Detection of Epidermal Growth Factor Receptor Mutations in Serum as a Predictor of the Response to Gefitinib in Patients with Non–Small-Cell Lung Cancer

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Abstract Cases of non–small-cell lung cancer (NSCLC) carrying the somatic mutation of epidermal growth factor receptor (EGFR) have been shown to be hyperresponsive to the EGFR tyrosine kinase inhibitor gefitinib (IRESSA). If EGFR mutations can be observed in serum DNA, this could serve as a noninvasive source of information on the genotype of the original tumor cells that could influence treatment and the ability to predict patient response to gefitinib. Serum genomic DNA was obtained from Japanese patients with NSCLC before first-line gefitinib monotherapy. Scorpion Amplified Refractory Mutation System technology was used to detect EGFR mutations. Wild-type EGFR was detected in all of the 27 serum samples. EGFR mutations were detected in 13 of 27 (48.1%) patients and two major EGFR mutations were identified (E746_A750del and L858R). The EGFR mutations were seen significantly more frequently in patients with a partial response than in patients with stable disease or progressive disease ($P = 0.046$, Fisher's exact test). The median progression-free survival was significantly longer in patients with EGFR mutations than in patients without EGFR mutations (200 versus 46 days; $P = 0.005$, log-rank test). The median survival was 611 days in patients with EGFR mutations and 232 days in patients without EGFR mutations ($P > 0.05$). In pairs of tumor and serum samples obtained from 11 patients, the EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. Thus, EGFR mutations were detectable using Scorpion Amplified Refractory Mutation System technology in serum DNA from patients with NSCLC. These results suggest that patients with EGFR mutations seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients without EGFR mutations.

Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (1). Targeting the epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of non–small-cell lung cancer (NSCLC) as EGFR has been found to be expressed, sometimes strongly, in NSCLC tumors (2). Mutations of EGFR tyrosine kinase have been reported in NSCLC patients with dramatic responses to gefitinib (IRESSA), an EGFR tyrosine kinase inhibitor (3, 4). Studies have reported that EGFR mutations are strong determinants of tumor response to EGFR tyrosine kinase inhibitors (5–7). Approximately 30 mutations in exons 18 to 21 of EGFR were detected in a lung tumor specimen (3–8). The two most common NSCLC-associated EGFR mutations are the 15-bp nucleotide in-frame deletion in exon 19 (E746_A750del) and the point mutation replacing leucine with arginine at codon 858 in exon 21 (L858R; refs. 5, 8). These two mutations account for ~90% of all EGFR mutations and could explain the dramatic responders to gefitinib. Most EGFR mutations have been identified retrospectively from operative resected tumor samples. However, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies; thus, it is necessary to establish a method to detect mutant EGFR, especially the two major mutations, from other more readily accessible patient samples.

Recently, PCR technology for the amplification of small amounts of DNA has made it possible to identify the same alterations, which are typically observed in DNA from resected or biopsied tumor cells, using serum samples from patients with various types of tumor, including NSCLC (9, 10). The detection of EGFR mutations in serum DNA may provide a noninvasive and repeatable source of genotypic information

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Received 10/27/05; revised 1/30/06; accepted 2/15/06.

Grant support: Research Resident Fellowship from the Foundation for Promotion of Cancer Research (Japan) for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control (H. Kimura).

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doi:10.1158/1078-0432.CCR-05-2324

that could influence treatment and prognosis, especially in patients with NSCLC treated with gefitinib. However, it is well known that interfusion of normal cells with tumor cells prevents the detection of mutations in the tumor cells. Therefore, it is necessary to enhance the sensitivity of the detection of EGFR mutations from tumor-derived DNA mixed with normal cells.

Scorpion primers are used in a fluorescence-based method for the specific detection of PCR products (11). A Scorpion is a specific probe sequence that is held in a hairpin loop configuration by complementary stem sequences at the 5′ and 3′ ends of the probe. Scorpion can be used in combination with the Amplified Refractory Mutation System (ARMS) to enable the detection of single-base mutations (11, 12). ARMS technology is used for allele discrimination and additional mismatches are introduced near the 3′ terminus of the primers to enhance specificity. For the detection of known mutations, the Scorpion-ARMS method is highly sensitive and fast (13). Our hypothesis was that the ARMS and Scorpion methods could enhance the sensitivity of the detection of EGFR mutations from the wild type.

The aims of this study were to develop a highly sensitive assay for the detection of EGFR mutations in serum DNA, to compare the mutation status in serum to tumors from a subset of their patients, and to clarify the relationship between the EGFR mutation status in serum DNA and clinical manifestations, and in particular the responsiveness to gefitinib.

Materials and Methods

Patients and clinical trials. This study was carried out as a correlative study in a multicenter clinical phase II trial of gefitinib monotherapy at the Department of Respiratory Medicine, Kanzawa University Hospital; the Department of Internal Medicine, Kouseiren Takaoka Hospital; the Department of Internal Medicine, Shinminato Municipal Hospital; the Department of Internal Medicine, Fukuiken Saiseikai Hospital; the Department of Respiratory Medicine, Toyama City Hospital; the Department of Respiratory Medicine, Ishikawa Prefectoral Hospital; and the Department of Respiratory Medicine, Kanazawa Municipal Hospital. According to Simon's minimax design, our study, with a sample size of 25, had an 80% power to support the hypothesis that the true objective response rate was >30% and a 5% significance to deny the hypothesis that the true objective response rate was <10%. Assuming an inevaluability rate of <20%, we projected an accrual of 30 patients. The study was conducted with the approval of the appropriate ethical review boards based on the recommendations of the Declaration of Helsinki for biomedical research involving human subjects. Japanese patients with stage IIIB or IV histologically or cytologically proven chemotherapy-naïve NSCLC were enrolled in this trial. Gefitinib was orally given to all patients at a fixed dosage of 250 mg/d. Efficacy was assessed using the Response Evaluation Criteria in Solid Tumors guidelines (14). The analysis of the samples in this study was done blinded to the clinical outcome.

Blood sample collection and DNA extraction. Blood samples from the 27 patients with NSCLC were collected before the initiation of gefitinib administration. Separated serum was stocked at −80°C until use. Serum DNA was extracted and purified using a Qiamp Blood Kit (Qiagen, Hilden, Germany) with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μL of sterile bidistilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stocked at −20°C until use.

Scorpion ARMS primers for the detection of E746_A750del and L858R. We used an EGFR Scorpion Kit (DxS Ltd., Manchester, United Kingdom), which combined two technologies (i.e., ARMS and Scorpion) to detect mutations in real-time PCR reactions. Four kinds of scorpion primers for the detection of E746_A750del, L858R, and wild type in both exon 19 and exon 21 were designed and synthesized by DxS. The sequences of the scorpion primer for E746_A750del and L858R were based on the GenBank-archived human sequence for EGFR (accession no. AY588246). All reactions were done in 25-μL volumes using 1 μL of template DNA, 7.5 μL of reaction buffer mix, 0.6 μL of primer mix, and 0.1 μL of Taq polymerase. All reagents are included in this kit. Real-time PCR was carried out using SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: initial denaturation at 95°C for 10 minutes, 50 cycles of 95°C for 30 seconds, and 62°C for 60 seconds with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was done with Cepheid SmartCycler software (Ver. 1.2b). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and maximum fluorescence (Fl) were used for interpretation of the results. Positive results were defined as follows: Ct ≤45 and Fl ≥50. These analyses were done in duplicate for each sample and reviewed by two investigators blinded to any clinical information. To confirm the sensitivities for the detection of E746_A750del and L858R, we used the standard DNA that was included in the EGFR Scorpion Kit. Standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000, or 10,000 pg and the mixture of standard DNA with wild type at 10,000 pg and standard DNA with E746_A750del and L858R at a volume of 1, 10, 100, 1,000 or 10,000 pg were used. For quantification, a standard curve was generated by plotting the cycle number of Ct against the log of the DNA volume of the known standards. The linear correlation coefficient (R^2) values and the formula of the slopes were calculated. DNA (10,000 pg) for the positive control was extracted from a Japanese human adenocarcinoma PC-9 cell line known to contain E746_A750del, a Japanese human adenocarcinoma 11_18 cell line known to contain L858R, and a human epidermoid carcinoma A431 cell line known to contain wild-type exon 19.

Tissue sample collection and DNA extraction. Tumor specimens were obtained on protocols approved by the Institutional Review Board. Twenty paraffin blocks of tumor material, obtained from 15 patients at the time of diagnoses (and before treatment), were collected retrospectively. Eleven tumor samples were collected from the primary cancer via transbronchial lung biopsy, one was resected intraoperatively, and nine were from metastatic sites (four from bone, three lymph nodes, one brain, and one colon). All specimens underwent histologic examination to confirm the diagnosis of NSCLC. DNA extraction from tumor samples was done using a DEXPAT kit (TaKaRa Biomedicals, Shiga, Japan).

PCR amplification and direct sequencing. Amplification and direct sequencing were done in duplicate for each sample obtained from serum and tissue specimens. PCR was done in 25-μL volumes using 15 μL of template DNA, 0.75 units of Ampli Taq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 2.5 μL of PCR buffer, 0.8 mmol/L deoxynucleotide triphosphate, 0.5 μmol/L of each primer, and different concentrations of $MgCl_2$, depending on the polymorphic marker. The sequences of primer sets and schedules of amplifications were followed as previously described (12). The amplification was done using a thermal cycler (Perkin-Elmer, Foster City, CA). Sequencing was done using an ABI prism 310 (Applied Biosystems, Foster City, CA). The sequences were compared with the GenBank-archived human sequence for EGFR (accession no. AY588246).

Statistical analysis. Fisher's exact test was used to assess the relationship between the presence of EGFR mutations in patients with NSCLC and different characteristics, including gender, tumor histology, and response to gefitinib. Regarding analyses of response to gefitinib, patients were categorized into the two groups: (*a*) partial response and (*b*) stable disease or progressive disease (Response Evaluation Criteria

in Solid Tumors criteria). We compared Kaplan-Meier curves for overall survival and progression-free survival using the standard log-rank test. Overall survival was defined as the time from the initiation of gefitinib administration to death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the time from the initiation of gefitinib administration to first appearance of progressive disease or death from any cause; patients known to be alive and without progressive disease at the time of analysis were censored at the time of their last follow-up. $P = 0.05$ was considered statistically significant. The statistical analyses were done using the StatView software package version 5.0.

Results

Patients and extracted DNA from serum. Twenty-eight patients were enrolled between October 23, 2002 and August 3, 2003 (Table 1). All patients were evaluated for response and followed for progression-free survival and overall survival. Blood samples (2 mL) were collected from 27 of these patients before the initiation of gefitinib administration. These 27 patients represented a subset of that phase II study. Serum DNA was extracted in all 27 samples at a median concentration of 70.0 ng/mL (range, 0-1,720.0 ng/mL).

Sensitivity of the EGFR Scorpion. Preliminary experiments were done to evaluate the sensitivity of the EGFR Scorpion kit (Fig. 1A-C). All curves using E746_A750del and L858R standard DNA (volumes of 1-10,000 pg) increased up to 45 cycles (Fig. 1A). When wild-type standard DNA and distilled water were used as negative controls, the curves did not increase and continued flat at 50 cycles (Fig. 1A and C). When diluted E746_A750del and L858R standard DNA were mixed with wild-type standard DNA at ratios from 10^0 to 10^{-5}, all curves that indicated the presence of E746_A750del and L858R increased up to 45 cycles (Fig. 1B and D). Standard curves in the range of measured volumes in this study were linear with r^2 values from 0.987 to 0.998. Both slopes of curves were almost parallel (Fig. 1E). The Ct of diluted mutant standard DNA mixed with wild-type DNA was close to that of mutant standard DNA alone. Although the peak fluorescence levels of diluted E746_A750del standard DNA mixed with wild-type DNA were lower than without wild-DNA standard, the presence of E746_A750del was clearly detected at ratios less than 10^{-4}. The peak fluorescence levels of diluted L858R standard DNA mixed with wild-type DNA were equivalent to those without wild-DNA standard. Curves of DNA with the mutations at an amount of up to 1 pg were unaffected by interfusion of DNA of wild-type EGFR. There were no significant differences between either the minimum detectable volume of the mutations or the minimum detectable ratio of wild type to the mutations.

