

CI) for these ERCC1 alterations was 0.53 (0.28-1.01, $p = 0.055$), although each study failed to show statistical significant association. Thus, ERCC1 may be a candidate for evaluation of the predictability of drug response in future clinical trials.

TP53, which is mutated or deleted in more than half of lung cancer cells, has a remarkable number of biological activities, including cell-cycle checkpoints, DNA repair, apoptosis, senescence, and maintenance of genomic integrity. Because most anticancer cytotoxic agents induce apoptosis through either DNA damage or microtubule disruption, mutated TP53 may decrease chemosensitivity by inhibiting apoptosis or, in contrast, may increase chemosensitivity by impairing DNA repair after drug-induced DNA damage.²⁹ This review showed that mutated TP53 was associated with poor drug response in patients with non-small-cell lung cancer (Table 6).

No other genes located downstream (including xeroderma pigmentosum group D, retinoblastoma 1, cyclin-dependent kinase inhibitor 1A, Kirsten rat sarcoma 2 viral oncogene homolog, B-cell CLL/lymphoma 2, and B-cell CLL/lymphoma 2-associated X protein) were associated with clinical drug response (Tables 5-7). The association was evaluated for only 8 of 43 *in vitro* chemosensitivity-associated downstream genes; therefore, key genes may be among the remaining 35 genes. Most clinical studies included a limited number of patients with various background characteristics such as tumor stage and chemotherapy regimen administered, which resulted in low statistical power to identify the association. Finally, because all but one study was retrospective, the quality of tumor samples may vary, and it is therefore unclear whether the gene alteration was detected in all samples. Thus, in future prospective clinical studies, the method of tumor sample collection and preservation, as well as immunohistochemistry and polymerase chain reaction-based methods, should be standardized, and the sample size of patients should be determined with statistical consideration.

The recently developed microarray technique enables investigators analyze mRNA expression of more than 20,000 genes at once, and as many as 100 to 400 genes were selected statistically as chemosensitivity-related genes.^{6-8,10} Among them, however, only a limited number of genes were functionally related to chemosensitivity, and only ABCB1 and BAX corresponded with the 80 chemosensitivity-associated genes identified in this literature review, which were picked because of their known function and contribution to *in vitro* chemosensitivity. Thus, it will be interesting to evaluate the role of expression profile of these genes using microarray analysis.

The association between the expression and alterations of genes and clinical drug responses should be studied further in prospective trials. ABCB1, GSTP1, ERCC1, and TP53, and other genes identified by exploratory microarray analyses should be evaluated in those trials. Simple methods to identify gene alterations, such as immunohistochemistry and polymerase chain reaction-based techniques, will be feasible in future clinical trials because of their simplicity, cost, and

time. The median number of patients in retrospective studies analyzed in this review was 50 (range, 28-108). In future prospective trials, sample size consideration for statistical power will also be important.

In conclusion, we identified 80 *in vitro* chemosensitivity-associated genes in a review of the literature; ABCB1, GSTP1, and ERCC1 expression and TP53 mutation were associated with drug responses among patients with lung cancer.

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Phase I pharmacokinetic and pharmacogenomic study of E7070 administered once every 21 days

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E7070 is a novel sulfonamide anticancer agent that disrupts the G₁/S phase of the cell cycle. The objectives of this phase I study of E7070 were to estimate the maximal tolerated dose (MTD), to determine the recommended dose for phase II, and to clarify the pharmacokinetic profile of E7070 and its relation to polymorphisms of CYP2C9 (*2, *3) and CYP2C19 (*2, *3) in Japanese patients. Patients received 1–2-h i.v. infusions of E7070 (400, 600, 700, 800 or 900 mg/m²) on day 1 of a 21-day cycle. Twenty-one patients received between one and eight cycles of E7070. The dose-limiting toxicities (DLT) comprised leukopenia, neutropenia, thrombocytopenia, elevation of aspartate aminotransferase, colitis, and ileus. The mean area under the plasma concentration–time curve (AUC) for successive dose levels increased in a non-dose-proportional manner. Two patients were heterozygous for the CYP2C9 mutation. For CYP2C19, eight patients were wild type and the remainder had heterozygous (*n* = 8) or homozygous mutations (*n* = 5). Regarding the CYP2C19 genotype, the AUC of patients with mutant alleles were higher than those of patients with wild type at a dose of 600 mg/m² or more. The severity of toxic effects, such as myelosuppression, seemed to depend on the AUC. No partial responses were observed. One patient treated at a dose of 700 mg/m² experienced a maximum tumor volume reduction of 22.5%. The MTD was estimated to be 900 mg/m². A dose of 800 mg/m² is recommended for further phase II studies. The pharmacokinetic/pharmacodynamic properties of E7070 seemed to be influenced by CYP2C19 genotype. The observed safety profile and preliminary evidence of antitumor activity warrant further investigation of this drug in monotherapy or in combination chemotherapy. (*Cancer Sci* 2005; 96: 721–728)

E7070, *N*-(3-chloro-7-indolyl)-1,4-benzenedisulfonamide, has been reported to exhibit a potent antitumor activity by blocking cell cycle progression. *In vitro* studies indicate that the drug disrupts the G₁/S phase, thereby inducing cell cycle arrest and apoptosis.⁽¹⁾ Although E7070 is not a direct inhibitor of cyclin-dependent kinases (CDK), it causes depletion of cyclin E, with a reduction in CDK2 catalytic activity.⁽²⁾ The exact mechanism of cyclin E/CDK2 inactivation is unclear. Transcriptional repression of cyclin H in a p53-independent manner also occurs in response to E7070.⁽³⁾ The reduction in G₁ CDK activity induces arrest at the G₁/S boundary, accompanied by hypophosphorylation of retinoblastoma (Rb) protein and decreases in CDK2, cyclin A, and cyclin B proteins.⁽¹⁾ E7070 activity is associated with upregulation of p53 and p21, which may contribute to the

reduced Rb phosphorylation, as well as subsequent apoptosis. In preclinical models, E7070 was cytotoxic to human HCT116 colon carcinoma and LX-1. E7070 exhibits more potent *in vivo* antitumor effects than 5-fluorouracil, mitomycin C, and irinotecan.⁽⁴⁾

E7070 has been the subject of four clinical phase I studies. In the first trial, E7070 was administered once every 21 days at doses between 50 and 1000 mg/m²,⁽⁵⁾ and in the second trial E7070 was administered five times per day once every 3 weeks at doses between 10 and 200 mg/m² per day.⁽⁶⁾ Other schedules used were a weekly infusion given over 4 consecutive weeks repeated every 6 weeks,⁽⁷⁾ and a continuous intravenous infusion for 5 days every 3 weeks.⁽⁸⁾ In the single-dose every 3 weeks study, neutropenia and thrombocytopenia were dose-limiting at 700 and 800 mg/m².⁽⁵⁾ In the second study, neutropenia and thrombocytopenia were dose-limiting at 160 and 200 mg/m².⁽⁶⁾ In the study that used a weekly dose schedule, neutropenia and thrombocytopenia were also dose-limiting toxicities (DLT) and other DLT included stomatitis, diarrhea, nausea, and fatigue.⁽⁷⁾ Partial responses were observed in patients with breast and endometrial cancer.^(6,7)

During a phase I trial, three patients receiving prophylactic daily oral maintenance therapy with acenocoumarol developed a hemorrhagic tendency and/or a prolonged prothrombin time following treatment with 700 and 800 mg/m² of E7070.⁽⁵⁾ The major metabolic enzyme for acenocoumarol is cytochrome P450 (CYP)2C9.⁽⁹⁾ *In vitro* studies have shown that E7070 has the potential to inhibit CYP2C9 and CYP2C19.⁽¹⁰⁾ The pharmacokinetic drug–drug interaction study indicated that primary interaction of the two drugs could occur via inhibition by E7070 of acenocoumarol metabolism.

Based on these results from the previous phase I and pharmacokinetic trials, the present phase I study was designed to evaluate ascending doses of E7070 administered as a single dose by 1–2-h i.v. infusion every 21 days. The objectives of the study were to determine the maximum tolerated dose (MTD) and the dose to be recommended for use in future phase II studies, as well as to assess the safety, pharmacokinetic profile and preliminary antitumor activity of the drug. We also evaluated the influence of genetic polymorphisms of CYP2C9 and CYP2C19 on the pharmacokinetics of E7070.

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Materials and Methods

Patient selection

Japanese patients with histologically or cytologically confirmed malignant solid tumors refractory to conventional chemotherapy, or tumors for which no effective therapy was available, were candidates for this study. Eligibility criteria included the following: age between 20 and 75 years; World Health Organization (WHO) performance status 0–1, life expectancy ≥ 3 months, absolute leukocyte count $\geq 4000/\mu\text{L}$ and $< 12\,000/\mu\text{L}$, absolute neutrophil count $\geq 2000/\mu\text{L}$, hemoglobin level ≥ 9 g/dL, platelet count $\geq 100\,000/\mu\text{L}$, serum creatinine level < 1.5 mg/dL or creatinine clearance ≥ 50 mL/min, and arterial partial pressure of oxygen of 65 torr or more. Additional entry criteria were serum bilirubin ≤ 1.5 mg/dL, and serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) ≤ 100 IU/L. Before study entry, a 6-week interval was required for patients previously treated with mitomycin C or nitrosoureas, a 4-week interval was required for other chemotherapy, endocrinotherapy, surgery, radiation therapy, immunotherapy treatments or other investigational agents, and a 2-week interval after blood transfusion or administration of granulocyte-colony stimulating factor (G-CSF). Patients were ineligible for the study if they had symptomatic central nervous system metastases, active infection, or other non-malignant disease, which was considered to be incompatible with the protocol. Patients who were receiving corticosteroids or coumarin anticoagulants less than 2 weeks prior to administration of E7070 were excluded from the study. The protocol was approved by the institutional review boards of the National Cancer Center, and all patients gave written informed consent prior to study entry.

