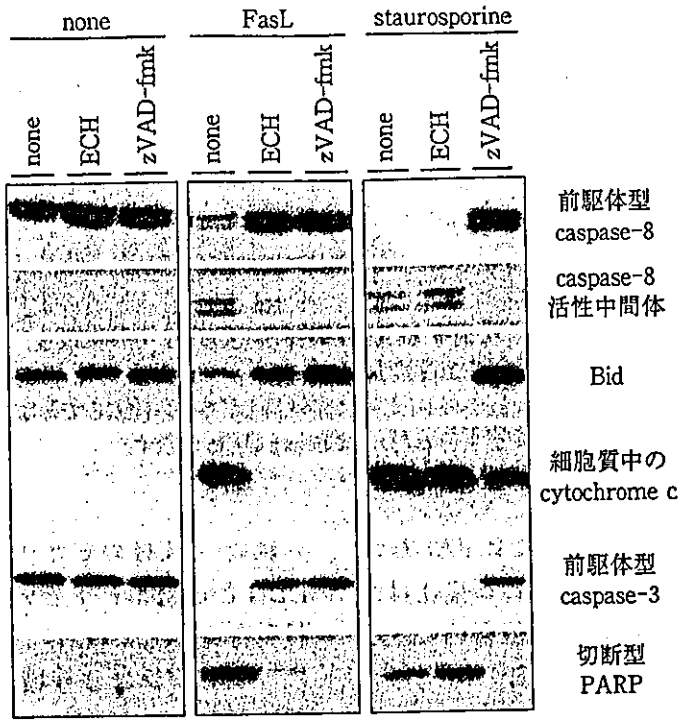

図3 アポトーシス関連蛋白質の検出(文献[13]より一部改変)
細胞をECH(20 μM)またはzVAD-fmk(20 μM)で30分間前処理後，FasL(500 ng/ml)またはstaurosporine(300 nM)を加えて4時間培養後に細胞を回収した．その細胞破砕液をSDS-PAGEで分離後，各抗体にてウエスタンブロッティングを行った．

Bcl-2ファミリーの一つであるBidを切断し，切断されたBidはミトコンドリアに移行し，cytochrome cの放出を誘導する．細胞質中に放出されたcytochrome cはApaf-1および前駆体型caspase-9，dATPとともにapoptosomeと呼ばれる複合体を形成し，caspase-9を活性化する．活性化したcaspase-9はcaspase-3を活性化し，活性化したcaspase-3が細胞内の様々な基質を分解することにより，DNAの断片化や核クロマチンの凝縮などのアポトーシスに特有の形態変化を引き起こす．ECHを処理した細胞では，この一連のシグナル伝達経路の最も上流であるcaspase-8の活性化が抑制され，それにより下流のシグナルの活性化はすべて抑制されていた．一方，化学薬剤やUV照射などのアポトーシス誘導シグナルはミトコンドリアに収束し，cytochrome cの放出を誘導する[11]．ECHは化学薬剤のstaurosporineによるアポトーシスに対しては，cytochrome cの放出およびそれより下流のcaspase群の活性化を全く抑制しなかった．ここで，コントロールとして用いたzVAD-fmkは両者の刺激によるアポトーシスに対してcaspase群の活性化を顕著に抑制した．これらの結果により，ECHはデスレセプター依存性アポトーシスに特異的なイニシエーターであるcaspase-8の活性化を抑制することにより，デスレセプター依存性アポトーシスを特異的に抑制することが明らかとなった．

次に，caspase-8の活性化を担うDISCに対する影響について検討を行った(図4)．FasにFasLが結合するとFasの細胞内領域のdeath domain(DD)にアダプター蛋白質であるFADDが自身のDDを介して結合し，更にFADDのdeath effector domain(DED)にcaspase-8が自身のDEDを介して結合し，Fas-FADD-caspase-8による複合体DISCが形成される[6]．それに伴いDISC中でcaspase-8が自己限定分解により活性化する．FasLで処理した細胞の破砕液からFasLを免疫沈降するとFADDおよびcaspase-8が共沈することによりDISCの形成が確認できる．またcaspase-8の活性中間体が出現し，caspase-8が活性化していることも確