In the cell-based experiments using genomic DNA of human cancer cell lines, the signal using DNA derived from the PC-9 cells was detected whereas the signal using DNA from the A431 cells was, as expected, not detected (Fig. 1D and E).

EGFR mutation status of serum DNA detected by EGFR scorpion. The E746_A750del or L858R status of serum DNA derived from 27 patients with NSCLC was examined. Wild-type exons 19 and 21 were detected from all serum samples. E746_A750del was detected in samples of 12 patients. L858R was detected in 1 patient (Table 2). In total, EGFR mutations were detected in 13 of 27 (48.1%) patients. The histologic subtypes of the original tumors are summarized in Table 3A in the 27 patients who were assessed for EGFR mutation in serum. Eleven of 23 (47.8%) cases of adenocarcinoma, one of two cases of squamous-cell carcinoma, and one of two cases of large-cell carcinoma were positive for EGFR mutations. An EGFR mutation was more frequently detected in the samples from female patients than those from males [7 of 10 (70%) versus 6 of 17 (35%); Table 3B].

EGFR mutation status in serum (EGFR Scorpion) and response to gefitinib. EGFR mutations were more frequently observed in the samples from the patients who showed a partial response (7 of 9 cases, 77.8%) than in samples from patients with stable disease or progressive disease (6 of 18 cases, 33.3%; $P = 0.046$, Fisher's exact test; Table 3C).

EGFR mutation status in serum (EGFR Scorpion) and effect on survival. Median progression-free survival and overall survival of all the patients treated with gefitinib were 98 and 306 days, respectively. Patients with EGFR mutations in serum showed a significantly longer median progression-free survival compared with the patients without EGFR mutations (200 versus 46 days, $P = 0.005$; Fig. 2A). The patients with EGFR mutations showed a longer median overall survival compared with the patients without EGFR mutations, although there was no statistical significance (611 versus 232 days, $P = 0.078$; Fig. 2B). These results suggest that patients who were serum EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative.

EGFR mutation in serum analyzed by direct sequencing and in comparison with EGFR Scorpion. The deletional mutation (E746_A750del) was detected by direct sequencing in serum DNA extracted from 10 of 27 patients (37.0%). No point mutation in exons 18, 19, and 21 was detected in the PCR

Table 1. Patient characteristics

	(*n*)
No. patients	27
Age (y)	
Median	64
Range	44-87
Sex	
Male	17
Female	10
Performance status	
0	19
1	6
2	2
Stage	
IIIB	3
IV	24
Histology	
Adenocarcinoma	23
Squamous-cell carcinoma	2
Large-cell carcinoma	2
Response	
Partial response	9
Stable disease	8
Progressive disease	10

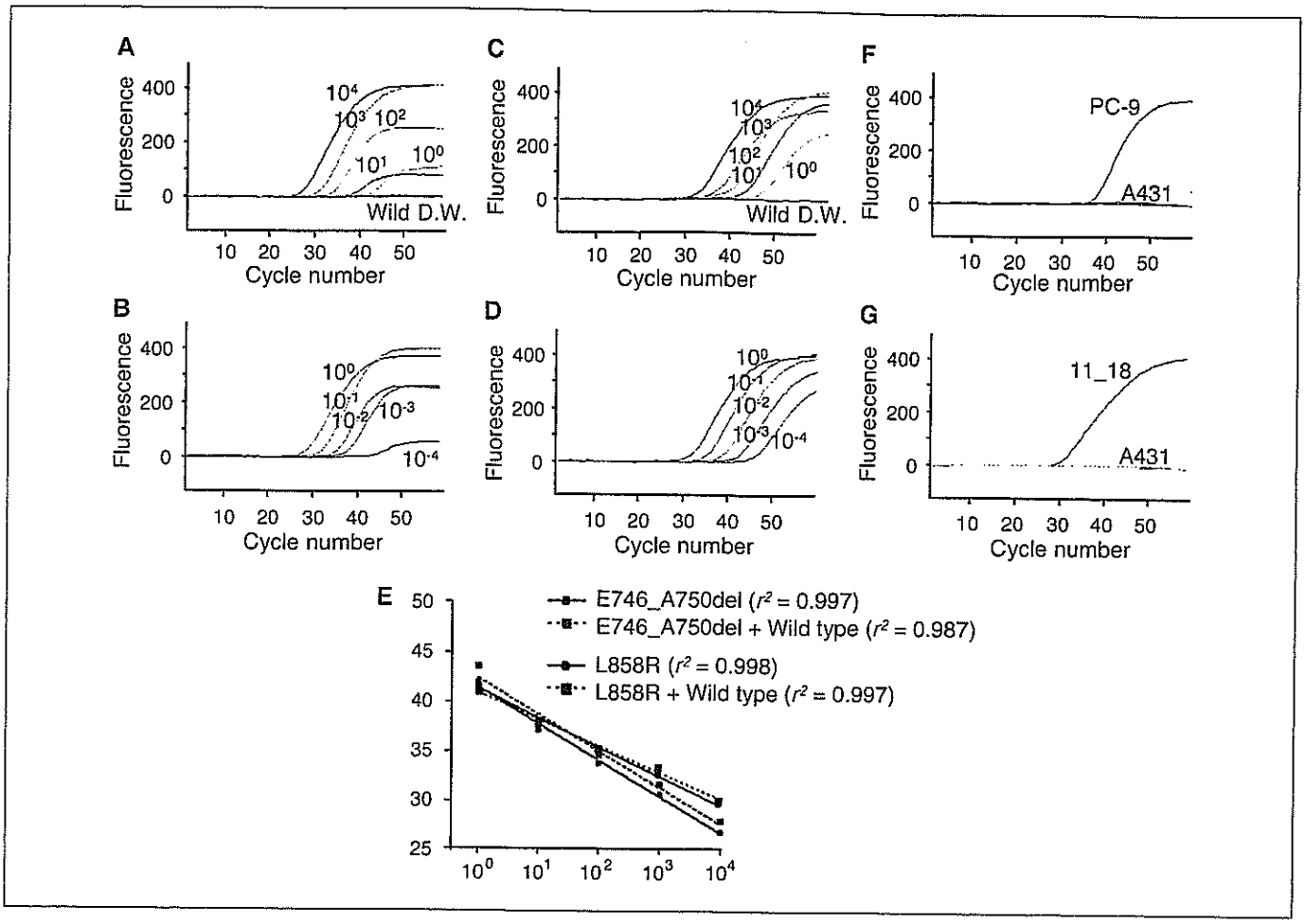

Fig. 1. Sensitivity of detection for mutations of E746_A750del and L858R using the EGFR Scorpion kit (*A* and *B*, E746_A750del; *C* and *D*, L858R). Standard DNA with E746_A750del (*A*) and L858R (*C*) were used at various volumes of 10,000 pg (10^4), 1,000 pg (10^3), 100 pg (10^2), 10 pg (10^1), and 1 pg (10^0). Standard DNA with wild-type (*Wild*) and distilled water (*D.W.*) were used as negative controls in the same experiment. Standard DNA with E746_A750del (*B*) and L858R (*D*) at concentrations from 1 to 10,000 pg were mixed with 10,000 pg of standard DNA with wild-type at a ratio of 1:1 (10^0), 1:10 (10^{-1}), 1:100 (10^{-2}), 1:1,000 (10^{-3}), and 1:10,000 (10^{-4}). *E*, standard curves were derived by plotting the Ct of each curve (shown in *A-D*) against the log of the standard DNA volume (*black lines*, E746_A750del; *blue lines*, L858R). *F*, PC-9 with E746_A750del and A431 with wild-type. *G*, 11_18 with L858R and A431.

products from serum samples. The serum EGFR status detected by direct sequence was not correlated statistically with histologic type, gender, response to gefitinib (Table 3), or survival (progression-free survival, $P = 0.277$; overall survival, $P = 0.859$). EGFR mutation status, as assessed by direct sequence, was consistent with those assessed by EGFR Scorpion in 15 of 27 (55.6%) of the paired samples. In four cases, EGFR mutation status (E746_A750del) was positive by direct sequence and negative by EGFR Scorpion. Eight cases were negative by direct sequence and positive by EGFR Scorpion. Thus, the sensitivity of EGFR Scorpion seems to be higher than that of direct sequencing due to the use of the specific primers for EGFR mutations in this kit.

EGFR mutations in tumors in comparison with those in serum. Twenty tumor samples were obtained from 15 patients retrospectively. Sequencing of EGFR exons 19 and 21 was done in samples from 12 of these under the same PCR conditions (Table 4; the other three samples were not evaluated because of low amplification of PCR products). EGFR mutations were detected in four cases (25.0%); three were the 15-bp deletion (E746_A750del) in exon 19 and one was the L858R point mutation in exon 21. Tumor histology of patients with EGFR mutations was adenocarcinoma in three and large-cell carcinoma in one. The responses to gefitinib in these four patients were two partial response, one stable disease, and one progressive disease.

Pairs of tumor samples and serum samples were obtained retrospectively from 11 patients (Table 4). The EGFR mutation status in the tumors was consistent with those in the serum of 8 of 11 (72.7%) of the paired samples. The E746_A750del mutation was positive in the tumor and negative in the serum in two patients, and the E746_A750del mutation was negative in the tumor and positive in the serum in one patient.

Discussion

Our findings have shown that EGFR mutations were detectable in serum samples obtained from patients with NSCLC and that the EGFR Scorpion kit consisting of ARMS and Scorpion technology is a useful method for detection of EGFR mutations. The EGFR mutation status in serum detected by the EGFR Scorpion was correlated statistically with responsiveness to, and the progression-free survival of, gefitinib treatment. Our finding supports the hypothesis that the EGFR

mutation status from serum DNA is useful to predict the responsiveness to gefitinib.

The mutation rate observed in our study seems to be relatively high (48%) although we have detected only two major mutations. EGFR mutations have been detected at a higher frequency in lung tumors from female patients, those with adenocarcinoma histology, nonsmokers, and patients of Asian origin (6, 8). However, previous reports show that the mutation rate of EGFR in operative samples of Japanese patients was from 26% to 59% (4, 6, 15, 16). The EGFR mutation rate in our study is equivalent to that observed in these reports. It can be speculated that the high sensitivity and specificity of the EGFR Scorpion allowed us to detect the EGFR mutations even in serum. Another possible reason is the high number of patients with adenocarcinoma in our study (23 of 27, 85.2%). Previous studies have shown that very few patients with nonadenocarcinoma, including squamous cell carcinomas and large-cell carcinomas, have EGFR mutations (3–8). Our results were in line with the previous studies and showed that no patients with squamous cell carcinoma or large-cell carcinoma had the mutations.