Dosage and dose escalation

E7070 was provided as a lyophilized powder in 500-mg vials by Eisai Co. (Tokyo, Japan). The starting dose of E7070 was set at 400 mg/m^2 because only mild to moderate grade 1 to grade 2 toxicity was observed at doses of 600 mg/m^2 or lower in the previous phase I study.⁽⁵⁾ Subsequent doses were to be escalated to 600, 700, 800 and 900 mg/m^2 . E7070 was dissolved in 20 mL of distilled water, then added to 500 mL of normal saline for injection, and this solution was administered by intravenous infusion over 1 h at doses of 400 and 600 mg/m^2 . Injection site reaction was observed in one of three patients at 400 mg/m^2 and three of three patients at 600 mg/m^2 . Therefore, E7070 was given in 1000 mL of normal saline over 2 h at 700, 800, and 900 mg/m^2 . Patients were hospitalized for the first course of E7070 and remained hospitalized for close observation for 21 days thereafter. Subsequent courses could be administered on an outpatient basis with weekly patient evaluations by the investigator.

Patients were enrolled in cohorts of three patients per dose level and observed for 21 days; the observation period was extended to 42 days if a longer recovery period was needed. If one of the three patients experienced DLT, then three additional patients were treated at the same dose. If two or more of three or six patients experienced DLT, that dose level was regarded as the MTD. If none of the first three patients demonstrated DLT, then the next three patients were treated at the

next (higher) dose level. Individual patients who did not demonstrate DLT and showed no evidence of disease progression could receive E7070 at the originally assigned dose.

After the MTD had been determined, a dose below the MTD was evaluated in a total of six patients for identification of the proposed recommended dose for phase II study. The recommended dose was the highest dose at which less than 33.3% of treated patients experienced DLT.

Definition of dose-limiting toxicity

The DLT was defined as the occurrence of any of the following events during cycle 1 that were attributable to E7070: National Cancer Institute Common Toxicity Criteria (NCI CTC) (version 2.0) grade 3 or 4 non-hematological toxicity (except nausea, vomiting effectively managed with symptomatic treatment, or alopecia), grade 4 leukopenia, grade 4 neutropenia accompanied by fever of $\geq 38.5^\circ\text{C}$, or that persisted ≥ 5 days, and platelet count $< 25\,000/\mu\text{L}$. Prophylactic use of colony-stimulating factors was not permitted during the first cycle; however, patients who had neutropenia that had met the criteria for DLT were permitted to receive concomitant treatment with G-CSF.

Evaluation of patients

Safety was evaluated on the basis of physical findings, vital signs, adverse events, and laboratory parameters obtained at baseline and periodically throughout the study. During the first cycle, hematology studies were performed at least twice a week, while vital signs, physical examinations (including evaluation of performance status) and serum biochemistry were measured on days 1, 8 and 15. Toxicity evaluations of subjective and objective findings were performed according to the NCI CTC (version 2.0) on days 1, 8 and 15. For the second and subsequent cycles, vital signs, laboratory tests and toxicity evaluations based on subjective and objective findings were performed on days 1, 8, and 15 of each cycle. Blood glucose was monitored before the dose of E7070 and after the end of infusion. Blood coagulation studies were carried out before each dose for all cycles and also at day 8 of the first cycle. Efficacy was assessed by the physician on the basis of antitumor effect according to the Response Evaluation Criteria in Solid Tumors (RECIST).⁽¹¹⁾ If an antitumor effect was observed, the disease site was re-evaluated at least 4 weeks later to confirm the response.

Pharmacokinetics

Pharmacokinetic studies were performed during the first cycle of treatment. On day 1, blood samples (4 mL each) were drawn from an indwelling intravenous cannula in the arm contralateral to that bearing the infusion line. Samples were collected in heparinized tubes, preinfusion, at 30 min after the start of the infusion, at the end of the 1- or 2-h infusion, and at 10, 30, and 60 min and 2, 4, 6, 10, 24, 48, 72, 96, 168, and 240 h after the infusion. The samples were centrifuged at $1500\times g$ for 10 min at 5°C , and the resulting plasma samples were stored at -20°C until analysis. Urine samples were collected before the start of E7070 infusion and over three 24-h intervals for 72 h after the start of the infusion. The concentrations of E7070 in plasma and in urine were analyzed at Eisai Co. by means of validated high-performance

liquid chromatography methods with UV detection (HPLC-UV). The lower limit of quantification was 20 ng/mL. *N*-(3-Chloro-7-indolyl)-4-(*N*-methylsulfamoyl)benzenesulfonamide (ER-67771)⁽¹²⁾ was used as an internal standard.

Plasma, the internal standard and 0.1 mol/L phosphate buffer (pH 6.8) were vortexed. After adding ethyl acetate, the mixture was shaken and centrifuged. The organic layer was collected and transferred into a glass tube, then evaporated under nitrogen flow in a drying block. The residue was dissolved in CH₃CN-6.7 mmol/L phosphate buffer (pH 6.6), and the solution was injected into an HPLC apparatus.

Chromatographic separation was achieved by using a column switching method with Asahipak C8P-50 (Showa Denko, Tokyo, Japan) as a separation column and YMC-pack ODS-AM-312 (YMC) as an analytical column. Mobile phases were CH₃CN: 6.7 mmol/L phosphate buffer (pH 6.6; 360:640 [v:v]) for separation and CH₃CN: 6.7 mmol/L phosphate buffer (pH 7.4; 360:640 [v:v]) for analysis. E7070 was monitored by UV detection at 280 nm.

Pharmacokinetic parameters of E7070 after a single dose administration during the first cycle were determined by non-compartmental analysis using WinNONLIN (Pharsight Corporation, CA, USA). The apparent elimination rate constant at the terminal phase (λ_z) was estimated by linear regression analysis from the terminal log-linear declining phase to the last quantifiable concentration. The elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln(2)/\lambda_z$. The area under the plasma concentration-time curve from zero to the last quantifiable sampling time, AUC_(0-n), was obtained by the log trapezoidal rule. The AUC from zero to infinity was calculated as AUC_(0-n) + C_n/ λ_z , where C_n was the last quantifiable concentration. The clearance was calculated as dose/AUC. The mean residence time (MRT) was estimated from AUMC/AUC, where AUMC is the first moment curve. The volume of distribution was calculated as MRT × clearance.

Genotyping procedures for CYP2C9 and CYP2C19

Genotyping was conducted using the Invader assay (BML, Tokyo, Japan). Genomic DNA was isolated from whole blood with the QIAamp DNA Blood Kit (Qiagen, CA, USA). The primary probes (wild type and mutant probes) were used to detect C430T (*2) and A1075C (*3) mutations of CYP2C9, and G681A (*2) and G636A (*3) of CYP2C19, respectively. The invader assay fluorescent resonance energy transfer (FRET)-detection 96-well plates (Third Wave Technologies, WI, USA) contained Cleavase enzyme, FRET probes, MOPS buffer and polyethylene glycol. Eight microliters of mixtures consisting of an appropriate primary probe, Invader oligonucleotide and MgCl₂ was added to the wells, followed by addition of 7 μ L of the heat-denatured genomic DNA, and this was overlaid with 15 μ L of mineral oil. For only CYP2C9*2 (C430T) detection, a dilution of the CYP2C9-specific polymerase chain reaction (PCR) product was used instead of genomic DNA, because the CYP2C9*2 (C430T) detection point has the same sequence on CYP2C19. The plates were incubated at 63°C for 4 h for genomic DNA or 1 h for the PCR product. The fluorescence intensities were measured on a Cytofluor 4000 fluorescence plate reader (Applied Biosystems, CA, USA) with excitation at 485/20 nm (wavelength/bandwidth) and emission at 530/

Table 1. Patients' characteristics

No. entered	21
Age (years)	
Median	57
Range	35-70
Male:female (no. patients)	15:6
WHO performance status (no. patients)	
0	7
1	14
Tumor type (no. of patients)	
Colorectal	15
SCLC	2
Gastric	1
NSCLC	1
Liposarcoma	1
Mesothelioma	1
Prior treatment	
Chemotherapy	
No. prior regimens (no. patients)	
0	0
1	1
2	5
>3	15
Surgery (no. patients)	18
Radiation therapy (no. patients)	7

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; WHO, World Health Organization.

25 nm for FAM dye detection, and excitation at 560/20 nm and emission at 620/40 nm for RED dye detection.

Subjects having either the *2 or *3 allele (*1/*2 or *1/*3) were defined as hetero extensive metabolizers (hetero EM), those with two mutated alleles (*2/*2, *2/*3 or *3/*3) as poor metabolizers (PM), and those with no mutated alleles (*1/*1) as homo EM.

Results

Patients' characteristics

Twenty-one patients (15 male and six female) were enrolled into the study (Table 1). All patients had a good performance status and had received previous chemotherapy regimens. The colon/rectum was the most commonly noted primary disease site. All patients were evaluable for toxicity during the first cycle, and for efficacy. Twenty-one patients received 42 cycles of treatment. The median number of cycles administered per patient was one (range, 1-8).