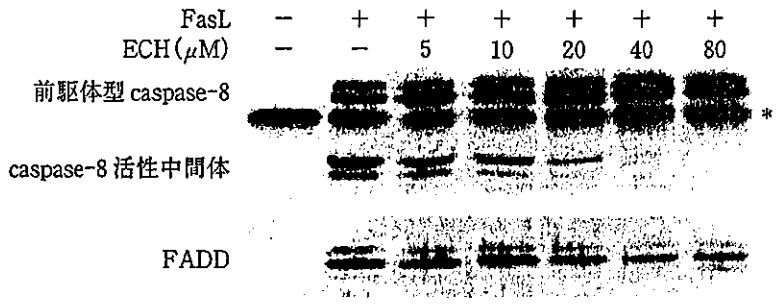

図4 DISCに対するECHの影響(文献[13]より一部改変)
細胞を各濃度のECHで30分間前処理後，FasL(2 μg/ml)を加え15分間処理後に細胞を回収した．その細胞破砕液からFasLを免疫沈降し，SDS-PAGEで分離後，各抗体にてウエスタンブロッティングを行った．*はheavy chainを示す．

認できる．ECHを処理した細胞ではFADDおよびcaspase-8のDISCへの会合には変化は認められなかったが，ECHの濃度依存的にcaspase-8の活性中間体が減少した．この結果から，ECHはDISCへのcaspase-8やFADDの会合には影響を与えずに，DISC中でのcaspase-8の活性化を阻害することが明らかとなった．

IV. ECHの細胞内標的分子

前述の結果よりECHの作用標的はcaspase-8である可能性が示唆された．そこで，ECHのcaspase-8に対する影響について検討を行った．caspaseは活性中心にシステインを有するシステインプロテアーゼであり，通常は不活性前駆体として存在し，アポトーシス誘導時に切断による構造変化により活性型となる[12]．ECHは*in vitro*において活性型caspase-8の酵素活性を阻害したが，その阻害濃度は比較対照として用いたzVAD-fmkと比べて1,000倍以上高濃度であった．細胞を用いた実験におけるECHの阻害濃度はzVAD-fmkとほぼ同程度であり，膜透過性や安定性などを考慮に入れても，*in vitro*での活性型caspase-8に対するECHの阻害濃度は実際の標的分子と考えるには高すぎた．そこで次に，細胞内のcaspase-8の酵素活性に対する影響について検討を行った．その結果，ECHは細胞内において前駆体型caspase-8の活性型への変換を阻害したが，いったん活性化した活性型caspase-8の酵素活性に対しては阻害効果を示さなかった．

次に，ECHのビオチン化誘導体を作製し，ECHとcaspase-8との直接的な結合について検討を行った．なお，ビオチン化ECHはHRP標識アビジンにより検出可能である．ECHは前駆体型および活性型caspase-8のどちらにも結合したが，前駆体型により高い結合性を示した(図5)．ECHは分子内にエポキシドやα，β-不飽和ケトンといった求核攻撃を受けやすい化学構造を有すること，およびcaspase-8の活性中心はシステインであることを考え合わせると，ECHがcaspase-8の活性中心のシステイン残基に結合している可能性が考えられた．実際，ECHのcaspase-8に対する結合および阻害活性はシステインやグルタチオンの添加により拮抗阻害された．

以上の結果から，ECHの細胞内標的分子は前駆体型caspase-8であり，活性中心のシステイン残基に結合することにより，caspase-8の活性化を抑制することが示唆された[13]．

V. ECHのバイオプローブとしての応用利用

本稿で紹介したECHは，デスレセプター依存性アポトーシスに対する高い特異性および，広い細胞種において抑制活性を示す普遍性を兼ね備えており，アポトーシス研究における有用なバイオプローブになり得ると考えられる．ここで，ECHのバイオプローブとしての応用利用について一例を紹介する．免疫細胞の一つである細胞傷害性T細胞(CTL)は2つの独立した

