Table 2. Toxicity profile

Toxicity	Incidence (n = 3808)	
	All adverse events	≧Grade 3 events
Leukopenia	26.0% (991)	2.6% (99)
Neutropenia	21.3% (810)	6.1% (231)
Anemia	15.7% (597)	4.6% (176)
Hemoglobin decreased	9.0% (343)	1.6% (60)
Red blood cell count decreased	8.1% (310)	1.9% (73)
Hematocrit decreased	5.2% (199)	0.8% (29)
Thrombocytopenia	8.3% (317)	1.5% (59)
Aspartate aminotransferase increased	5.2% (198)	0.6% (22)
Alanine aminotransferase increased	4.6% (175)	0.4% (16)
Nausea / Vomiting	19.3% (734)	2.2% (85)
Anorexia	26.4% (1004)	5.9% (226)
Fatigue	17.5% (666)	3.5% (132)
Diarrhea	16.5% (629)	2.0% (77)
Stomatitis	12.5% (476)	1.2% (46)
Pigmentation	14.6% (557)	1.1% (43)
Rash	8.4% (318)	0.9% (34)
Overall	74.3% (2831)	25.0% (952)

Actual numbers of cases are shown in parentheses

Table 3. Incidence of toxicities in three patient groups classified by administration eligibility status

Toxicity		Incidence in patient groups according to eligibility status			Hazard ratio (95% CI)*	P value*
		Appropriate (n = 2778)	Careful-use (n = 909)	Inappropriate (n = 121)		
Leukopenia/Neutropenia	All	29.7%	32.5%	32.2%	2.314	P = 0.0001
	≧Grade 3	5.5%	11.9%	10.7%	(1.823–2.938)	
Anemia	All	25.5%	28.3%	24.8%	2.325	P = 0.0001
	≧Grade 3	5.2%	11.0%	9.9%	(1.816–2.977)	
Thrombocytopenia	All	7.5%	10.7%	9.1%	1.824	P = 0.0243
	≧Grade 3	1.3%	2.2%	2.5%	(1.081–3.079)	
Aspartate/alanine aminotransferase increased blood bilirubin increased	All	12.3%	10.1%	4.1%	1.352	P = 0.2428
	≧Grade 3	1.7%	2.2%	1.7%	(0.815–2.244)	
Nausea, vomiting, anorexia	All	31.5%	33.2%	34.7%	1.42	P = 0.0095
	≧Grade 3	5.9%	7.6%	10.7%	(1.089–1.852)	
Fatigue	All	16.3%	20.2%	23.1%	1.637	P = 0.0064
	≧Grade 3	3.0%	4.4%	6.6%	(1.148–2.335)	
Diarrhea	All	15.9%	17.9%	19.0%	1.276	P = 0.3219
	≧Grade 3	1.9%	2.3%	2.5%	(0.788–2.067)	
Stomatitis	All	12.3%	13.4%	10.7%	1.225	P = 0.5269
	≧Grade 3	1.2%	1.4%	0.8%	(0.654–2.296)	
Pigmentation	All	14.9%	13.6%	14.9%	1.225	P = 0.5417
	≧Grade 3	1.1%	1.0%	3.3%	(0.639–2.348)	
Rash	All	8.4%	7.9%	9.9%	0.86	P = 0.7097
	≧Grade 3	0.9%	0.7%	1.7%	(0.389–1.900)	

* Appropriate group vs careful-use and inappropriate groups

coagulopathy; 1 died of neutropenic sepsis; 1 died of hepatic failure; and 1 died of hyperglycemia with metabolic acidosis. Of these 5 patients, those patients who died of neutropenic sepsis and hyperglycemia were in the appropriate group; and the 2 patients who died of serious thrombocytopenia and the patient who died of hepatic failure were in the careful-use group.

The incidences of hematological toxicities (leukopenia, neutropenia, anemia thrombocytopenia) in relation to creatinine clearance, calculated using the Cockcroft-

Table 4. Incidence of hematological toxicities in four patient groups classified by creatinine clearance[a]

Creatinine clearance (ml/min)	Patients administered at standard initial dose		Patients administered at reduced initial dose	
	Overall incidence	≧Grade 3	Overall incidence	≧Grade 3
<30	70.0% (14/20)	45.0% (9/20)	41.2% (7/17)	23.5% (4/17)
≧30 to <50	56.4% (206/365)	23.3% (85/365)	45.5% (70/154)	18.8% (29/154)
≧50 to <80	47.6% (640/1345)	13.8% (185/1345)	40.1% (173/431)	12.1% (52/431)
≧80	40.7% (429/1054)	9.2% (97/1054)	36.3% (115/317)	10.7% (34/317)

[a] Patients with unknown baseline creatinine level or who received inadequate dose were excluded from this analysis

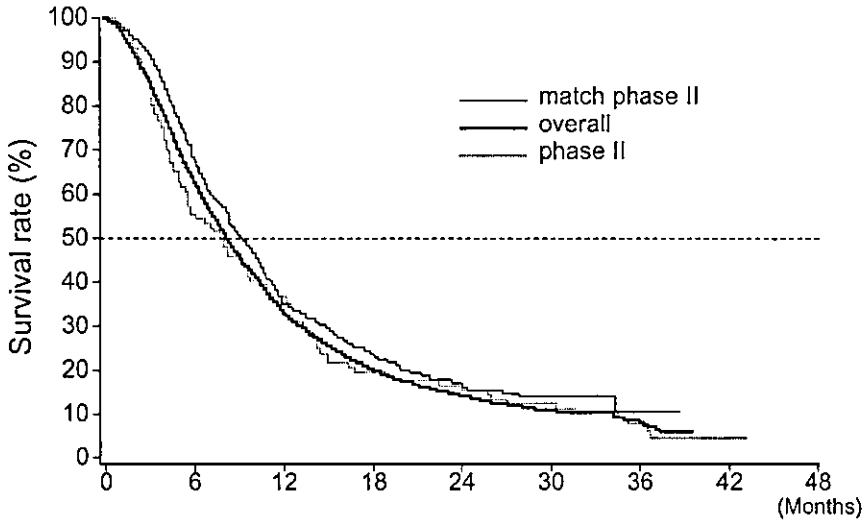

Fig. 1. Survival curves, for patients *overall* (n = 3801), for those with criteria that matched phase II criteria (*match phase II*; n = 483), and for patients in the original phase II study (*phase II*; n = 101)

Gault formula, are shown in Table 4. In the patients with lower creatinine clearance, the incidences of adverse reactions were higher for all grades combined, as well as for grades 3 or worse. Additionally, in the patients who initially received administration at a reduced dose, the incidence of adverse reactions was lower.

Efficacy results

For the efficacy analysis, 280 (7.3%) of the 3801 patients suitable for the analysis were lost to follow up. The MST of these 3801 patients was 8.3 months (95% confidence interval [CI], 8.0–8.6 months), and the 1-year survival rate was 33.3% (95% CI, 31.8%–34.9%) (Fig. 1). Among these 3801 patients, 1540 patients had had no prior chemotherapy and 483 of these patients had baseline data which met the eligibility criteria of the prior registration phase II studies (age, 20–74 years; PS, 2 or better; WBC, 4000–12000/μl; hemoglobin, >9.0 g/dl; platelets, >100000/μl; total bilirubin, ≦1.5 mg/dl; AST and ALT, ≦100 IU/l; alkaline phosphatase [ALP], within two times the upper limit; serum creatinine, within the normal upper limit; and no history of prior chemotherapy [2,3]). The MST and 1-year survival rate of these 483 patients were 9.3 months (95% CI, 8.4–10.3 months) and 36.1% (95% CI, 31.7%–40.6%), respectively.

Discussion

There have been several scandals around new drug approvals in Japan. Eighteen people died as a result of the combined use of sorivudine (an anti-herpes drug that completely inhibits dihydropyrimidine dehydrogenase) with fluorouracil-based anticancer drugs. Twenty of 477 patients died of toxicity during phase I and phase II registration studies of CPT-11, which was approved in 1994. These unfortunate results prompted the MHLW to change the Japanese drug approval system; new guidelines, "the Revised Good Clinical Practice", which recommended very strict safety monitoring during the registration studies, were adopted in 1997. In these guidelines, the MHLW requires two independent phase II studies for new drug approval, while the applicant is required to perform a post-marketing survey and studies to demonstrate the clinical benefit of the approved drug. In accordance with the Japanese approval system,

the company (Taiho Pharmaceutical Company) sponsored two independent phase II studies of S-1, which demonstrated high activity for gastric cancer, and achieved an accelerated approval in Japan. Then, as recommended by the MHLW, the company conducted a nationwide post-marketing survey in a strict manner to prevent the improper use of this agent from causing serious toxicities in general use.

Limitations are inevitable in obtaining information (particularly regarding the safety profile) for a new agent before its approval. In the Japanese system, the safety data of this new agent were obtained from only about 100 patients before marketing. In addition, only 31% (483/1540) of the chemo-naive patients in this present survey met the eligibility criteria of the prior registration study. Considering these limitations, it seems essential to keep the introduction of this agent under careful survey. Discrepancies in safety profiles are likely to occur between clinical studies and general use, particularly when the agent is indicated for gastric cancer. There are major differences in patient populations between these two settings. Although patients with peritoneal dissemination constitute a major proportion of gastric cancer patients, they are usually excluded as candidates for phase II studies because of difficulties in measuring the size of their metastatic lesions. These patients also have serious associated complications, such as bowel obstruction, hydronephrosis, and ascites, due to the peritoneal dissemination; these undesirable conditions are likely to delay the elimination of pharmacological components and may cause serious toxicities. Additionally, there are only a limited number of chemotherapy experts in Japan; the number of medical oncologists is still small in this country. Therefore, the use of newly approved agents, particularly for gastric cancer, should be carefully monitored under survey by the pharmaceutical company and the MHLW.

The overall incidences of adverse events in the present survey and in the phase II studies were 74.3% and 75.2%, respectively [2,3]. These results suggest that the power of the present post-marketing survey was similar to that of the phase II studies. On the other hand, the incidences of adverse events of grade 3 or worse were 25.0% and 14.9%, respectively. A possible cause of this discrepancy was the exclusion of patients potentially in the careful-use or inappropriate groups from the clinical studies, which rejected patients with a PS of 3 or WBC of less than 3500/μl. This interpretation fits well with the higher incidences of grade 3 or worse hematological toxicities in the careful-use and inappropriate groups than in the appropriate group in the present study. Another compromising factor could be impaired renal function; the incidences of grade 3 or worse hematological toxicities were also higher in the low-creatinine-clearance group. CDHP, a component of S-1 that sustains the concentration of tegafur-derived 5-FU by inhibiting dihydropyrimidine dehydrogenase, is known to be eliminated by renal excretion [7,8]. Therefore, S-1 should be particularly carefully administered in patients with impaired renal function, particularly in those with creatinine clearance of 50 ml/min or less.

As for the efficacy analyses, the MST and 1-year survival rate in the present survey were 8.3 months and 33.3%, respectively. Although the present population included patients with a prior history of chemotherapy and those with peritoneal dissemination, these survival results were comparable to those obtained in the phase II studies, i.e., 8.0 months and 36.6%, respectively. The efficacy of S-1 seen in the clinical studies was thus shown to translate into real clinical benefits. These results have proven the utility of this post-marketing survey in assessing the reproducibility of the efficacy results obtained from prior clinical studies.

Acknowledgments We are grateful to Drs. M. Fukushima and C. Hamada for their kind advice. Our gratitude is also extended to the physicians and institutions who participated in the present survey. This survey was supported by Taiho Pharmaceutical Co Ltd, Tokyo.