Table 2. Patients' characteristics and EGFR mutant status detected from serum DNA using the EGFR ARMS-Scorpion method

Response	Gender	Histology	Exon 19		Exon 21	
			Wild	E746_A750del	Wild	L858R
PR	M	Ad	+	–	+	+
PR	F	Ad	+	+	+	–
PR	M	Ad	+	–	+	–
PR	F	Ad	+	+	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	–	+	–
PR	M	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
PR	F	Ad	+	+	+	–
SD	M	Large	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Ad	+	–	+	–
SD	F	Ad	+	+	+	–
SD	M	Scc	+	+	+	–
PD	F	Scc	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Large	+	+	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	–	+	–
PD	M	Ad	+	+	+	–
PD	M	Ad	+	–	+	–

Abbreviations: SD, stable disease; PD, progressive disease; PR, partial response; M, male; F, female; Ad, adenocarcinoma; Large, large-cell carcinoma; Scc, squamous-cell carcinoma; +, curve detected by SmartCycler; –, curve not detected by SmartCycler.

Table 3. Frequency of EGFR mutations in serum DNA from patients with NSCLC according to histology (A), gender (B), and response to gefitinib (C)

	EGFR Scorpion kit			Direct sequence		
	+	–		+	–	
(A) Histology and EGFR mutant states						
Ad	11	12		8	15	
Non-Ad	2	2	$P > 0.999$	2	2	$P > 0.999$
(B) Gender and EGFR mutant states						
Female	7	3		5	5	
Male	6	11	$P = 0.120$	5	12	$P = 0.415$
(C) Response to gefitinib and EGFR mutant states						
PR	7	2		4	5	
SD/PD	6	12	$P = 0.046$	6	12	$P = 0.683$

NOTE: A total of 27 samples were obtained from 28 patients before treatment.

We identified 12 deletion mutations and a single point mutation (L858R). Previous reports have shown that the frequency of detection of E746_A750del is almost equivalent to that of L858R (15, 16). It seems that the rate of detection of L858R in our study was very low compared with the rate of E746_A750del. The sensitivity for detection of L858R using the Scorpion ARMS method is very high and equivalent to that of E746_A750del. We thus consider that it is unlikely that the low-frequency L858R mutation could be due to assay-related false-negative findings. On the other hand, it also seems unlikely that either sampling method or the patients' eligibility criteria are biased toward the high rate of E746_A750del. Therefore, we have not been able to clarify the moot point. Further analyses in much larger groups of patients will be necessary to clarify the frequency of the major two mutations in serum DNA. Unfortunately, parallel tumor tissue investigations were done only on a small subset of the participating patients. Furthermore, findings in the serum were divergent from those obtained from the primary tissue in 3 of 11 patients from whom the paired samples were obtained. Therefore, this study is at best hypothesis-forming and will require follow-up analysis in much larger groups of patients.

Some investigators reported that mutations in the EGFR tyrosine kinase domain enhanced responsiveness to the EGFR tyrosine kinase inhibitors gefitinib and erlotinib, and seemed to be associated with the prolonged survival of the patients who received these drugs (7, 8). In a placebo controlled study showing a survival advantage for NSCLC patients who received erlotinib, Tsao et al. (17) showed that the presence of an EGFR mutation might increase responsiveness to erlotinib, but was not indicative of a survival benefit, and concluded that EGFR mutation analysis was not necessary to identify patients in whom treatment with EGFR inhibitors was appropriate. Our results are not in line with their conclusions. In their study, the rate of mutation analysis was low and 107 of 731 patients

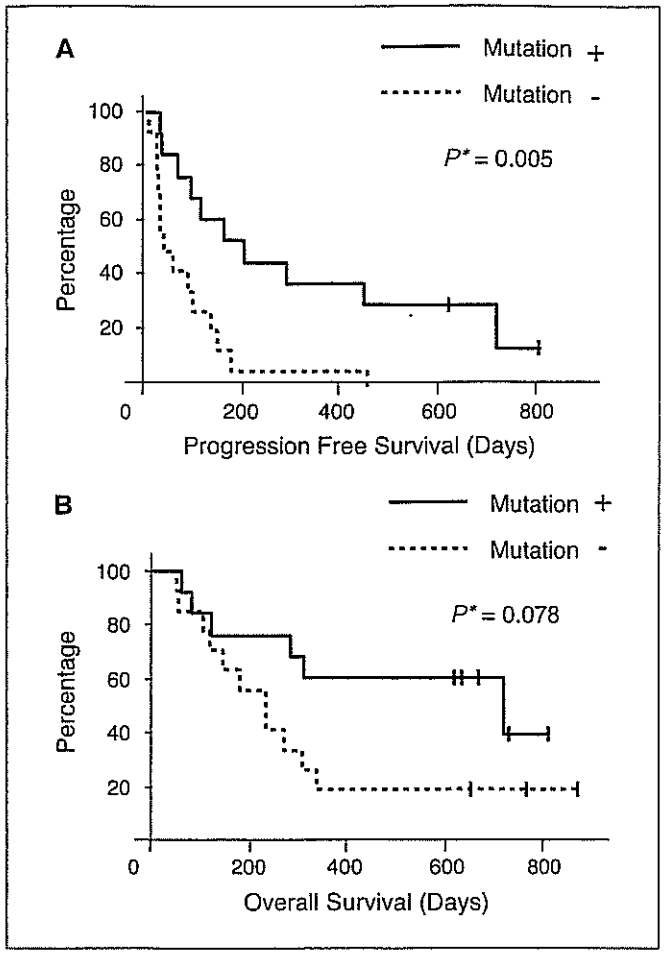

Fig. 2. Progression-free survival (*A*) and overall survival (*B*) with respect to the EGFR mutation status of NSCLC. *, log-rank test.

enrolled in their study were successfully analyzed for EGFR mutation. Sensitivity for detecting EGFR mutation in their study might be unstable as interfusion of normal cells in tumor cells decreases the sensitivity for detecting tumor-derived mutations using direct sequencing. They propose that additional processes (such as microdissection) to enrich tumor cell DNA might increase the rate of detection of new mutations; however, it seems that their results are insufficiently robust to reach this conclusion. Therefore, we propose the use of EGFR mutation analysis from serum DNA, which is easily collected and repeatable, to show that EGFR mutation status using the EGFR Scorpion kit correlates with the responsiveness to gefitinib.

EGFR mutation in NSCLC is reported to be somatic (3, 4). It is well known that the concentration of free circulating DNA in serum is higher in patients with tumors than in healthy volunteers (18) and it seems that the detected mutational EGFR in serum was tumor derived. This is the first report analyzing EGFR mutations from serum DNA and evaluating EGFR mutation status and clinical outcome (response and survival) with gefitinib. No other studies have analyzed EGFR mutations from samples other than actual tumor samples. The mutation in two patients was positive in the tumor and negative in the serum, and the mutation in one patient was negative in the tumor and positive in the serum. We have tried to explain the discrepancy why tumor and serum were not better correlated as follows. In cases of positive in the tumor and negative in the serum, the volumes of mutant DNA extracted from the serum were under the detectable limit using the Scorpion ARMS method, or a very small amount of DNA derived from an actual tumor was circulating in the bloodstream. A previous study showed that 73% of patients with at least one molecular event, such as a hypermethylation of the tumor suppressor gene *p16*, in their tumor DNA had the same alteration in plasma DNA (10). In a case of negative in the tumor and positive in the serum, wild-type DNA interfered with

Table 4. EGFR mutation status in tumor samples and serum samples. Pairs of both tumor samples and serum samples were obtained from 11 patients

Gender	Histology	Response	EGFR mutation status				
			Tumor sample	EGFR Scorpion kit (serum sample)			
				Exon 19		Exon 21	
				Wild	Mutation	Wild	Mutation
M	Large	SD	Wild	+	–	+	–
F	Scc	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PR	L858R	+	–	+	+
F	Ad	SD	Wild*	+	+	+	–
M	Large	PD	E746-A750del	+	+	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	PD	Wild	+	–	+	–
M	Ad	SD	E746-A750del*	+	–	+	–
F	Ad	PR	E746-A750del*	+	–	+	–
M	Ad	PD	Wild	+	–	+	–

*Patients who have different states of EGFR mutation from tumor-derived DNA and serum-derived DNA.

the detection of mutant DNA in the tumor samples using the direct sequencing method. The rate of the mutations in serum DNA detected by the Scorpion ARMS was compared with that in tumor tissues detected by the direct sequencing method as a current standard method. DNA from tumor samples consisted of a mixture of the mutant DNA and wild-type DNA because the EGFR mutation status was always heterogeneous, and the complete removal of normal cells, such as normal epithelial cells and inflammatory cells, from tumor specimens is very difficult. Parallel tumor tissue investigations were done on only a small subset of these patients, which is a recognized limitation in the present study. A larger study is necessary to evaluate the consistency of the mutation status from tumor and serum. On the other hand, it is sometimes difficult to obtain tumor samples from patients with inoperable NSCLC in prospective studies. We showed that patients who were EGFR mutation positive in the serum DNA using the Scorpion ARMS method seem to have better outcomes with gefitinib treatment in terms of progression-free survival, overall survival, and response, despite the nonconformity between the mutation states of tumor and serum DNA in some of the patients. We anticipate that the detection of EGFR mutations in serum DNA using the Scorpion ARMS will be equivalently useful as a feasible approach for predicting tumor response to gefitinib.

Two groups have reported alternative methods for detection of EGFR mutations. One group used the LightCycler PCR assay (19) and the other postulated that the SSCP assay was more sensitive than direct sequencing and was a rapid method (20). Further studies are needed to validate these assays for detection of EGFR mutations and to clarify the most sensitive assay. Although the direct sequence method is common in reported studies, the EGFR mutation status in serum DNA by direct sequencing did not correlate with the responsiveness to and survival benefit of gefitinib in our study. These results indicate that the EGFR Scorpion kit is superior to the direct sequencing method for detection of an EGFR mutation in serum as a predictive marker.

One limitation of the EGFR Scorpion kit is that it is only able to detect mutations targeted by the designed Scorpion primers. EGFR mutations are not solely at these two sites but clustered around the ATP-binding site in exons 18, 19, and 21 (3–8). Moreover, the secondary mutation, a substitution of methionine for threonine at position 790 (T790M), leads to gefitinib resistance in NSCLC patients who have EGFR mutations and are responsive to treatment with gefitinib (21–23). Mutations in *K-ras*, a known downstream signaling molecule in the EGFR signaling pathway, are more frequent in patients who develop disease progression with treatment with either gefitinib or erlotinib (24). These mutation states may also be critical factors for the treatment of gefitinib. To clarify the usefulness of serum DNA as a source of genotypic information, the Scorpion primers need to be designed for detection of these mutations, and further studies using these primers are required.

In conclusion, the two major mutations of EGFR, E746_A750del and L858R, were detected in serum DNA with the EGFR Scorpion kit from patients with NSCLC. These results suggest that patients who were EGFR mutation positive seem to have better outcomes with gefitinib treatment, in terms of progression-free survival, overall survival, and response, than those patients who were EGFR mutation negative. In the near future, a controlled clinical trial is necessary to confirm these conclusions.