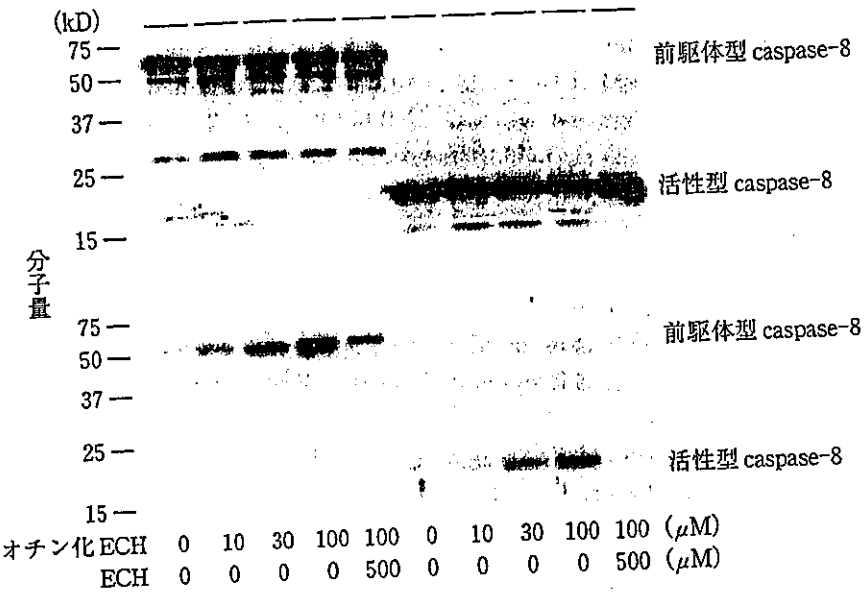

図5 ECHとcaspase-8との結合(文献[13]より一部改変)
リコンビナント前駆体型caspase-8および活性型caspase-8に各濃度のビオチン化ECHを加え2時間氷上にて反応させた．その後，SDS-PAGEで分離し，抗caspase-8抗体(上段：添加したcaspase-8の量)またはavidin-HRP(下段：caspase-8に結合したECHの量)にて検出した．

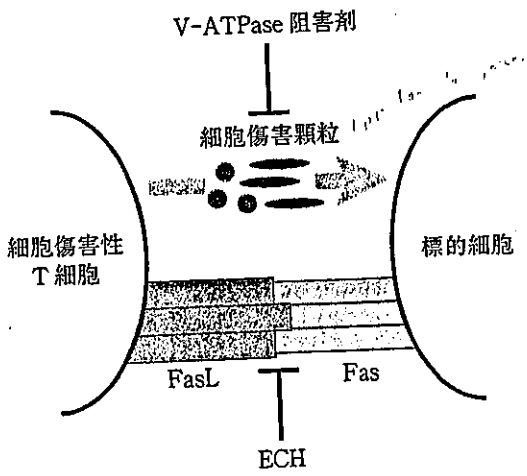

図6 ECHによる細胞傷害性T細胞の制御

経路，すなわちFas/FasLシステムおよび細胞傷害顆粒(perforin/granzyme B)により標的細胞を殺傷する(図6)[14]．片岡らはconcanamycin AなどのV-ATPase阻害剤がFas/FasL経路には影響を与えずに，細胞傷害顆粒の経路を特異的に阻害することを報告している[15]．今回，片岡らは著者らとの共同研究の過程で，ECHのCTLの殺細胞機構に対する影響について検討を行い，V-ATPase阻害剤とは異なり，ECHは細胞傷害顆粒の経路には影響を与えずに，Fas/FasL経路を特異的に阻害することを明らかにした[16]．これによりV-ATPase阻害剤とECHを用いることで，CTLの2つの殺細胞経路をそれぞれ特異的に制御することが可能となり，ECHのCTL研究におけるバイオプローブとしての活躍も期待される．

おわりに

デスレセプター依存性アポトーシスは癌細胞の除去以外にも，自己免疫疾患や劇症肝炎，関節リウマチなど様々な疾患に関与している．ECHはそれらの疾病に対する治療薬としての可能性も有しているが，ECHには作用濃度の高さ，長時間使用における細胞毒性などの問題点も残されている．そこで，著者らは引き続きECHの構造活性相関研究の過程でECHとほぼ同程度の活性を有する誘導体RKTS-33 & 34(図1-a)の創製に成功するとともに[17]，逆にアポトーシス誘導活性を有するECH類縁化合物の抗癌剤への可能性を検討している．

