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

Toxicity	Grade	Dose (mg/m²)				
		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

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

Toxicity	Grade	Dose (mg/m²)				
		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/m²)	No. patients	C_{max} (μg/mL)	AUC (μg·h/mL)	CL (mL/min per m²)	MRT (h)	$t_{1/2}$ (h)	V_{ss} (L/m²)	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.

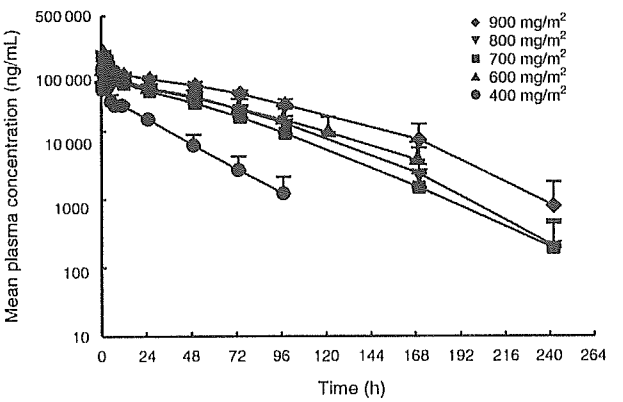

Fig. 1. Mean plasma concentrations of E7070 after single intravenous infusion at each dose level. Circles, 400 mg/m²; triangles, 600 mg/m²; squares, 700 mg/m²; inverted triangles, 800 mg/m²; diamonds, 900 mg/m². Each point represents the mean with standard deviation.

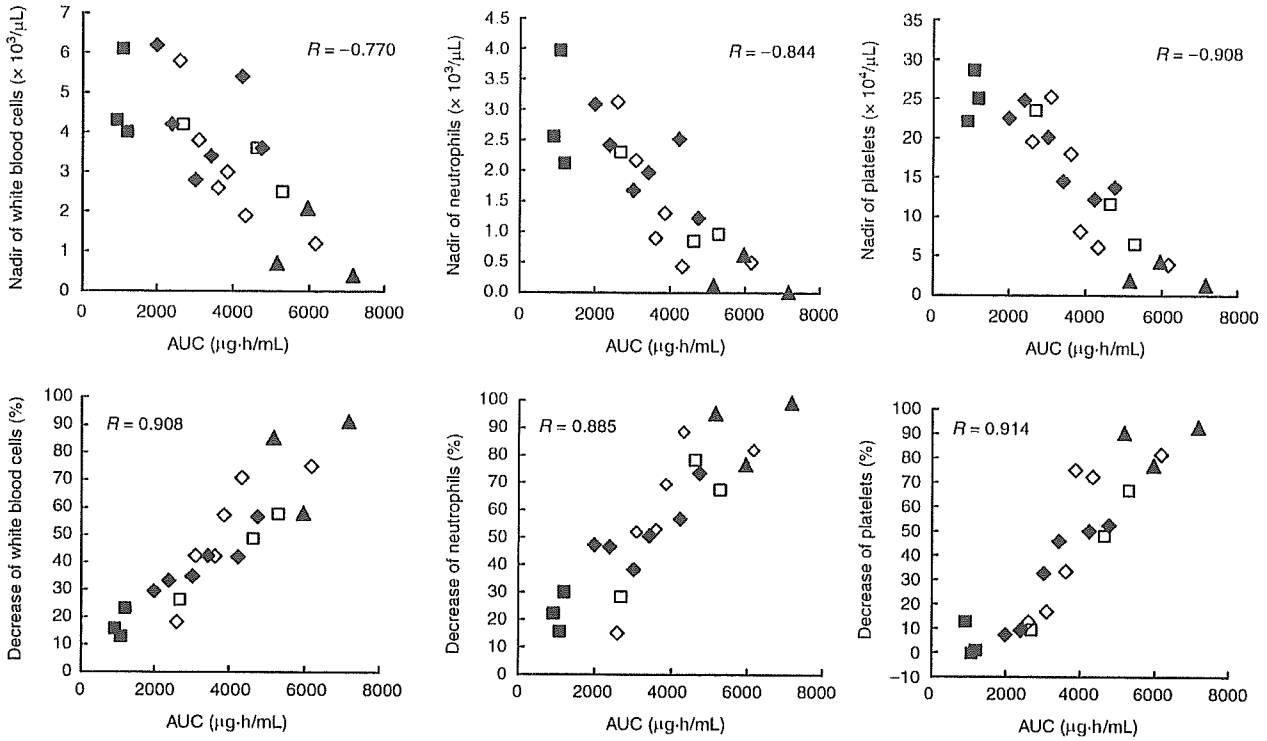

Fig. 2. Relationship between the area under the plasma concentration–time curve (AUC) of E7070 and white blood cells, neutrophil and platelet counts. Closed squares, 400 mg/m²; open squares, 600 mg/m²; closed diamonds, 700 mg/m²; open diamonds, 800 mg/m²; closed triangles, 900 mg/m². R, Pearson's correlation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

