biopsy specimen of tumor obtained before chemotherapy, not only p53 but also integrin β1 should be examined for analysis of SCLC prognosis, in spite of the fact that integrin β1 expression in tumor cells appeared to be less closely related to SCLC prognosis than p53 expression.

Both p53 and integrin β1 expression are more closely related to SCLC prognosis than is the clinical stage of the disease. Because each gene is related to cell apoptosis after treatment with anticancer drugs, identification of the apoptotic pathway mediating integrin β1- and p53-dependent survival signals may provide new therapeutic strategies to improve the response of SCLC to chemotherapy.

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Genomic-wide cDNA microarray screening to correlate gene expression profile with chemoresistance in patients with advanced lung cancer

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We conducted a study using cDNA microarray analysis to determine whether expression levels of genes in tumors were correlated with tumor response to chemotherapy. Between September 2000 and December 2001, 47 patients were registered in the study. Eighteen patients had small cell lung cancer (SCLC), and others had non-small cell lung cancer (NSCLC). All patients except three received platinum-based chemotherapy. Sixteen of the 18 patients with SCLC (89%) and 13 of the 29 patients with NSCLC (45%) responded to chemotherapy, respectively. Transbroncheal biopsy specimens of tumors were obtained before chemotherapy. The expression levels of 1176 genes in tumor specimens were analyzed using the Atlas™ Human Cancer 1.2 Array. When we analyzed the data for correlations between gene expression levels and tumor response to chemotherapy, there was a significant increase in the expression of nine genes in non-responders compared with responders to chemotherapy ($p < 0.01$). Multivariate regression analysis revealed that allogenic inflammatory factor, HLA-DR antigen associated invariant subunit and MHC class II HLA-DR-beta precursor were independent chemo-resistant factors ($p<0.0001$). When we analyzed the differences in gene expression levels between patients with SCLC and NSCLC, expression levels of one or more resistant genes were increased in comparison with the mean expression of control house keeping genes in five of 18 SCLC patients and 19 of 29 NSCLC patients, respectively ($p=0.012$).

In conclusion, some chemo-resistant genes were detected in the tumor tissue of lung cancer patients using cDNA microarray analysis. A prospective study is required to confirm whether expression levels of these genes reflect chemosensitivity.

Key words: microarray, chemoresistance, gene, lung, cancer

INTRODUCTIONN

Lung cancer is a leading cause of cancer death and most patients with this disease are candidates for chemotherapy. Small cell lung cancer (SCLC) is one of the most chemosensitive tumors, and reduces by chemotherapy in 80 to 90% of the patients. On the other hand, non-small cell lung cancer (NSCLC) is moderately effective responsive to chemotherapy and reduces by chemotherapy in 30 to 40% of the patients. The mechanisms of the difference of in chemosensitivity between SCLC and NSCLC patients have not been sufficiently enough examined, although responders to chemotherapy may have a better prognosis than non-responders (1). On the contrary, a large proportion of cancer patients suffer adverse

effects of with chemotherapy while showing no effective response in terms of tumor regression. Accordingly, it is important to be able to predict likely responders before subjecting patients to chemotherapy. Unfortunately, no reliable predictor of response has not yet been found.

A linear correlation was reported between the extent of gene-specific damage in human adenocarcinoma cells in pleural effusions and in mononuclear cells of peripheral blood (MNC) from lung cancer patients exposed to cisplatin *in vitro* prior to chemotherapy (2). When we examined the extent of gene-specific damage in MNC incubated with cisplatin *in vitro* before chemotherapy, the DNA damage to MNC was greater in responders in to chemotherapy showed that DNA damage in MNC was greater than that in non-responders (3). The PCR-stop assay measures DNA damage in specific genes and can be applied used to the measurement of DNA damage caused by a variety of anticancer agents. We have also demonstrated that this assay can detect the difference in DNA damage between VP-16-sensitive and resistant cells (4), and that it may be able to detect differences in other topoisomerase- related anticancer drugs such as CPT-11 (4). But use of this assay requires that cells should be treated with anticancer drugs before analysis. Moreover, the assay detects only DNA damage in treated cells and could does not clarify which genes influence to a patient's response in to chemotherapy.

The properties of cancer cells are determined by complicated interactions among all gene products expressed in cancer cells, and it is certain that many proteins – including enzymes involved in apoptosis, in DNA repair, and in the metabolism and detoxification of drugs – have individual responses.