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Cancer Chemother Pharmacol (2006) 58: 173–182
DOI 10.1007/s00280-005-0149-6

ORIGINAL ARTICLE

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Phase I and pharmacokinetic study of edotecarin, a novel topoisomerase I inhibitor, administered once every 3 weeks in patients with solid tumors

Received: 7 June 2005 / Accepted: 29 October 2005 / Published online: 25 November 2005

Abstract *Purpose*: Edotecarin (J-107088) is a potent indolocarbazole topoisomerase I inhibitor which is structurally distinct from the camptothecins. This study aimed to determine the maximum tolerated dose (MTD), the recommended dose for future Phase II studies and the safety, pharmacokinetic profile, and preliminary antitumor activity of edotecarin in a population of patients with advanced solid tumors. *Experimental design*: Edotecarin was administered as a single dose by IV infusion over 2 h every 21 days (with 1 week permitted for recovery from toxicities, if needed) in patients with advanced solid tumors. Doses ranged from 8 to 15 mg/m^2. Pharmacokinetic assessments were performed during and after the first administration. *Results*: Twenty-four patients received 61 cycles of therapy. Dose-limiting toxicities (infection, febrile neutropenia, constipation, ileus, and prolonged grade 4 granulocytopenia) were observed in 3 of 5 evaluable patients at the 15 mg/m^2 dose, defining the MTD. The most commonly reported non-hematologic toxicities were anorexia, nausea, malaise, and constipation. Diarrhea was neither frequent nor severe. Neutropenia was the most common hematologic toxicity (grade 3–4 in 21/23 patients during cycle 1). Plasma concentrations of edotecarin rose rapidly following the start of the 2-hour infusion, reaching C_{max} values of 103 ± 17 ng/ml at the 13 mg/m^2 dose, and decreased steeply after the end of the infusion. Plasma concentrations declined to approximately 1–2 ng/ml at 26 h post start of infusion, the last PK sampling time point. The mean apparent plasma half-life of the drug was 20 h, which should be considered a preliminary estimate until results from studies with a longer duration of plasma sampling are available. A mean of 1.4–3.6% of the dose was recovered as unchanged drug in the urine over 48 h. Unconfirmed tumor regression ≥50% was observed in 2 patients, 1 with metastatic gastric carcinoma and 1 with esophageal cancer. *Conclusions*: The MTD of edotecarin administered IV over 2 h every 21 days was 15 mg/m^2. The recommended dose for Phase II studies with a 3-week schedule (with 1 week permitted for recovery from toxicities, if needed) is 13 mg/m^2. The observed safety profile and preliminary evidence of antitumor activity warrant further investigation of this drug in solid tumors.

Keywords Edotecarin · Pharmacokinetics · Maximum tolerated dose · Dose-limiting toxicity · Topoisomerase I inhibitor

Presented at the American Society of Clinical Oncology 2002 Annual Meeting (abstract no. 385).

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Introduction

DNA damage mediated by topoisomerase I (topo I) inhibitors is an important mechanism of antineoplastic activity [1]. During DNA replication, topo I relieves torsional strain by causing a reversible single-strand

break in the DNA [2]. Topo I binds to DNA at the break site to form a cleavable complex. Topo I inhibitory drugs bind to the topo I-DNA complex, stabilizing it and preventing the religation of the single-strand breaks. Cell death appears to be due to double-strand DNA damage that occurs during DNA synthesis when replication enzymes interact with the stabilized cleavable complex. Currently in the USA, two topo I inhibitors, both camptothecin derivatives, are available for the treatment of cancer patients: topotecan, approved for ovarian carcinoma and small cell lung cancer indications, and irinotecan, approved for metastatic colorectal carcinoma [3–6]. The remarkable anticancer activity of the campothecins and the possibility that other topo I inhibitors could exhibit different activity, better tolerability, or a more favorable pharmacokinetic profile have led to the search for new topo I inhibitors.

Edotecarin (J-107088) is a new derivative of NB-506, an indolocarbazole antitumor agent with a chemical structure completely different from the camptothecins (Fig. 1) [7]. Like camptothecin and its derivatives, edotecarin inhibits topo I [7, 8], but because of the distinct structure of this agent, its interaction with the target enzyme differs significantly from the camptothecin derivatives [9]. Edotecarin is a more potent inhibitor of topo I than camptothecin. The cleavable complex formed with edotecarin is more stable than that with camptothecin and persists significantly longer after removal of drug from cell culture medium [7]. The activity of edotecarin does not appear to be cell-cycle dependent. In vitro edotecarin has demonstrated a wide spectrum of activity against human cancer cell lines [7] and is active in vivo against a variety of human and murine tumor-derived xenografts as well as experimental liver metastases [10, 11]. Preclinical studies in animal species have shown that edotecarin is largely eliminated as unchanged parent drug via biliary excretion (unpublished Banyu data), in marked contrast to the camptothecin analog, irinotecan, which is characterized by a very complex disposition and metabolic pathways. In vitro studies showed that edotecarin was not metabolized by liver microsomes or by hepatocytes from humans or several animal species (unpublished Banyu data). The unique in vitro and in vivo pharmacological profile of edotecarin relative to other topo I inhibitors makes this compound a potentially useful antineoplastic agent.

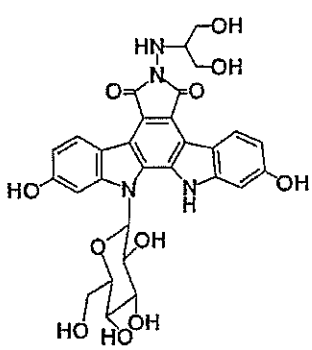

Fig. 1 Structure of edotecarin, an indolocarbazole. Molecular weight: 608.56 Da

When this study was initiated in Japan, edotecarin had been previously investigated in 2 Phase I studies in the USA. In the first [12], edotecarin was administered as a 2 h IV infusion once every 21 days (with an additional week permitted for recovery to ≥grade 1 toxicity, if necessary) at doses of 6, 8, 11, 13 and 15 mg/m^2. Nausea, vomiting, headache, fatigue, febrile neutropenia and neutropenia were dose-limiting at 15 mg/m^2. Treatment could be administered repeatedly for 2 or more courses in 24 of 29 patients (83%). One patient with metastatic bladder cancer had a confirmed partial response of long duration and 12 patients showed stabilization of disease. In the second study [13], edotecarin was administered as a 1 h IV infusion twice-weekly (on days 1, 4, 8, and 11) in cycles of at least 28 days at doses of 2, 4, 5.5, and 7.5 mg/m^2/day. Mucositis, neutropenia and thrombocytopenia were dose-limiting at 7.5 mg/m^2/day. Only two patients received treatment beyond cycle 2.

Based on these results, this Phase I study was designed to evaluate ascending doses of edotecarin administered by 2 h IV infusion every 21 days. A more sensitive assay was utilized in this study compared to the earlier study, of similar design, conducted in the USA. Objectives of the study were to determine the MTD (where the MTD is the maximum dose administered) and the dose to be recommended in future Phase II studies, and to assess its safety, pharmacokinetic profile, and the preliminary antitumor activity in a population of Japanese patients with advanced solid tumors.

Patients and methods

Patients

Japanese patients with histologically or cytologically confirmed evaluable malignant solid tumors refractory to conventional chemotherapy or tumors for which no effective therapy existed were candidates for this study. Inclusion criteria also included the following: age ≥20 years; Eastern Cooperative Oncology Group (ECOG) performance status 0, 1, or 2; life expectancy ≥12 weeks; absolute granulocyte count ≥1,500/mm^3, platelet count ≥100,000/mm^3, hemoglobin ≥9 g/dl, and serum creatinine < 1.5 mg/dl. Additional entry criteria were serum total bilirubin within the normal limit and serum AST, ALT, and alkaline phosphatase less than twice the upper limit of normal. A 4-week interval was required for chemotherapy, radiation therapy, or immunotherapy treatments (6-week interval for patients previously treated with mitomycin C or nitrosoureas). A 2-week interval after major surgery was required.

Patients had to have fully recovered from toxicity associated with previous therapy. Patients were ineligible for the study if they had symptomatic central nervous system metastases, neurological symptoms, fever, or unstable significant clinical conditions. Patients who were receiving corticosteroids, anticoagulants, immunotherapy, biological response modifiers, or other investigational agents; or who had received prior radiation therapy to 20% greater of bone marrow; or who had received a bone marrow stem cell transplant, were excluded from the study.

The protocol was approved by the institutional review boards of the National Cancer Center Hospital and the Nagoya Medical Center, and all patients gave written informed consent prior to study entry.

Dosage and dose escalation

Edotecarin (J-107088) was provided as an injectable preparation in plastic infusion bags by Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). Each bag contained 37.5 mg edotecarin in a 250 ml 5% glucose solution (final edotecarin concentration of 0.15 mg/ml). Edotecarin was administered by intravenous (IV) infusion over 2 h every 21 days. Patients were hospitalized for the initial course of edotecarin and remained hospitalized for close observation for 21–28 days thereafter. Subsequent courses could be administered on an outpatient basis with a weekly evaluation by the investigator.

The initial dose of edotecarin was 8 mg/m^2, and subsequent doses were escalated in approximately 33% increments (to 11 and 15 mg/m^2). Patients were enrolled in cohorts of 3 patients per dose and observed for 21 days; the observation period was extended to 28 days if a longer recovery period was needed. If only one of the three patients experienced a dose-limiting toxicity (DLT), then three additional patients were treated at the same dose. If none of the first three patients in a cohort, or if only one of six patients demonstrated DLT, then the next three patients were treated at the next higher dose. If at least two patients in the cohort experienced DLT, that dose level was regarded as the MTD. The dose for the next cohort would then be reduced by approximately 15%. The recommended dose for future Phase II studies was to be evaluated in a total of nine patients and was to be the highest dose at which fewer than a third of treated patients experienced a DLT. Individual patients who did not experience DLT and had no evidence of disease progression could receive up to four courses of edotecarin at the dose originally assigned. No intrapatient dose escalation was permitted. Patients who had a DLT that had recovered to grade 2 or less could continue treatment with edotecarin at a dose below the dose at which DLT was demonstrated. If DLT occurred at the reduced dose, no further treatment was to be administered. Patients with progressive disease were to discontinue treatment.

Definition of dose limiting toxicity (DLT)

For the purpose of this study, DLT was defined as the occurrence of pre-specified severe adverse events [severity defined according to the National Cancer Institute Common Toxicity Criteria (NCI CTC) version 2.0], occurring during cycle 1 and attributed to edotecarin. Criteria that are relevant to DLT's observed in this study were: grade 3 or 4 non-hematologic toxicity (except nausea, vomiting, fever and fatigue effectively managed with symptomatic treatment, and alopecia); grade 4 granulocytopenia accompanied by fever of ≥39.1°C, or accompanied by an infection requiring antibiotic or antifungal treatment based on fever of ≥38.0°C, or that persisted ≥5 days; grade 4 leukopenia that persisted ≥5 days; or failure of granulocyte and platelet counts to return to ≥1,500/mm^3 and ≥100,000/mm^3, respectively, within 28 days after edotecarin administration.

Supportive care

Each patient received granisetron 3 mg and dexamethasone 20 mg IV pretreatment on day 1 and dexamethasone 8 mg IV on days 2–4 post treatment for prevention of nausea, vomiting, and general malaise. Granisetron 3 mg IV was also administered on days 2–4 if needed. Routine use of colony-stimulating factors was not permitted during cycle 1. However, patients who had granulocytopenia that had met the criteria for DLT were permitted to receive filgrastim in cycle 1 and subsequent cycles.

Patient evaluation

Patients were evaluated at baseline and periodically throughout the study. During the first cycle, vital signs were measured every 1–3 days, hematology determinations were performed every 2–3 days, and serum biochemistries on days 3, 8, and 15. Physical examinations, including evaluation of performance status and measurement of palpable tumors, were done on days 8 and 15. During the second and the subsequent cycles, vital signs, laboratory tests and toxicity evaluations were performed on days 1, 8, and 15 of each cycle. For cycle 1, blood coagulation studies were done before each dose and on days 8 and 15. Measurements for subsequent cycles were on day 1, and finally, within 2 weeks after terminating the study. Performance status was assessed by the physician according to the ECOG criteria [14]. Tumor responses were based on WHO criteria. Radiographic evaluations of tumor size were performed every two cycles and were to be repeated after 4 weeks in case of response. Safety events were recorded on the basis of changes in signs and symptoms, physical findings, vital signs, and laboratory abnormalities. Weekly severity

assessments of subjective and objective findings were performed according to the NCI CTC version 2.