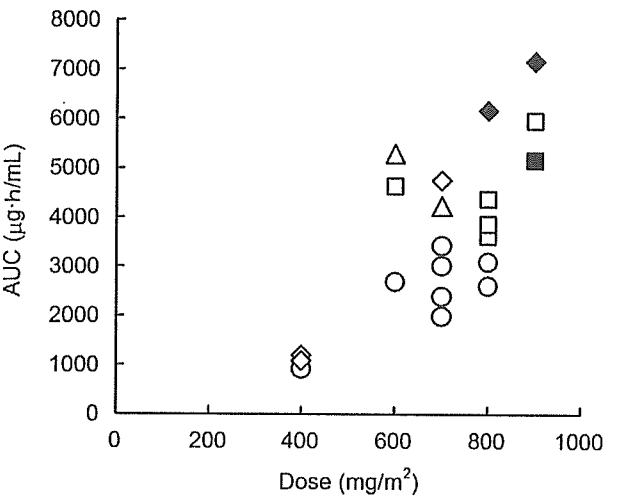

Fig. 3. Relationship between dose and area under the plasma concentration–time curve (AUC) of E7070 in relation to CYP2C9 and CYP2C19 genotypes. Circles, homo extensive metabolizers (EM) for both CYP2C9 and CYP2C19; squares, homo EM for CYP2C9 and hetero EM for CYP2C19; triangles, hetero EM for both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

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MTD in this study was estimated to be 900 mg/m^2, and the recommended dose for phase II studies is 800 mg/m^2. DLT observed at 900 mg/m^2 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/m^2. At the recommended dose of 800 mg/m^2, 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/m^2; 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/m^2 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/m^2 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/m^2 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 chloroquinoxaline 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/m^2. 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/m^2 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/m^2. This was probably because metabolic capacity was less saturated at the low dose of 400 mg/m^2 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/m^2 and the recommended dose for a phase II study is 800 mg/m^2. At 800 mg/m^2, 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.

References

1 Fukuoka K, Usuda J, Iwamoto Y *et al.* Mechanism of action of the novel sulfonamide anticancer agent E7070 on cell cycle progression in human non-small cell lung cancer cells. *Invest New Drugs* 2001; **19**: 219–27.

2 Sugi N, Ozawa Y, Watanabe T *et al.* A novel agent ER-35744, targeting G1 phase. II: Antitumor activities *in vitro* and *in vivo*. *Proc Am Assoc Cancer Res* 1996; **37**: A2668.

3 Yokoi A, Kuromitsu J, Kawai T *et al.* Profiling novel sulfonamide antitumor agents with cell-based phenotypic screens and array-based gene expression analysis. *Mol Cancer Ther* 2002; **1**: 275–86.

4 Ozawa Y, Sugi NH, Nagasu T *et al.* E7070, a novel sulphonamide agent with potent antitumour activity *in vitro* and *in vivo*. *Eur J Cancer* 2001; **37**: 2275–82.

5 Raymond E, ten Bokkel Huinink WW, Taieb J *et al.* Phase I and pharmacokinetic study of E7070, a novel chloroindolyl sulfonamide cell-cycle inhibitor, administered as a one-hour infusion every three weeks in patients with advanced cancer. *J Clin Oncol* 2002; **20**: 3508–21.

6 Punt CJ, Fumoleau P, van de Walle B, Faber MN, Ravic M, Campone M. Phase I and pharmacokinetic study of E7070, a novel sulfonamide, given at a daily times five schedule in patients with solid tumors. A study by the EORTC-early clinical studies group (ECSG). *Ann Oncol* 2001; **12**: 1289–93.

7 Dittrich C, Dumez H, Calvert H *et al.* Phase I and pharmacokinetic study of E7070, a chloroindolyl-sulfonamide anticancer agent, administered on a weekly schedule to patients with solid tumors. *Clin Cancer Res* 2003; **9**: 5195–204.

8 Terret C, Zanetta S, Roche H *et al.* Phase I clinical and pharmacokinetic study of E7070, a novel sulfonamide given as a 5-day continuous infusion repeated every 3 weeks in patients with solid tumours. A study by the EORTC Early Clinical Study Group (ECSG). *Eur J Cancer* 2003; **39**: 1097–104.

9 Thijssen HH, Flinois JP, Beaune PH. Cytochrome P4502C9 is the principal catalyst of racemic acenocoumarol hydroxylation reactions in human liver microsomes. *Drug Metab Dispos* 2000; **28**: 1284–90.

10 Van den Bongard HJGD, Sparidans RW, Critchley DJP, Beijnen JH, Schellens JHM. Pharmacokinetic drug-drug interaction of the novel anticancer agent E7070 and acenocoumarol. *Invest New Drugs* 2004; **22**: 151–8.

11 Therasse P, Arbuck SG, Eisenhauer EA *et al.* New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205–16.

12 Owa T, Yoshino H, Okauchi T *et al.* Discovery of novel antitumor sulfonamides targeting G1 phase of the cell cycle. *J Med Chem* 1999; **42**: 3789–99.

13 Rigas JR, Tong WP, Kris MG, Orazem JP, Young CW, Warrell RP Jr. Phase I clinical and pharmacological study of chloroquinoxaline sulfonamide. *Cancer Res* 1992; **52**: 6619–23.

14 Van den Bongard HJGD, Pluim D, van Waardenburg RC, Ravic M, Beijnen JH, Schellens JHM. *In vitro* pharmacokinetic study of the novel anticancer agent E7070: red blood cell and plasma protein binding in human blood. *Anticancer Drugs* 2003; **14**: 405–10.

15 Aithal GP, Day CP, Kesteven PJ, Daly AK. Association of polymorphisms in the cytochrome P450 CYP2C9 with warfarin dose requirement and risk of bleeding complications. *Lancet* 1999; **353**: 717–9.

16 Takahashi H, Echizen H. Pharmacogenetics of warfarin elimination and its clinical implications. *Clin Pharmacokinet* 2001; **40**: 587–603.

17 De Morais SM, Wilkinson GR, Blaisdell J, Meyer UA, Nakamura K, Goldstein JA. Identification of a new genetic defect responsible for the polymorphism of (*S*)-mephenytoin metabolism in Japanese. *Mol Pharmacol* 1994; **46**: 594–8.