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[CANCER RESEARCH 64, 9101–9104, December 15, 2004]

Small In-Frame Deletion in the Epidermal Growth Factor Receptor as a Target for ZD6474

Tokuzo Arao,[1] Hisao Fukumoto,[1] Masayuki Takeda,[1] Tomohide Tamura,[2] Nagahiro Saijo,[2] and Kazuto Nishio[1,3]

[1]*Shien-Lab,* [2]*Medical Oncology, National Cancer Center Hospital, Tsukiji, Japan; and* [3]*Pharmacology Division, National Cancer Center Research Institute, Tokyo, Japan*

ABSTRACT

ZD6474 is an inhibitor of vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) tyrosine kinase, with additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase. ZD6474 inhibits angiogenesis and growth of a wide range of tumor models *in vivo*. Gefitinib ("Iressa") is a selective EGFR tyrosine kinase inhibitor that blocks signal transduction pathways implicated in cancer cell proliferation. Here, the ability of gefitinib and ZD6474 to inhibit tumor cell proliferation was examined directly in eight cancer cell lines *in vitro*, and a strong correlation was noted between the IC_{50} values of gefitinib and ZD6474 ($r = 0.79$). No correlation was observed between the sensitivity to ZD6474 and the level of EGFR or VEGFR expression. The NSCLC cell line PC-9 was seen to be hypersensitive to gefitinib and ZD6474, and a small (15-bp) in-frame deletion of an ATP-binding site (exon 19) in the EGFR was detected (delE746-A750–type deletion). To clarify the involvement of the deletional mutation of EGFR in the cellular sensitivity to ZD6474, we examined the effect of this agent on HEK293 stable transfectants expressing deletional EGFR that designed as the same deletion site observed in PC-9 cells (293-pΔ15). These cells exhibited a 60-fold higher sensitivity to ZD6474 compared with transfectants expressing wild-type EGFR. ZD6474 inhibited the phosphorylation of the mutant EGFR by 10-fold compared with cells with wild-type EGFR. In conclusion, the findings suggested that a small in-frame deletion in the EGFR increased the cellular sensitivity to ZD6474.

INTRODUCTION

Gefitinib ("Iressa") is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks the signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting cancer cell growth (1–3). Mutation of the EGFR tyrosine kinase in human non–small-cell lung carcinoma (NSCLC) and hypersensitiveness to gefitinib in patients with NSCLC with this mutation recently were reported (4, 5). The mutations were small, in-frame deletions or substitutions clustered around the ATP-binding site in exons 18, 19, and 21 of the EGFR. The mutant receptors were significantly more sensitive to gefitinib than the wild-type receptor (IC_{50} 0.015 versus 0.1 μmol/L). However, of the 95 other primary tumors and 108 cell lines derived from other tumor types studied, none showed any mutations of this receptor (4). Conversely, Ohm *et al.* (6) reported that all four patients with gefitinib-responsive NSCLC were shown to have mutations of the EGFR near the ATP-binding site compared with none of seven cases showing no response to this drug. These results clearly suggest that the EGFR mutation may be a strong determinant of the tumor response to gefitinib.

ZD6474 is an inhibitor of VEGFR-2 and EGFR signaling that inhibits angiogenesis and tumor growth in a diverse range of tumor models (7). We previously have shown that the NSCLC cell line PC-9 is hypersensitive to gefitinib, with an IC_{50} value of ~0.02 μmol/L (8, 9). It subsequently was established that the PC-9 cells also showed hypersensitivity to ZD6474.

In this report, we discuss the presence of an EGFR deletional mutation and its ability to determine sensitivity to ZD6474.

MATERIALS AND METHODS

Reagents. ZD6474 and gefitinib (Iressa) were provided by AstraZeneca (Cheshire, United Kingdom).

Cell Culture. The human NSCLC cell lines PC-9 and PC-14 were established at the Tokyo Medical University (10, 11). The human epidermal carcinoma cell line A431, breast carcinoma cell line SK-BR-3, ovarian carcinoma cell line SK-OV-3, and colon carcinoma cell lines WiDr and LoVo were obtained from the American Type Culture Collection (Manassas, VA). The SBC-3 cells were supplied by Okayama University School of Medicine. All of the cell lines were maintained in Roswell Park Memorial Institute 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Rockville, MD), except for the LoVo (F12; Nissui Pharmaceutical, Tokyo, Japan), WiDr (modified Eagle's medium; Nissui Pharmaceutical), and A431 cells (Dulbecco's modified Eagle's medium; Nissui Pharmaceutical). The HEK293 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

***In vitro* Growth-Inhibition Assay.** The cell growth-inhibitory effect of gefitinib and ZD6474 was determined using the thiazolyl blue tetrazolium bromide (MTT) assay (Sigma). Briefly, 180 μL/well of the cell suspension were seeded onto Sumilon 96-well microculture plates (Sumitomo Bakelite, Akita, Japan) and incubated in 10% FBS-containing medium for 24 hours. The cells were treated with gefitinib or ZD6474 at various concentrations (4 nmol/L to 80 μmol/L) and cultured at 37°C in a humidified atmosphere for 72 hours. After the culture period, 20 μL of MTT reagent were added, and the plates were further incubated for 4 hours. After centrifugation of the plates, the culture medium was discarded, and wells were filled with dimethyl-sulfoxide. The absorbance of the cultures was measured at 562 nm using Delta-soft on a Macintosh computer (Apple, Cupertino, CA) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ). This experiment was conducted in triplicate. The statistical analysis was performed using KaleidaGraph (Synergy Software, Reading, PA).

Plasmid Construction and Transfection. Construction of expression plasmid vector of mock (empty vector), wild-type EGFR, and the 15-bp deletional EGFR (delE746-A750–type deletion; ref. 4) that possess the same deletion site observed in PC-9 cells (Fig. 2*A*) in detail was described in another paper.[4] The plasmids were transfected into the HEK293 cells, and the transfectants were selected by Zeosin (Sigma). The stable transfectants (pooled cultures) of the empty vector, wild-type EGFR, and its deletion mutant were designated as Mock, 293-pEGFR, and 293-pΔ15, respectively.

Immunoblot Analysis. Immunoblot analysis was performed as described previously (3). EGFR antibody was purchased from Santa Cruz Biotechnology (no. 1005; Santa Cruz, CA) and Cell Signaling (Beverly, MA). Phospho-EGFR antibody (specific for Tyr-1068), human epidermal growth factor receptor 2, p44/p42 mitogen-activated protein kinase (MAPK), phospho-p44/p42 MAPK, AKT, phospho-AKT, and antirabbit horseradish peroxidase–conjugated antibody all were purchased from Cell Signaling. The transfected cells cultured in

Received 7/1/04; revised 9/10/04; accepted 10/14/04.

Grant support: Funds for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12217165). T. Arao is the Recipient of a Research Resident Fellowship from the Foundation of Promotion of Cancer Research in Japan.

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[4] Unpublished observation.

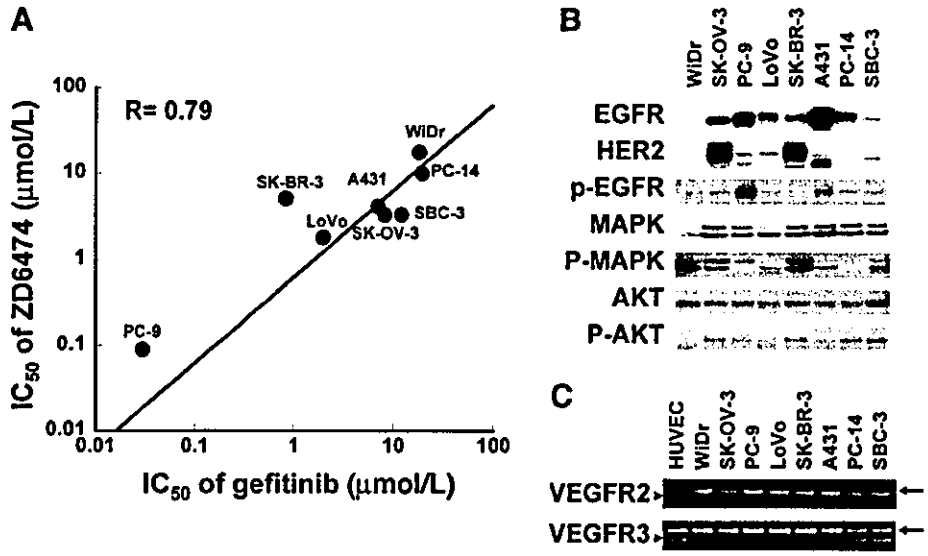

Fig. 1. The cellular characteristics and growth-inhibitory effect of gefitinib and ZD6474. *A*, correlation plot of the IC_{50} values of gefitinib and ZD6474 in human cancer cell lines. The growth-inhibitory effect against PC-9, WiDr, LoVo, PC-14, A431, SK-OV-3, SK-BR-3, and SBC-3 cells was determined by MTT assay (72-hour exposure). The data were obtained from three independent experiments. *B*, expression and phosphorylation status of EGFR and downstream molecules in human cancer cell lines. Data were obtained by immunoblot analysis with anti-EGFR, anti–phospho-EGFR, anti-HER2, anti–phospho-p44/p42 MAPK, anti-p44/p42 MAPK, anti-AKT, anti–phospho-AKT, and anti-AKT. *C*, The mRNA expression level of VEGFR-2 and VEGFR-3 was determined by reverse transcription-PCR. Human umbilical vascular endothelial cell (*HUVEC*) was used as the positive control. Whereas VEGFR-2 expression was not detected in any of the cancer cell lines, VEGFR-3 expression was detected in the PC-14 and SBC-3 cells; *arrows*, β-actin; *arrowheads*, VEGFR-2 or VEGFR-3.

the serum-free medium for 24 hours were stimulated by the addition of EGF (Sigma) at a final concentration of 10 ng/mL. After a 30-minute incubation, the cells were incubated for an additional 3 hours in the presence of ZD6474 and then collected for immunoblot analysis. The subconfluent cancer cell lines were cultured in medium containing 10% FBS and collected for immunoblot analysis.

Reverse-Transcription PCR. Five micrograms of total RNA from each cultured cell line were converted to cDNA using a GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA). The primers used for the PCR were as follows: VEGFR-2, 5′-CAGACGGACAGTGGTATGGTTC-3′ (forward) and 5′-ACCTGCTGGTGGAAAGAACAAC-3′ (reverse); and VEGFR-3, 5′-AGCCATTCATCAACAAGCCT-3′ (forward) and 5′-GGCAACAGCTGGATGTCATA-3′ (reverse). As a control, the following human β-actin primers were used: 5′-GGAAATCGTGCGTGACATT-3′ and 5′-CATCTGCTGGAAGGTGGACAG-3′. PCR amplification consisted of 35 cycles (95°C for 45 seconds, 62°C for 45 seconds, and 72°C for 60 seconds) followed by incubation at 72°C for 7 minutes. The bands were visualized by ethidium bromide staining.

Sequencing. Sequencing of exons 18 through 21 of EGFR cDNA in the tumor cell lines was performed. The cDNAs were amplified using the following primers: 5′-TCCAAACTGCACCTACGGATGC-3′ (forward) and 5′-CATCAACTCCCAAACGGTCACC-3′ (reverse). PCR amplification consisted of 25 cycles (95°C for 45 seconds, 55°C for 30 seconds, and 72°C for 60 seconds). The sequences of the PCR products were determined using ABI prism 310 (Applied Biosystems). Amplification and sequencing were performed in duplicate for each tumor cell line. The sequences were compared with the GenBank-archived human sequence of EGFR (accession no. NM 005228.3).

RESULTS

Growth-Inhibitory Activity of Gefitinib and ZD6474. We examined the *in vitro* growth-inhibitory activities of gefitinib and ZD6474 on eight cancer cell lines by MTT assay. The IC_{50} values of gefitinib and ZD6474 for each cell line were compared and plotted as shown in Fig. 1*A*. Good correlation ($r = 0.79$) was observed between the IC_{50} values of gefitinib and ZD6474, suggesting that the mechanisms underlying the growth-inhibitory activities of the two drugs *in vitro* might be similar. To clarify the correlation between the cellular sensitivity for gefitinib and ZD6474 and the EGFR status, we examined the expression and phosphorylation levels of EGFR in the cell lines by immunoblot analysis (Fig. 1*B*). No correlation was found between the expression status or the phosphorylation level of EGFR and the IC_{50} value of either drug. There also was no correlation between the cellular sensitivity and the phosphorylation status of any downstream molecules, such as phosphorylated MAPK and phospho-AKT (Fig. 1*B*). To determine the correlation between the VEGFR expression levels and cellular sensitivity, we examined the mRNA levels of the VEGFR-2 and VEGFR-3 in the cell lines by reverse transcription-PCR and detected VEGFR-3 transcripts in PC-14 and SBC-3 cells (Fig. 1*C*). VEGFR-2 was not detectable in all of the cancer cell lines. The results suggested that there was no correlation between the cellular sensitivity to ZD6474 and the VEGFR-2 and VEGFR-3 expression level. Among all of the cell lines examined, the PC-9 cell line was found to be hypersensitive to gefitinib (IC_{50} = 0.03 ± 0.002 μmol/L) and ZD6474 (IC_{50} values = 0.09 ± 0.01 μmol/L). The respective IC_{50} values of gefitinib and ZD6474 for the other cell lines were as follows: WiDr, 18.7 ± 2.5 μmol/L and 17.7 ± 2.3 μmol/L; SK-OV-3, 8.3 ± 1.5 μmol/L and 3.3 ± 0.2 μmol/L; LoVo, 2.0 ± 0.3 μmol/L and 1.8 ± 0.2 μmol/L; A431, 7.1 ± 0.9 μmol/L and 4.1 ± 0.2 μmol/L; PC-14, 20 ± 2.1 μmol/L and 10 ± 1.2 μmol/L; SK-BR-3, 0.8 ± 0.15 μmol/L and 5.2 ± 0.1 μmol/L; and SBC-3, 12.3 ± 2.1 μmol/L and 3.3 ± 0.3 μmol/L.