The cDNA microarray method is now widely used to analyze the expression of thousands of genes simultaneously in cancer tissues, and its development has facilitated the analysis of genome-wide expression profiles that can generate a large body of information concerning genetic networks related to the response of tumors response to various drugs and the identification ofto identify genes involved in pathological conditions. Thus, the cDNA microarray analysis is a promising method for identifying genes associated with the sensitivity of tumors to various anticancer drugs, using amplified RNA extracted from a very small piece of biopsy sample from cancer patients. (5,6)

Large-scale gene expression microarray studies of lung cancer (7,8) have shown that altered expression of various genes is associated with a significantly worse prognosis. Theose data were under the influenced by of several factors, such as the response of the tumor effect ofto chemotherapy, the adverse effects of chemotherapy on patients, by chemotherapy and tumor progression and metastasis. The genetic informations are is required with regard to not only survival but also tumor response to effect and adverse effects by with chemotherapy.

Staunton *et al.* identified putative predictive markers of chemosensitivity and showed the feasibility of chemosensitivity prediction by transcriptional profiling (7). Different sets of genes were identified which may act as predictive markers for chemosensitivity to drugs in human cancer cell lines or tumor tissues using cDNA microarray (8-10). However, in these studies the predictive markers were identified as using *in vitro* or animal experiments, although the markers are required to be able to predict chemosensitivity in human cancer chemotherapy.

Therefore, we used cDNA microarray screening in the following study to examine the expression levels of specific genes expressions in tumor tissue, which was obtained through by transbroncheal biopsy, in order to determine any correlations withe tumor effect response to chemotherapy using cDNA microarray.

PATIENTS AND METHODS

Patients

This study was approved by the Institutional Review Boards of Kanagawa Cancer Center. The patients with histologically proven lung cancer treated with chemotherapy were entered into the present study. All were eligible for treatment. They had an expected survival of at least 6 six weeks,; measurable lesions,; Eastern Cooperative Oncology Group (ECOG) performance status (PS) score ≤ 3; white blood count ≥ 4,000/μl; hemoglobin ≥ 10 g/dl; platelet count ≥ 100,000/μl,; total serum bilirubin < 2 mg/d; aspartate aminotransferase and alanine aminotransferase less than twice the upper limit of the normal range; serum creatinine ≤ 1.5 mg/dl; and creatinine clearance > 50 ml/min. None of the patients had received prior chemotherapy for the primary lesion. Written informed consent for chemotherapy and a genetic analysis of tumor tissue was obtained in every case.

Chemotherapy

All patients with non-progressive cancer were treated with two or more courses of chemotherapy. Response criteria were evaluated according to the World Health Organization (WHO) criteria (11).

Tumor samples

Transbroncheal biopsy specimens of tumors were obtained before chemotherapy. The half of the

specimens were fixed in formalin-fixed for use for pathological diagnosis and another the other half were immediately frozen for storage at -80°c until genetic analysis.

Extraction and Purification of RNA and Preparation of Probes

The total RNA of each sample was isolated and treated with DNase I to avoid contamination of genomic DNA by silica membrane affinity chromatography using Macherey-Nagel's total RNA isolation kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). One hundred nanograms of the total RNA for each sample was reverse transcribed into cDNA and amplified by SMART polymerase chain reaction (PCR) technology (Chenchik et al.12) with using the Super SMART™ PCR cDNA Synthesis kit (BD Biosciences Clontech, CA, USA) according to the manufacturer's instructions. To represent the expression profile of the starting initial total RNA material, the optimal conditions for PCR cycling determined for each sample by testing the amplified cDNA with gel electrophoresis. All samples were amplified for 19 to 23 cycles. Each cDNA sample was subjected to microarray expression profioling with using the BD Atlas™ Human Cancer 1.2 Array (Clontech) based on the manufacturer's protocol. The following is a brief overview of the procedures used is as follows. A Rradioactively labeled probe mixture for hybridization with array membranes was synthesized from each cDNA sample by using the CDS Primer Mix specific for the Atlas™ Human Cancer 1.2 Array and [α-^{32}P] dATP.

cDNA Microarray

Each of the labeled probe was then hybridized into a separate Atlas Array. After appropriate washing, array membranes were exposed to a phosphor screen and the signal intensity for each spot, which corresponds to each gene examined, was determined by using a STORM image analyzer (Amersham Bioscience, Picataway, NJ). The hybridization pattern and signal intensity were analyzed to determine changes in gene expression levels using AtlasImage™ 2.01 software (CLONTECH, Laboratory, Inc., Japan).