Dose escalation and identification of DLT, MTD, and the recommended phase II dose

The DLT in this study were leukopenia, neutropenia, thrombocytopenia, elevation of AST, colitis, and ileus. None of the patients treated at any dose of less than 800 mg/m² experienced DLT. At a dose of 900 mg/m², two of three patients experienced dose-limiting leukopenia, neutropenia, and thrombocytopenia, identifying this dose level as the MTD. At the same dose, grade 3 colitis and grade 3 AST elevation were observed in one patient. Therefore, three additional patients were enrolled at 800 mg/m². One of the additional three patients evaluated for safety at 800 mg/m² experienced

Table 2. Hematological toxicities during the first cycle of treatment with E7070

Toxicity	Grade	Dose (mg/m ²)				
		400 (n = 3)	600 (n = 3)	700 (n = 6)	800 (n = 6)	900 (n = 3)
Neutropenia	1/2	0	0	3	1	0
	3/4	0	2	0	3	3
Leukopenia	1/2	1	2	3	3	1
	3/4	0	0	0	2	2
Thrombocytopenia	1/2	0	2	1	2	0
	3/4	0	0	0	1	3
Anemia	1/2	1	2	4	2	2
	3/4	0	0	0	1	1

DLT of grade 3 ileus. This patient had previously undergone intestinal surgery for colon cancer. On the basis of these findings, a total of six patients were treated at the dose of 800 mg/m² and one of the six patients experienced DLT. Thus, based on protocol-defined criteria, the MTD was estimated to be 900 mg/m². Therefore, a dose of 800 mg/m² is the recommended dose for single-agent phase II studies.

Hematological toxicity

Neutropenia, leukopenia, and thrombocytopenia were the hematological toxicities observed most commonly during the first cycle (Table 2). Neutropenia was the principal hematological toxicity in this study and was dose-limiting at 900 mg/m². Eight patients treated at 600, 800 and 900 mg/m² experienced grade 3 or more neutropenia. In these patients the median times to nadir neutrophil counts were 12.5 (8–25) days in the first cycle and 15.5 (8–25) days in all cycles, and the median times to recovery from nadir to grade 1 were 5.0 (3–15) days in the first cycle and 6.0 (3–15) days in all cycles. Neutrophil counts recovered to grade 1 within 21 days after E7070 infusion in all patients treated with 400 mg/m², but had not recovered by day 22 in two, one and two patients at 600, 700 and 800 mg/m², respectively. Neutrophil counts recovered by day 29 after E7070 infusion in all patients. G-CSF support was provided during cycle 1 in two of three patients treated at 900 mg/m². One patient treated at 800 mg/m² and three patients treated at 900 mg/m² experienced grade 3 thrombocytopenia. In patients treated at 800 and 900 mg/m², the median time to nadir platelet counts was 10.0 (7–12) days, and the median time to recovery from nadir to grade 1 was 5.0 (2–9) days in the first cycle. Anemia, reported in 13 (62%) patients, did not exceed grade 1–2 severity except in two patients at 800 and 900 mg/m². The numbers of patients with blood cell count toxicity did not tend to increase with increasing number of courses of treatment, suggesting that the hematological toxicity of E7070 is not cumulative.

Non-hematological toxicity

The non-hematological toxicities reported commonly during the first cycle were rash, fatigue, stomatitis, alopecia, injection site reaction, diarrhea and constipation (Table 3). These toxicities were generally mild. Grade 3 and grade 4 toxicities were reported in patients treated with 800 or 900 mg/m². Grade 3 ileus and grade 4 constipation associated with the

ileus developed in one patient at 800 mg/m². Grade 3 AST elevation, grade 3 colitis and grade 3 diarrhea accompanying the colitis were observed in one patient at 900 mg/m². The toxicities reported most commonly in subsequent cycles were similar in terms of number of patients affected and severity to those reported during the first cycle of treatment.

Gastrointestinal toxicity, usually mild, was the most common non-hematological toxicity associated with E7070. Diarrhea (grades 1–3) was noted in eight (38%) patients during the first cycle, and the incidence was greater at the 800 mg/m² (5/6) and 900 mg/m² (3/3) doses than at the 400–600 mg/m² doses (none). Severe diarrhea (grade 3) was observed in only one patient, who received 900 mg/m² and had previously undergone surgery for primary colorectal cancer. In almost all cases, nausea and vomiting responded well to antiemetic therapies and patients were able to maintain good oral intake. Mild constipation (grades 1–2) was noted at 600–900 mg/m², except for one patient with grade 4 constipation associated with grade 3 ileus at 800 mg/m². Alopecia was observed in nine (43%) patients. Grades 1–2 injection site reaction, including irritation, pain, or phlebitis, developed in one patient at 400 mg/m² and three patients at 600 mg/m². Therefore, E7070 in 1000 mL of normal saline was given over 2 h at 700, 800, and 900 mg/m². However, three patients at 700 mg/m² and two patients at 800 mg/m² showed injection site reaction, and thus E7070 was given to patients at 900 mg/m² through a central vein. There were no deaths within 28 days of E7070 administration, and none of the deaths that occurred after the study was considered to have been treatment-related.

Pharmacokinetics

Complete pharmacokinetic data sets were obtained in 21 patients. The mean (+SD) plasma concentration-time curves of E7070 are shown in Figure 1. The mean (±SD) pharmacokinetic parameters derived from the plasma concentration are listed in Table 4. After the end of the infusion, plasma concentration of E7070 decreased rapidly for several hours, followed by a slower elimination phase (Fig. 1). During the elimination phase, the E7070 plasma concentration-time profile was convex, which is characteristic of non-linear pharmacokinetics. Maximum plasma concentrations (C_{max}) of E7070 at the 700–900 mg/m² doses were lower than that at 600 mg/m² (Table 4). This is probably related to the change of the infusion time of E7070 from 1 h to 2 h at doses over

Table 3. Non-hematological toxicities during the first cycle of treatment with E7070

Toxicity	Grade	Dose (mg/m ²)				
		400 (n = 3)	600 (n = 3)	700 (n = 6)	800 (n = 6)	900 (n = 3)
Diarrhea	1/2	0	0	0	5	2
	3	0	0	0	0	1
Constipation	1/2	0	1	2	3	1
	3/4	0	0	0	1	0
Nausea	1/2	0	1	3	1	1
	3	0	0	0	1	0
Vomiting	1/2	0	1	0	0	0
	3	0	0	0	1	0
Anorexia	1/2	0	1	2	1	1
	3	0	0	0	1	0
Stomatitis	1/2	1	1	2	3	3
	3	0	0	0	0	0
Injection site reaction	1/2	1	3	3	2	0*
	3	0	0	0	0	0*
Rash	1/2	1	1	5	2	3
	3	0	0	0	0	0
Fatigue	1/2	1	2	2	3	3
	3	0	0	0	0	0
Headache	1/2	1	1	4	2	0
	3	0	0	0	0	0
Alopecia	1/2	0	1	2	3	3

*E7070 was administered through a central vein at a dose of 900 mg/m².

Table 4. Pharmacokinetic parameters of E7070

Dose (mg/m ²)	No. patients	C _{max} (µg/mL)	AUC (µg·h/mL)	CL (mL/min per m ²)	MRT (h)	t _{1/2} (h)	V _{ss} (L/m ²)	Urinary excretion (%)
400	3	82.2 ± 15.4	1066 ± 140	6.3 ± 0.9	26 ± 8	20 ± 5	9.8 ± 1.6	0.82 ± 0.22
600	3	142.8 ± 12.3	4204 ± 1353	2.6 ± 1.0	53 ± 17	32 ± 11	7.6 ± 0.0	1.67 ± 0.13
700	6	116.1 ± 11.3	3300 ± 1058	3.9 ± 1.3	41 ± 12	21 ± 7	8.7 ± 0.9	1.57 ± 0.39
800	6	117.7 ± 8.6	3943 ± 1243	3.6 ± 1.0	45 ± 11	22 ± 4	9.2 ± 0.8	1.77 ± 0.80
900	3	133.8 ± 0.7	6095 ± 1009	2.5 ± 0.4	59 ± 10	27 ± 8	8.7 ± 0.7	2.47 ± 1.33

C_{max}, maximum plasma concentration; AUC, area under the plasma concentration–time curve; CL, clearance; MRT, mean residence time; t_{1/2}, terminal elimination half-life; V_{ss}, distribution volume at steady state; urinary excretion, cumulative excreted amount of E7070 in urine.

600 mg/m² because of injection site reaction. The AUC increased more than expected with increasing dose. The clearance decreased between 400 and 900 mg/m², with mean values of 6.3 mL/min per m² to 2.5 mL/min per m². The mean plasma half-life (t_{1/2}) was between 20 and 32 h at the examined doses. Mean 72-h urinary excretion was 0.82% to 2.47% of the administered dose of E7070 in the five cohorts.

Pharmacodynamics

The pharmacodynamic analysis was performed by focusing on leukopenia, neutropenia and thrombocytopenia, because these were the DLT of E7070. Figure 2 shows that the nadirs of white blood cells (WBC), neutrophils, and platelets were related to the AUC of E7070. The percentage decrease rate from the value before dosing to the nadir of WBC, neutrophil or platelet count also showed a good correlation with the AUC of E7070.