Fifteen-Base Pair In-Frame Deletion of EGFR in PC-9 Cells. To determine the cellular determinants of the hypersensitivity of the PC-9 cells to gefitinib, we determined the sequence of the EGFR mRNA in the PC-9 cells. The analysis revealed a 15-bp in-frame deletion around the ATP-binding site in exon 19 (Fig. 2*A*). No deletion or mutation was found in the other cell lines. The 15-bp in-frame deletion in the EGFR was consistent with the observations of Ohm *et al.* (6) in four patients with lung cancer.

Deletional Mutation of EGFR Increases the Cellular Sensitivity to ZD6474. We hypothesized that the cellular hypersensitivity of the PC-9 cells to ZD6474 was attributable to the deletional mutation of EGFR in these cells. To confirm the validity of this hypothesis, we examined ZD6474 sensitivity to HEK293 transfectant expressing the 15-bp deletion mutant EGFR or wild-type EGFR. The sequencing of EGFR cDNA obtained from 293-pEGFR and 293-pΔ15 cells was shown (Fig. 2*B*). The sensitivity of the transfectants was examined by 72-hour exposure of ZD6474 using MTT assay. The 293-pΔ15 cells were found to be 60-fold more sensitive to ZD6474 than the mock and wild-type EGFR transfectants (Fig. 3*A*). The IC_{50} of ZD6474 for the 293-pΔ15 cells, 293-pEGFR cells, and the mock transfectants were 0.08, 5.2, and 6.3 μmol/L, respectively.

The EGFR expression levels in the transfectants were quantified by immunoblot analysis using anti-EGFR antibody recognizing the

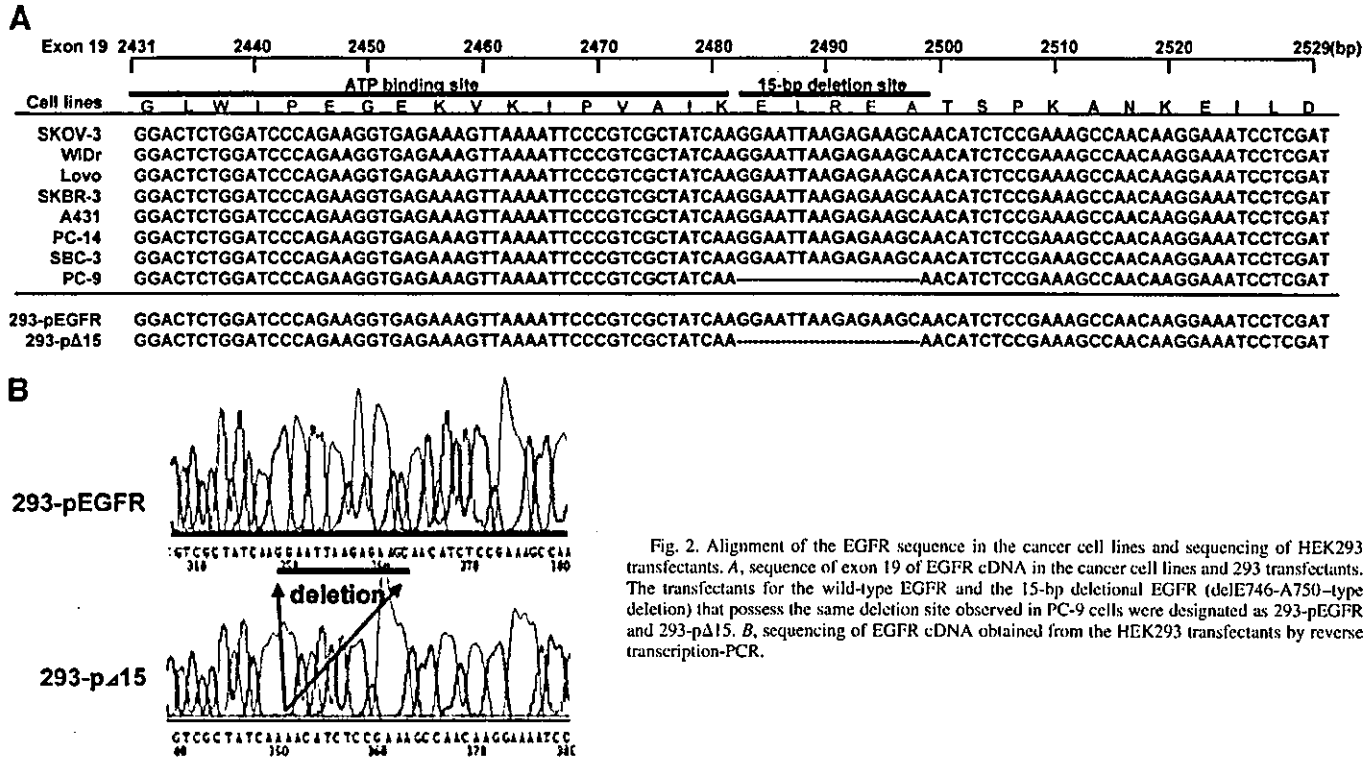

Fig. 2. Alignment of the EGFR sequence in the cancer cell lines and sequencing of HEK293 transfectants. *A*, sequence of exon 19 of EGFR cDNA in the cancer cell lines and 293 transfectants. The transfectants for the wild-type EGFR and the 15-bp deletional EGFR (delE746-A750–type deletion) that possess the same deletion site observed in PC-9 cells were designated as 293-pEGFR and 293-pΔ15. *B*, sequencing of EGFR cDNA obtained from the HEK293 transfectants by reverse transcription-PCR.

COOH-terminus of EGFR. High expression of EGFR proteins was detected in the 293-pΔ15 cells and 293-pEGFR cells but not in the mock cells (Fig. 3*B*). Exposure to ZD6474 did not affect the expression of levels of either the wild-type or the mutant EGFR. EGFR status was quantified by measuring the phosphorylation level of the Tyr-1068 residue, commonly used as a marker of the autophosphorylation of EGFR (12).

Under the condition of serum starvation, wild-type EGFR did not show any autophosphorylation, whereas the addition of EGF activated the receptor. However, marked autophosphorylation of the mutant EGFR was observed, even without the addition of EGF (Fig. 3*B*). ZD6474 exposure inhibited the phosphorylation of wild-type EGFR and mutant EGFR in a dose-dependent manner, with 2 μmol/L and 0.2 μmol/L of ZD6474 completely inhibiting phosphorylation of the wild-type EGFR and mutant EGFR, respectively. These results suggest that cells expressing the deletion mutant of EGFR are markedly more sensitive to the inhibitory effect of ZD6474 than those expressing wild-type EGFR.

DISCUSSION

Recent reports by Paez and Lynch have indicated that deletional mutations of EGFR impact on the therapeutic effects of the molecular-

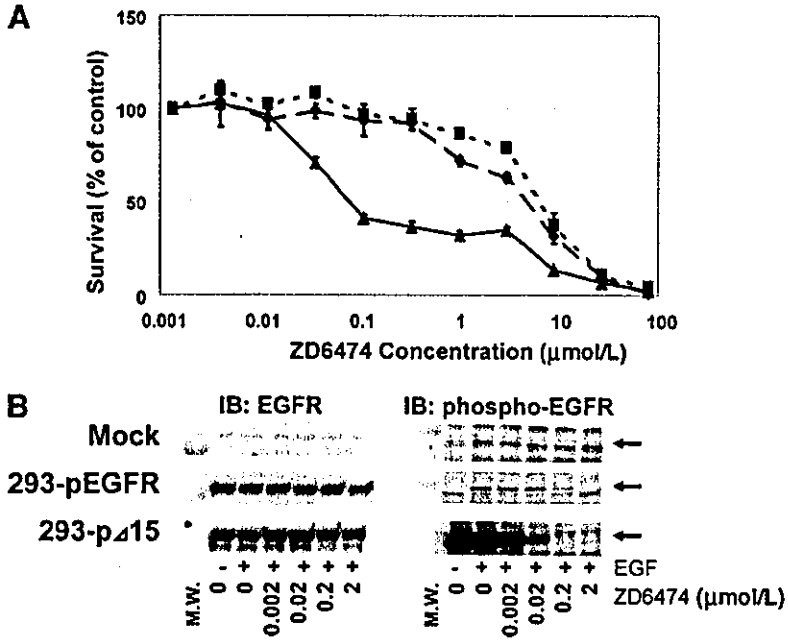

Fig. 3. Effect of ZD6474 on cellular growth inhibition and phosphorylated status of EGFR in the HEK293 transfectants. *A*. The cellular sensitivity of the transfectants against ZD6474 was determined by MTT assay (72-hour exposure). The mean values and SD represent the values obtained from the growth-inhibition curves in three independent experiments; ♦, mock (empty vector); ■, 293-pEGFR (wild-type EGFR); ▲, 293-pΔ15 (deletional-mutant EGFR). *B*, effect of EGF stimulation and ZD6474 exposure on mock, wild-type EGFR, and deletional mutant EGFR-transfected HEK293 cells determined by immunoblot analysis. Cells cultured under serum starvation for 24 hours were exposed to 10 ng/mL EGF for 30 minutes and then treated with 0.002 to 2 μmol/L ZD6474 for 3 hours in the presence or absence of EGF. *Left*, EGFR expression levels; *right*, EGFR phosphorylation levels.

targeted EGFR inhibitor gefitinib (4, 5). Here, we show that a 15-bp deletional mutation residing near the ATP binding site of EGFR in cancer cells also increases the sensitivity of the cells to ZD6474.

ZD6474 is a small molecule inhibitor of VEGFR-2 tyrosine kinase that is in Phase II clinical evaluation. *In vivo*, this compound inhibits VEGF signaling, tumor-induced angiogenesis, and the growth of a histologically diverse panel of tumor xenografts. This includes highly significant activity against tumor xenografts with intrinsic or acquired resistance to EGFR inhibitors (13). However, ZD6474 also has activity against EGFR tyrosine kinase that may give additional therapeutic benefit when tumors have a high dependency on EGFR signaling for growth and/or survival. This has been shown in PC-9 cells that are hypersensitive to treatment with gefitinib (9). PC-9 tumor cells also are hypersensitive to ZD6474 *in vitro* and regress in response to ZD6474 treatment when grown as tumor xenografts *in vivo* (14).

We have shown that PC-9 cells contain a 15-bp in-frame deletional mutation in EGFR, and this mutation may confer increased sensitivity to ZD6474 and gefitinib. The difference in ZD6474 concentration required for complete inhibition of wild-type and mutant EGFR phosphorylation was relatively small (2 versus 0.2 μmol/L), whereas difference in sensitivity to ZD6474 was large (60-fold).

The deletional EGFR was constitutively phosphorylated, and the addition of EGF to the cultures did not result in any additional increase in phosphorylation (Fig. 3*B*). These observations contradict data reported by Lynch *et al.* (4), who showed that a receptor with a similar deletion was still regulated by EGF.

The most possible explanation for this contradiction is that the expression level of deletional EGFR in the 293-pΔ15 cells is much higher than that of the transient transfectant of Del L747-P753insS reported by Lynch *et al.* Ligand-independent oligomerization of the receptor and phosphorylation may have occurred in the 293-pΔ15 cells as a result. This hypothesis is consistent with the result that PC-9 cells harboring the same 15-bp deletion showed a stronger phosphorylation of the EGFR in a 10% FBS medium than other nonhypersensitive cell lines (Fig. 1*B*).