Plasma edotecarin measurements and assay

Pharmacokinetic analysis

Pharmacokinetic studies were performed during the first cycle of treatment. On day 1, blood samples (6 ml each) were drawn into heparinized tubes from an indwelling IV cannula in the arm contralateral to the arm bearing the infusion line. Samples were collected before infusion, at 15 and 60 min after the start of the infusion, at the end of the 2-h infusion, and at 5, 15, 30, and 60 min and 2, 4, 6, 8, 10 and 24 h after the end of the infusion. Urine samples were collected over two 24-h intervals for 48 h after the start of the infusion.

The concentrations of edotecarin in plasma and in urine were analyzed by Mitsubishi Chemical BCL (Tokyo) using validated, high-performance liquid chromatography. J-109404, a chemical analog of edotecarin, was the internal standard. BondElut CH cartridges were pre-conditioned with successive 1-ml washes of dichloromethane, methanol, and water. Plasma samples (1 ml) were mixed with 0.1 ml of 10% acetonitrile, 0.1 ml of the internal standard (1 μg/ml) in 10% acetonitrile, and 1 ml of 50 mM, pH 7.0 phosphate buffer and applied to pre-washed BondElut cartridges. The cartridges were then washed with 1 ml of water, spun at 1,000 rpm for 1 min at 4°C, washed again with 1-ml of 20% methanol, and spun again. To each column was then added 0.5 ml of 60% methanol followed by centrifugation to elute the retained compounds of interest. Eluates were dried under a nitrogen stream, reconstituted in 0.1 mL of mobile phase, and transferred to Ultrafree MC (0.2 μm) centrifugal filter units. The filter units were spun at 15,000 rpm for 5 min at 4°C and 60 μl of the filtrate was chromatographed. Chromatography was carried out on a Superiorex ODS S-5 μm, 4.6 mm ID×250 mm column with a Capcell C18 UG120, 4 mm ID×10 mm guard cartridge (source of columns was Shiseido, Tokyo) eluted with water/acetonitrile/methanol/trifluoroacetate (TFA) (67/18/15/0.1) flowing at 1.0 ml/min. The ultraviolet absorbance of the effluent was monitored at 334 nm. Linearity was demonstrated over the range of 1–500 ng/ml. Assays of quality control samples (each assay run included 2 replicate QC samples each at 2.5, 40.0, and 400.0 ng/ml) were within ±20% of nominal concentrations, with one exception: a single 2.5 ng/ml replicate assayed at 1.6 ng/ml in 1 assay run. The lower limit of quantitation (LLOQ) was 1.0 ng/ml. The interday and intraday coefficients of variation for plasma are 2.0~5.9% and 2.0~4.0%, respectively.

Extraction and chromatography of urine samples was carried out with slight modifications of the plasma method. The urine volume assayed was 0.1 ml, which was mixed with 0.1 ml of 10% acetronitrile, 0.1 ml of 2 μg/ml internal standard in 10% acetonitrile, and 0.2 ml of mM phosphate buffer, pH 7.0. Extraction was carried out using pre-washed BondElut CN columns. The mobile phase consisted of acetonitrile/water/TFA (75/25/0.1) flowing at 0.8 ml/min. The absorbance of the effluent was monitored at 430 nm. LLOQ was 50 ng/ml. Performance of the urine assay was comparable to that of the plasma assay. The interday and intraday coefficients of variation for urine are 1.1~8.5 and 3.3~6.6%, respectively.

Adequate freeze/thaw, frozen storage, and autosampler stability were demonstrated for both plasma and urine samples. Stability for plasma and urine was demonstrated after three cycles of freezing and thawing; at −70°C for 3.5 months; and for 24 h while in the autosampler during the assays.

Statistical analysis

Noncompartmental pharmacokinetic parameters were computed using WinNonlin, version 3.1 software (Pharsight Corporation, Mountain View, CA, USA) [15]. An apparent terminal half-life ($t_{1/2}$) was calculated from plasma concentrations observed at 8, 10, and 24 h after the end of the infusion. Dose proportionality was analyzed by means of a power model: $AUC_{0-\infty} = A \times dose^B$ and $C_{max} = C \times dose^D$, where A and C are proportionality constants, dose units are milligram/patient, and the 95% confidence limits around B and D include 1 if AUC and C_{max} are directly proportional to dose. Nonlinear regression of $AUC_{0-\infty}$ and C_{max} versus dose and 95% confidence limits on computed parameters B and D were computed using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA, USA). Descriptive statistics (Table 6) were also computed using GraphPad Prism.

Results

Patient characteristics

Twenty-four Japanese patients were enrolled into the study (Table 1) and received at least one dose of edotecarin. The median age of the patients was 56 years (range 33–72), 22 (92%) had an ECOG performance status of 1 at baseline, and 16 (67%) patients were male. The most common tumor types were lung and colorectal cancer. All patients except one had previously received chemotherapy with a median number of chemotherapy regimens of 2 (range 0–4). Patients treated at various dose levels had similar baseline characteristics.

All 24 patients were assessed for both safety and efficacy.

A total of 61 cycles of treatment were administered in these patients. The majority of cycles were administered every 3 weeks, with 10 cycles administered at day 28. Three cycles were administered beyond this to accommodate patients' personal reasons and schedules. The

Table 1 Patient characteristics

Number enrolled	24
Age (years)	
Median	56
Range	33–72
Male:female (no. of patients)	16:8
ECOG performance status (no. of patients)	
0	2
1	22
Tumor type (no. of patients)	
Lung	7
Colorectal	4
Uterine sarcoma	3
Gastric	2
Bile duct	2
Primary unknown	2
Other	4
Prior treatment	
Chemotherapy	
No. of prior regimens (no. of patients)	
0	1
1	11
2	8
3	3
4	1
Median (no. of regimens)	2
Surgery (no. of patients)	11
Radiation therapy (no. of patients)	5
Chemotherapy + radiation therapy (no. of patients)	5

median number of cycles at the 13 mg/m^2 dose was 2 (range 1–6). The most common reasons for treatment discontinuation were progressive disease or best response achieved (i.e., further treatment effect not expected) (nine patients each) (Table 2).

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

At the initial dose of 8 mg/m^2, no patient experienced DLT. At the next dose of 11 mg/m^2, 1 of 6 patients experienced DLTs (grade 3: infection, febrile neutropenia, and hypoxia). Six patients were enrolled at 15 mg/m^2 . Three patients experienced DLTs (grade 3: infection, febrile neutropenia, and constipation in 1 patient; grade 3: ileus and constipation in the second; and grade 4 granulocytopenia persisting for 5 days or more in the third). Both patients who had grade 3 constipation had previous intestinal surgery for rectal and colon cancer, respectively. One patient with superior vena caval syndrome at baseline required radiotherapy at day 7 for progression of this syndrome. The dose of 15 mg/m^2 was defined as the MTD.

Consequently, 9 patients were treated at 13 mg/m^2. Two of the nine patients treated at this dose experienced DLTs (grade 4 granulocytopenia persisting for 5 days or more in both patients). Therefore, based on protocol-predefined criteria, 13 mg/m^2 is the dose recommended for future single-agent Phase II studies with an administration every 21 days (with an additional week permitted for recovery from toxicities, if needed).

Non-hematologic toxicity

The non-hematologic adverse events reported most commonly (occurrence in ≥50% of all patients treated) during the first cycle were anorexia, nausea, malaise, and constipation (Table 3). No grade 4 non-hematologic toxicities were reported in any treatment group. During subsequent cycles, adverse events were similar in terms of frequency and severity to those reported during the first cycle of treatment and no cumulative toxicity was observed.

Gastrointestinal toxicity, usually mild, was the most common non-hematologic toxicity associated with edotecarin administered with an antiemetic regimen. The median time to onset of constipation during the first cycle was 3 days (range: 2–4 days). None of the patients who received antiemetic prophylaxis and who were treated at the Phase II recommended dose of 13 mg/m^2 had grade 2 or higher nausea, vomiting, or anorexia. Diarrhea (grade 1–2) was observed in only a few patients (8 mg/m^2, 1 patient; 11 mg/m^2, 2; 13 mg/m^2, 2; and 15 mg/m^2, 1). Alopecia was not reported. Injection site phlebitis (grade 2) was reported in 11 (48%) of the patients. The frequency of this event was not dose-dependent, suggesting that it was related to the infusion

Table 2 Number of treatment cycles, number of patients with dosing interval extensions and reasons for treatment discontinuation

	Edotecarin dose (mg/m^2)			
	8	11	13	15
Number of patients	3	6	9	6
Median number of treatment cycles (range)	3 (1–4)	2 (1–4)	2 (1–6)	1 (1–5)
Dose delay				
Number of patients (number of days in dosing interval)	0	1 (45)	2 (31–34)	0
Reasons for treatment discontinuation				
Progressive disease	1	3	4	1
Further treatment effect not expected	2	2	3	2
DLT	0	1	1	2
Adverse events other than DLT	0	0	1	0
Prohibited concomitant therapy	0	0	0	1

Table 3 Most common non-hematologic toxicities occurred in ≥50% of all patients treated during cycle 1

Adverse event	Grade	Dose (mg/m^2)				All doses ($N=24$)
		8 ($N=3$)	11 ($N=6$)	13 ($N=9$)	15 ($N=6$)	
Anorexia	1/2	3 (100%)	6 (100%)	7 (78%)	4 (67%)	20 (83%)
	3/4	0	0	0	0	0
Nausea	1/2	2 (67%)	2 (33%)	5 (56%)	6 (100%)	15 (63%)
	3/4	0	1 (17%)	0	0	1 (4%)
Malaise	1/2	3 (100%)	4 (66%)	5 (56%)	4 (67%)	16 (67%)
	3/4	0	0	0	0	0
Constipation	1/2	1 (33%)	1 (17%)	6 (67%)	3 (50%)	11 (46%)
	3/4	0	0	0	2 (33%)	2 (8%)

procedure rather than the drug. There were no deaths within 28 days of edotecarin administration, and none of the deaths that occurred after the study were considered treatment-related.

One adverse event deserves a more detailed description. A 44 year-old-male with esophageal cancer with liver and lung metastases developed grade 2 interstitial pneumonitis 2 weeks after administration of edotecarin 13 mg/m^2. He had undergone radiation therapy for the primary lesion approximately 4 months before edotecarin infusion. Most lesions of the interstitial pulmonary lesions were in the field of radiation therapy, and radiation pneumonitis was diagnosed. However, the rapid onset of pneumonitis after edotecarin infusion suggested a recall phenomenon induced by edotecarin and the event was judged to be possibly related to the study treatment.

Hematologic toxicity

Neutropenia/granulocytopenia, leukopenia, anemia, and lymphocytopenia were the most common (occurrence in ≥50% of all patients treated at the 3 highest dose levels) hematologic toxicities reported during the first cycle (Table 4). Neutropenia was the principal hematologic toxicity in this study and granulocytopenia was dose-limiting at 13 and 15 mg/m^2 (Table 5). At 11–15 mg/m^2, the median time to nadir granulocyte count was 11–14 days, and the median time to recovery from nadir was 5 to 7 days. In all patients, granulocyte counts recovered to grade 1 within 21 days after edotecarin infusion at 8 and 15 mg/m^2, but had not recovered by day 22 in 1 patient at 11 mg/m^2 and in 4 patients at 13 mg/m^2. However, by day 26 after edotecarin infusion, granulocyte counts were within normal limits in all patients. Given this frequent and severe neutropenia, G-CSF support was provided during cycle 1 in 2 patients at 13 mg/m^2 and 1 at 15 mg/m^2,after DLT confirmation. Grade 4 granulocytopenia persisting for more than 5 days led to dose reduction in the second cycle in 2 patients, one each at 13 and 15 mg/m^2.