文　献

1) Osada H(ed): Trends in Bioprobe Research. In: Bioprobes, p1-13, Springer, Tokyo, 2000.
2) Watabe M, et al: Activation of MST/Krs and c-Jun N-terminal kinases by different signaling pathways during cytotrienin A-induced apoptosis. J Biol Chem **275**: 8766-8771, 2000.
3) Machida K, et al: A novel adenine nucleotide translocase inhibitor, MT-21, induces cytochrome c release by a mitochondrial permeability transition-independent mechanism. J Biol Chem **277**: 31243-31248, 2000.
4) Garcia M, et al: Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem **273**: 32608-32613, 1998.
5) Bando M, et al: Actin cytoskeleton is required for early apoptosis signaling induced by anti-Fas antibody but not Fas ligand in murine B lymphoma A20 cells. Biochem Biophys Res Commun **290**: 268-274, 2002.
6) Ashkenazi A, et al: Death receptors: signaling and modulation. Science **281**: 1305-1308, 1998.
7) Nagata S: Apoptosis by death factor. Cell **88**: 355-365, 1997.
8) 神林嘉和ほか：日本公開特許公報, p222456, 1999.
9) Yonehara S, et al: A cell-killing monoclonal antibody(anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med **169**: 1747-1756, 1989.
10) Kischkel C, et al: Cytotoxicity-dependent APO-1(Fas/CD95)-associated proteins form a death-inducing signaling complex(DISC) with the receptor. EMBO J **14**: 5579-5588, 1995.
11) Debatin M, et al: Chemotherapy: targeting the mitochondrial cell death pathway. Oncogene **21**: 8786-8803, 2002.
12) Cohen M: Caspases: the executioners of apoptosis. Biochem J **1**: 1-16, 1997.
13) Miyake Y, et al: Epoxycyclohexenone inhibits Fas-mediated apoptosis by blocking activation of procaspase-8 in the death-inducing signaling complex. J Biol Chem **278**: 11213-11220, 2003.
14) Berke G: The CTL's kiss of death. Cell **81**: 9-12, 1995.
15) Kataoka T, et al: Concanamycin A, a powerful tool for characterization and estimation of contribution of perforin- and Fas-based lytic pathways in cell-mediated cytotoxicity. J Immunol **156**: 3678-3686, 1996.
16) Mitsui T, et al: ECH, an epoxycyclohexenone derivative that specifically inhibits Fas ligand-dependent apoptosis in CTL-mediated cytotoxicity. J Immunol **172**(6): 3428-3436, 2004.
17) Kakeya H, et al: Novel non-peptide inhibitors targeting death receptor-mediated apoptosis. Bioorg Med Chem Lett **13**: 3743-3746, 2003.

[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 hyperresponsiveness 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.

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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Shien-Lab, Medical Oncology, National Cancer Center Hospital, Tsukiji 5-1-1, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511; Fax: 81-3-3547-5185; E-mail: knishio@gan2.res.ncc.go.jp.

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

[4] Unpublished observation.

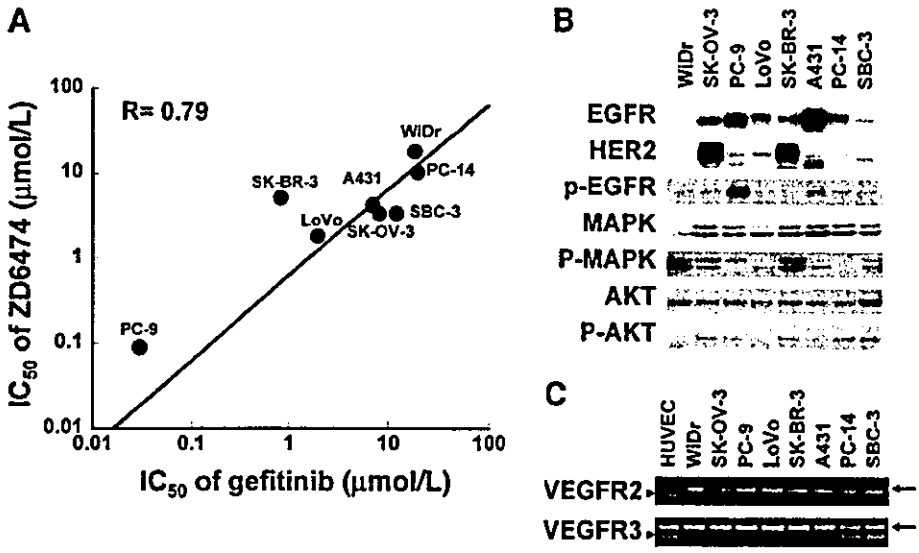

Fig. 1. The cellular characteristics and growth-inhibitory effect of gefitinib and ZD6474. *A*, correlation plot of the IC_{50} values of gefitinib and ZD6474 in human cancer cell lines. The growth-inhibitory effect against PC-9, WiDr, LoVo, PC-14, A431, SK-OV-3, SK-BR-3, and SBC-3 cells was determined 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′-GGCAACAGCTG-GATGTCATA-3′ (reverse). As a control, the following human β-actin primers were used: 5′-GGAAATCGTGCGTGACATT-3′ and 5′-CATCTGCTG-GAAGGTGGACAG-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