The other possible explanation is that apparent distinct amino acid sequences of EGFR exist between our mutant and that of Lynch *et al.* (293-pΔ15, VAIKELREATSPK>VAIKTSPK; delL747-P753insS, VAIKELREATSPK>VAIKESK). Five amino acids are simply deleted in the 293-pΔ15 cells, whereas six amino acids are deleted and serine is inserted in the delL747-P753insS cells. This small difference may be critical to the ATP-binding properties of 293-pΔ15 and delL747-P753insS, determining whether EGFR is constitutively active. Therefore, it is not surprising that our constitutive active form of EGFR is out of ligand regulation.

The mock-transfected 293 cells and 293-pEGFR cells were not sensitive to the growth-inhibitory effect of ZD6474 (Fig. 3*A*), indicating that these cells were independent of EGFR signaling. The 293 cells are oncogenic transformant. Therefore, the 293 cells were considered to have acquired the dependency on the oncogenic signal. Conversely, the overexpression of the deletional EGFR transduces the excess signal to downstream of EGFR in the 293-pΔ15 cells. If the downstream mutant EGFR signaling pathway were shared with that of the oncogenic signaling pathway in the cells, the excess and constitutive signal from the mutant EGFR would dominate the downstream pathway, possibly influencing the dependency of the cells on the EGFR signal.

A recent report by Sordella *et al.* (15) showed the mutant EGFRs (delL747-P753insS and L858R) expressing a stable transfectant selectively activate AKT and STAT signaling pathways. They also showed that NSCLC cell lines that harboring mutant EGFR transduce survival signals and depend on the acquisition of these signals. Their evidence is consistent with our present speculations. We now are investigating the downstream pathways of the mutant EGFR signaling in the 293-pΔ15 cells.

In summary, inhibition of VEGFR-2 tyrosine kinase by ZD6474 may potentially confer activity against tumors that are not dependent on EGFR signaling. Nevertheless, the additional activity of ZD6474 against EGFR tyrosine kinase could provide further benefit, particularly when EGFR is mutated. Patients with lung adenocarcinoma showing EGFR mutations are likely to be highly sensitive to gefitinib and ZD6474 treatment.

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Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib ("Iressa")-sensitive and resistant xenograft models

Fumiko Taguchi,[1] Yasuhiro Koh,[1] Fumiaki Koizumi,[1,2] Tomohide Tamura,[3] Nagahiro Saijo[3] and Kazuto Nishio[1,2,4]

[1]Pharmacology Division, National Cancer Center Research Institute; and [2]Shien-Lab and [3]Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045

(Received August 6, 2004/Revised September 27, 2004/Accepted October 8, 2004)

ZD6474 is a novel, orally available inhibitor of vascular endothelial growth factor (VEGF) receptor-2 (KDR) tyrosine kinase, with additional activity against epidermal growth factor receptor (EGFR) tyrosine kinase. ZD6474 has been shown to inhibit angiogenesis and tumor growth in a range of tumor models. Gefitinib ("Iressa") is an selective EGFR tyrosine kinase inhibitor (TKI) that blocks signal transduction pathways. We examined the antitumor activity of ZD6474 in the gefitinib-sensitive lung adenocarcinoma cell line, PC-9, and a gefitinib-resistant variant (PC-9/ZD). PC-9/ZD cells showed cross-resistance to ZD6474 in an *in vitro* dye formation assay. In addition, ZD6474 showed dose-dependent inhibition of EGFR phosphorylation in PC-9 cells, but inhibition was only partial in PC-9/ZD cells. ZD6474-mediated inhibition of tyrosine residue phosphorylation (Tyr992 and Tyr1045) on EGFR was greater in PC-9 cells than in PC-9/ZD cells. These findings suggest that the inhibition of EGFR phosphorylation by ZD6474 can contribute a significant, direct growth-inhibitory effect in tumor cell lines dependent on EGFR signaling for growth and/or survival. The effect of ZD6474 (12.5–50 mg/kg/day p.o. for 21 days) on the growth of PC-9 and PC-9/ZD tumor xenografts in athymic mice was also investigated. The greatest effect was seen in gefitinib-sensitive PC-9 tumors, where ZD6474 treatment (>12.5 mg/kg/day) resulted in tumor regression. Dose-dependent growth inhibition, but not tumor regression, was seen in ZD6474-treated PC-9/ZD tumors. These studies demonstrate that the additional EGFR TKI activity may contribute significantly to the antitumor efficacy of ZD6474, in particular in those tumors that are dependent on continued EGFR-signaling for proliferation or survival. In addition, these results provide a preclinical rationale for further investigation of ZD6474 as a potential treatment option for both EGFR-TKI-sensitive and EGFR-TKI-resistant tumors. (Cancer Sci 2004; 95: 984–989)

ZD6474 is a novel, orally available inhibitor of VEGF receptor-2 (KDR) tyrosine kinase, with additional activity against EGFR tyrosine kinase, and it inhibits angiogenesis and tumor growth in a diverse range of tumor models.[1,2] Phase I clinical evaluation has shown ZD6474 to be generally well tolerated, and tumor responses in patients with non-small cell lung cancer (NSCLC) have been documented.[3,4] Thus, ZD6474 is considered to be a multi-target tyrosine kinase inhibitor active against solid tumors. The purpose of this study is to clarify the mode of antitumor action of ZD6474 as compared with that of gefitinib ("Iressa," ZD1839). Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor (EGFR-TKI) that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting tumor growth.[5–7] Gefitinib is now available clinically for non-small cell lung cancer patients. In order to elucidate the mode of action of ZD6474, the antitumor activity and pharmacodynamics were investigated in an established human lung cancer cell line resistant to gefitinib (PC-9/ZD cells).[8] This approach allowed us to clarify the common and differential modes of actions of gefitinib and ZD6474 in lung cancer, and this will be important for deciding how to use ZD6474 in non-small cell lung cancer patients in combination with gefitinib.

Materials and Methods

Reagents and cell culture. ZD6474 and gefitinib ("Iressa," ZD1839) were provided by AstraZeneca (Macclesfield, UK). Human NSCLC cell lines PC-9 and PC-14 were used.[9,10] In addition, a gefitinib-resistant subline, PC-9/ZD, was derived from PC-9 cells by short-term exposure to the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, continuous exposure to 0.2–0.5 μM gefitinib for 28 days, and subcloning. The resistant phenotype has been stable for at least 6 months under drug-free conditions.[8] The PC-9/ZD cell line shows no cross-resistance to conventional anticancer drugs.[8] Cells were maintained in RPMI-1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY).

Antibodies. Anti-vonWillebrand Factor (vWF) antibody was purchased from Chemicon, Temecula, CA. Affinity-purified antibody to EGFR was purchased from Santa Cruz, CA and affinity-purified antibodies to phospho-EGFR specific for Tyr845, Tyr992, Tyr1045, and Tyr1068 were purchased from Cell Signaling Technology, Beverly, MA.

Growth inhibition assay. Cell sensitivity to ZD6474 and gefitinib was estimated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.[11] Briefly, PC-9, PC-9/ZD, or PC-14 cells were exposed to 0–10 μM ZD6474 or gefitinib for 72 h before measuring absorbance. Optical density was assessed at 562–630 nm using an EL340 96-well microtiter plate reader (Bio-Tek, Winooski, VT).

Xenograft studies in athymic mice. Suspensions of PC-9 cells (5×10^6) or PC-9/ZD cells (3×10^6) were injected subcutaneously into the backs of 5-week-old female athymic mice (Japan Charles River Co., Atsugi, Japan). After 1 week (tumors >95 mm^3), mice were randomly allocated into groups of six animals to receive ZD6474 (12.5, 25, or 50 mg/kg/day), gefitinib (12.5, 25, or 50 mg/kg/day) or vehicle only by oral gavage. Tumor diameter and body weight were measured twice weekly. The tumor volume was calculated (width^2×length/2) and is presented as a percentage of the pretreatment value. A tumor volume below 100% of the pretreatment volume was defined as "tumor reduction." Experiments were performed in accordance with the UK Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (second edition). After 3 weeks of treatment, tumors were removed.

[4]To whom correspondence should be addressed.
E-mail: knishio@gan2.res.ncc.go.jp

Abbreviations: VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

Two tumor specimens per group were processed for immunohistochemical analysis.

Immunohistochemical analysis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections as reported previously.[1,5] An anti-Ki67 monoclonal antibody (clone MIB1; DBA, Milan, Italy) was used and the proportion of positive (proliferating) cells was assessed. At least 1000 cancer cells were counted and scored per slide. Both the percentage of specifically stained cells and the intensity of immunostaining were recorded. Blood vessels were detected with an anti-von Willebrand Factor (vWF) antibody (Chemicon). Microvessel density was determined by calculating the proportion of vWF-positive cells.

Evaluation of apoptosis (TUNEL). Sections were stained with an *in situ* Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined. The TUNEL-positive tumor cell nuclei were counted, and the apoptotic index was calculated as the proportion of cells with apoptotic nuclei.

Immunoprecipitation and immunoblotting. Cells were maintained in medium without serum for 12 h. Then serum-starved cells were exposed to ZD6474 or gefitinib, incubated for 1 h and stimulated in medium including 10% fetal bovine serum for 30 min. The cells were subsequently washed twice with ice-cold PBS, scraped in lysis buffer (50 m*M* Tris-HCl [pH 8.0], 120 m*M* NaCl, 0.5% Nonidet P-40, 100 m*M* NaF, 200 μ*M* Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin, and PMSF), and incubated on ice for 60 min. The lysates were centrifuged at 8000*g* for 20 min, and total protein was obtained from the supernatants. Protein concentration was measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Cell lysates for immunoprecipitates contained 2 mg of total protein. Anti-EGFR antibody (3 μg) was incubated overnight with the lysates at 4°C, and the precipitates were collected with 40 μliters of Protein G Sepharose beads over a 1 h period. Antibody-complexed proteins were washed with lysis buffer, analyzed by SDS-PAGE and visualized using an enhanced chemiluminescence solution (ECL; Amersham Pharmacia Biotech UK, Buckinghamshire, UK). Quantative analysis was performed using Kodak software. Quantified values of phospho-EGFR bands were standardized according to those of EGFR bands.

Results

***In vitro* evaluation of ZD6474 and gefitinib inhibition of tumor cell growth.** The IC_{50} values of gefitinib for growth inhibition of PC-9 and PC-9/ZD cells were 0.038 μ*M* and 6.8 μ*M*, respectively. The IC_{50} values of ZD6474 were 0.14 μ*M* and 5.92 μ*M*, respectively (Fig. 1A). PC-9 cells were 180-fold more sensitive to gefitinib than PC-9/ZD cells, and PC-9/ZD cells were cross-resistant to ZD6474. Experiments with another VEGFR-TKI, SU5416, and PDGFR-TKI, Tyrphostin 9, revealed no cross-resistance (data not shown).

In a separate experiment, the IC_{50} values of gefitinib were 0.006 μ*M* and 20.5 μ*M*, in PC-9 and PC-14 (another human NSCLC cell line), respectively (Fig. 1B). PC-9 cells were therefore approximately 3400-fold more sensitive to gefitinib than PC-14 cells. Corresponding IC_{50} values for ZD6474 were 0.11 μ*M* and 9.81 μ*M*, demonstrating cross-resistance to ZD6474.

Other workers have examined the ability of gefitinib or ZD6474 to inhibit serum-dependent tumor cell growth *in vitro*, and have demonstrated IC_{50} values of gefitinib[12] and ZD6474[13] of >1 μ*M* for tumor cell lines. Therefore, PC-9 is particularly sensitive to *in vitro* growth inhibition by both gefitinib and ZD6474, whereas the sensitivities of both gefitinib-resistant PC-9/ZD and PC-14 fall within the normal range reported for other tumor cell lines.