Grade 4 neutropenia (not necessarily lasting > 5 days), was reported in 15 (65%) patients during the first cycle of therapy, and was observed at all dose levels but did not increase in incidence with additional cycles.

Anemia, reported in 15 (65%) patients, did not exceed grade 1 or 2 in severity. Thrombocytopenia was not reported in this study. The numbers of patients with abnormal laboratory values did not tend to increase with increasing courses of treatment, suggesting that the toxicity of edotecarin was not cumulative.

No significant changes were demonstrated by the blood coagulation studies, which assessed prothrombin time (PT) and activated partial thromboplastin time (aPTT).

Pharmacokinetics

Plasma pharmacokinetic parameters are listed in Table 6. Edotecarin plasma concentrations rose rapidly

Table 4 Most common hematologic toxicities occurred in ≥50% of patients at each of the three highest dose levels during cycle 1

Adverse event	Grade	Dose, mg/m^2				All doses ($N=23$)
		8 ($N=3$)	11 ($N=6$)	13 ($N=9$)	15 ($N=5$)	
Neutropenia	1/2	1 (33%)	0	0	0	1 (4%)
	3/4	1 (33%)	6 (100%)	9 (100%)	6 (100%)	22 (92%)
Granulocytopenia	1/2	1 (33%)	0	0	1 (17%)	2 (8%)
	3/4	1 (33%)	6 (100%)	9 (100%)	5 (83%)	21 (88%)
Leukopenia	1/2	1 (33%)	4 (67%)	5 (56%)	2 (33%)	12 (50%)
	3/4	0	2 (33%)	4 (44%)	4 (67%)	10 (42%)
Anemia	1/2	3 (100%)	4 (67%)	5 (56%)	4 (67%)	16 (67%)
	3/4	0	0	0	0	0
Lymphocytopenia	1/2	1 (33%)	4 (67%)	4 (44%)	2 (33%)	11 (46%)
	3/4	0	0	1 (11%)	2 (33%)	3 (13%)

Table 5 Granulocytopenia during cycle 1

Dose (mg/m²)	No. of patients	Grade				Nadir (no./mm³)[a]	Days to nadir[a]	Days to recovery (grade 0) from nadir			
								Without G-CSF		With G-CSF	
		1	2	3	4			No. of patients	No. of days[a]	No. of patients	No. of days[a]
8	3	0	0	1 (33%)	0	2,193 (550–993)	18 (14–21)	1	7	0	–
11	6	0	0	2 (33%)	4 (67%)	440 (150–923)	14 (11–16)	6	7 (3–12)	0	–
13	9	0	0	4 (44%)	5 (56%)	480 (46–912)	14 (9–21)	7	7 (2–13)	2	5 (4–5)
15	6	0	1 (17%)	2 (33%)	3 (50%)	529 (88–1,017)	11 (11–14)	4	5 (2–7)	1	5

G-CSF granulocyte colony-stimulating factor
[a]Expressed as median (range)

Table 6 Pharmacokinetic parameters in cycle 1 (mean ± SD)

Dose (mg/m²)	No. of patients[c]	C_{max}, (ng/ml)	$AUC_{0\text{-}26h}$, (ng h/ml)	$AUC_{0\text{-}\infty}$ (ng h/ml)	Percentage of area extrapolated	CL		$t_{1/2}$ (h)	V_{SS} (l/m²)	48-h urinary recovery (%)
						ml/min/m²	ml/min			
8	2	57 ± 17[a]	132, 192	330, 198	42, 23	404, 673	735, 1043	45.5, 29.0	972, 715	1.4 ± 2.3[a]
11	5	86 ± 10[b]	211 ± 38	294 ± 54	25 ± 8	624 ± 114	962 ± 287	26.1 ± 3.3	670 ± 277	3.6 ± 2.7
13	9	103 ± 17	262 ± 43	330 ± 44	19 ± 6	657 ± 88	1154 ± 138	22.9 ± 2.1	569 ± 256	2.9 ± 3.8
15	5	113 ± 17[b]	269 ± 56	352 ± 62	20 ± 5	711 ± 123	1057 ± 190	20.2 ± 1.6	561 ± 258	1.8 ± 3.4[b]

C_{max}, maximum plasma concentration; *AUC* area under the plasma concentration-time curve; *CL* clearance; $t_{1/2}$, apparent terminal half-life; V_{ss}, steady-state volume of distribution
[a]$N = 3$
[b]$N = 6$
[c]As drug concentrations were below the detectable range at one or more time points beyond the 8 h after the administration, some parameters are not available for all patients

at the start of the infusion and dropped sharply at discontinuation of the infusion (Fig. 2), reaching levels of 1–2 ng/ml at the last sampling point.

In order to compare PK data from this study, to PK data from the similar US study, a noncompartmental model for calculating PK parameters was used.

Peak plasma concentrations of edotecarin and $AUC_{0\text{-}26h}$ increased with increasing dose (Fig. 3). Power model fitting to $AUC_{0\text{-}\infty}$ and C_{max} vs dose data suggested that C_{max} was proportional to dose while $AUC_{0\text{-}\infty}$ was somewhat less than dose-proportional (results not presented).

Clearance (CL) values were comparable at 11, 13 and 15 mg/m² as were volumes of distribution.

An apparent terminal half-life ($t_{1/2}$) was calculated from plasma concentrations observed at 8, 10, and 24 h after the end of the infusion. In three patients, the plasma concentration at 1 or more of these time points was below the lower limit of quantification of 1 ng/ml; consequently, values of $t_{1/2}$, $AUC_{0\text{-}\infty}$, and CL were not calculated in these patients. The mean apparent $t_{1/2}$ was approximately 20 h. Since the PK sampling duration was only 26 h post start of infusion (i.e., for only about 1 half-life), the computed $AUC_{0\text{-}\infty}$, CL, and $t_{1/2}$ values should be considered preliminary until data are available from trials measuring plasma levels through at least 3–4 times the apparent terminal $t_{1/2}$.

Mean 48-h urinary excretion accounted for 1.4–3.6% of the administered dose of edotecarin in the 4 cohorts.

Coefficient of interpatient variation in edotecarin C_{max} and AUC values was 15–20%.

Antitumor activity

All 24 patients were assessable for efficacy, and 21 patients had evaluable lesions. No objective and confirmed responses were observed. However, stable disease (SD) was reported in 12 patients (57%), with a maximum duration of 5 cycles. Eight (38%) patients had tumor volume reductions ranging from 10.5 to 65.4% based on the sum of the products of the dimensions of measurable lesions. Two patients had a decrease in tumor volume of at least 50%. One patient, enrolled at 13 mg/m², had esophageal cancer with liver metastases. After cycle 1, at day 18, tumor volume was reduced by 65%. Unfortunately, treatment was discontinued because of pneumonitis and the disease had progressed 28 days later. The second patient, who received 11 mg/m², had gastric cancer with measurable metastatic lesions in the lung

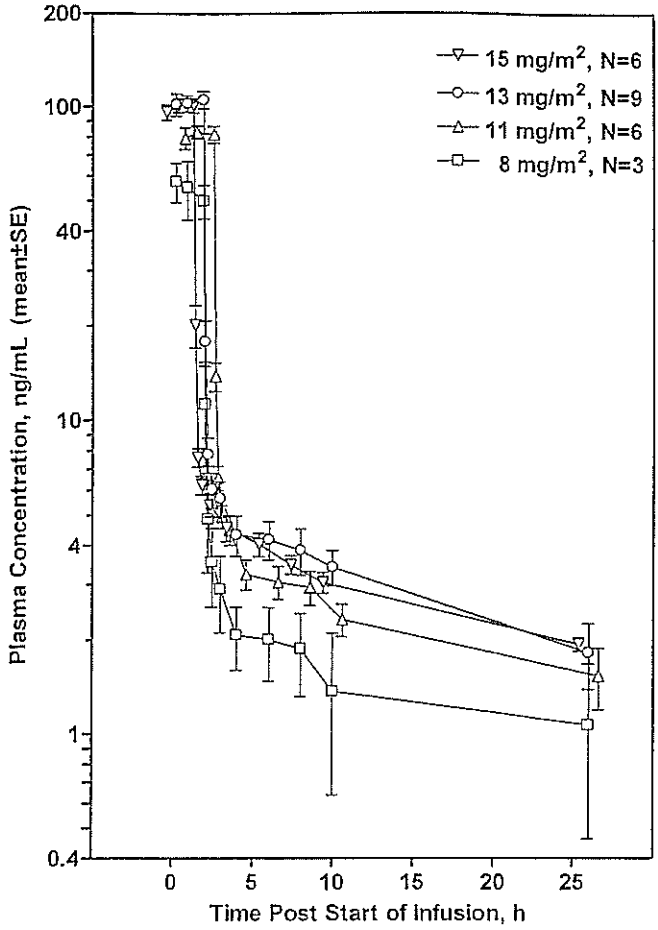

Fig. 2 Edotecarin cycle-1 plasma concentration-time profiles (mean ± SE) in patients with advanced solid tumors. Data at 11 and 13 mg/m^2 have been offset by + 0.6 and −0.6 h, respectively, in the *x*-direction to prevent overlapping *data points* and *error bars*

and mediastinal lymph nodes. These lesions decreased gradually in size, with the reduction from baseline reaching 53% by cycle 3. For administrative reasons, cycle 4 was delayed (45 days). The response was no longer present and the treatment was discontinued. Three of four patients with colorectal cancer, all with lung lesions and having received prior 5-fluorouracil treatment, showed evidence of tumor stabilization.

Discussion

The first objective of this Phase I clinical study was to determine the MTD of single-agent edotecarin administered by IV infusion over 2 h once every 21 days with pre- and post-treatment supportive medication (steroids and 5-HT$_3$ antagonists). The MTD was 15 mg/m^2 with this schedule of administration. At this dose, hematologic (neutropenia, febrile neutropenia) and non-hematologic (constipation, ileus, infection without neutropenia) grade 3 and 4 toxicities were observed in 3 of the 6 patients of the MTD cohort.

In order to make a recommendation regarding the dose of edotecarin to be studied in future single-agent Phase II studies, 9 patients were treated at 13 mg/m^2 with supportive medication. Grade 4 granulocytopenia, lasting for 5 days or more, was observed in only 2 patients. Therefore this dose is recommended, with supportive medication, for future Phase II studies. A similar Phase I edotecarin trial conducted in the USA recommended the same dose [12].