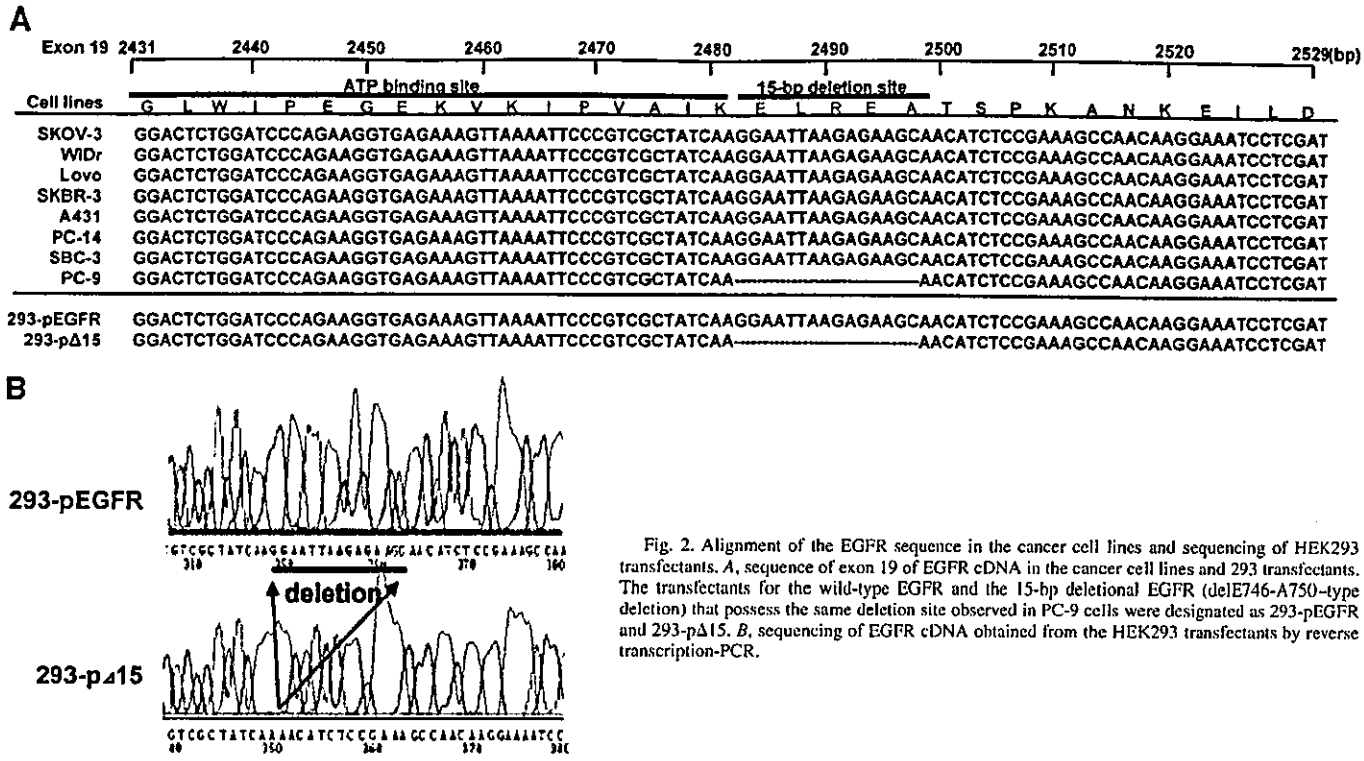

Fig. 2. Alignment of the EGFR sequence in the cancer cell lines and sequencing of HEK293 transfectants. *A*, sequence of exon 19 of EGFR cDNA in the cancer cell lines and 293 transfectants. The transfectants for the wild-type EGFR and the 15-bp deletional EGFR (delE746-A750–type deletion) that possess 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-