***In vivo* antitumor effects.** ZD6474 treatment (12.5–50 mg/kg/day) resulted in inhibition of PC-9 tumor growth, with robust tumor regression seen even at the lowest dose tested. ZD6474 treatment also resulted in dose-dependent inhibition of PC-9/ZD tumor xenograft growth, although in this case, regression was not seen (Fig. 2, A and B). This antitumor effect of ZD6474 was very similarly to that of gefitinib we previously reported (Fig. 2, C and D).[8]

Effect of treatment on cell proliferation, apoptosis, and vascularization. ZD6474 treatment resulted in a dose-dependent decrease in the proportion of proliferating cells in the PC-9 tumors, but not in PC-9/ZD xenografts (Fig. 3). No significant

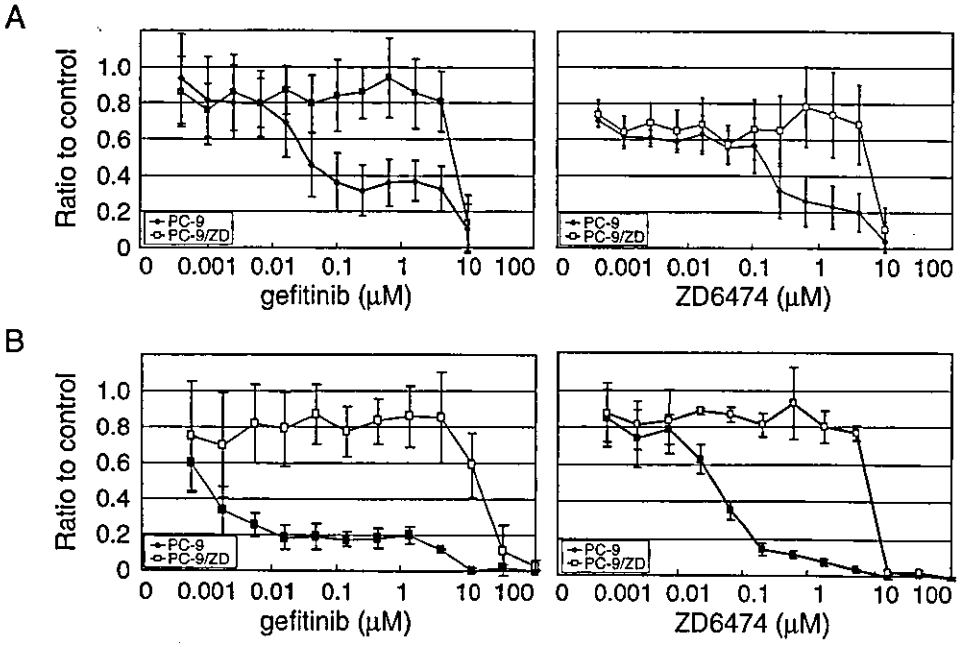

Fig. 1. Growth inhibitory effect of gefitinib (ZD1839) and ZD6474. A: PC-9 and PC-9/ZD, B: PC-9 and PC-14 cells. Data shown are mean values from three experiments (±SD).

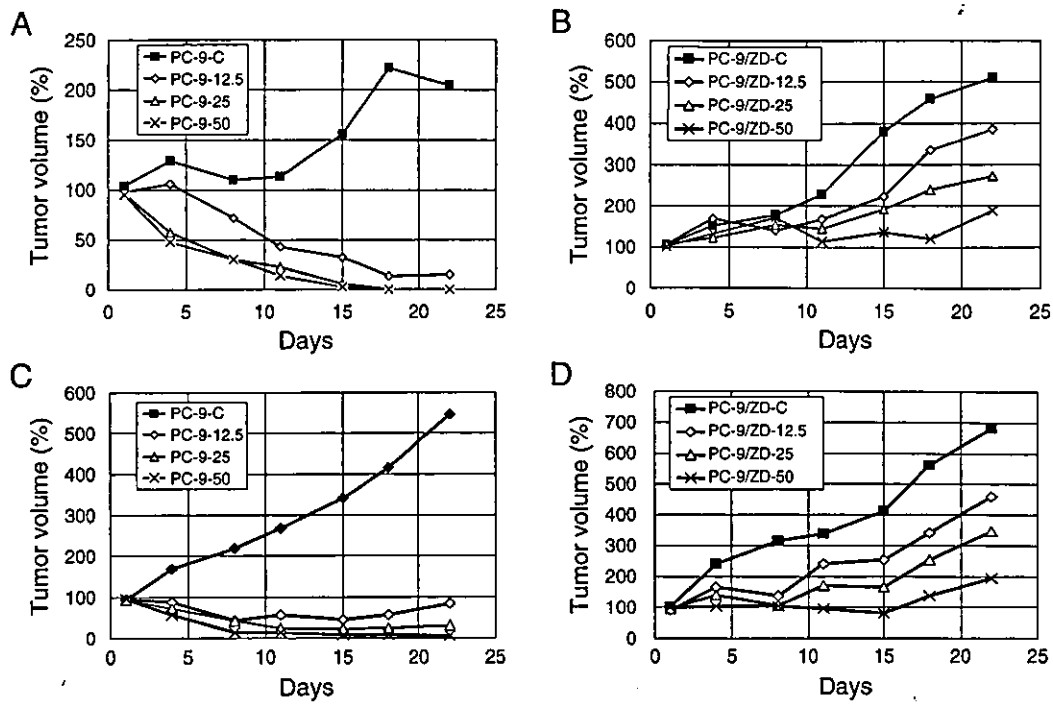

Fig. 2. Antitumor activity of ZD6474 (A, B) and gefitinib (C, D) on established PC-9 (A, C) and PC-9/ZD (B, D) human lung cancer xenografts.

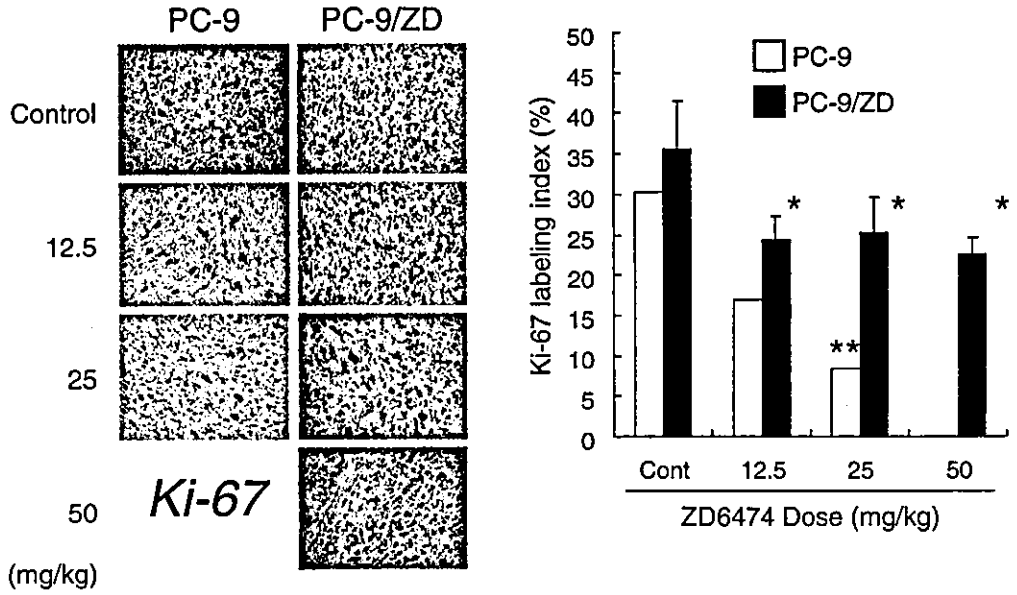

Fig. 3. Effect of ZD6474 on the Ki67 labeling index of PC-9 and PC-9/ZD tumors *in vivo*. Data represent mean values (±SD). Significant difference from control shown by the Dunnett test (* $P<0.05$, ** $P<0.01$).

increase in apoptosis was observed in either tumor type (Fig. 4).

Assessment of tumor vascularization showed a significant reduction in vascular density following ZD6474 treatment of PC-9 tumor xenografts, although no effect was seen in PC-9/ZD tumors (Fig. 5). Differences in the action of ZD6474 on PC-9 and PC-9/ZD tumors are summarized in Table 1.

Inhibition of EGFR activity. It is possible that the antitumor activity of ZD6474 is partly attributable to EGFR inhibition based on the evidence of cross-resistance to gefitinib (Figs. 1–3). Therefore, site-specific anti-phosphorylated-EGFR antibodies were used to investigate inhibition of EGFR phosphorylation by ZD6474 in PC-9 and PC-9/ZD cells at four different tyrosine phosphorylation sites (Tyr845, Tyr992, Tyr1045, and Tyr1068; Fig. 6). ZD6474 dose-dependently inhibited phosphorylation of the four EGFR tyrosine residues in PC-9 cells (Fig. 6). In PC-9/ZD cells, drug-related inhibition of phosphorylation at the Tyr992 site was highly resistant to ZD6474 treatment (Fig. 6), and the Tyr845 and Tyr1045 sites were moderately resistant, while the effect of phosphorylation at the Tyr1068 site did not differ significantly between the sensitive and resistant cell lines (Table 1). The spectrum of activity of ZD6474 on the

four EGFR tyrosine residues examined in PC-9/ZD cells differed from that of gefitinib. ZD6474 displayed a variety of actions on each tyrosine residue, which may be responsible for the wide range of biological activities.

Discussion

In the NSCLC xenograft model reported here, ZD6474 treatment significantly inhibited PC-9 tumor growth, inducing tumor regression. In addition, ZD6474 caused dose-dependent PC-9/ZD tumor growth inhibition. These data indicate that ZD6474 exerts potent antitumor activity against gefitinib-sensitive and resistant lung cancers *in vivo*. Although PC-9/ZD cells are less sensitive to gefitinib than PC-9 cells, the *in vitro* sensitivity of these cells falls within the normal range for other tumor cell lines. Accordingly, gefitinib has significant *in vivo* activity against PC-9/ZD, producing a dose-dependent inhibition of xenograft growth, rather than the tumor regression seen with PC-9 xenografts. Therefore, the antitumor activity of ZD6474 appeared to parallel that of gefitinib in PC-9 and PC-9/ZD tumor cells, both *in vitro* and *in vivo*. Since gefitinib is a TKI with a high degree of selectivity for EGFR,[1,2,4] inhibition of EGFR autophosphorylation is likely to contribute to the antitumor activity of ZD6474, particularly in tumor cells which are dependent on EGFR signaling for continued growth and survival. This was shown *in vitro*, as ZD6474 inhibited EGFR

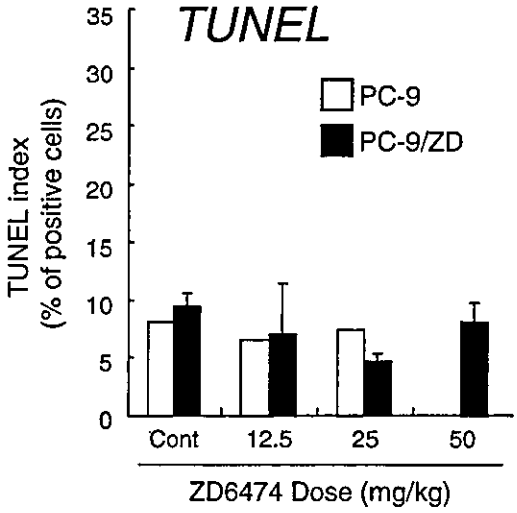

Fig. 4. Effect of ZD6474 on the TUNEL index of PC-9 or PC-9/ZD tumors *in vivo*. Data represent mean values (±SD).

Table 1. Site-specific effect of ZD6474 on EGFR tyrosine residues in PC-9 and PC-9/ZD cells

Tyr residue of EGFR	Inhibition of phosphorylation			
	ZD6474		Gefitinib	
	PC-9	PC-9/ZD	PC-9	PC-9/ZD
845	++	+	++	+
992	++	–	++	++
1045	++	+	–	–
1068	++	++	++	+

++ strong; + moderate; – not significant.