18 Mainwarning PN, van Cutsem E, van Laethem JL *et al.* A multicentre randomised phase II study of E7070 in patients with colorectal cancer who have failed 5-fluorouracil based chemotherapy. *Proc Am Soc Clin Oncol* 2002; **21**: 611.

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

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KEYWORDS
Neuroendocrine carcinoma;
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Cisplatin

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-naive 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-naive 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 carcionoid 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 these161 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%; ±, 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.

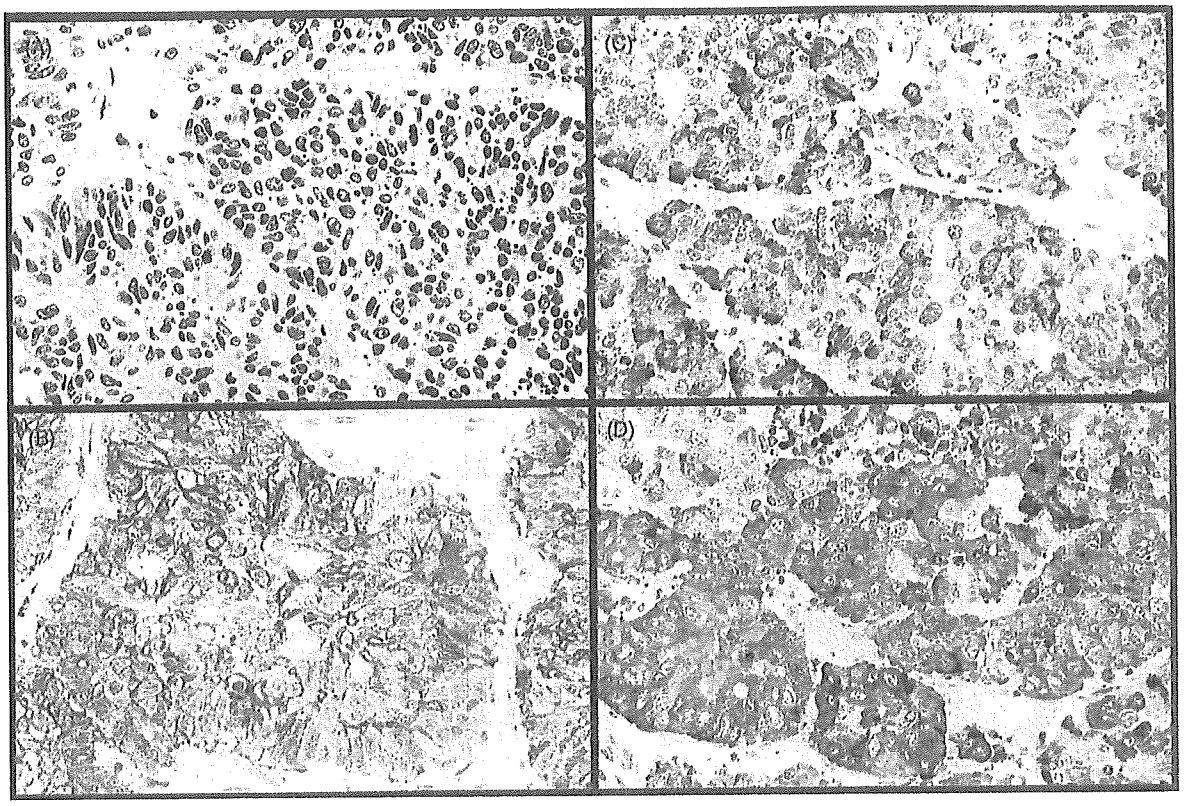

Fig. 1 Case no. 2, 57-year-old man. (A) The tumor cells which are large-sized, polygonal in shape and have a low nuclear-cytoplasmic ratio, are arranged in organoid nests and trabeculae (H&E stain, ×200). Positive staining for neural cell adhesion molecule (B), chromogranin A (C), and synaptophysin (D)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

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.

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 patients. The chemotherapy regimens used were as follows: cisplatin (80 mg/m^2, day 1) and etoposide (100 mg/m^2, days 1–3) ($n = 9$), cisplatin (80 mg/m^2, day 1), vindesine (3 mg/m^2, days 1 and 8) and mitomycin (8 mg/m^2, day 1) ($n = 6$), cisplatin (80 mg/m^2, day 1) and vindesine (3 mg/m^2, days 1 and 8) ($n = 4$), or cisplatin (100 mg/m^2, 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-naive 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 objective 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-naive 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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References

[1] Travis WD, Linnoila RI, Tsokos MG, Hitchcock CL, Cutler Jr GB, Nieman L, et al. Neuroendocrine tumors of the lung with proposed criteria for large-cell neuroendocrine carcinoma. An ultrastructural, immunohistochemical, and flow cytometric study of 35 cases. Am J Surg Pathol 1991;15:529–53.

[2] Travis W, Corrin B, Shimosato Y, Brambilla E. Histological typing of lung and pleural tumours. Berlin: Springer-Verlag; 1999.

[3] Rusch VW, Klimstra DS, Venkatraman ES. Molecular markers help characterize neuroendocrine lung tumors. Ann Thorac Surg 1996;62:798–809 [discussions 09–10].

[4] Dresler CM, Ritter JH, Patterson GA, Ross E, Bailey MS, Wick MR. Clinical-pathologic analysis of 40 patients with large cell neuroendocrine carcinoma of the lung. Ann Thorac Surg 1997;63:180–5.