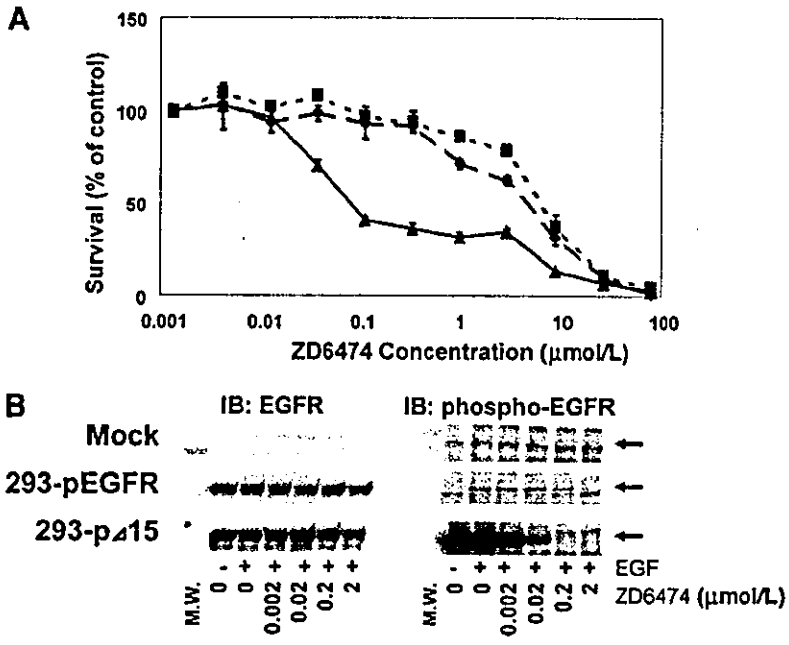

Fig. 3. Effect of ZD6474 on cellular growth inhibition and phosphorylated status of EGFR in the HEK293 transfectants. *A*, The cellular sensitivity of the transfectants against ZD6474 was determined by MTT assay (72-hour exposure). The mean values and SD represent the values obtained from the growth-inhibition 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.

REFERENCES

1. Ciardiello F, Caputo R, Tortora G, et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. Clin Cancer Res 2000;6: 2053–63.
2. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibitor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. Cancer Res 2001;61:7184–8.
3. Koizumi F, Kanzawa F, Nishio K, et al. Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. Int J Cancer 2004;108:464–72.
4. Lynch TJ, Bell DW, Haber DA, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
5. Paez JG, Janne PA, Meyerson M, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
6. Ohm JE, Amann JM, Carbone DP. Acquired EGFr TKI resistance associated with mutation of the EGFr. Presented at the 95th Annual Meeting of the American Association of Cancer Research, November 17–21, 2004, Bonita Springs, FL.
7. Ciardiello F, Caputo R, Tortora G, et al. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. Clin Cancer Res 2003;9:1546–56.
8. Naruse I, Ohmori T, Nishio K, et al. Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line in vitro and in vivo. Int J Cancer 2002;98:310–5.
9. Koizumi F, Taguchi F, Shimoyama T, Saijo N, Nishio K. Mechanism of resistance to epidermal growth factor receptor inhibitor ZD1839: a role for inhibiting phosphorylation of EGFR at Tyr1068. Presented at the 94th Annual Meeting of the American Association of Cancer Research, July 11–14, 2003, Washington, DC.
10. Kawamura-Akiyama Y, Kusaba H, Nishio K, et al. Non-cross resistance of ZD0473 in acquired cisplatin-resistant lung cancer cell lines. Lung Cancer 2002;38:43–50.
11. Nishio K, Arioka H, Saijo N, et al. Enhanced interaction between tubulin and microtubule-associated protein 2 via inhibition of MAP kinase and CDC2 kinase by paclitaxel. Int J Cancer 1995;63:688–93.
12. Nielsen UB, Cardone MH, Sinskey AJ, MacBeath G, Sorger PK. Profiling receptor tyrosine kinase activation by using Ab microarrays. Proc Natl Acad Sci USA 2003;100:9330–5.
13. Ciardiello F, Bianco R, Tortora G, et al. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. Clin Cancer Res 2004;10:784–93.
14. Taguchi F, Koh Y, Nishio K, et al. Anticancer effects of ZD6474, a VEGF receptor tyrosine kinase inhibitor, in gefitinib (Iressa) sensitive and resistant xenograft models. Cancer Sci 2004;in press.
15. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science 2004;305:1163–7.