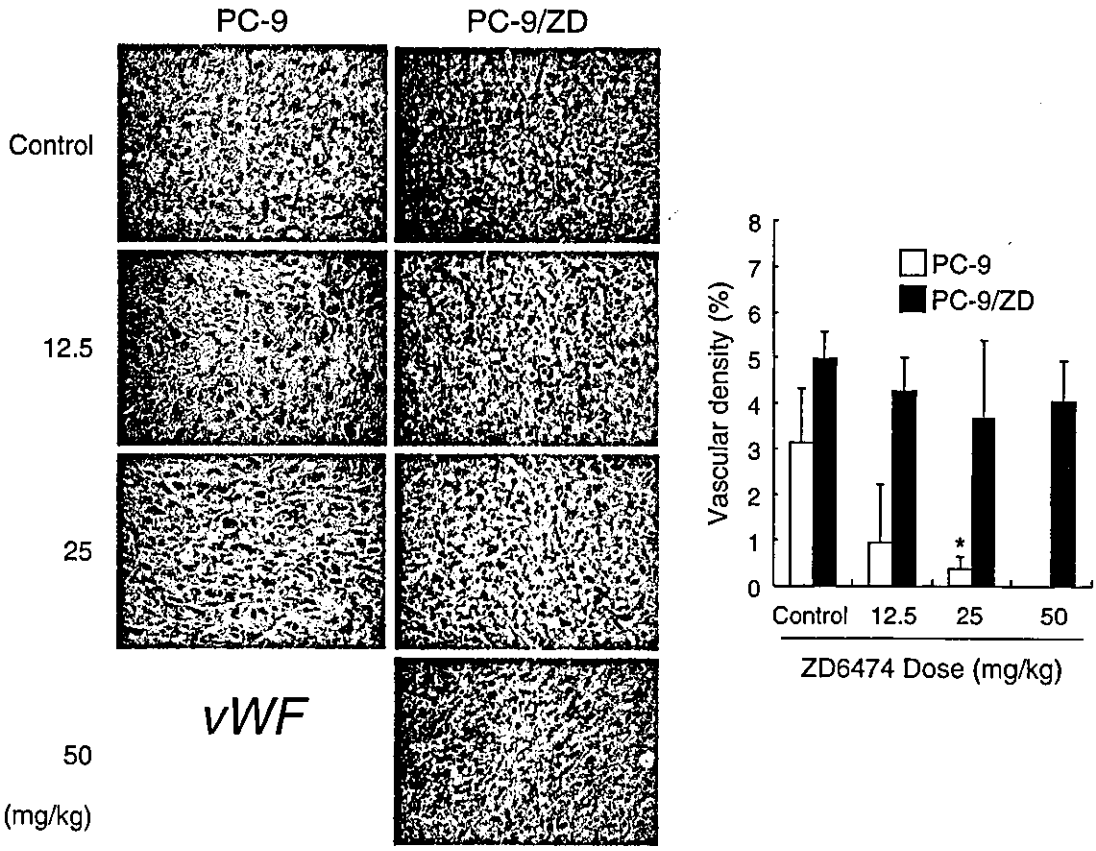

Fig. 5. Effect of ZD6474 on the vascular density of PC-9 and PC-9/ZD tumors stained *in vivo* with anti-vWF. Values are means±SD. Significant difference from the control by the Dunnett test (* $P<0.05$).

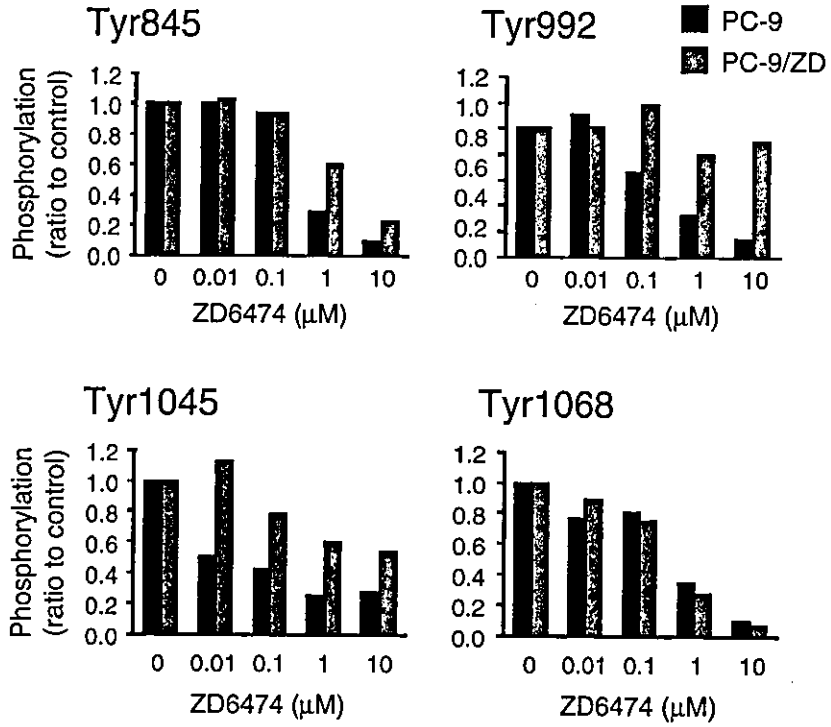

Fig. 6. Phosphorylation of EGFR tyrosine residues in PC-9 and PC-9/ZD cells after exposure to ZD6474.

phosphorylation in a dose-dependent manner. These results are consistent with previous reports[1] and indicate that ZD6474 is a potent EGFR TKI. *In vivo*, ZD6474 decreased vascular density in PC-9 tumors but not in PC-9/ZD cells, suggesting that ZD6474 may affect the angiogenic process via EGFR blockade. This could be mediated by inhibition of EGFR-induced paracrine production of angiogenic growth factors, such as VEGF, bFGF, and TGF from cancer cells, but the exact mechanism of action is unclear. This activity is, however, likely to be of less significance than the VEGFR-2-mediated antiangiogenic effect, since ZD6474 has been shown to have consistent *in vivo* antitumor activity in a range of histologically diverse human tumor xenografts, including activity in tumor models which do not respond to treatment with an EGFR TKI.[13] In addition, any change, or lack of change in microvessel density needs to be interpreted with caution as a either positive or negative indication of antiangiogenic activity, since the efficacy of antiangiogenic agents may not be related to microvessel density measurements.[14] ZD6474 was expected to induce increased apoptosis in tumor cells; although no induction of apoptosis was in fact observed, this may have been due to experimental factors.

Phosphorylations of Tyr845 and Tyr1045 of PC-9 and PC-9/ZD cells are similarly inhibited by ZD6474. On the other hand, while the inhibition pattern of Tyr845 phosphorylation by ZD6474 is coincident with that by gefitinib, the patterns at Tyr1045 are different. Therefore, we considered that the Tyr1045 is more important than Tyr845 for assessing the distinctive mode of action of ZD6474. In searching for a common mode of action of ZD6474 and gefitinib, Tyr845 seems to be the most promising site.

Phosphorylation of Tyr992 has been reported to transduce the signal to phospholipase C and protein kinase C.[15–17] In contrast, no inhibition of pan-phospho-PKC (the downstream signal of Tyr992) by gefitinib or ZD6474 was observed (data not shown). Tyr1045 has been reported to be linked to the Cbl-ubiquitin signaling pathway.[18] We have previously reported that Tyr1068 is a possible target site of EGFR for gefitinib[7], and gefitinib inhibited phosphorylation of Tyr1068 to varying degrees in PC-9 and PC-9/ZD cells, whereas ZD6474 inhibited Tyr1068 in both cell lines. These results suggest that the mode of inhibition of phosphorylation of EGFR by ZD6474 is subtly different to that of gefitinib. Therefore, although ZD6474 shows cross-resistance to gefitinib in these PC-9/ZD tumor cells, it has the potential for activity against gefitinib-resistant tumors through at least two mechanisms: (i) inhibition of EGFR-dependent downstream signaling pathways through differential effects on the phosphorylation status of tyrosine residues in the intracellular domain of EGFR, and (ii) inhibition of tumor angiogenesis through inhibition of VEGFR2 tyrosine kinase activity, which has not been examined in the present study. Site-directed mutagenesis studies are now under way to elucidate the biological significance of these sites.

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British Journal of Cancer (2004) 91, 659–665

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Multi-institutional phase II trial of irinotecan, cisplatin, and etoposide for sensitive relapsed small-cell lung cancer

K Goto*[,1], I Sekine[2], Y Nishiwaki[1], R Kakinuma[1], K Kubota[1], T Matsumoto[1], H Ohmatsu[1], S Niho[1], T Kodama[2], T Shinkai[2], T Tamura[2], Y Ohe[2], H Kunitoh[2], N Yamamoto[2], H Nokihara[2], K Yoshida[3], T Sugiura[3], K Matsui[4] and N Saijo[2]

[1]Division of Thoracic Oncology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa, Chiba 277-8577, Japan; [2]Internal Medicine and Thoracic Oncology Division, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan; [3]Department of Internal Medicine, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan; [4]Department of Internal Medicine, Osaka Prefectural Habikino Hospital, 3-7-1 Habikino, Habikino, Osaka 583-0872, Japan

Clinical

Irinotecan (CPT-11) has been shown to exhibit excellent antitumour activity against small-cell lung cancer (SCLC). A multi-institutional phase II study was therefore conducted to evaluate the efficacy and toxicity of CPT-11 combined with cisplatin (CDDP) and etoposide (ETOP) (PEI regimen) for the treatment of sensitive relapsed SCLC. Patients who responded to first-line chemotherapy but relapsed more than 8 weeks after the completion of first-line therapy ($n = 40$) were treated using the PEI regimen, which consisted of CDDP (25 mg m^{-2}) weekly for 9 weeks, ETOP (60 mg m^{-2}) for 3 days on weeks 1, 3, 5, 7, and 9, and CPT-11 (90 mg m^{-2}) on weeks 2, 4, 6, and 8 with granulocyte colony-stimulating factor support. Five complete responses and 26 partial responses were observed, and the overall response rate was 78% (95% confidence interval 61.5–89.2%). The median survival time was 11.8 months, and the estimated 1-year survival rate was 49%. Grade 3/4 leucocytopenia, neutropenia, and thrombocytopenia were observed in 55, 73, and 33% of the patients, respectively. Nonhaematological toxicities were mild and transient in all patients. In conclusion, the PEI regimen is considered to be highly active and well tolerated for the treatment of sensitive relapsed SCLC.
British Journal of Cancer (2004) **91**, 659–665. doi:10.1038/sj.bjc.6602056 www.bjcancer.com
Published online 27 July 2004

Keywords: irinotecan; etoposide; small-cell lung cancer; sensitive relapse; second line; salvage chemotherapy

Small-cell lung cancer (SCLC) is one of the most chemosensitive solid tumours, and first-line combination chemotherapy improves survival. However, despite a high response rate to chemotherapy, the majority of SCLC patients relapse. At the time of recurrence, the tumour is broadly resistant to second-line chemotherapy and is lethal within a few to several months (Glisson, 2003). The further development of not only first-line chemotherapy but also of effective salvage chemotherapies is needed.

In predicting the efficacy of salvage chemotherapy, two major factors are important: the response to the initial chemotherapy and the duration of time between the last exposure to chemotherapy and the confirmation of recurrence (Postmus *et al*, 1987; Giaccone *et al*, 1988; Ardizzoni *et al*, 1997; Ebi *et al*, 1997). Based on these factors, relapsed SCLC is now commonly classified into two main groups. Patients who both respond to the initial chemotherapy and relapse more than 2 or 3 months after the completion of chemotherapy are considered to be 'sensitive relapse' patients, while patients whose tumour is stable or progresses during the initial chemotherapy or who have a recurrence within 2 or 3 months after the completion of chemotherapy are considered to be 'refractory relapse' patients (Giaccone *et al*, 1988). Since the outcomes of salvage chemotherapy for relapsed SCLC patients are different between these two groups, the ratios of sensitive and refractory cases must be carefully considered when evaluating the results of clinical trials for second-line chemotherapy.

The combination of cisplatin (CDDP) and etoposide (ETOP) (PE regimen) has been the standard chemotherapeutic regimen for SCLC (Fukuoka *et al*, 1991; Ihde, 1992; Roth *et al*, 1992; Aisner, 1996). Moreover, PE is a reasonable second-line chemotherapy for relapsed SCLC after combination chemotherapy consisting of cyclophosphamide, doxorubicin (ADM), and vincristine (VCR) (CAV regimen); the likelihood of a response to this regimen is 40–50% (Evans *et al*, 1984; Porter *et al*, 1985). Since PE has a relatively mild toxicity profile, other cytotoxic agent can be combined with PE.

Irinotecan (CPT-11), a camptothecin derivative topoisomerase I inhibitor, has been shown to exhibit excellent antitumour activity against SCLC in monotherapy and in combination with CDDP (Masuda *et al*, 1992; Kudoh *et al*, 1998). Based on these results, the Japan Clinical Oncology Group (JCOG) conducted a randomised phase III trial comparing CPT-11 and CDDP (IP regimen) with standard PE for previously untreated extensive stage (ED) SCLC (JCOG 9511) (Noda *et al*, 2002). The response rates were significantly higher for IP than for PE, and overall survival was also significantly better for IP than for PE. This was the first study to show the superiority of any one regimen over PE for the

*Correspondence: Dr K Goto; E-mail: kgoto@east.ncc.go.jp
Received 14 April 2004; revised 1 June 2004; accepted 2 June 2004; published online 27 July 2004

treatment of ED SCLC, and IP has become one of the standard regimens for ED SCLC in Japan. Thereafter, several clinical trials of CPT-11-containing regimens for patients with limited disease (LD), ED, and relapsed SCLC have been conducted by Japanese clinical study groups (Masuda *et al*, 1998; Mori *et al*, 2002; Sekine *et al*, 2002).