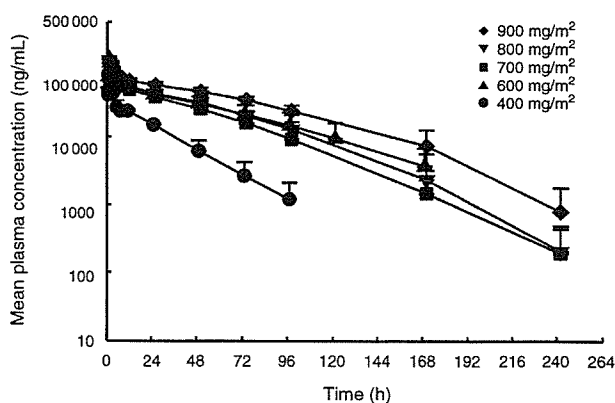


Fig. 1. Mean plasma concentrations of E7070 after single intravenous infusion at each dose level. Circles, 400 mg/m²; triangles, 600 mg/m²; squares, 700 mg/m²; inverted triangles, 800 mg/m²; diamonds, 900 mg/m². Each point represents the mean with standard deviation.

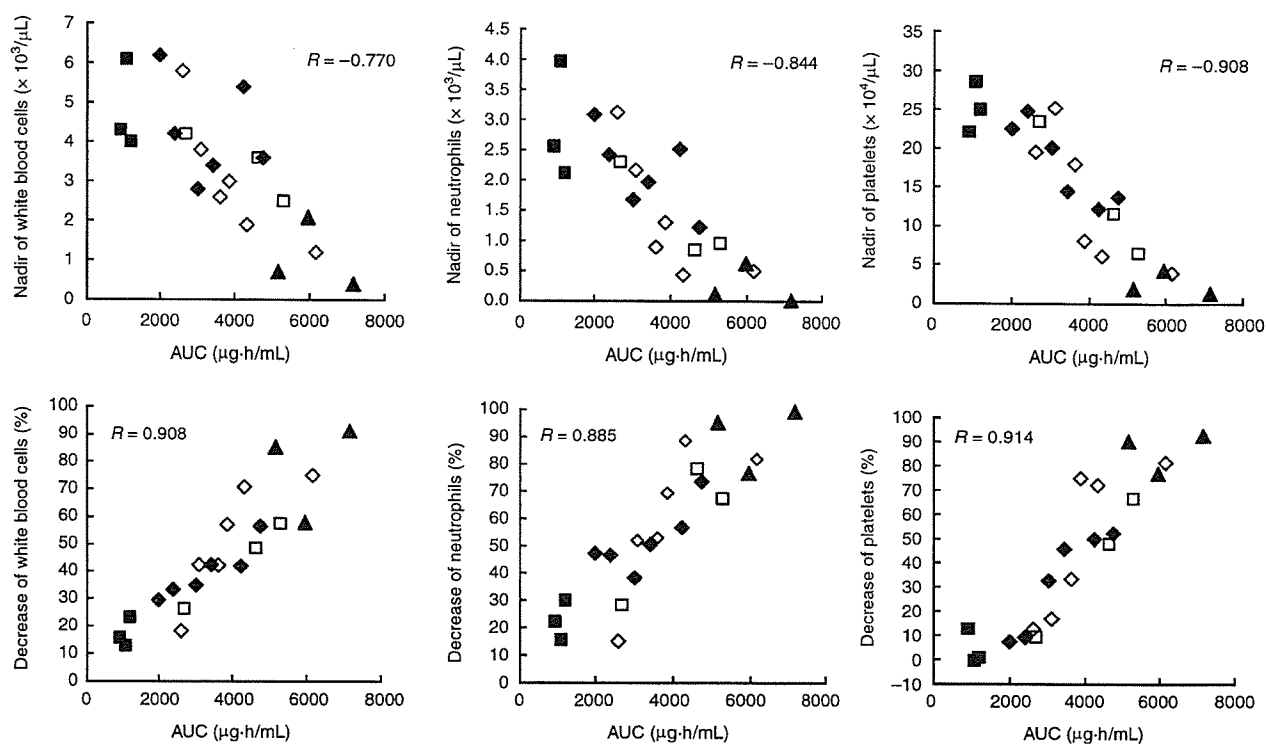


Fig. 2. Relationship between the area under the plasma concentration–time curve (AUC) of E7070 and white blood cells, neutrophil and platelet counts. Closed squares, 400 mg/m²; open squares, 600 mg/m²; closed diamonds, 700 mg/m²; open diamonds, 800 mg/m²; closed triangles, 900 mg/m². R, Pearson's correlation coefficient.

Genotyping of CYP2C9 and CYP2C19

The CYP2C9 and CYP2C19 genotypes were studied in 21 patients. Two (10%) were hetero EM for CYP2C9 (*1/*3), and 19 (90%) were homo EM for CYP2C9 (*1/*1). Five (24%) were PM for CYP2C19 (*2/*2 or *2/*3), eight (38%) were hetero EM for CYP2C19 (*1/*2 or *1/*3) and eight (38%) were homo EM for CYP2C19 (*1/*1). Figure 3 shows the relationship between dose and AUC of E7070 with respect to CYP2C9 and CYP2C19 genotypes. At a dose level of 600 mg/m² or more, the AUC of patients with mutant allele(s) (PM and hetero EM) of CYP2C9 or CYP2C19 were higher than those of the patients without mutant alleles (homo EM). DLT was observed in one CYP2C19 PM patient at 800 mg/m² and two CYP2C19 hetero EM or PM patients at 900 mg/m².

Antitumor activity

No objective clinical responses were observed, but liver metastasis was reduced by 22.5% at the 8th cycle of 700 mg/m² in one colorectal cancer patient, who had previously received 5-fluorouracil.

Discussion

This phase I study was conducted to determine the MTD of E7070 administered by intravenous infusion over 1–2 h every 21 days, to determine the recommended single-agent dose for phase II studies, and to characterize the safety, pharmacokinetic and pharmacodynamic profiles of E7070. The

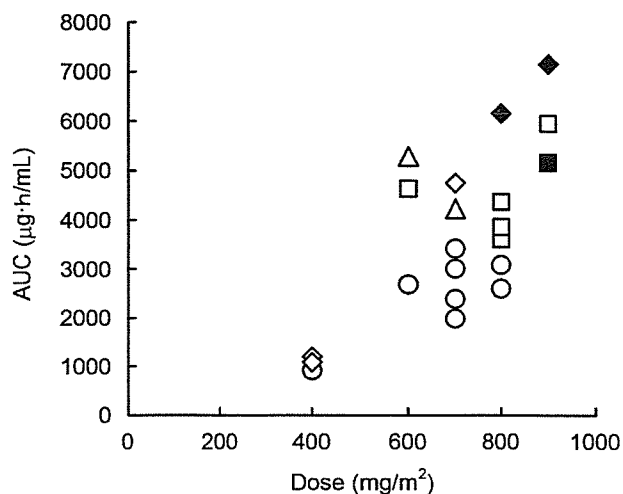


Fig. 3. Relationship between dose and area under the plasma concentration–time curve (AUC) of E7070 in relation to CYP2C9 and CYP2C19 genotypes. Circles, homo extensive metabolizers (EM) for both CYP2C9 and CYP2C19; squares, homo EM for CYP2C9 and hetero EM for CYP2C19; triangles, hetero EM for both CYP2C9 and CYP2C19; diamonds, homo EM for CYP2C9 and poor metabolizers (PM) for CYP2C19. Patients with dose-limiting toxicity are indicated with closed symbols.

MTD in this study was estimated to be 900 mg/m², and the recommended dose for phase II studies is 800 mg/m². DLT observed at 900 mg/m² included leukopenia, neutropenia, thrombocytopenia, elevation of AST, and colitis.

The hematological abnormalities most commonly reported during the first cycle of treatment were neutropenia, leukopenia, and thrombocytopenia. Neutropenia, which tended to be dose-dependent, but not course-dependent, was the principal hematological toxicity in this study and was dose-limiting at 900 mg/m². At the recommended dose of 800 mg/m², the mean recovery time of neutrophils from day 1 to grade 1 neutropenia was 24.0 ± 6.1 days. Therefore, bone marrow recovery should be confirmed before the start of successive treatment cycles. Hematological toxicities were also dose-limiting in four previous phase I trials of E7070.⁽⁵⁻⁸⁾

The non-hematological toxicities most commonly reported during the first cycle of treatment were rash, fatigue, stomatitis, alopecia, injection site reaction, diarrhea and constipation. The type and incidence of the frequently noted events were generally consistent across dosages and cycles of treatment. Gastrointestinal toxicity, the most common non-hematological type of toxicity associated with E7070, was usually mild and well-controlled with medication. The frequency of diarrhea increased with dose, and grade 3 severe diarrhea and colitis were observed only in one patient at 900 mg/m²; this patient had previously undergone intestinal surgery for colon cancer. Diarrhea was a dose-limiting toxicity in two previous phase I trials of E7070.^(6,7) Because of the relatively high frequency and dose-dependency of diarrhea in this study, patients receiving E7070 should be carefully monitored for diarrhea. Grade 3 ileus followed by grade 4 constipation was reported in one patient treated with 800 mg/m² of E7070. Although this event appeared to be related to peritoneal dissemination, its onset after 7 days of E7070 infusion suggested that it might have been induced by E7070. None of the patients treated at less than 800 mg/m² had grade 2 or higher nausea, vomiting, or anorexia.