[5] Travis WD, Rush W, Flieder DB, Falk R, Fleming MV, Gal AA, et al. Survival analysis of 200 pulmonary neuroendocrine tumors with clarification of criteria for atypical carcinoid and its separation from typical carcinoid. Am J Surg Pathol 1998;22:934–44.

[6] Takei H, Asamura H, Maeshima A, Suzuki K, Kondo H, Niki T, et al. Large cell neuroendocrine carcinoma of the lung: a clinicopathologic study of eighty-seven cases. J Thorac Cardiovasc Surg 2002;124:285–92.

[7] Graziano SL, Mazid R, Newman N, Tatum A, Oler A, Mortimer JA, et al. The use of neuroendocrine immunoperoxidase markers to predict chemotherapy response in patients with non-small-cell lung cancer. J Clin Oncol 1989;7:1398–406.

[8] Schleusener JT, Tazelaar HD, Jung SH, Cha SS, Cera PJ, Myers JL, et al. Neuroendocrine differentiation is an independent prognostic factor in chemotherapy-treated nonsmall cell lung carcinoma. Cancer 1996;77:1284–91.

[9] Carles J, Rosell R, Ariza A, Pellicer I, Sanchez JJ, Fernandez-Vasalo G, et al. Neuroendocrine differentiation as a prognostic factor in non-small cell lung cancer. Lung Cancer 1993;10:209–19.

[10] Ginsberg RJ, Vokes EE, Rosenzweig K. Non-small cell lung cancer. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer: principles & practice of oncology. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 925–83.

[11] Murren J, Glatstein E, Pass HI. Small cell lung cancer. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer: principles & practice of oncology. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 983–1018.

[12] Fushimi H, Kikui M, Morino H, Yamamoto S, Tateishi R, Wada A, et al. Histologic changes in small cell lung carcinoma after treatment. Cancer 1996;77:278–83.

[13] Sobin LH, Wittenkind C. TNM classification of malignant tumors. New York: Wiley-Liss; 1997.

[14] Handbook for reporting results of cancer treatment. Geneva: World Health Organization (WHO); 1979.

[15] Kaplan ELMP. Non-parametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–81.

[16] Jiang SX, Kameya T, Shoji M, Dobashi Y, Shinada J, Yoshimura H. Large cell neuroendocrine carcinoma of the lung: a histologic and immunohistochemical study of 22 cases. Am J Surg Pathol 1998;22:526–37.

[17] Iyoda A, Hiroshima K, Toyozaki T, Haga Y, Fujisawa T, Ohwada H. Clinical characterization of pulmonary large cell neuroendocrine carcinoma and large cell carcinoma with neuroendocrine morphology. Cancer 2001;91:1992–2000.

[18] Paci M, Cavazza A, Annessi V, Putrino I, Ferrari G, De Franco S, et al. Large cell neuroendocrine carcinoma of the lung: a 10-year clinicopathologic retrospective study. Ann Thorac Surg 2004;77:1163–7.

[19] Yang YJ, Steele CT, Ou XL, Snyder KP, Kohman LJ. Diagnosis of high-grade pulmonary neuroendocrine carcinoma by fine-needle aspiration biopsy: nonsmall-cell or small-cell type? Diagn Cytopathol 2001;25:292–300.

[20] Wiatrowska BA, Krol J, Zakowski MF. Large-cell neuroendocrine carcinoma of the lung: proposed criteria for cytologic diagnosis. Diagn Cytopathol 2001;24:58–64.
[21] Mazieres J, Daste G, Molinier L, Berjaud J, Dahan M, Delsol M, et al. Large cell neuroendocrine carcinoma of the lung: pathological study and clinical outcome of 18 resected cases. Lung Cancer 2002;37:287–92.
[22] Travis WD, Gal AA, Colby TV, Klimstra DS, Falk R, Koss MN. Reproducibility of neuroendocrine lung tumor classification. Hum Pathol 1998;29:272–9.

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ORIGINAL REPORT

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 (Arcturus 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′-CACCG-CGACCTGGCAGCCA-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 ≥ 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 (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).

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, bronchioloalveolar 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.

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

patient developed new lesions and died on day 71. These patients were included in the analysis as a responder and a nonresponder, respectively. The overall response rate was 53%. Forty-one patients died, and the median follow-up time for the 25 survivors was 14.6 months (range, 10.3 to 32.3 months). Eleven patients were still receiving gefitinib without progression at the time of the analysis. The median TTP and the median survival time (MST) for all patients were 5.2 and 16.3 months, respectively.

EGFR *and* ERBB2 *Mutations*

Forty-three mutations in the *EGFR* tyrosine kinase domain were detected in 39 (59%) of the 66 patients. All the mutations detected in this study are shown in Table 2. Twenty patients had deletional mutations in exon 19, and 17 patients had missense mutations (L858R) in exon 21. In exons 18 and 20, five types of missense mutations were detected. Two of them (G719S and G719C) occurred at a codon considered to be a third hotspot.[6,7,9-12] The others (L703V, E709K, and S768I) were detected in patients who also had mutations at the hotspots. Because these mutations were not detected in the normal lung tissues from the same patients, they were considered to be somatic mutations. No somatic mutations were detected in exons 22 to 24. Silent single nucleotide polymorphisms were identified at nucleotides 2361 (G/A; Q787Q), 2370 (G/A; T790T), and 2457 (G/A; V819V) in exon 20, and at nucleotide 2709 (C/T; T903T) in exon 23, but the association between these polymorphisms and the somatic mutations was not observed. In this study, no mutations and no polymorphisms were detected in exons 18 to 24 of *ERBB2*.