Statistical methods

T-tests were used to identify differences in mean expression levels between responders and non-responders to chemotherapy. Fisher's exact and χ^2 tests were used to assess whether the frequency of gene - expression was associated with an objective response to chemotherapy. $P < 0.001$ was considered to be significant.

Table I. Patient characteristics

				No. of patients
Total				47
Gender	Male			36
	Female			11
Smokers				38
PS(ECOG)	0			5
	1			30
	2			9
	3			3
Pathology	SCLC	Stage	LD	2
			ED	16
	NSCLC	Stage	IIB/IIIA	4
		IIIB		8
		IV		17

PS, performance status; ECOG, Eastern Cooperative Oncology Group;

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer;

LD, limited disease; ED, extensive disease.

RESULTS

Between September 2000 and December 2001, 47 patients were registered in the study. Patient characteristics are summarized in Table I. Thirty-six patients were male and eleven were female, with a median age of 66 years (range 35–81 years). Thirty-eight patients were smokers. The PS was 0 for five patients; 1 for 30 patients; 2 for nine; and 3 for three patients. Eighteen patients had SCLC, and the remaining had NSCLC. Of the patients with SCLC, 2 two had limited disease and the other 16 had extensive SCLC. Of the patients with NSCLC, four had stage IIB/IIIA, eight had stage IIIB, and 17 had stage IV. No patients had receivedno prior chemotherapy.

All patients except three who had been sub-scribedreceived paclitaxel and irinotecan received were given platinum-based chemotherapy. Three of patients with SCLC and seven of patients with NSCLC received thoracic radiotherapy concurrently or sequentially with chemotherapy (Table II). 16 of the 18 patients with SCLC (89%) and 13 of the 29 patients with NSCLC (45%) responded to chemotherapy, respectively.

The expression levels of 1176 genes expression in the tumor specimens were analyzed using cDNA microarray screening. Four housekeeping genes which were expressed in every all 47 tumor samples in the present study were used as controls for gene

Table II. *Therapeutic regimens*

		No. of patients
SCLC	cisplatin + etoposide	6
	cisplatin + etoposide + TRT	2
	cisplatin + irinotecan	4
	cisplatin + irinotecan + etoposide	2
	carboplatin + etoposide	3
	cisplatin + TRT	1
NSCLC	cisplatin + gemcitabine	7
	cisplatin + vinorelbine	3
	cisplatin + vinorelbine + TRT	2
	cisplatin + vindesine + TRT	3
	cisplatin + irinotecan	1
	cisplatin +TRT	2
	carboplatin + etoposide	1
	carboplatin + paclitaxel	1
	nedaplatin + irinotecan	6
	paclitaxel + irinotecan	3

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; TRT, thoracic radiation therapy.

expression, ubiquitin, liver glyceraldehydes 3-phosphate dehydrogenase, 23-kDa highly basic protein, 60S ribosomal protein L13A and 40S ribosomal protein S9. When we analyzed the data for correlations between gene expression levels and tumor response to chemotherapy, nine genes showed the a significantly different expression in responders to chemotherapy compared with non-responders to chemotherapy (Table III, $p<0.01$). Stepwised multivariate regression analysis revealed that allogenic inflammatory factor, HLA-DR antigen associated invariant subunit and MHC class II HLA-DR-beta precursor were independent chemo-resistant factors (Table IV, $p<0.0001$). Expression levels of the each of these three genes were significantly elevated in non-responders compared with responders, and thus these three independent genes were defined as resistant genes. Furthermore, the expression levels of one or more independent resistant genes were elevated compared to the mean expression level of control genes expression in ten out of 29 responders and 14 out of 18 non-responders, respectively (Table V, $p=0.0039$).

When we analyzed the differences in independent resistant gene expression levels between patients with SCLC and NSCLC, the expression levels of one or more independent resistant genes were elevated compared with the mean expression level of control genes in five out of 18 SCLC patients and 19 of the 29 NSCLC patients, respectively (Table V, $p=0.012$).