Consequently, a phase I trial of CPT-11 combined with weekly CDDP (25 mg m^{-2}) and biweekly ETOP (60 mg m^{-2}) (PEI regimen) was conducted, and the recommended dose of 90 mg m^{-2} of CPT-11 was repeated every 2 weeks (JCOG 9507) (Sekine *et al*, 2003). This regimen showed promising antitumour activity in patients with untreated ED SCLC (response rate, 91%, 1-year survival rate 46%). Moreover, since the drug dose and treatment schedule can be easily modified in a weekly regimen, this protocol is considered to be suitable for relapsed SCLC patients, who usually present with severe haematological toxicities during salvage chemotherapy because of poor bone marrow reserve (Masuda *et al*, 1990; Faylona *et al*, 1995).

Based on these results, we conducted two phase II trials to evaluate the efficacy and toxicities of PEI in patients with sensitive and refractory relapsed SCLC, separately. In this paper, the final results for the sensitive relapsed SCLC group are reported.

PATIENTS AND METHODS

Patient selection

Patients with histologically or cytologically confirmed SCLC who respond to first-line chemotherapy or chemoradiotherapy and relapsed more than 8 weeks after the completion of first-line treatment were candidates for the present study. Additional eligibility criteria were as follows: (1) age of 75 years or younger; (2) performance status of 0–2 on the Eastern Cooperative Oncology Group scale; (3) measurable disease; (4) adequate organ function as documented by a $4.0 \times 10^9 l^{-1} \leqslant$ WBC count $\leqslant 12.0 \times 10^9 l^{-1}$, haemoglobin level of $\geqslant 9.0$ g dl^{-1}, platelet count of $\geqslant 100 \times 10^9 l^{-1}$, total serum bilirubin level of $\leqslant 1.5$ mg dl^{-1}, a hepatic transaminase level of $\leqslant 2$ times the institutional upper limit of normal, a serum creatinine level of $\leqslant 1.5$ mg dl^{-1}; and (5) written informed consent. Patients were not eligible for the study if they had experienced any of the following events: (1) massive pleural effusion requiring drainage; (2) prior radiotherapy with an irradiated area larger than one-third of the bone marrow volume; (3) active infection; (4) contraindications for the use of CPT-11, including diarrhoea, ileus, interstitial pulmonary fibrosis, massive ascites, or hypersensitive reaction to CPT-11; (5) serious concomitant medical illness, including severe heart disease, uncontrollable diabetes mellitus or hypertension; or (7) pregnancy or lactation. This study was approved by the institutional review board at each participating institution.

Treatment schedule

Figure 1 shows the treatment schema of the PEI regimen. CDDP (25 mg m^{-2}) was administered intravenously (i.v.) over 60 min on day 1 and at 1-week intervals for 9 weeks; ETOP (60 mg m^{-2}) was administered i.v. over 60 min on days 1–3 of weeks 1, 3, 5, 7, and 9; and CPT-11 (90 mg m^{-2}) was administered i.v. over 90 min on day 1 on weeks 2, 4, 6, and 8. Hydration (2000 ml) and granisetron (40 μg kg^{-1}) were given on day 1. After day 1 on week 2, granulocyte colony-stimulating factor (G-CSF) (50 μg m^{-2}) was administered routinely according to JCOG 9507 on days when the cytotoxic drugs were not given, unless the WBC count exceeded $10.0 \times 10^9 l^{-1}$. Patients were expected to complete at least six cycles of this regimen; if the toxicities were acceptable and the tumour responded to the treatment, a maximum of nine cycles of chemotherapy were performed.

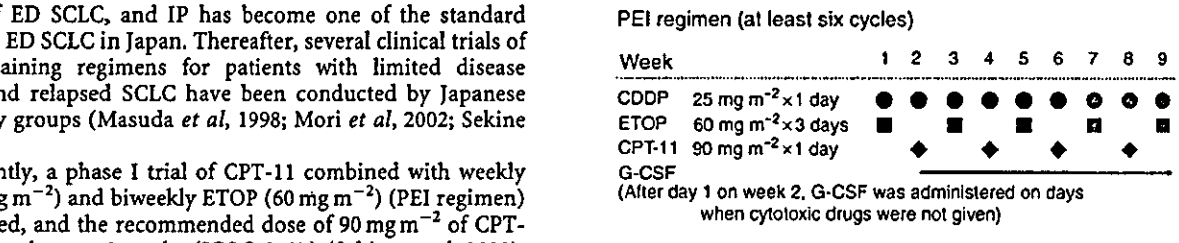

Figure 1 Treatment schedule.

Toxicity assessment and treatment

During the course of treatment, complete blood cell counts and differential counts were analysed twice a week, and routine chemistry measurements and a chest X-ray were performed once a week. Toxicity was graded according to the toxicity criteria of the JCOG (Tobinai *et al*, 1993), a modified version of the NCI Common Toxicity Criteria issued in 1991. Grade 4 neutropenia was defined as $<0.5 \times 10^9 l^{-1}$, and grade 3 neutropenia was defined as between (and including) $0.5-1.0 \times 10^9 l^{-1}$, according to the JCOG criteria. The second and subsequent cycles of chemotherapy were delayed for 1 week if one of the following toxicities was noted on day 1: a WBC count of $<2.0 \times 10^9 l^{-1}$, a platelet count of $<50 \times 10^9 l^{-1}$, a serum creatinine level of $\geqslant 2.0$ mg dl^{-1}, an elevated hepatic transaminase level or total serum bilirubin of grade 2 or higher, diarrhoea of grades 1–2, fever $\geqslant 38°C$, or a performance status of 3. The treatment was terminated if the above-mentioned criteria did not disappear in 3 weeks or if one of the following severe nonhaematological toxicities was noted: diarrhoea of grade 2 lasting for more than 1 week, diarrhoea of grade 3, neurotoxicity of grade 3, or drug-induced pneumonitis.

Dose modifications for toxicity

The CPT-11 dosage was reduced to 67.5 mg m^{-2} (25% reduction) in subsequent cycles if one of the following toxicities was noted: a WBC count of $<1.0 \times 10^9 l^{-1}$, or a platelet count of $<25 \times 10^9 l^{-1}$. If the above-mentioned toxicities reappeared after a 25% reduction in the dosage, the CPT-11 dosage was further reduced to 50 mg m^{-2} (44% reduction). Since CDDP (25 mg m^{-2}) and ETOP (60 mg m^{-2}) in this regimen were relatively low dose, no dose modifications for these drugs were permitted.

Pretreatment evaluation

Pretreatment assessment included a complete blood cell count, differential counts, routine chemistry measurements, creatinine clearance, blood gas analysis, electrocardiogram, chest X-rays, computed tomography (CT) scan of the chest, brain CT scan or magnetic resonance imaging (MRI), abdominal CT scan or ultrasound sonography, radionuclide bone scan, and bone X-rays, if indicated.

Response evaluation

Objective tumour responses were evaluated in all enrolled patients according to the WHO criteria issued in 1979 (WHO, 1979). A complete response (CR) was defined as the disappearance of all known disease for at least 4 weeks with no new lesions appearing. A partial response (PR) referred to a decrease in the total tumour size of at least 50% for at least 4 weeks without the appearance of new lesions. No change (NC) was defined as the absence of a partial or complete response and the appearance of no progressive or new lesions for at least 4 weeks. Progressive disease (PD) was

defined as a 25% or greater increase in the size of any measurable lesion or the appearance of new lesions. Patients whose responses were not evaluated were included in the analysis as not evaluable (NE).

Statistical methods

The primary end point of this study was the response rate, defined as the proportion of patients whose best response was CR or PR among all eligible patients, and its confidence interval was based on an exact binomial distribution. Simon's two-stage minimax design was used to determine the sample size and decision criteria. Assuming that a response rate of 40% in eligible patients would indicate a potential usefulness of the regimen while a rate of 20% would be the lower limit of interest and that alpha = 0.05 and beta = 0.20, the estimated number of required patients was 33 (Simon, 1989). Finally, this regimen would be considered worthy of further testing if 11 (33%) or more eligible patients showed an objective response. At the first stage decision, this regimen would be rejected if four (22%) or fewer of 18 eligible patients had an objective response. Thus, we determined that the sample size would be 35 registered patients. The planned accrual period was 2 years, and the follow-up period was set as 1 year after the completion of accrual. Secondary end points were toxicity and overall survival. The duration of overall survival was measured from the date of registration to the date of death from any cause or the last follow-up examination. Progression-free survival was calculated from the date of registration until evidence of PD. All patients started the treatment within 1 week of registration. The survival distribution was estimated by the method of Kaplan and Meier (1958).

RESULTS

Patient characteristics

From October 1998 to March 2001, 40 patients were enrolled in this study. The first-stage decision was made in October 1999, when 22 patients were registered. Three CRs and 13 PRs were observed in 18 analysed patients, resulting in a response rate of 89% (95% confidence interval (CI), 65.3–98.6%). This result did not meet the criteria for stopping the study as defined in the protocol, and the study was continued. At the time of the final analysis, there were three censored cases (8%). The median follow-up period for these cases was 25.5 months (range, 4.4–46.1 months).

The clinical characteristics of the enrolled patients are listed in Table 1. Of the 40 patients in the total, 29 (73%) were male and 11 (27%) were female; the median age was 67 years. A total of 39 patients (97%) had a good performance status of 0 or 1. The extent of the disease at the time of recurrence was LD in five patients (12%) and ED in 35 (88%). All 40 patients had been previously treated using platinum-based chemotherapy, such as PE in 11 patients, carboplatin plus ETOP in 11, PE plus weekly CDDP/VCR/ADM/ETOP (CODE) in six, CDDP plus CPT-11 in six, PEI in two, and other regimens in four. Eight (20%) of these patients received thoracic radiotherapy. All patients were eligible, and the toxicity and efficacy of the regimen was evaluated in all 40 patients.

Compliance with treatment

A total of 251 treatment cycles were administered, with a median of six cycles per patient (range, 1–9 cycles). A total of 32 patients (80%) completed six or more cycles of chemotherapy, and the median number of weeks for completing six cycles of chemotherapy was 7 weeks (range 6–10 weeks). Eight patients could not complete the planned six or more cycles for the following reasons: toxicities in four cases (grades 4 and 3 diarrhoea, grade 3 liver dysfunction, and grade 3 erythema); patient refusal in three cases; and PD in one case. Six patients (15%) had their dosage of CPT-11 reduced because of leucocytopenia in three, thrombocytopenia in two, and both in one.

Clinical response and survival

All the patients were included in the analyses of tumour response and survival. Five CRs (13%) and 26 PRs (65%) were observed, for an overall response rate of 78% (31 out of 40 patients; 95% CI, 61.5–89.2%). Four NC, four PD, and one NE were also observed. One patient was lost to follow-up and only two patients were still alive as of April 16, 2003. The median survival time (MST) was 11.8 months (95% CI, 10.1–13.5 months), and the estimated 1-year survival rate was 49% (Figure 2).

Table 1 Patient characteristics

Total no. of patients	40
Age, median (range)	67 (41–74)
Sex	
Male	29
Female	11
ECOG performance status	
0	9
1	30
2	1
Disease extent at relapse	
Limited disease	5
Extensive disease	35
Prior chemotherapy	
CDDP/ETOP	11
CBDCA/ETOP	11
CDDP/ETOP/CODE	6
CDDP/CPT-11	6
PEI	2
Others	4
Prior thoracic radiotherapy	8

ECOG = Eastern Cooperative Oncology Group; CDDP = cisplatin; ETOP = etoposide; CBDCA = carboplatin; CODE = cisplatin/vincristine/doxorubicin/etoposide; CPT-11 = irinotecan; PEI = cisplatin/etoposide/irinotecan.