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 μ*M* 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 μ*M* 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

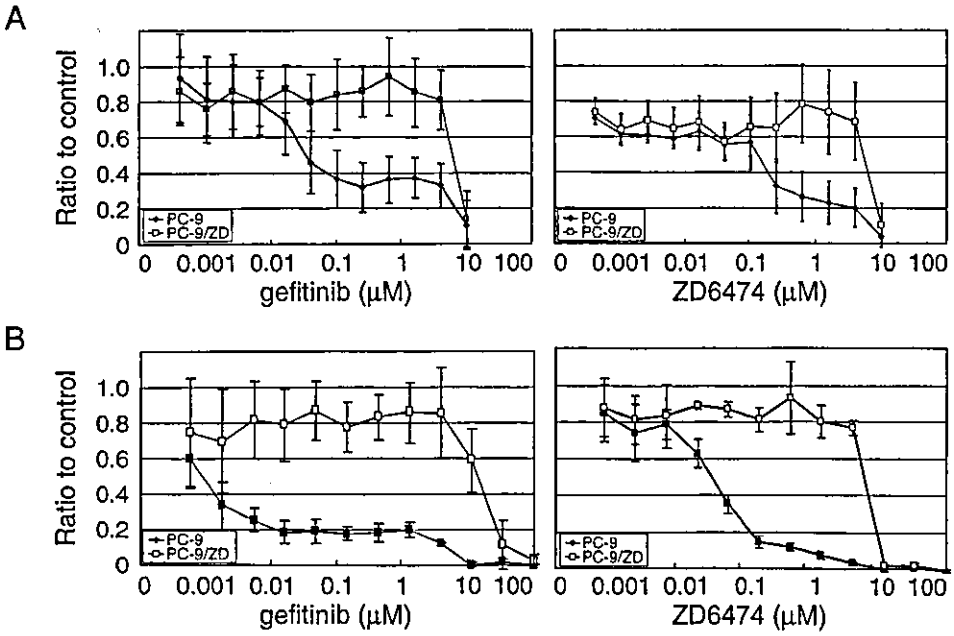

Fig. 1. Growth inhibitory effect of gefitinib (ZD1839) and ZD6474. A: PC-9 and PC-9/ZD, B: PC-9 and PC-14 cells. Data shown are mean values from three experiments (±SD).