DISCUSSION

We examined cancer-related gene expressions in lung cancer samples obtained before chemotherapy using cDNA microarray screening, and analyzed the relationship between gene expression levels and clinical outcome after chemotherapy. We identified three specific genes whose expression levels were correlated with the response of the tumor to chemotherapy. These three resistant genes identified as

Table III. *Genes closely associated with sensitivity in chemotherapy for lung cancer.*

		Expression of each genes compared to control					
		Responder			*Non-responder*		
Description	**Symbol**	**n**	**mean**	**SD**	**n**	**mean**	**SD**	**—**
allograft inflammatory factor 1 (AIF1); ionized calcium-binding adapter molecule 1	U19713	18	1.67	21.13	29	-10.17	7.59	0.0084
lymphocyte antigen	M81141	18	11.39	26.37	29	-4.76	13.85	0.0085
hepatocyte growth factor-like protein; macrophage-stimulating protein (MSP)	M74178	18	15.17	20.48	29	3.55	8.36	0.0092
HLA-DPB1 precursor; HLA class II histocompability antigen SB beta chain	K01615; M83664;	18	3.17	16.99	27	-7.56	8.67	0.0078
IgG receptor FC large subunit P51 precursor (FCRN); neonatal FC receptor; IgG FC fragment receptor transporter alpha chain	U12255	8	28.50	31.89	17	4.47	11.02	0.0096
HLA-DR antigen-associated invariant subunit	X00497	18	130.44	190.18	28	-8.25	57.39	0.0007
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	M20430	15	25.60	31.81	25	-11.08	12.96	<0.0001
HLA class II histocompatibility antigen alpha chain precursor	K01171	18	-3.67	194.09	29	-161.86	65.00	0.0002
vimentin (VIM)	X56134; M14144	18	15.72	252.30	29	-150.83	126.75	0.0043

Four housekeeping genes were used as controls for gene expression.

Table IV. Stepwized multivariate regression analysis on chemotherapeutic response.

Description	coefficient	SE
allograft inflammatory factor 1	-0.014	0.002
HLA-DR antigen-associated invariant subunit	-0.001	0.0003
MHC class II HLA-DR-beta (DR2-DQW1/DR4 DQW3) precursor	-0.010	0.002

Coefficient; responder 1, non-responder 0

Table V. Correlation between resistance gene expression and objective response to chemotherapy.

		Cases of elevated gene expression of independent resistant genes			*p*
		1 to 3 genes	0 gene	Total	
Pathology	SCLC	5	13	18	0.012
	NSCLC	19	10	29	
Response to chemotherapy	Responder	10	19	29	0.0039
	Non-responder	14	4	18	

Elevated gene expression was defined as higher expression than mean of expressions of 4 house keeping genes. SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer.

predictive markers of chemotherapy response in the present study had a role in host immunity. Anticancer drugs cause rapid and persistent depletion of lymphocytes, possibly by the direct induction of apoptosis in mature T and B cells. *In vivo* chemotherapy induces a significant increase in lymphocyte apoptosis *ex vivo*. Chemotherapy-induced lymphocyte depletion involves distinct mechanisms of apoptosis induction, such as direct mitochondrial and caspase-dependent pathways in resting lymphocytes and p53-dependent pathways in cycling lymphocytes (13). Furthermore, we have previously shown that responders to chemotherapy demonstrate greater gene-specific damage in MNC compared with non-responders (3). These data support the observation that most of the genes identified as resistant genes in the present study were involved in host immunity. The expression level of each resistant gene in our study was elevated in non-responders, and was expected to oppose apoptosis induction by anticancer drugs *in vivo*. Upon reference to other studies, it was confirmed that three genes involved in host immunity had some influence on a patient's response to treatment.

Some researchers have described an activation of these immunity-related genes with chemotherapy, such as interferon (IFN) treatment. One investigation demonstrated that IFN-treated renal cell carcinoma (RCC) cells induced HLA-DR expression. A significant correlation was found between the expression of an MHC antigen-associated invariant chain and the degree of lymphocyte infiltration (14). A previous genetic analysis study has demonstrated that MHC class II genes influence the outcome of chronic C hepatitis treatment with IFN (15). The findings of this study are not applicable to the treatment of cancer with chemotherapy; some human cancers such as melanomas and RCC are also treated with IFN, and the gene may be implicated in the mechanism of chemosensitivity of cancer cells. Keratinocyte-bound HLA-DR antigens were observed after treatment with IFN in melanomas and RCC (16). The allograft inflammatory factor-1, which is encoded within the HLA class III genomic region, is a modulator of the immune response during macrophage activation (17,18). From these data, it may be suggested that host immune response is closely related to tumor depression by anticancer drugs.