All 43 mutations were detected in LCM samples, but 11 (26%) of these mutations were not detected in the bulk tumor samples. In 13 patients, LCM was performed at separate areas with different histologic subtypes, but no heterogeneity was identified; the same mutations were detected in nine patients, and no mutations were detected in four patients. Mutational analyses of synchronous double lung cancers were performed in two patients; one patient had a tumor with wild-type *EGFR* and a more invasive tumor with L858R + S768I, and the other patient had a tumor with a 9-bp deletion (del L747-E749) and a more invasive tumor with a 15-bp deletion (del E746-T751insA) + L703V.

Among the 39 patients with *EGFR* mutations, the proportion of mutant alleles ranged from 29% to 94%. Nineteen patients showed a BH pattern and 20 patients showed an MD pattern.

EGFR *Copy Number*

The *EGFR* copy number in the laser-captured tumor cells ranged from 1.27 to 31.2 per cell, and increased *EGFR* copy numbers (≥ 3.0 per cell) were observed in 29 patients (44%). The relation between the copy number and the proportion of mutant alleles is shown in Figure 1. Increased copy numbers were observed more frequently in patients with *EGFR* mutations than in patients with wild-type *EGFR* (56% [22 of 39 patients] *v* 26% [seven of 27 patients]; $P = .014$). High copy numbers (≥ 6.0 per cell) were observed only in patients with an MD pattern of mutations. The copy number and the proportion of mutant alleles among patients with *EGFR* mutations was positively correlated (Spearman correlation coefficient = 0.643; $P < .001$), implying that the mutant alleles were selectively amplified in patients with an MD pattern. One patient with an MD pattern had a tumor with only approximately one copy per cell, indicating a hemizygous mutation with a loss of wild-type allele. No alterations in the gene copy number were observed in normal lung tissues.

Table 2. *EGFR* Mutations Detected in the Present Study

Exons	Amino Acids	Nucleotides	No. of Patients
19	del E746-A750	del 2235-2249	12
	del E746-A750	del 2236-2250	5
	del E746-T751insA	del 2237-2251	1
	del L747-E749	del 2239-2247	1
	del E746-S752insV	del 2237-2255 + ins T	1
21	L858R	T → G at 2573	17
18	G719S	G → A at 2155	1
	G719C	G → T at 2155	1
	L703V	C → G at 2107	1*
	E709K	G → A at 2125	1†
20	S768I	G → T at 2303	2‡

Abbreviations: *EGFR*, epidermal growth factor receptor; del, deletion; ins, insertion.
*A patient with del E746-T751insA.
†A patient with L858R.
‡A patient with L858R and a patient with G719C.

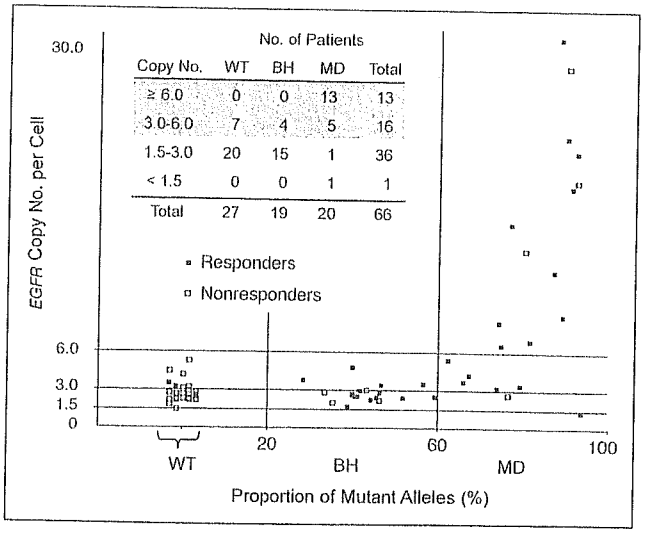

Fig 1. Relation between the epidermal growth factor receptor (*EGFR*) copy number and the proportion of mutant alleles. WT, patients with wild-type *EGFR*; BH, patients with a balanced heterozygous pattern of *EGFR* mutations; MD, patients with a mutant-allele-dominant pattern of *EGFR* mutations.

EGFR *Mutations,* EGFR *Copy Number, and Clinical Outcome*

The tumor responses to gefitinib according to the mutational status of *EGFR* are shown in Table 3. The response rates of patients with mutant and wild-type *EGFR* were 82% and 11%, respectively ($P < 10^{-7}$). Seven patients with *EGFR* mutations were nonresponders; three patients had PD at 0.3 (early death), 2.3, and 2.3 months, and four patients had SD. Three of the four patients with SD had MR (TTP, 2.5, 5.2, and 6.9 months), and the other patient continued to receive gefitinib therapy without progression for 24.2 months, whereas all SD tumors with wild-type *EGFR* progressed within 5 months without MR. Meanwhile, three patients with wild-type *EGFR* exhibited PR, and two of these patients were still receiving gefitinib therapy without progression at 10.9+ and 21.1+ months. The Kaplan-Meier plots of TTP and OS according to the presence of the *EGFR* mutations are shown in Figures 2 and 3, respectively. Patients with *EGFR* mutations had a significantly longer TTP and OS compared with those with wild-type *EGFR*.

Univariate analyses were performed to assess the correlations among patient characteristics, *EGFR* mutations, *EGFR* copy number, and clinical outcome (Tables 4 and 5). The response rates were significantly higher in women, never/former smokers, and patients with BAC features and were marginally higher in patients with a papillary-dominant subtype. The response rates among these subgroups were approximately consistent with the rates of *EGFR* mutations. An increased *EGFR* copy number was also significantly associated with a higher response rate and a longer TTP.