Grades 1-2 rash, commonly localized to the face, anterior chest, and upper back, with mild itching, was observed in 12 patients given 400-900 mg/m² of E7070. Its frequency and severity were not dose-dependent. Rashes recovered within a week after the administration of E7070, and skin toxicity did not interrupt the therapy in any patient. Injection site reaction (grades 1-2) was reported in nine (43%) patients. The frequency of this event did not seem to be dose-dependent, suggesting that it was related to infusion irritation by E7070, rather than hemolysis or thrombosis. E7070 shows similarities to chloroquine sulfonamide, which is known to cause hypoglycemia and cardiac tachycardia.⁽¹³⁾ However, no hypoglycemia or cardiac arrhythmia was observed in this phase I trial of E7070.

The results of pharmacokinetic analysis suggested that the AUC of E7070 was non-linearly related to dose within the dose range of 400-900 mg/m². The clearance seemed to decrease, with a disproportionate increase in AUC. These results were in agreement with those obtained in other phase I trials with Caucasian patients.⁽⁵⁻⁸⁾ This non-linearity was prominent at higher dose levels and is likely to be a complex consequence of saturation of metabolism, protein binding and distribution of E7070.⁽¹⁴⁾ The absolute values of nadirs and the decrease ratios of WBC, neutrophil and platelet

counts were apparently correlated with the AUC of E7070. *In vitro* experiments have shown that E7070 has the potential to inhibit CYP2C9 and CYP2C19, suggesting that these CYP may be involved in the metabolism of E7070.⁽¹⁰⁾ In fact, other *in vitro* experiments have shown that CYP2C9 and CYP2C19 are responsible for the metabolism of E7070 (unpublished data). Since these CYP show genetic polymorphism, there is a possibility that subjects with one or more mutant alleles of these CYP have decreased clearance for any compound that is mainly metabolized by these polymorphic CYP. Therefore, we were prompted to investigate the relationship of the pharmacokinetics of E7070 with CYP2C9 and CYP2C19 genotype in this trial. At a dose level of 600 mg/m² or more, the AUC of patients with mutant allele(s) (PM and hetero EM) of CYP2C19 were higher than those of the patients without mutant alleles (homo EM). These results imply that the presence of mutant allele(s) of CYP2C19 may result in a decrease in the clearance of E7070 (Fig. 3), and support the involvement of CYP2C19 in the metabolism of E7070, as suggested from *in vitro* studies. The difference of AUC between CYP2C19 homo EM and PM was not clear at a dose of 400 mg/m². This was probably because metabolic capacity was less saturated at the low dose of 400 mg/m² compared with the higher doses, and so intergenotypic differences did not appear. The influence of the CYP2C9 genotype on the AUC of E7070 was not clarified because only two subjects had a mutant allele of this gene. The incidence of CYP2C9 PM is known to be less in Asian (< 1%) than in Caucasian (< 10%) people,^(15,16) whereas CYP2C19 PM is more frequent in Asian (20%) than in Caucasian (< 1%) people.⁽¹⁷⁾ Due to the low frequency of mutation of CYP2C9 in Asian populations, investigation of the effect of CYP2C9 on the pharmacokinetics of E7070 might be difficult in Japanese subjects. Research on subjects with various racial origins would be necessary for evaluation of the clinical impact of the CYP2C9 genotype. In any case, because of the small number of subjects in the present study, further studies should be taken into consideration to assess whether either the CYP2C9 or CYP2C19 genotype is of any clinical significance from the viewpoints of safety and efficacy of E7070. Urinary excretion of unchanged E7070, up to 72 h after the start of administration, was only 0.82-2.47% of the administered dose, indicating that renal clearance plays only a minor role in the elimination of E7070.

Although clinical efficacy (in terms of confirmed partial or complete responses) of E7070 was not demonstrated in this study, one patient with liver metastasis from colon cancer had a reduction in tumor size of ≤ 22.5% and demonstrated stable disease lasting 5 months. A phase II trial of E7070 as a single agent in 5-fluorouracil-resistant or refractory colorectal cancer showed limited activity with a 4% response rate,⁽¹⁸⁾ and thus further clinical studies of combination therapy with irinotecan (CPT-11) are ongoing for the treatment of this tumor type.

In conclusion, the MTD of E7070 administered intravenously in a 1-2 h infusion every 3 weeks was estimated to be 900 mg/m² and the recommended dose for a phase II study is 800 mg/m². At 800 mg/m², hematological toxicities were manageable. Gastrointestinal toxicity was the most common non-hematological toxicity associated with E7070, but was generally well controlled with premedication. However, this recommended dose might be influenced by the CYP2C19

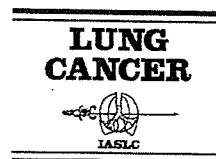
genotype and possibly by the CYP2C9 genotype as well. E7070 seems to be an interesting agent with novel cell-cycle-inhibitory effects. Additional phase I and II studies are currently ongoing in various tumor types to explore further the antitumor activity of this drug as a single agent and in combination with other chemotherapeutic agents.

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Clinical responses of large cell neuroendocrine carcinoma of the lung to cisplatin-based chemotherapy

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KEYWORDS

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Summary

Background: The efficacy of chemotherapy in patients with large cell neuroendocrine carcinoma of the lung (LCNEC) remains unclear.

Methods: Patients with LCNEC who received cisplatin-based chemotherapy were identified by reviewing 567 autopsied and 2790 surgically resected lung cancer patients. The clinical characteristics and objective responses to chemotherapy in these patients were analyzed.

Results: Overall, 20 cases of LCNEC were identified, including stage IIIA ($n=3$), stage IIIB ($n=6$), stage IV ($n=6$) and postoperative recurrence ($n=5$) cases. Six patients had received prior chemotherapy, and 14 were chemo-naïve patients. The patients had received a combination of cisplatin and etoposide ($n=9$), cisplatin, vindesine and mitomycin ($n=6$), cisplatin and vindesine ($n=4$), or cisplatin alone ($n=1$). One patient showed complete response and nine showed partial response, yielding an objective response rate of 50%. The response rate did not differ between patients with the initial diagnosis of SCLC and those with the initial diagnosis of NSCLC, however, the response rate in chemo-naïve patients (64%) was significantly different from that in previously treated patients (17%).

Conclusions: Our results suggest that the response rate of LCNEC to cisplatin-based chemotherapy was comparable to that of SCLC.

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1. Introduction

Pulmonary neuroendocrine tumors include a spectrum of four clinicopathological entities classified on the basis of the morphological and biological features: typical carcinoid and atypical carcinoid, which are tumors of low to intermediate grade malignancy, and large cell neuroendocrine carcinoma (LCNEC) and small cell carcinoma (SCLC), which are high-grade malignant tumors. Travis et al. proposed the term LCNEC in 1991 [1], for classifying a type of poorly differentiated high-grade carcinoma characterized by a neuroendocrine appearance under light microscopy. LCNEC exhibits more prominent cellular pleomorphism and higher mitotic activity than the atypical carcinoid (AC), and is distinguished from SCLC by the tumor cell size and chromatin morphology. Although several different terminologies and classifications have been proposed previously, and even the present classification of pulmonary neuroendocrine tumors lacks uniform definition criteria, this class of tumors could become widely accepted and included in the updated histological classification of the World Health Organization [2].

The clinical features of LCNEC have not yet been completely clarified. The prognosis of patients with surgically resected LCNEC is reported to be intermediate between that of AC and SCLC [3–5], and the same as that of resected NSCLC, except that stage I LCNEC has a poorer prognosis than stage I non-small cell lung cancer (NSCLC) [6]. To the best of our knowledge, however, there are no studies that have examined the role of chemotherapy for LCNEC and the prognosis of patients with unresectable LCNEC, even though several reports have been published on the association between response to chemotherapy and the neuroendocrine differentiation of NSCLC [7–9]. The appropriate treatment of unresectable LCNEC, therefore, remains unclear. In the present study, we attempted to investigate the effectiveness of chemotherapy with cisplatin-based regimens for LCNEC in patients with unresectable and recurrent LCNEC.

2. Materials and methods

Eighty-seven of 2790 patients with primary lung cancer who underwent tumor resection from 1982 to 1999 at the National Cancer Center Hospital were found to have tumors with the histological characteristics of LCNEC [6]. Of these, five had received cisplatin-based chemotherapy at the time

of recurrence, and were enrolled as subjects of this study. In addition, 303 of 567 patients who were autopsied from 1983 to 1997 at the National Cancer Center Hospital who had the following histological diagnoses were first selected: SCLC ($n=112$), poorly differentiated adenocarcinoma ($n=99$), large cell carcinoma ($n=58$), poorly differentiated squamous cell carcinoma ($n=29$), poorly differentiated adenosquamous carcinoma ($n=2$), LCNEC ($n=2$), and carcinoid ($n=1$). Of these, 161 had received cisplatin-based chemotherapy were selected for a pathological review. Finally, specimens from 17 of these 161 cases were found to have histological characteristics consistent with the diagnosis of LCNEC, and were selected as subjects of this study. We focused on cisplatin, because since the 1980s, cisplatin has been the only anticancer agent with proven efficacy against both SCLC and NSCLC [10,11]; we, therefore, considered that the effectiveness of chemotherapy for LCNEC could be reasonably evaluated if cisplatin were included in the regimen. Cases which had received adjuvant chemotherapy without evaluable lesions were excluded from the analysis.