Using computational analysis, in the study reported here, we selected genes likely to be associated with chemo-resistance, and were able to distinguish SCLC from NSCLC according to the different biological natures of the genes. The expression levels of resistant genes were elevated in about two thirds of NSCLCs, but not in most of the SCLCs in the present study. The data indicated that SCLC is highly sensitive to chemotherapy, while NSCLC is only moderately sensitive.

We need to undertake prospective evaluations to determine whether the selected genes in this study are truly important and potentially useful for predicting chemoresistance. It is also necessary to determine whether administration of drugs will result in changes to the expression levels of the resistant genes we identified, and if any such changes are related to tumor response. If the expression level of a gene changes with treatment, that gene will be the new target of

cancer chemotherapy. In this study we measured the expression levels of genes in patients treated with platinum-based chemotherapy. Recently, patients with NSCLC have been treated with non-platinum chemotherapy. It is thus also necessary that the expression levels of our resistant genes can be used to predict clinical outcome with non-platinum chemotherapy. Accumulation of these data could eventually lead to the prescription of "personalized chemotherapy" with effective anticancer drugs.

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Prognostic Impact of Survivin, Cyclin D1, Integrin β1, and VEGF in Patients With Small Adenocarcinoma of Stage I Lung Cancer

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Abstract: The purpose of this study was to investigate the impact of survivin, cyclin D1, integrin β1, and vascular endothelial growth factor (VEGF) in tumor on survival of patients with small adenocarcinoma of the lung. Seventy-two patients with pathologic stage I resected tumors <2 cm in diameter were entered into the study. Each patient underwent curative surgical resection for lung cancer between July 1992 and November 1999. The resected tumors were subjected to immunostaining for each gene. Thirty-five, 26, 6, and 16 patients had tumors with >10% survivin-, >20% cyclin D1-, >10% integrin β1-, and >10% VEGF-positive cells, respectively. When the survival of 72 patients was compared according to each gene expression, the overall survival of patients with positive expression of survivin, cyclin D1, and integrin β1 was significantly worse than that of individuals whose tumors had negative expression of each gene. By multivariate analysis controlling for each gene expression, no gene expression was an independent marker of poor prognosis, however, the overall survival of the complex gene expression (2 or more gene-positive) group (n = 35) was significantly worse than that of 0 or 1 gene-positive group (n = 37; log-rank test, $P = 0.0011$; Wilcoxon test, $P = 0.0011$). When the association between survival and pathologic factors, including lymphatic invasion, venous invasion, type of bronchioalveolar carcinoma, and complex gene positive expression was analyzed, only complex gene-positive expression was found to be a significant independent factor (hazard ratio = 0.085, $P = 0.0299$). It can be concluded that multiple increased expression of oncogene is a poor prognostic factor in patients with small adenocarcinoma of the lung.

Key Words: oncogene, lung cancer, prognostic factor

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Non–small-cell lung cancer (NSCLC) is the leading cause of cancer death in Japan. To improve the prognosis of lung cancer patients, attempts have been made to develop tests that will facilitate the early diagnosis and treatment of lung cancer and thereby decrease the mortality from this disease. Many chest roentgenogram-negative lung cancers can be detected on chest computed tomography scans, but a significant number of patients with early stage disease show aggressive tumors. Although locoregional control of NSCLC can be achieved by surgery, more than 70% of relapses in patients with stage I disease occur at distant sites.[1] Thus, most patients with NSCLC must have systemic disease, even at the earliest stage. Recent efforts at improving the management and outcome of patients with this disease have been directed at neoadjuvant and adjuvant chemotherapy to reduce the high systemic relapse sites.

New therapeutic strategies for NSCLC are required a better understanding of the cell biology of early stage NSCLC. Several molecular markers have been evaluated in association with established histologic and clinical prognostic parameters of early stage NSCLC,[2–7] and it is suspected that tumor invasion and metastasis involve complex alterations of gene expression that may be selective for specific cancer types. However, none is currently being used in treatment decision making.

Initiated cancer cells at early stage disease are considered to acquire other gene alterations in addition to early genetic alteration, and progress to locally advanced or metastatic tumors. Many genetic alterations, which relates to cell proliferation, apoptosis, vascularization, and tumor invasion, were reported as prognostic factors in resected NSCLC. However, there is no study showing which gene alterations mostly influence tumor progression and metastasis in the early stage of NSCLC. Clarification of the gene alterations that influence tumor progression from early to advanced stage in NSCLC is required when considering new therapeutic strategies for resectable NSCLC.