The results of multivariate analyses among 62 patients with adenocarcinoma are shown in Table 6. The presence of *EGFR* mutations was strongly associated with a higher response rate, a longer TTP, and a longer OS. An increased *EGFR* copy number was also a significant or marginally significant predictor of a higher response rate and a longer TTP. These results did not change substantially if any combinations of variables were included in the models.

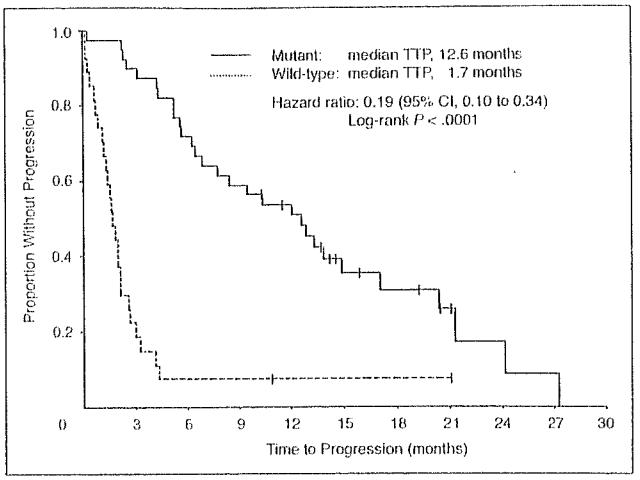

Fig 2. Kaplan-Meier plot of time to progression (TTP) according to epidermal growth factor receptor (*EGFR*) mutation status.

Among patients with wild-type *EGFR*, TTP was significantly longer in patients with increased *EGFR* copy numbers (median, 3.0 *v* 1.4 months; log-rank $P = .021$), and both of the two long-term responders had tumors with moderately increased *EGFR* copy numbers (3.20 and 3.45/cell). Among patients with *EGFR* mutations, TTP and OS were not significantly different according to the types of mutations, the presence of additional mutations, the proportion of mutant alleles, or the *EGFR* copy number (data not shown).

DISCUSSION

This study strongly implies that the mutational status of *EGFR* is a major determinant of gefitinib sensitivity in patients with NSCLC. The response rate was 82%, the median TTP was 12.6 months, and the MST was 20.4 months in gefitinib-treated patients with *EGFR*-mutant NSCLC. *EGFR* mutations might be a good prognostic factor independent of treatment, but these remarkable results suggest a

Table 3. *EGFR* Mutations and Tumor Response to Gefitinib

	Responders		Nonresponders			Responders/Total Patients	Response Rates (%)
	CR	PR	MR	SD	PD		
Mutant	2	30*	3	1	3†	32/39	82
DEL	0	18*	2	0	0	18/20	90
L858R	2	11	1	1	2†	13/17	76
G719	0	1	0	0	1	1/2	50
Wild-type	0	3	0	5	19	3/27	11
Total	2	33	3	6	22	35/66	53

Abbreviations: *EGFR*, epidermal growth factor receptor; CR, complete response; PR, partial response; MR, minor response; SD, stable disease without MR; PD, progressive disease; DEL, deletional mutations in exon 19; G719, G719S, or G719C.
*Including a clinical responder without measurable lesions.
†Including a patient who had no measurable lesions at baseline.

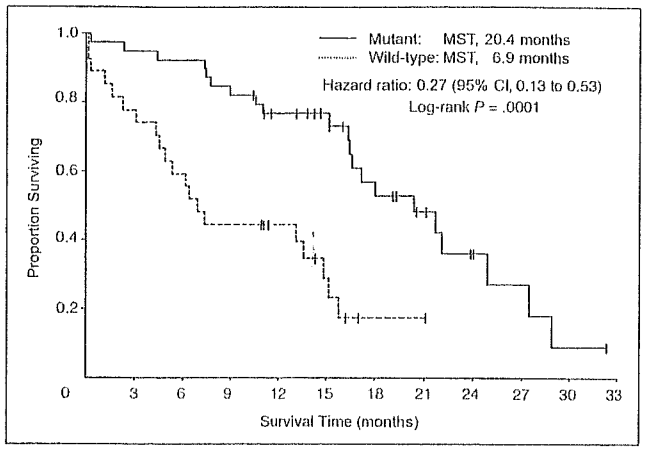

Fig 3. Kaplan-Meier plot of overall survival according to epidermal growth factor receptor (*EGFR*) mutation status. MST, median survival time.

survival benefit from gefitinib therapy in patients with *EGFR* mutations. Four of seven nonresponders with *EGFR* mutations also seemed to experience some clinical benefits because they had MR or a long SD (≥ 6 months). Among nine patients with SD, MR, or a long SD was observed only in patients with *EGFR* mutations. Although the sample size was too small to draw a firm conclusion, this finding suggests that *EGFR* mutations are also associated with clinical benefits in SD.

Table 4. *EGFR* Mutations Among Patient Subgroups

	EGFR Mutations		
	No. of Patients	%	*P*
Total	39/66	59	
Sex			.18
Female	18/26	69	
Male	21/40	53	
Smoking history			.003†
Never smokers	21/31	68	
Former smokers	10/12	83	
Current smokers	8/23	35	
Histologic diagnosis			—
Adenocarcinoma	38/62	61	
Squamous cell carcinoma	0/3	0	
Pleomorphic carcinoma	1/1	100	
Dominant subtype*			.059‡
Papillary	22/30	73	
Acinar	10/18	56	
BAC	5/9	56	
Solid	1/5	20	
BAC features*			.002
Yes	34/47	72	
No	4/15	27	

Abbreviations: *EGFR*, epidermal growth factor receptor; BAC, bronchioloalveolar carcinoma.
*Only patients with adenocarcinoma (n = 62).
†Comparison between never/former smokers and current smokers.
‡Comparison between patients with papillary-dominant adenocarcinoma and patients with other adenocarcinoma.