At the recommended dose of 13 mg/m^2, edotecarin, infused IV for 2 h with supportive medication, had manageable toxicities. Neutropenia was dose-dependent

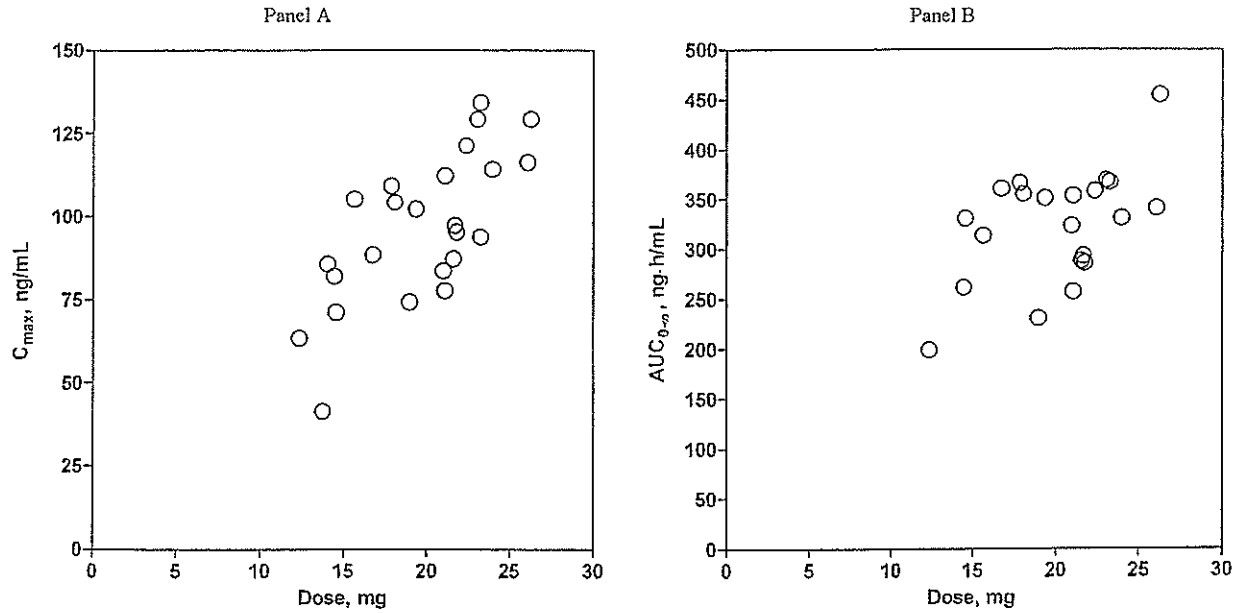

Fig. 3 Peak plasma concentrations (*left panel*) and $AUC_{0-\infty}$ values (*right panel*) as a function of dose in milligram in patients with advanced solid tumors

but was not cycle-dependent. At the recommended dose, the median time to neutrophil nadir was 14 days with a median recovery time of 7 days. This suggests that administration of edotecarin every 3 weeks is possible, but bone marrow recovery has to be verified before the administration of the next cycle. Other hematologic toxicities were observed (anemia, lymphocytopenia), but thrombocytopenia was not reported.

Gastrointestinal toxicity, the most common non-hematologic toxicity observed, was usually mild. At the recommended dose and with antiemetic premedication, nausea, vomiting or anorexia never exceeded grade 1. The incidence of constipation was dose dependent and dose-limiting at 15 mg/m^2 in 2 patients that had previously undergone intestinal surgery for cancer. However, at the recommended dose, constipation was never more severe than grade 2. In two previous Phase 1 trials of edotecarin [12, 13], constipation was not dose-limiting. The reason for these discrepant observations is unclear, but constipation is not infrequent in colorectal cancer patients receiving chemotherapy. For instance, the incidence of constipation in colorectal patients treated with irinotecan single agent in second line therapy is estimated between 8 and 10% [16]. Because of the relatively high frequency and the dose dependency observed in this study, frequency of bowel movements should be monitored in patients enrolled in future studies with edotecarin. Alopecia and severe diarrhea frequently observed in association with other chemotherapy drugs (e.g., camptothecins) were not seen in this trial.

At the recommended dose, 1 patient, who had received thoracic radiotherapy for lung metastasis of an esophageal carcinoma, had a grade 2 interstitial pneumonitis shortly after edotecarin infusion, suggesting a recall phenomenon. Interstitial pneumonitis has also been observed after irinotecan [17, 18]. Pulmonary toxicity should be closely monitored in patients previously treated with thoracic radiation therapy in future Phase II trials of edotecarin.

Conclusions, based on the pharmacokinetic data, are tentative because of the narrow dose range and small number of patients studied in this trial, as well as the relatively short duration of PK sampling. While plasma concentrations appeared to reach a plateau during the 2-h infusion, attainment of a steady state concentration (C_{ss}) would not be expected for a drug with the multi-compartmental disposition behavior exhibited in Fig. 2. In theory, an infusion duration of 3–4 times the apparent terminal $t_{1/2}$ of 20 h would be required to achieve C_{ss}. This is supported by the fact that an attempt to compute CL by the equation $CL = k_0/C_{max}$, where k_0 is infusion rate and C_{max} is used as an estimate of C_{ss}, yielded CL values much higher than those calculated by means of $CL = dose/AUC_{0-\infty}$ (results not presented).

Within the limited dose range of 11–15 mg/m^2, C_{max} and AUC appeared to increase roughly linearly with dose, and CL was not relatively changed. These results suggest that edotecarin exhibits linear pharmacokinetics within 11–15 mg/m^2.

Urinary recovery did not appear to vary with the dose, which indicates that renal elimination contributes minimally to total body CL.

The pharmacokinetic profile of edotecarin appears to be simple with relatively little interpatient variability compared to that of irinotecan (a prodrug topo-I inhibitor with a very complex disposition) and to many other cytotoxic drugs.

Efficacy was a secondary endpoint of this trial conducted in a population of heavily pretreated cancer patients. No partial or complete confirmed responses were observed. However 8 patients had minor reductions in tumor size, including 2 patients with > 50% regression of tumor size, however these reductions were not confirmed. Twelve patients qualified as having SD. Further clinical work is necessary to quantify precisely the level of edotecarin antitumor activity.

In conclusion, this study showed that the MTD of edotecarin, administered as a 2 h infusion with supportive medication of steroids and antiemetics, is 15 mg/m^2 and the dose to be studied in future Phase II trials as a single agent with an administration every 21 days is 13 mg/m^2. At 13 mg/m^2, toxicities, mainly neutropenia, are manageable and are characterized by the lack of severe diarrhea. The pharmacokinetic profile is attractive. Edotecarin is a promising new anticancer agent, especially in colorectal cancer, and deserves further clinical evaluation.

Acknowledgements We acknowledge Ms. Sayaka Miyahara, Dr. Mami Matsumoto, Ms. Yoko Kawamura, Mr. Katsuhiro Mizuno, and Mr. Naoyoshi Yatsuzuka for pharmacokinetic analyses, data management, and clinical research coordination, and Drs. Claude George and Samit Hirawat for their contribution in preparation of this manuscript.

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Biochem. J. (2006) 397, 537–543 (Printed in Great Britain) doi:10.1042/BJ20051952

In-frame deletion in the EGF receptor alters kinase inhibition by gefitinib

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The existence of an in-frame deletion mutant correlates with the sensitivity of lung cancers to EGFR (epidermal growth factor receptor)-targeted tyrosine kinase inhibitors. We reported previously that the in-frame 15-bp deletional mutation (delE746–A750 type deletion) was constitutively active in cells. Kinetic parameters are important for characterizing an enzyme; however, it remains unclear whether the kinetic parameters of deletion mutant EGFR are similar to those of wild-type EGFR. We analysed autophosphorylation in response to ATP and inhibition of gefitinib for deletion mutant EGFR and wild-type EGFR. Kinetic studies, examining autophosphorylation, were carried out using EGFR fractions extracted from 293-pΔ15 and 293-pEGFR cells transfected with deletion mutant EGFR and wild-type EGFR respectively. We demonstrated the difference in activities between unstimulated wild-type (K_m for ATP $= 4.0 \pm 0.3$ μM) and mutant EGFR (K_m for ATP $= 2.5 \pm 0.2$ μM). There was no difference in K_m values between EGF-stimulated wild-type EGFR (K_m for ATP $= 1.9 \pm 0.1$ μM) and deletion mutant EGFR (K_m for ATP $= 2.2 \pm 0.2$ μM). These results suggest that mutant EGFR is active without ligand stimulation. The K_i value for gefitinib of the deletion mutant EGFR was much lower than that of wild-type EGFR. These results suggest that the deletion mutant EGFR has a higher affinity for gefitinib than wild-type EGFR.

Key words: autophosphorylation, epidermal growth factor receptor (EGFR), gefitinib, kinase inhibition, tyrosine kinase.

INTRODUCTION

EGFR [EGF (epidermal growth factor) receptor] is among the most important targets for lung cancer therapy, and many EGFR-targeted inhibitors have been developed [1]. These EGFR-targeted compounds inhibit the tyrosine kinase activity of EGFR by competing at the ATP-binding site [2]. Many EGFR-targeted tyrosine kinase inhibitors such as gefitinib and erlotinib have been assessed clinically [3,4]. Recently, an EGFR mutation was found in patients who responded to gefitinib, and mutant EGFR has been reported to be a determinant of the response to EGFR tyrosine kinase inhibitors [5,6]. To date, over 30 EGFR mutations including delE746–A750, L858R and delL747–P753insS, have been reported in lung cancer. These EGFR mutations, except for T790M, are considered to be of the 'gain-of-function' type. Differences exist among them; for example, constitutively active in delE746–A750 compared with hyperresponsive to ligand stimulation in L858R and delL747–P753insS, although these mutant EGFRs increase sensitivity to EGFR-targeted tyrosine kinase inhibitors [7–9]. In general, the observation of hyperresponsiveness to ligand stimulation, as in the case of L858R, raises the possibility of high affinity for ATP. We reported previously that deletion mutant EGFR was constitutively phosphorylated under unstimulated conditions, whereas wild-type EGFR was not phosphorylated until ligand stimulation [7]. The differences in cellular phenotype and sensitivity to gefitinib between deletion mutant EGFR and wild-type EGFR raise the possibility that the enzymatic properties of the deletion mutant EGFR may differ from those of wild-type EGFR. However, it remains unclear whether the kinetic parameters of deletion mutant EGFR are different from those of wild-type EGFR. In the present study, we focused on the autophosphorylation of deletion mutant EGFR, and investigated the inhibition constant of gefitinib. Technically, we used deletion mutant EGFR and wild-type EGFR extracted from ectopically expressed HEK-293 (human embryonic kidney) cells. The autophosphorylation assay reflects the native behaviour of EGFR in maintaining cellular functions.

MATERIALS AND METHODS

Reagents

Gefitinib (Iressa®, ZD1839) was provided by AstraZeneca.

Cell culture

The HEK-293 cell line was obtained from the A.T.C.C. (Manassas, VA, U.S.A.) and was cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated foetal bovine serum (Life Technologies).

Plasmid construction and transfection

Construction of the expression plasmid vector of wild-type EGFR and the 15-bp deletion mutant EGFR (delE746–A750 type deletion), which has the same deletion site as that observed in detail in PC-9 cells, has been described elsewhere [7,10,11]. The plasmids were transfected into HEK-293 cells and the transfectants were selected using Zeosin (Sigma). The stable transfectants (pooled cultures) of the wild-type EGFR and its deletion mutant were designated 293-pEGFR and 293-pΔ15 cells respectively.

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; HEK-293, human embryonic kidney; 293-pEGFR, HEK-293 cells transfected with wild-type EGFR; 293-pΔ15, HEK-293 cells transfected with deletion mutant EGFR; TBS-T, Tris-buffered saline with Tween 20; TGF-α, transforming growth factor-α.

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Immunoblotting

The 293-pΔ15 and 293-pEGFR cells were treated with or without gefitinib for 3 h, stimulated with EGF (100 ng/ml) under serum-starvation conditions and then lysed for immunoblot analysis. Immunoblot analysis was performed as described previously [12]. Equivalent amounts of protein were separated by SDS/PAGE (2–15 % gradient) and transferred to a PVDF membrane (Millipore). The membrane was probed with a mouse monoclonal antibody against EGFR (Transduction Laboratories), a phospho-EGFR antibody (specific for Tyr^{1068}) (Cell Signaling Technology) as the first antibody, followed by a horseradish-peroxidase-conjugated secondary antibody. The bands were visualized with ECL® (enhanced chemiluminescence) (Amersham Biosciences).

Determination of ligand secretion by ELISA

The 293-pΔ15 and 293-pEGFR cells were cultured in 12-well plates under serum-starvation conditions. The cell culture supernatant was collected for each cell line and stored at −80 °C for further analysis. Amounts of EGF and TGF-α (transforming growth factor α) in the culture medium from each cell line were determined with a DuoSet ELISA development kit (R&D Systems). The assay was performed in triplicate according to the manufacturer's instructions.