All the available paraffin-embedded tissue sections stained with hematoxylin–eosin were reviewed. We classified LCNEC according to the histopathological criteria in the WHO classification [2]. Immunohistochemical analysis was performed to confirm the neuroendocrine features of the tumors. For this purpose, formalin-fixed paraffin sections were stained for a panel of neuroendocrine markers, including chromogranin A (CGA), synaptophysin (SYN), and neural cell adhesion molecule (NCAM), using standard methods. The intensity of immunostaining for these markers was scored as follows: +, when the proportion of stained tumor cells was $>50\%$; \pm , when 10–50% of tumor cells were stained; and –, when $<10\%$ of tumor cells were stained, as previously described [6]. One case included in this study had the typical histological features of LCNEC, but no neuroendocrine features as determined by the immunohistochemical analysis. For specimens obtained after treatment, we routinely confirmed that the histopathological and morphological features showed no changes due to treatment as compared with the pretreatment biopsy or cytologic specimens. Such cases for which no pretreatment samples were available were excluded from the study; since it has been reported that histological changes may occur after treatment in SCLC [12], we were concerned that misdiagnosis might occur if the same were also true for LCNEC.

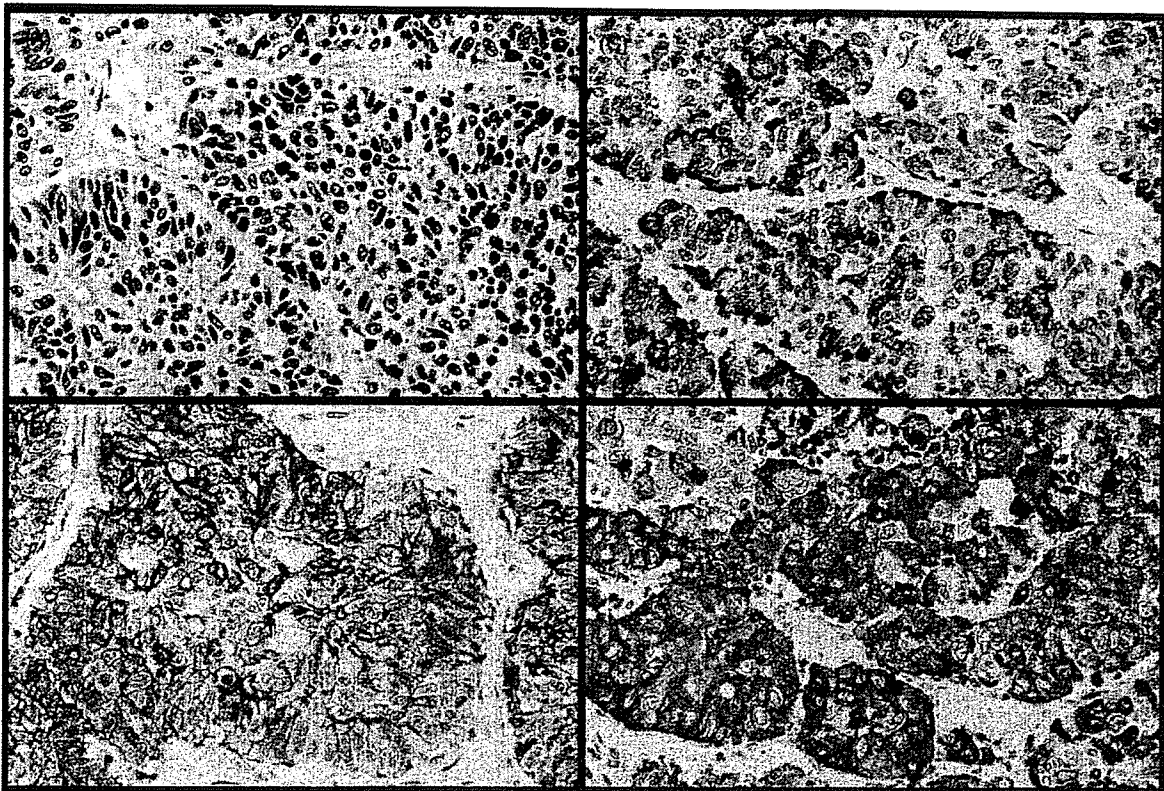


Fig. 1 Case no. 2, 57-year-old man. (A) The tumor cells which are large-sized, polygonal in shape and have a low nuclear-cytoplasmic ratio, are arranged in organoid nests and trabeculae (H&E stain, $\times 200$). Positive staining for neural cell adhesion molecule (B), chromogranin A (C), and synaptophysin (D) (immunostain, $\times 400$).

Clinical information about the cases was obtained from the medical records. The clinical disease staging was reassessed according to the latest International Union Against Cancer (UICC) staging criteria [13]. The response to chemotherapy and overall survival rate were assessed retrospectively. The objective tumor response was evaluated according to the WHO criteria published in 1979 (WHO, 1979) [14]. The survival time was measured from the date of start of chemotherapy with a cisplatin-containing regimen. Survival curves were drawn using the Kaplan–Meier method [15]. Drug toxicity could not be assessed as the study was a retrospective one and records were often incomplete.

3. Results

Overall, 22 cases were recognized as having tumors with histological characteristics consistent with LCNEC among the autopsied and surgically resected

cases of primary lung cancer that had received cisplatin-based chemotherapy and had evaluable lesions; of these 17 were autopsied cases and five were surgically resected cases. Two of the autopsied cases were excluded, because no pre-treatment pathological or cytological samples were available. The typical microscopic appearance of the tumor specimens is shown in Fig. 1A. The specimen sources for the prechemotherapy-diagnosis included surgically resected specimens ($n=5$), biopsy specimens ($n=9$), and cytology specimens ($n=6$). The histological and cytological findings in the specimens obtained before chemotherapy were consistent with those in the specimens obtained after chemotherapy. We therefore finally enrolled 20 cases in this study. The initial pathologic diagnoses in these patients were as follows: small cell carcinoma ($n=10$), poorly differentiated adenocarcinoma ($n=6$), large cell carcinoma ($n=2$), undifferentiated carcinoma ($n=1$), and poorly differentiated carcinoma ($n=1$) (Table 1). None of the cases had been labeled as LCNEC at the time of initial diagnosis, probably because the concept of LCNEC

Table 1 Patient characteristics

Characteristics	N	%
No. of patients	20	
Sex		
Male	18	90
Female	2	10
Age, median (range)	58 (37–74)	
Smoking history		
Yes	19	95
No	1	5
Performance status		
1–2	19	95
>2	1	5
Initial pathological diagnosis		
Small cell carcinoma	10	50
Adenocarcinoma	6	30
Large cell carcinoma	2	10
Others	2	10
Clinical stage at the start of chemotherapy		
IIIA	3	15
IIIB	6	30
IV	6	30
Postoperative recurrence	5	25
Prior treatment		
None	10	50
Surgery	4	20
Radiotherapy	2	10
Chemotherapy without cisplatin	6	30

was not completely accepted at our hospital at that time.

The results of the immunohistochemical staining are shown in Table 2, and a typical case showing positive staining is shown in Fig. 1B and D. Of the 20 LCNECs, 19 expressed at least one of the three general neuroendocrine markers, namely CGA, SYN, and NCAM. Sixteen of the 20 LCNECs exhibited positive staining for NCAM, while one showed equivocal staining. Twelve of the 20 LCNECs showed positive staining for CGA. Thirteen LCNECs showed positive staining for SYN and three showed equivocal staining. Only one case was negative for all the three general neuroendocrine markers, however, this case exhibited the typical histological features of LCNEC on light microscopy.

The clinical characteristics of the patients are summarized in Table 1. The extremely high predominance of men and smokers in this study was comparable to the demographic features of our LCNEC patients treated by surgical resection [6]. Previous chemotherapy was given in six patients: nedaplatin in one and cyclophosphamide-based regimen in five

Table 2 Staining for neuroendocrine markers in 20 LCNECs

Case	NCAM	CGA	SYN
1	+	+	+
2	+	+	+
3	+	+	+
4	±	+	+
5	+	+	+
6	+	+	+
7	–	+	–
8	+	–	–
9	–	–	–
10	–	+	±
11	+	–	+
12	+	+	+
13	+	+	+
14	+	–	±
15	+	+	+
16	+	–	NA
17	+	–	+
18	+	–	NA
19	+	–	+
20	–	+	+

NCAM, neural cell adhesion molecule; CGA, chromogranin A; SYN, synaptophysin; NA, not assessed.

patients. The chemotherapy regimens used were as follows: cisplatin (80 mg/m², day 1) and etoposide (100 mg/m², days 1–3) (n = 9), cisplatin (80 mg/m², day 1), vindesine (3 mg/m², days 1 and 8) and mitomycin (8 mg/m², day 1) (n = 6), cisplatin (80 mg/m², day 1) and vindesine (3 mg/m², days 1 and 8) (n = 4), or cisplatin (100 mg/m², day 1) alone (n = 1). The median (range) number chemotherapy cycles were 2 (1–6). Of the 20 patients, one showed CR and nine showed PR, yielding an overall response rate of 50% (95% confidence interval, 27.2–72.8%). One CR and four PRs were observed among the cases treated with cisplatin and etoposide, two PRs were found among those treated with cisplatin, vindesine and mitomycin, and three PRs were found among those treated with cisplatin and vindesine. Seven patients showed NC, and three showed progressive disease. While the response rate did not differ between patients with an initial diagnosis of SCLC and those patients with an initial diagnosis of NSCLC, previous chemotherapy affected the response to cisplatin: the response rate in chemo-naïve patients was 64%, whereas that in previously treated patients was 17%. The median progression-free survival in the 20 patients was 103 days, median survival was 239 days, 1-year survival rate was 35%, and 2-year survival rate was 15%.