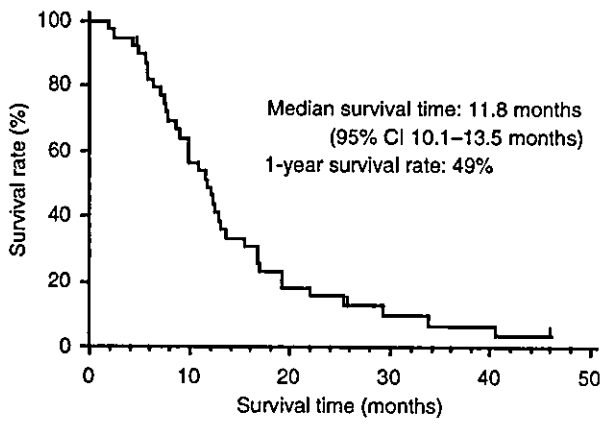

Figure 2 Overall survival ($n = 40$).

clinical

Site of first relapse and progression-free survival

The majority of patients ($n = 30$, 75%) experienced a systemic relapse after completing PEI, including 17 patients (43%) with central nerve metastases. Six patients (15%) developed only a locoregional recurrence, and one had no recurrence and died of acute myocardial infarction. No data on recurrence patterns were available in three patients because these patients were followed up at other hospitals. In all, 13 patients received additional chemotherapy treatment after recurrence (no data on response to third-line chemotherapy were available), while four patients underwent palliative chest radiotherapy and 18 underwent whole-brain irradiation for cerebral metastases. One patient, who achieved a CR by this regimen, developed a locoregional recurrence and underwent a right upper lobectomy. He has not experienced any further relapse and is still alive. The median progression-free survival period was 5.0 months (95% CI, 4.1–5.9 months) (Figure 3).

Toxicities

All the patients were included in the toxicity analysis. Severe toxicities were mainly haematological. Grades 3–4 leucopenia, neutropenia, and thrombocytopenia were observed in 22 (55%), 29 (73%), and 13 (33%) patients, respectively (Table 2). Nonhaematological toxicities were mild and transient in all patients. Grades 3–4 diarrhoea was noted in only three patients (8%) (Table 3). No treatment-related deaths occurred.

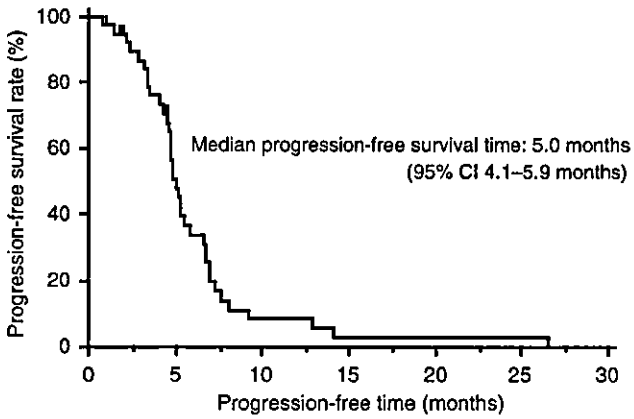

Figure 3 Progression-free survival ($n = 40$).

Table 2 Haematological toxicities (JCOG toxicity criteria)

	0	1	2	3	4	% of Grs 3 and 4
Leucocytopenia	2	3	13	17	5	55
Neutropenia	3	4	4	12	17	73
Anemia	2	4	16	18	—	45
Thrombocytopenia	10	7	10	7	6	33
Elevated total bilirubin	33	—	6	1	0	3
Elevated GOT	32	7	0	1	0	3
Elevated GPT	30	7	2	1	0	3
Elevated creatinine	37	3	0	0	0	0
Hyponatremia	28	4	6	0	2	5
Hypokalemia	32	5	3	0	0	0

Grs = grades; GOT = glutamic oxaloacetic transaminase; GPT = glutamic pyruvic transaminase.

Table 3 Nonhaematological toxicities (JCOG toxicity criteria)

	0	1	2	3	4	% of Grs 3 and 4
PS	1	30	4	5	0	13
Infection	28	4	7	1	0	3
Fever	29	7	4	0	0	0
Nausea/vomiting	11	15	11	3	—	8
Diarrhoea	15	16	6	2	1	8
Mucositis	36	4	0	0	0	0
Arrythmia	36	2	0	1	1	5
Eruption	37	1	1	1	0	3
Alopecia	16	17	7	—	—	—
Allergy	39	0	1	0	0	0

Grs = grades; PS = performance status.

DISCUSSION

Despite a high response rate to first-line chemotherapy, most patients with SCLC experience a relapse within a year of the completion of therapy (Hansen, 1992). Although many relapsed patients in good physical condition undergo second-line chemotherapy, the results are disappointing. The obtained response is usually brief, and the median survival period is generally less than 4 months (Albain *et al*, 1993; Glisson, 2003).

Although one phase III trial for patients with relapse SCLC comparing the use of topotecan with CAV has been reported (von Pawel *et al*, 1999), a standard treatment for relapsed SCLC has not been agreed upon. However, the repeated use of the original induction regimen is the most popular treatment for sensitive relapsed patients. Reinduction chemotherapy has been reported to produce a response rate of 50%, and patients who relapsed more than 3 months after the end of their previous chemotherapy regimen were sensitive to reinduction chemotherapy (Giaccone *et al*, 1987; Postmus *et al*, 1987). Giaccone *et al* (1988) suggested that sensitive tumour cells, which were not completely eradicated by the induction chemotherapy, regrow spontaneously after the suspension of chemotherapy, eventually constituting a clinically significant part of the tumour burden. In the present study, two patients received the PEI regimen as a reinduction chemotherapy, and both patients showed PRs.

Many clinical trials of salvage chemotherapy for relapsed SCLC have been reported. In these studies, the single administration of CPT-11 or ETOP produced good results, with response rates of 16–47% and an MST of 3.5–6.2 months (Einhorn *et al*, 1990; Johnson *et al*, 1990; Masuda *et al*, 1992; Le Chevalier *et al*, 1997). Moreover, CPT-11 or ETOP-containing combined chemotherapy regimens showed favourable results, with response rates of 20–88% and an MST of 4.7–8.7 months (Table 4) (Evans *et al*, 1985; Masuda *et al*, 1990; Sculier *et al*, 1990; Gridelli *et al*, 1991; Roth *et al*, 1992; Faylona *et al*, 1995; Kubota *et al*, 1997; Masuda *et al*, 1998; Groen *et al*, 1999; Nakanishi *et al*, 1999; von Pawel *et al*, 1999; Domine *et al*, 2001; Kosmas *et al*, 2001). Therefore, these two drugs are considered to be key drugs for the treatment of relapsed SCLC. In particular, the combination of CPT-11 and ETOP (a combination of topoisomerase I and II inhibitors) produced a high response rate (71%) and the best survival results (MST, 8.7 months) (Masuda *et al*, 1998). In addition, a weekly chemotherapy regimen containing ETOP (CODE) was highly active in patients with relapsed SCLC, with a favourable response rate (88%) and survival duration (MST, 8.2 months) (Kubota *et al*, 1997). In the two studies mentioned above, four patients (16%) with refractory relapsed SCLC were included in the CPT-11 and ETOP study, and six patients (35%) with refractory relapsed SCLC were included in the CODE study. Three and five of these patients achieved PR, respectively.

Table 4 Combination chemotherapy studies for relapsed small-cell lung cancer

Author	Regimen	No. of pts	% of ref pts (%)	RR (%)	RR in ref pts (%)	MST (month)
Sculier	CAV	61	75	21	5	6.2–7.5
von Pawel	CAV	104	20	18	5	6.2
Roth	CAV	41	32	12	8	NM
Roth	PE	59	46	22	15	NM
Evans	PE	78	50	55	28	NM
Masuda	PE	20	NM	50	NM	4.7
Gridelli	CCNU/MTX	33	100	21	21	4.0
Faylona	PE/IFO	46	41	55	50	6.8
Kubota	CODE	17	35	88	83	8.2
Masuda	CPT-11/ETOP	25	16	71	75	8.7
Nakanishi	CPT-11/CDDP	5	100	20	20	NM
Domine	GEM/PTX	31	58	50	40	NM
Groen	CBDCA/PTX	35	100	74	74	7.2
Kosmas	CDDP/IFO/PTX	33	61	73	70	6.5

Pts = patients; ref = refractory; RR = response rate; MST = median survival time; CAV = cyclophosphamide/doxorubicin/vincristine; PE = cisplatin/etoposide; CCNU = lomustine; MTX = methotrexate; IFO = ifosfamide; CODE = cisplatin/vincristine/doxorubicin/etoposide; CPT-11 = irinotecan; ETOP = etoposide; CDDP = cisplatin; GEM = gemcitabine; PTX = paclitaxel; CBDCA = carboplatin; NM = not mentioned.

The response and survival data from Japanese clinical trials for relapsed SCLC were generally better than those obtained in western countries. We have no proof that this difference depends on either drug metabolism or tumour sensitivity. It is possibly related to the difference in patient follow-up interval between Japan and western countries. Since intensive follow up after completion of first-line treatment is common in Japan, relapses can be detected in the early stage by CT or MRI before becoming symptomatic. Therefore, relapsed patients had a relatively good performance status, and showed good responses to second-line chemotherapy as well as better survival results.

The weekly regimen was designed to increase the overall relative dose intensity of the chemotherapeutic drugs (Murray *et al*, 1991). However, several phase III trials have made it clear that intensive weekly chemotherapy does not improve the survival of patients with SCLC (Furuse *et al*, 1998; Murray *et al*, 1999). On the other hand, drug dosages and treatment schedules are easy to modify in weekly chemotherapy regimens. Since patients with relapsed SCLC may have lower bone marrow reserve, a high-dose regimen or intensified dosage can lead to treatment-related death (Masuda *et al*, 1990; Faylona *et al*, 1995). In the PEI regimen, the individual dosage of each drug is within the commonly used range and the dose given at one time is lower than that of a standard 3-week cycle regimen. The PEI regimen therefore permits greater flexibility in dosage adjustment and treatment delays based on laboratory data or the physical condition of patients. Thus, this regimen is considered to be suitable for the treatment of patients with relapse SCLC. In addition, this weekly schedule may be of great advantage for enabling the synergistic effects of ETOP (a topoisomerase II inhibitor) and CPT-11 to be realised because the development of resistance to topoisomerase II inhibitors has been reported to increase tumour sensitivity to subsequent treatment with topoisomerase I inhibitors (Vasey and Kaye, 1997).

Three cytotoxic drugs were used in this PEI regimen. However, three-drug combination chemotherapy was reportedly associated with more severe toxicity and showed no survival benefit as compared with the two-dug combination (Mavroudis *et al*, 2001; Niell *et al*, 2002). The main reason for mild toxicities was that the PEI regimen consists of a weekly schedule. With a weekly chemotherapy regimen, drug dosages and treatment schedules can easily be adjusted according to haematological data and the patient's physical condition. These careful modifications resulted in a mild toxicity profile with the PEI regimen. Moreover, the PEI regimen did not consist of concomitant administration of three drugs but rather weekly alternative administration of a two-drug combination chemotherapy, that is, PE and IP. As a result, the toxicity profile was similar with that of two-drug combination chemotherapy.

Although all the patients in this study were sensitive relapsed cases, the overall response rate of 78% is one of the best results reported for relapsed SCLC. Moreover, although only selected patients with a good performance status were included in this study, it is notable that the median survival time was 11.8 months and the 1-year survival rate was 49%. In JCOG- 9511, the MST was 12.8 months in the IP arm and 9.4 months in the PE arm for chemotherapy naive ED SCLC patients (Noda *et al*, 2002). Our survival data for PEI is almost equivalent to that of first-line treatment. Salvage chemotherapy may be possible to prolong the survival of sensitive relapsed SCLC patients who are in good physical condition.

Since second-line chemotherapy for relapsed SCLC patients is a palliative treatment, a reasonable toxicity profile is essential. The main toxicities of the PEI regimen were haematological. Although G-CSF was routinely administered, Grades 3–4 leucopenia and neutropenia were observed in 55 and 73% of patients, respectively. Grades 3–4 thrombocytopenia was observed in 33% of patients. However, the frequencies of these haematological toxicities were approximately equal to that of first-line PE treatment (Noda *et al*, 2002). Nonhaematological toxicities were mild and transient in all patients. Grades 3–4 diarrhoea was noted in only three patients (8%). Irinotecan dose modifications as a result of haematological toxicities were only performed in six patients (15%). All toxicities were easily manageable, and no treatment-related deaths occurred.

In conclusion, PEI is a highly active and well-tolerated treatment for sensitive relapsed SCLC. Another phase II trial restricted to refractory relapsed SCLC patients is presently being performed by our clinical group. Further phase III studies comparing PEI regimen with rechallenges of the same drugs used in the first-line chemotherapy regimen should clarify the role of second-line chemotherapy for sensitive relapsed SCLC and are now being planned.

ACKNOWLEDGEMENTS

This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health Labour and Welfare of Japan.

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