The *EGFR* mutations detected in this study were concentrated in three hotspots, deletions around codons 747 to 749, L858R, and G719S (or G719C), similar to the results of previous reports.[6-13] Some genetic variations existed among these mutations. Together with one of the hotspot mutations, additional missense mutations in exons 18 or 20 were detected in four patients. Among the 39 patients with *EGFR* mutations, an MD pattern was observed in 20 patients. Because the *EGFR* copy number in their tumor cells increased as the proportion of mutant alleles increased, this pattern was assumed to be caused not by homozygous mutations but by the selective amplification of the mutant alleles. Because one patient had a hemizygous mutation without amplification, the loss of wild-type alleles was also thought to be responsible for the pattern. The moderately increased copy number in patients with a BH pattern or wild-type *EGFR* can be explained by *EGFR* amplification and/or polysomy of chromosome 7.

Among the patients with *EGFR* mutations, three patients had PD and eight of the other 36 patients had tumor regrowth within 6 months. This suggests the presence of other factors associated with intrinsic or acquired resistance to gefitinib. Although any genetic alterations of *EGFR*-mutant tumors at the time of primary surgery were not significantly associated with clinical outcome, that might be because further alterations occurred after the primary surgery or after gefitinib administration. Recently, a secondary mutation (C → T at nucleotide 2369; T790M) in exon 20 was detected in patients with *EGFR*-mutant NSCLC who had tumor regrowth during gefitinib therapy after exhibiting an initial response to the agent; this mutation was thought to be associated with acquired resistance.[28,29] To elucidate the determinants and the mechanism of resistance to gefitinib, genetic analyses of tumor samples obtained after gefitinib treatment are needed.

In this study, three (11%) of the 27 patients with wild-type *EGFR* responded to gefitinib. Various explanations for this result are possible: (1) the mutational analyses of the responders were false-negative, (2) the *EGFR* mutations occurred in their tumors after the primary surgery, (3) the recurrent tumors originated from a source other than the analyzed tumor cells, or (4) other determinants of gefitinib sensitivity were present.

The results of multivariate analyses suggest that the *EGFR* copy number is another independent predictor of gefitinib sensitivity. It is noteworthy that an increased *EGFR* copy number was observed in two of the three responders with wild-type *EGFR*, and was significantly associated with a longer TTP among patients with wild-type *EGFR*. Because patients with *EGFR* mutations had favorable clinical outcome regardless of *EGFR* copy numbers, the impact of increased copy numbers on *EGFR*-mutant NSCLC was unclear. In the overall population, an increased *EGFR* copy number was significantly associated with a higher response

Table 5. Clinical Outcome Among Patient Subgroups (univariate analyses)

	Response Rate			Time to Progression		Overall Survival	
	No.	%	*P*	Median (months)	Log-Rank *P*	Median (months)	Log-Rank *P*
Total	66	53		5.2		16.3	
Sex			.033		.35		.30
Female	26	69		6.2		16.5	
Male	40	43		3.3		15.1	
Smoking history			.007		.026		.37
Never/former smokers	43	65		6.9		16.4	
Current smokers	23	30		2.6		15.1	
Dominant subtype*			.070		.28		.65
Papillary	30	67		7.7		16.4	
Others	32	44		4.2		15.7	
BAC features*			.012		.12		.19
Yes	47	64		6.5		16.5	
No	15	27		2.1		15.7	
Performance status			.77		.012		< .0001
0-1	50	52		5.2		17.1	
2-3	16	56		3.1		6.1	
EGFR mutations			< .0001		< .0001		.0001
Yes	39	82		12.6		20.4	
No	27	11		1.7		6.9	
EGFR copy number			.005		.038		.33
≥ 3.0	29	72		9.4		16.4	
< 3.0	37	38		2.6		15.7	

Abbreviation: BAC, bronchioloalveolar carcinoma; *EGFR*, epidermal growth factor receptor.
*Only patients with adenocarcinoma (n = 62).

rate and a longer TTP, but not with a longer OS, which might be because an increased copy number had an unfavorable impact on prognosis, as suggested by another study.[15] In chronic myeloid leukemia, as well as *BCR-ABL* mutations that were structurally corresponding to T790M in *EGFR*, an increased *BCR-ABL* gene copy number was reported as a determinant of resistance to imatinib, a BCR-ABL tyrosine kinase inhibitor.[30] Therefore, we should consider the possibility that an increased *EGFR* copy number is associated with not only sensitivity but also resistance to gefitinib.

Among adenocarcinomas, the presence of BAC features was significantly associated with gefitinib sensitivity and *EGFR* mutations, but the BAC component was relatively small in most of the responders. The dominant subtype associated with a higher response rate was not BAC but papillary; both of the two patients with BwFI had PD, and all three patients with pure papillary adenocarcinoma without BAC features had PR. The association between pathologic features and gefitinib sensitivity or *EGFR* mutations is also the subject of further investigation.