4. Discussion

In this extensive review of over 3000 lung cancer patients, we found considerable difficulty in evaluating the response of LCNEC to systemic chemotherapy. The pathological diagnosis of LCNEC was established in 87 (3.1%) of 2790 patients treated by surgical resection. This low incidence of LCNEC in surgically treated lung cancer patients is comparable to that in other previously published reports: 2.4% (50/2070), 2.9% (22/766), and 3.6% (53/1530) [16–18]. Of the 87 patients, only five who had received cisplatin-based chemotherapy for recurrent tumor that was evaluable for the response. While LCNEC is difficult to diagnose prior to the start of treatment on the basis of the findings in biopsy or cytological specimens, the architectural neuroendocrine features may, more or less, be reflected in these small samples [19,20]. We, therefore, conducted a review of 567 autopsy cases of lung cancer, and identified 15 cases of LCNEC who had received cisplatin-based chemotherapy. We obtained a response rate to cisplatin-based chemotherapy of 50% in these 20 patients with LCNEC, however, the clinical characteristics of patients with medically treatable advanced LCNEC would still remain to be clarified, because autopsy is conducted only in highly selective cases.

Travis et al. suggested that immunohistochemical or electron-microscopic evidence of neuroendocrine features were important to diagnose LCNEC [1]. We assessed the neuroendocrine marker expression by immunohistochemical staining for CGA, SYN, and NCAM. Our cases included one that was negative for all the three neuroendocrine markers examined, but showed the typical histological features of LCNEC, which could be attributable to technical staining problems. Immunohistochemical staining for neuroendocrine tumors is generally recognized as only a supplementary diagnostic tool. In addition, the post-surgical survival rate did not differ between histologically diagnosed cases of LCNEC with neuroendocrine differentiation in marker expression as assessed by immunohistochemical staining and large cell carcinoma with neuroendocrine morphology where the neuroendocrine markers were negative (data not shown). Thus, we decided to include the case with negative staining as LCNEC on the basis of its typical neuroendocrine morphology.

To the best of our knowledge, only one study on the efficacy of chemotherapy in patients with LCNEC has been reported previously. In the study, 13 patients with LCNEC received chemotherapy when relapse was noted after surgical resection, and two (20%) of 10 evaluable patients showed an objec-

tive response. The evaluable lesion in these patients, however, was the brain in seven, liver in two, and bone in one patient [21]. Thus, the relatively low response rate in the report may be due to the site of the evaluable lesion. In addition, reports on the correlation between response to chemotherapy and neuroendocrine differentiation of NSCLC may be helpful. Graziano et al. reported that the proportion of NSCLC positive for neuroendocrine markers was higher in responders than in non-responders among 52 NSCLC patients treated by chemotherapy, and that the result suggested a correlation between positivity for neuroendocrine marker expression and the likelihood of response to chemotherapy [7]. On the other hand, others have reported the absence of any correlation between the presence of neuroendocrine differentiation and the response to chemotherapy [8,9]. The neuroendocrine differentiation in NSCLCs in the aforementioned studies was confirmed only by immunohistochemical staining and not on the basis of the morphological definition of LCNEC. Therefore, these groups might have potentially included heterogeneous subtypes of lung carcinoma, such as adenocarcinoma or squamous cell carcinoma, with components of neuroendocrine differentiation. The conflicting conclusions of these studies may, therefore, reflect differences in the biological characteristics of the tumors included in the analysis. Since the definition of LCNEC is based on morphological criteria as well as positivity for neuroendocrine marker expression, LCNEC is may be considered to be a clinically homogeneous group. Therefore, our study of LCNEC may endorse the former reports about the relationship between neuroendocrine differentiation and the sensitivity to chemotherapy.

Objective response to chemotherapy can be observed in only 15–30% of NSCLCs, even when they are treated with regimens containing cisplatin [10]. In SCLC, however, effective combination regimens yield objective response rates in the range of 80–90% [11]. Our study showed an overall response rate of LCNEC of 50% to cisplatin-based chemotherapy, and a response rate of 64% in chemo-naïve patients, which seemed to be higher than the response rate of NSCLC to chemotherapy. Considered together, these results suggest that the chemosensitivity of LCNEC is intermediate between that of NSCLC and SCLC, although we were unable to obtain firm evidence from this retrospective study, which included only a small cohort of patients.

Since LCNEC is a relatively rare subtype of lung cancer, a prospective study is difficult to perform, and may only be possible as a multicenter study.

For this purpose, it is an urgent task to establish diagnostic criteria for LCNEC based on examination of biopsy or cytologic specimens. Although the histological definition of LCNEC in surgically resected specimens proposed by Travis et al. is commonly accepted, its diagnostic reproducibility is not satisfactory [22]. It is also difficult to apply the definition to biopsy specimens, in which artifacts can easily be produced and detailed examination may be difficult due to insufficient specimen size. Thus, definitive diagnostic criteria also applicable to biopsy and cytologic specimens are required.

Our study did not include any cases labeled as LCNEC at the time of initial diagnosis. One half of the cases was originally diagnosed as SCLC and the other half as NSCLC, including poorly differentiated adenocarcinoma and large cell carcinoma. This was attributed to the fact that the concept of LCNEC was not clearly defined prior to its being proposed by Travis et al. [1]. Thus, it is possible that patients with LCNEC were included in earlier clinical trials for NSCLC or SCLC. If LCNEC shares the poor prognosis of NSCLC, the reported results of chemotherapy for NSCLC may have been worse in studies in which cases of LCNEC were included. Similarly, the results of clinical studies of SCLC to study their objective response to chemotherapy may also have been worse because of the confounding effects of the inclusion of LCNECs among the cases.

In conclusion, our results suggest that the response rate of LCNEC to cisplatin-based chemotherapy was comparable to that of SCLC. However, because of the retrospective nature of this study and the small sample size, we could not arrive at any definitive conclusion; we, therefore, propose to conduct a prospective study in the future aimed at elucidating the efficacy of chemotherapy for LCNEC. To that end, firm diagnostic criteria for LCNEC need to be established, even when the diagnosis must be based only on examination of biopsy and cytology specimens.

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Epidermal Growth Factor Receptor Gene Mutations and Increased Copy Numbers Predict Gefitinib Sensitivity in Patients With Recurrent Non–Small-Cell Lung Cancer

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ABSTRACT

Purpose

To evaluate epidermal growth factor receptor (*EGFR*) mutations and copy number as predictors of clinical outcome in patients with non–small-cell lung cancer (NSCLC) receiving gefitinib.

Patients and Methods

Sixty-six patients with NSCLC who experienced relapse after surgery and received gefitinib were included. Direct sequencing of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* was performed using DNA extracted from surgical specimens. Pyrosequencing and quantitative real-time polymerase chain reaction were performed to analyze the allelic pattern and copy number of *EGFR*.

Results

Thirty-nine patients (59%) had *EGFR* mutations; 20 patients had deletional mutations in exon 19, 17 patients had missense mutations (L858R) in exon 21, and two patients had missense mutations (G719S or G719C) in exon 18. No mutations were identified in *ERBB2*. Response rate (82% [32 of 39 patients] v 11% [three of 27 patients]; $P < .0001$), time to progression (TTP; median, 12.6 v 1.7 months; $P < .0001$), and overall survival (median, 20.4 v 6.9 months; $P = .0001$) were significantly better in patients with *EGFR* mutations than in patients with wild-type *EGFR*. Increased *EGFR* copy numbers (≥ 3 /cell) were observed in 29 patients (44%) and were significantly associated with a higher response rate (72% [21 of 29 patients] v 38% [14 of 37 patients]; $P = .005$) and a longer TTP (median, 9.4 v 2.6 months; $P = .038$). High *EGFR* copy numbers (≥ 6 /cell) were caused by selective amplification of mutant alleles.

Conclusion

EGFR mutations and increased copy numbers were significantly associated with better clinical outcome in gefitinib-treated NSCLC patients.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the *ErbB* family that has been implicated in cell proliferation and survival and is frequently overexpressed in many solid tumors, including non–small-cell lung cancer (NSCLC). Gefitinib (Iressa; AstraZeneca, Osaka, Japan) is an orally active, selective EGFR tyrosine kinase inhibitor that binds to the adenosine triphosphate-binding

pocket of the EGFR kinase domain and blocks downstream signaling pathways. Two phase II studies, IRESSA Dose Evaluation in Advanced Lung Cancer 1 and 2 (IDEAL 1 and 2), have demonstrated that gefitinib monotherapy exerts an antitumor activity in patients with advanced NSCLC who had previously received platinum-based chemotherapy.^{1,2} Gefitinib was approved in Japan for the treatment of inoperable or recurrent NSCLC in July 2002.

The IDEAL trials and retrospective studies have revealed that women, never smokers, patients with adenocarcinoma, and Japanese patients have higher response rates to gefitinib.¹⁻⁴ Among patients with adenocarcinoma, histologic subtypes have been studied; one study showed that responses were more frequent in patients with bronchioloalveolar carcinoma (BAC) features (38% v 14%; $P < .001$),³ whereas another study showed that the response rate was higher in patients with a papillary-dominant subtype (76% v 21%; $P = .002$).⁵

Although no predictive molecular markers had been identified at the time of approval, somatic mutations in the kinase domain of *EGFR* have been subsequently linked to gefitinib sensitivity. According to three initial reports, 20 of 24 gefitinib-responsive tumors contained *EGFR* mutations, whereas 19 nonresponsive tumors did not contain any mutations.⁶⁻⁸ The mutations were detected in exons 18 to 21 of *EGFR*, close to the region coding the adenosine triphosphate-binding pocket of the kinase domain, and most of them were observed in two hotspots: in-frame deletions including amino acids at codons 747 to 749 in exon 19 and an amino acid substitution at codon 858 (L858R) in exon 21. Analyses of surgically resected NSCLC tumors revealed that such mutations were more frequent among women, never smokers, patients with adenocarcinoma, and Japanese or East Asian patients,⁷⁻¹³ consistent with the known clinical predictors of gefitinib sensitivity.