Preparation of cell lysates for EGFR autophosphorylation

Cultivated cells, after reaching 70–80 % confluence, were starved in serum-free medium for 24 h, with or without EGF (100 ng/ml) stimulation. The cells were washed twice with ice-cold PBS containing 0.33 mM $MgCl_2$ and 0.9 mM $CaCl_2$ [PBS(+)], then lysed with lysis buffer [50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 0.25 % Triton X-100, 5 mM EDTA, protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Sigma)]. For the preparation of gefitinib-treated cell lysates, cultivated cells were starved in serum-free medium for 24 h, and were then pre-incubated with 2 μM gefitinib for 3 h. Either with or without EGF stimulation (100 ng/ml), the cells were washed twice with ice-cold PBS(+) and lysed with lysis buffer. The cell lysate was centrifuged at 20000 *g* for 10 min, and the protein concentration of the supernatant was measured with a BCA (bicinchoninic acid) protein assay (Pierce).

Autophosphorylation assay

The amount of EGFR in 293-pΔ15 and 293-pEGFR cells was determined by quantitative immunoassay (R&D Systems) according to the manufacturer's instructions. The autophosphorylation assay was carried out with a quantitative immunoassay system. Wells in a 96-well immunomodule (Nalge Nunc International) were incubated with 0.8 μg/ml goat anti-(human EGFR) antibody in PBS (provided with the EGFR quantitative immunoassay system) and incubated at 4 °C overnight. The plates were washed three times with TBS-T (Tris-buffered saline with Tween 20; 20 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.05 % Tween 20) and were then filled with blocking buffer (PBS containing 1 % BSA and 5 % sucrose) and incubated for 2 h at room temperature (25 °C). The wells were washed three times with TBS-T and incubated with cell lysates of 293-pEGFR or 293-pΔ15 including equal amounts of EGFR (130 ng of EGFR/well) diluted with lysis buffer. After a 2 h incubation at room temperature, the 96-well plate was washed with TBS-T. Autophosphorylation of EGFR was initiated by addition of ATP (0–32 μM in 50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$ and phosphatase inhibitor) followed by incubation for 5 min. In some experiments, various concentrations of gefitinib were added to the wells before the addition of ATP. Following the autophosphorylation reaction, the wells were washed with TBS-T. Next,

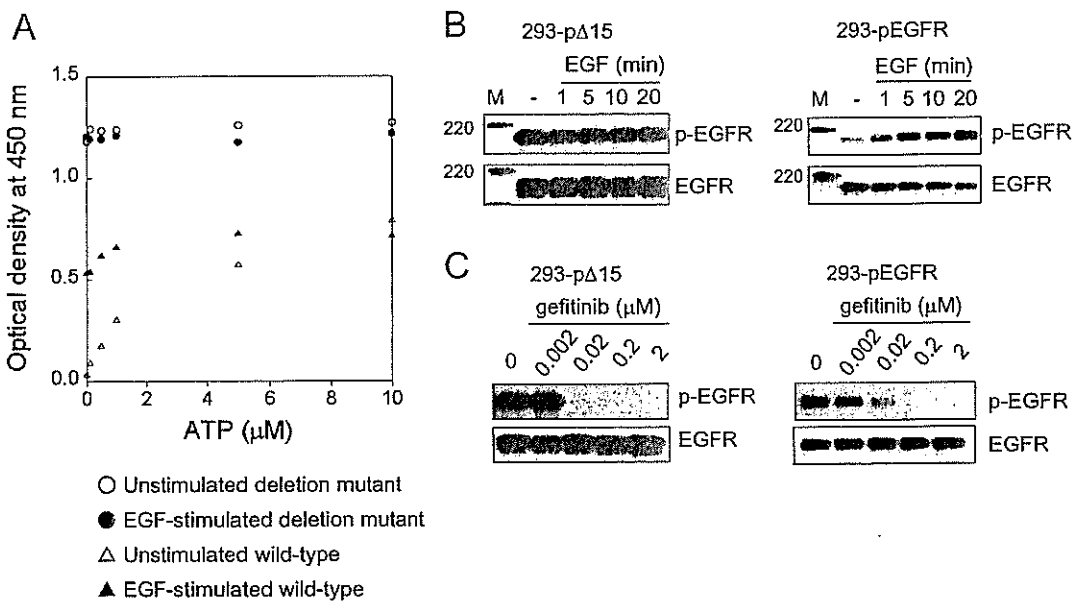

Figure 1 Autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR

(**A**) The 293-pΔ15 and 293-pEGFR cells were treated with or without EGF (100 ng/ml) for 10 min after serum-starvation. EGFR was extracted from the cells and immobilized on wells with anti-EGFR antibody. Autophosphorylation reactions were initiated by the addition of ATP, and autophosphorylation was detected using horseradish-peroxidase-conjugated phosphotyrosine antibody, measuring the absorbance ('optical density') at 450 nm. Autophosphorylation was seen for unstimulated (○) and EGF-stimulated (●) deletion mutant EGFR, and unstimulated (△) and EGF-stimulated (▲) wild-type EGFR. Results are representative of at least three independent experiments. (**B**) The 293-pΔ15 and 293-pEGFR cells were treated with or without EGF (100 ng/ml) for the indicated times after serum-starvation. Phosphorylation of EGFR and total EGFR was determined by immunoblotting. (**C**) The 293-pΔ15 and 293-pEGFR cells were exposed to gefitinib (0.002–2 μM) for 3 h under serum-starvation conditions, and stimulated with EGF (100 ng/ml) for 10 min. The cells were then lysed and subjected to immunoblot analysis.

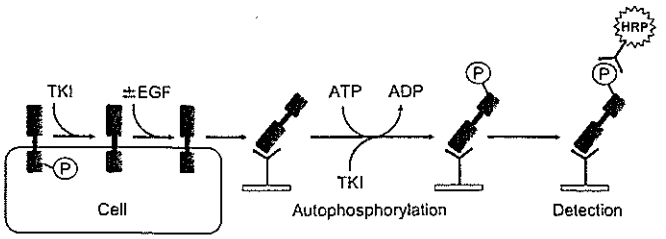

Figure 2 Schematic illustration of the cell-based autophosphorylation assay

The 293-pΔ15 and the 293-pEGFR cells overexpressing deletion mutant EGFR and wild-type EGFR respectively were treated with 2 μM gefitinib for 3 h and stimulated with or without EGF (100 ng/ml) under serum-starvation conditions. EGFR was extracted from cells and immobilized on wells with anti-EGFR antibody. The autophosphorylation reaction was initiated by the addition of ATP with or without gefitinib, and horseradish-peroxidase-conjugated anti-phosphotyrosine antibody was used to detect the phosphorylation of EGFR. TKI, tyrosine kinase inhibitor.

horseradish-peroxidase-conjugated anti-phosphotyrosine antibody, PY-99-HRP (0.4 μg/ml in PBS containing 1% BSA and 0.1% Tween 20) (Santa Cruz Biotechnology) was added to the wells for 2 h at room temperature. The wells were washed three times with TBS-T. Bound phosphotyrosine antibody was detected colorimetrically after adding 100 μl of substrate (tetramethylbenzidine and H_2O_2) to each well. After a 10 min incubation, the colour reaction was quenched by the addition of 100 μl of 1M H_2SO_4. The absorbance readings for each well were determined at 450 nm with Delta-soft on an Apple Macintosh computer interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics).

Data analysis

For kinetic analysis, an Eadie–Hofstee plot was applied for the calculation of K_m (Michaelis constant) and V_{max} (maximum velocity). The data obtained were plotted as velocity against velocity/substrate concentration (V/ATP). The slope of the line is equal to $-K_m$ and the x-intercept is V_{max}. The K_i value was calculated as follows:

$$K_i = (K_m \times [I])/(K_{m,I} - K_m) \quad (1)$$

in which K_m is the Michaelis constant for ATP, $K_{m,I}$ is the Michaelis constant for ATP in the presence of gefitinib and [I] is the concentration of gefitinib. The statistical analysis was performed using KaleidaGraph (Synergy Software).

RESULTS

Autophosphorylation of deletion mutant EGFR and wild-type EGFR

We performed the autophosphorylation assay and immunoblot analysis using lysates extracted from 293-pΔ15 and 293-pEGFR cells under unstimulated and EGF-stimulated conditions (Figures 1A and 1B). Under unstimulated conditions, deletion mutant EGFR was highly phosphorylated in the absence of ATP. Addition of ATP did not affect the autophosphorylation of deletion mutant EGFR. On the other hand, autophosphorylation of wild-type EGFR was barely detectable without ATP, and proceeded in an ATP-dependent manner. In the EGF-stimulated case, wild-type EGFR was phosphorylated to a greater extent in the absence of ATP than unstimulated wild-type EGFR. The autophosphorylation of EGF-stimulated wild-type EGFR additively increased with the addition of ATP. These findings indicate that the deletion mutant retains the constitutive activity in our autophosphorylation assay. In the immunoblot analysis, phosphorylation of deletion mutant EGFR was detected in 293-pΔ15 cells without ligand stimulation. Addition of EGF increased phosphorylation of EGFR in the 293-pEGFR cells. Taken together, these results indicate that the deletion mutant has constitutive autophosphorylation activity.

In addition, we examined the secretion of major ligands for EGFR such as EGF and TGF-α from transfected HEK-293 cells by ELISA. No detectable EGF and TGF-α secretion was observed in the cultivation medium used for HEK-293 transfectants (results not shown), indicating that these transfectants are not activated via EGF-mediated autocrine loops. We considered that autophosphorylation using unstimulated EGFR represents a

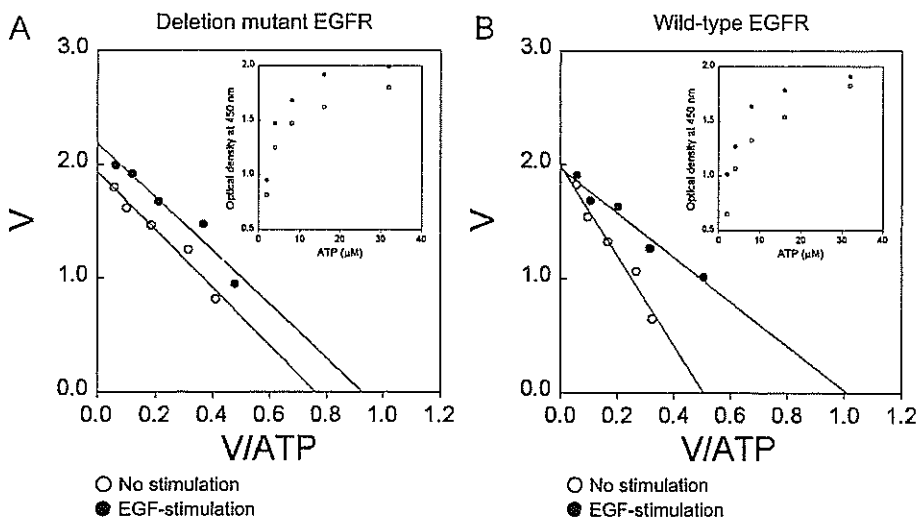

Figure 3 Autophosphorylation activities of deletion mutant EGFR and wild-type EGFR

Plots of absorbance ('optical density') against ATP concentration (inset) were fitted to an Eadie–Hofstee plot to calculate the values of kinetic parameters (K_m and V_{max}) for deletion mutant EGFR (**A**) and wild-type EGFR (**B**) under unstimulated (○) and EGF-stimulated conditions (●). Results are representative of at least three independent experiments with similar results.