Table 6. Univariate and Multivariate Analyses of the Association Between Biomarkers and Clinical Outcome in Patients With Lung Adenocarcinoma (n = 62)

	Odds Ratios for Response		Hazard Ratios for TTP		Hazard Ratios for OS	
	Univariate	Multivariate*	Univariate	Multivariate*	Univariate	Multivariate*
EGFR mutations, yes *v* no	31.0	27.9	0.21	0.13	0.30	0.16
95% CI	7.2 to 134	3.7 to 209	0.11 to 0.38	0.06 to 0.29	0.15 to 0.62	0.06 to 0.39
P	< .001	.001	< .001	< .001	.001	< .001
EGFR copy number, ≥ 3.0 *v* < 3.0	4.0	4.6	0.57	0.42	0.80	0.59
95% CI	1.4 to 12	0.84 to 25	0.32 to 1.0	0.21 to 0.84	0.42 to 1.5	0.26 to 1.4
P	.011	.079	.050	.014	.49	.22

Abbreviations: TTP, time to progression; OS, overall survival; *EGFR*, epidermal growth factor receptor.
*In the multivariate analyses, age (continuous variable), sex (women *v* men), smoking history (never/former smokers *v* current smokers), dominant subtype (papillary *v* others), bronchioloalveolar carcinoma features (yes *v* no), performance status (0 to 1 *v* 2 to 3), prior chemotherapy (yes *v* no), *EGFR* mutations (yes *v* no), and *EGFR* copy number (≥ 3.0 *v* < 3.0) were included as factors.

In never/former smokers, both the *EGFR* mutation rate and the response rate were significantly higher than in current smokers. We speculate that *EGFR* mutations occur equally throughout the entire population, regardless of smoking history, and account for smoking-unrelated carcinogenesis. Because many other genetic alterations, like *KRAS* mutations, occur and induce lung adenocarcinoma more frequently in smokers, the *EGFR* mutation rate seems to be relatively lower in smokers with lung adenocarcinoma.

The response rate of 53% and the *EGFR* mutation rate of 59% observed in this study were higher than previously reported rates. These results can partially be attributed to the fact that the physicians tended to select patients with characteristics known to be predictive for gefitinib sensitivity: women, never-smokers, and patients with adenocarcinoma. Consequently, this cohort was not necessarily representative of unselected NSCLC populations in Japan. However, other recent studies have also shown relatively high frequencies (32% to 55%) of *EGFR* mutations in Japanese or East Asian patients with lung adenocarcinoma who underwent surgical resection.[7,9-11,13] The reason why such somatic mutations occur selectively in East Asian people remains unknown. Environmental or genetic factors common among East Asian populations should be investigated to answer this question.

Recently, no significant survival benefit of gefitinib was reportedly observed in the initial analysis of the IRESSA Survival Evaluation in Lung Cancer (ISEL) trial, a phase III trial comparing gefitinib monotherapy to a placebo as a second- or third-line treatment for patients with advanced NSCLC.[31] Because subgroup analyses of the trial suggested survival benefits in never smokers or Asian patients, the selection of patients is thought to be crucial when considering gefitinib treatment. Because the present study showed that the *EGFR* mutation status is a major determinant of gefitinib sensitivity, mutational analyses in patients with advanced NSCLC should be considered before deciding on a course of treatment.

In this study, we performed LCM and direct sequencing using methanol-fixed surgical specimens to obtain high-quality data. If we had analyzed only bulk tumor samples without LCM, nine of the 39 patients with *EGFR* mutations would have been misjudged as having wild-type *EGFR*. Thus such procedures with LCM are presently recommended for the detection of *EGFR* mutations. However, obtaining appropriate tumor samples is often difficult in patients with advanced NSCLC, and performing LCM and direct sequencing in all patients is not practical. Thus more practical methods for detecting the major *EGFR* mutations using small tumor samples contaminated with normal tissue should be developed and validated.

Other than *EGFR* mutations, some candidate predictive biomarkers have been studied. The *EGFR* copy number is the leading candidate, and it can also be detected by FISH. Practicality and accuracy should be assessed comparing FISH and quantitative real-time PCR. The impact of *ERBB2* mutations on clinical outcome remains to be investigated because we could not detect any mutations in *ERBB2* in the present study. Protein expression analyses by IHC are easier to perform than the genetic analyses, but their significance is still controversial. Further studies are required to evaluate the predictive values of these biomarkers and to determine whether they are independent predictors of gefitinib sensitivity or surrogate markers of *EGFR* mutations.

In conclusion, this study indicates that *EGFR* mutations and increased copy numbers predict better clinical outcome in patients with NSCLC treated with gefitinib. Further research and clinical trials are needed to incorporate these markers into clinical practice appropriately.

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Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

REFERENCES

1. Fukuoka M, Yano S, Giaccone G, et al: A multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small cell lung cancer (The IDEAL 1 Trial). J Clin Oncol 21:2237-2246, 2003

2. Kris MG, Natale RB, Herbst RS, et al: Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: A randomized trial. JAMA 290:2149-2158, 2003

3. Miller VA, Kris MG, Shah N, et al: Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non–small-cell lung cancer. J Clin Oncol 22:1103-1109, 2004

4. Takano T, Ohe Y, Kusumoto M, et al: Risk factors for interstitial lung disease and predictive factors for tumor response in patients with advanced non-small cell lung cancer treated with gefitinib. Lung Cancer 45:93-104, 2004

5. Kim YH, Ishii G, Goto K, et al: Dominant papillary subtype is a significant predictor of the response to gefitinib in adenocarcinoma of the lung. Clin Cancer Res 10:7311-7317, 2004

6. Lynch TJ, Bell DW, Sordella R, et al: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small-cell lung cancer to gefitinib. N Engl J Med 350:2129-2139, 2004

7. Paez JG, Janne PA, Lee JC, et al: *EGFR* mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304:1497-1500, 2004