To evaluate the exact predictive value, we studied consecutive patients with recurrent NSCLC who received gefitinib therapy. To insure high-quality genetic analyses of the archived tissues, we used methanol-fixed, paraffin-embedded surgical specimens, which are known to preserve DNA better than formalin-fixed tissues,¹⁴ and performed laser capture microdissection (LCM).

Recently, some other biomarkers of NSCLC have been studied. The *EGFR* and chromosome 7 copy numbers in NSCLC were assessed using fluorescence in situ hybridization (FISH), and more than 3.0 *EGFR* copies per cell (balanced polysomy or gene amplification) were detected in 39 (22%) of 183 patients.¹⁵ A correlation between an increased *EGFR* copy number and gefitinib sensitivity was also proposed in another study.¹⁶ In yet other studies, mutations in the kinase domain of *ERBB2* (*HER2*), a gene coding another receptor tyrosine kinase of the ErbB family, were detected in 16 (3.6%) of 445 patients with lung adenocarcinoma.^{17,18} In the current study, we also analyzed the *EGFR* copy number and the presence of *ERBB2* mutations to assess their impact on clinical outcome.

The expression of *EGFR* and related proteins has been more widely studied using immunohistochemistry. Some studies suggested that high expression of phosphorylated Akt^{19,20} or low expression of phosphorylated mitogen-activated protein kinase^{20,21} was associated with better outcome in gefitinib-treated patients, but in general, methods,

criteria, and results were inconsistent among studies. We thought that protein expression should be analyzed in another exploratory study, and in the current study, we focused on the genetic analyses.

PATIENTS AND METHODS

Patients

After searching the pharmaceutical records of the National Cancer Center Hospital, 279 patients with NSCLC who had begun receiving gefitinib monotherapy (250 mg/d) between July 2002 and May 2004 were identified. Seventy-three of these patients had undergone surgical resection of primary NSCLC at the hospital and subsequently relapsed. Recurrences were not necessarily confirmed pathologically but were diagnosed clinically. Seven patients were ineligible for inclusion in this study because methanol-fixed tissues were not available ($n = 5$) or their informed consent to the genetic analysis was not obtained ($n = 2$); consequently, 66 patients were included.

Genetic Analyses of *EGFR* and *ERBB2*

On a protocol approved by the institutional review board of the National Cancer Center, we performed mutational analyses of exons 18 to 24 of *EGFR* and exons 18 to 24 of *ERBB2* and analyzed the *EGFR* copy number. Methanol-fixed, paraffin-embedded surgical specimens of primary NSCLC were collected retrospectively, and DNA was extracted from bulk tumor tissue, laser capture microdissected tumor tissue, and normal lung tissue from each patient. LCM was performed using a PixCell II LCM system (Arcurus Engineering Inc, Mountain View, CA) according to a previously described method.²² If appropriate, tumor cells were captured separately from two areas with different histologic subtypes, such as an area with a BAC subtype and another area with stromal invasion. Nested polymerase chain reaction (PCR) was performed to amplify exons 18 through 24 of *EGFR* using previously described primers,⁶ and standard PCR was used to amplify exons 18 through 24 of *ERBB2*. Direct sequencing of the PCR products was performed using ABI PRISM 3700 and 3100 DNA Sequencers (Applied Biosystems, Foster City, CA). All sequencing reactions were performed in both forward and reverse directions, and single nucleotide substitutions, insertions, and deletions were detected using an application program named NAMIHEI.²³ Pyrosequencing was performed to verify the sequencing data of the hotspots of *EGFR* and to assess the proportion of mutant alleles in the laser-captured tumor cells using a Pyrosequencing PSQ 96MA (Pyrosequencing, Uppsala, Sweden).²⁴ On the basis of the proportion of mutant alleles, *EGFR* mutations were divided into two patterns: balanced heterozygous (BH) pattern ($< 60\%$) and mutant-allele-dominant (MD) pattern ($\geq 60\%$). The cutoff level of 60% was decided because if more than 60%, the superiority of the mutant over the wild-type sequences was obvious on the direct sequencing chromatograms. Quantitative, real-time, TaqMan duplex PCR was performed to analyze the *EGFR* copy number using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The *EGFR* primers were 5'-GGAGGACCGTCGCTTGGT-3' and 5'-AACACCGCAGCATGTCAAGA-3'; the probe (5'-CACCGCGACCTGGCAGCCA-3') was labeled with the reporter dye 6-carboxyfluorescein (FAM). RNaseP was coamplified in the same reaction mixture as the endogenous reference gene using TaqMan RNaseP Control Reagents (6-carboxyrhodamine [VIC] dye; Applied

Biosystems). The average *EGFR* copy number per cell was calculated from the differences in the threshold amplification cycles between *EGFR* and *RNaseP*. Peripheral-blood samples obtained from healthy volunteers were analyzed as normal controls. Decreased, normal, moderately increased, and highly increased *EGFR* copy numbers were defined as less than 1.5, 1.5 to 3.0, 3.0 to 6.0, and ≥ 6.0 copies per cell, respectively.

Pathologic Evaluation

We reviewed the histologic features of the 66 patients using hematoxylin and eosin-stained slides of tumor samples. Two board-certified pathologists (K.T. and Y.M.) who were unaware of the patients' outcome and mutational status examined all the specimens independently; in case of discrepancy, final diagnoses were established by consensus. Adenocarcinoma was categorized in two ways. The first categorization was based on the WHO's classification of lung tumors,²⁵ which includes four major subtypes of adenocarcinoma: papillary, acinar, BAC, and solid; the dominant subtype in the total tumor mass of each case was documented. The second categorization was based on a report from the Memorial Sloan-Kettering Cancer Center,²⁶ in which adenocarcinomas were classified into adenocarcinoma without BAC features (Ad), adenocarcinoma with BAC features (AwBF), BAC with focal invasion (BwFI), and pure BAC (PBAC). If two or more tumors were present in one patient, the diagnosis of the most invasive tumor in each case was documented.

Radiologic Evaluation

In patients who had measurable lesions, imaging studies were performed at baseline, approximately 4 weeks after the initiation of gefitinib treatment, and periodically thereafter throughout the treatment. One board-certified radiologist (U.T.) who was unaware of the patients' mutational status reviewed the baseline, first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements.²⁷ Responders were defined as patients with CR or PR. In this study, SD was subdivided into minor response (MR) and no response. MR was defined as a $\geq 25\%$ decrease in the sum of the products of the perpendicular diameters of all measurable lesions at any point during gefitinib treatment. Time to progression (TTP) was defined as the time from the start of gefitinib administration to confirmed disease progression or death.

Statistical Analysis

The associations among mutational status, *EGFR* copy number, patient characteristics, and tumor response to gefitinib were assessed using a χ^2 test. The differences in TTP and overall survival (OS) according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. Multivariate analyses using logistic regression models and Cox proportional hazard models were performed to assess the association between the biomarkers and clinical outcome while adjusting for the baseline patient characteristics. All analyses were performed using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS Inc, Chicago, IL).

RESULTS

Patient Characteristics

The patient characteristics are listed in Table 1. All of the patients were Japanese. The proportions of women

Table 1. Patient Characteristics

	Patients (n = 66)	
	No.	%
Age, years		
Median	65	
Range	32-80	
Sex		
Female	26	39
Male	40	61
Smoking history*		
Never smokers	31	47
Former smokers	12	18
Current smokers	23	35
Histologic diagnosis		
Adenocarcinoma	62	94
Papillary/acinar/BAC/solid†	30/18/9/5	45/27/14/8
Ad/AwBF/BwFI/PBAC	15/45/2/0	23/68/3/0
Squamous cell carcinoma	3	5
Pleomorphic carcinoma	1	2
Performance status		
0/1	22/28	33/42
2/3	12/4	18/6
Prior chemotherapy regimens		
0	37	56
1	14	21
≥ 2	15	23

Abbreviations: BAC, bronchioalveolar carcinoma; Ad, adenocarcinoma without BAC features; AwBF, adenocarcinoma with BAC features; BwFI, BAC with focal invasion; PBAC, pure BAC.

*Never smokers were defined as subjects who have never had a smoking habit, and former smokers were defined as subjects who had stopped smoking at least 1 year before diagnosis.

†Dominant subtype.

(39%), never smokers (47%), and patients with adenocarcinoma (94%) in this study were higher than those in a database of more than 1,000 patients with advanced or recurrent NSCLC treated at our hospital during the four most recent years (27%, 27%, and 73%, respectively). Twenty-two patients (33%) had been included in our phase II trial for first-line gefitinib therapy for patients with recurrent NSCLC, and the others had been treated with gefitinib in clinical practice settings. The operations for primary NSCLC were performed between February 1994 and August 2003, and the median time from the operations to the start of gefitinib was 2.3 years (range, 0.6 to 9.1 years).

Clinical Outcome

Sixty-four patients had measurable lesions at the start of gefitinib administration. CR and PR were observed in two and 32 patients, respectively. MR was observed in three of nine patients with SD. Twenty-one patients had PD, including six patients who died before the first follow-up imaging studies. Two patients had only unmeasurable bone lesions at baseline; one patient showed rapid symptom improvement and continued to receive gefitinib therapy without progression for 13.8+ months, whereas the other