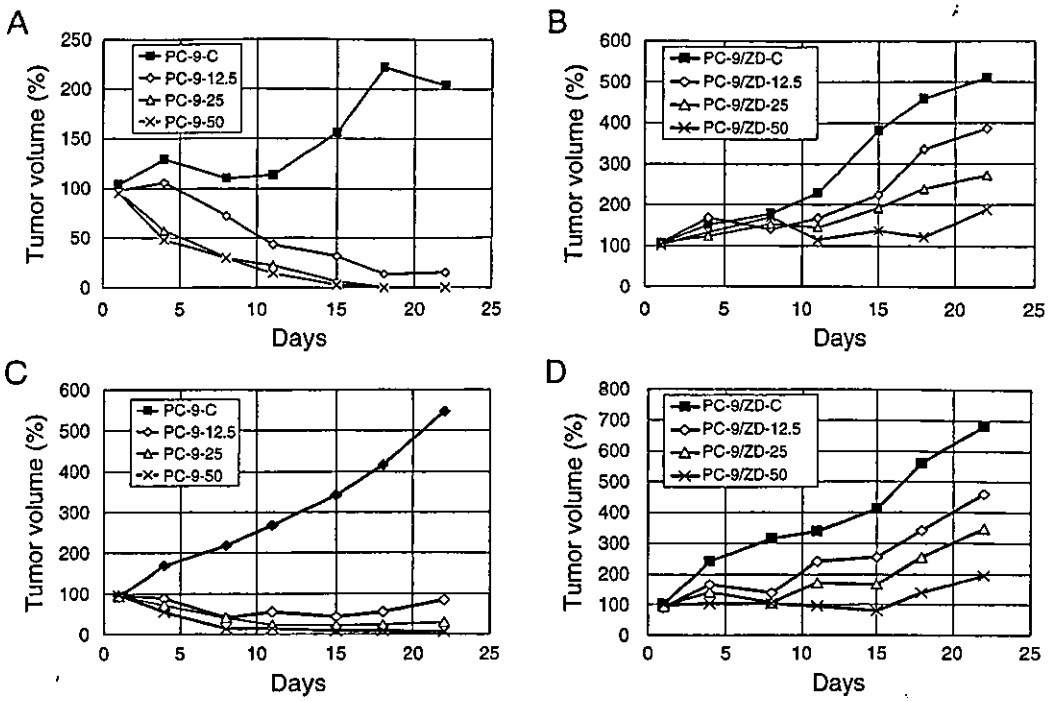

Fig. 2. Antitumor activity of ZD6474 (A, B) and gefitinib (C, D) on established PC-9 (A, C) and PC-9/ZD (B, D) human lung cancer xenografts.

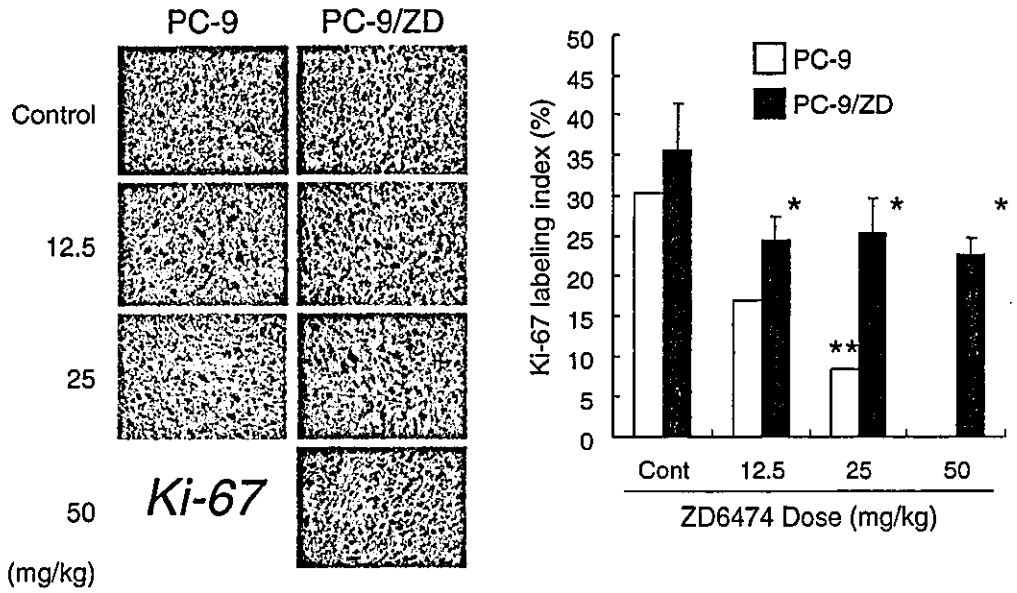

Fig. 3. Effect of ZD6474 on the Ki67 labeling index of PC-9 and PC-9/ZD tumors *in vivo*. Data represent mean values (±SD). Significant difference from control shown by the Dunnett test (* $P<0.05$, ** $P<0.01$).

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

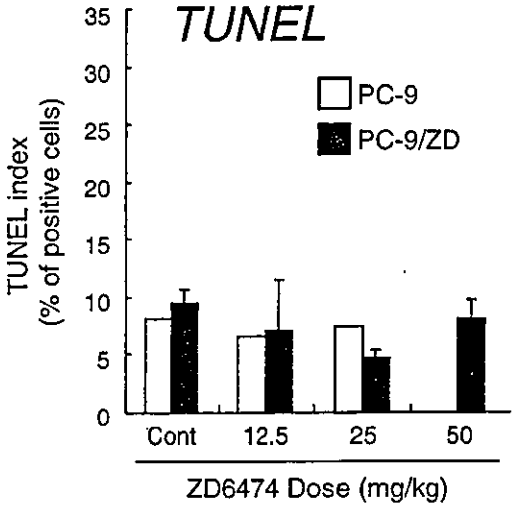

Fig. 4. Effect of ZD6474 on the TUNEL index of PC-9 or PC-9/ZD tumors *in vivo*. Data represent mean values (±SD).

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.

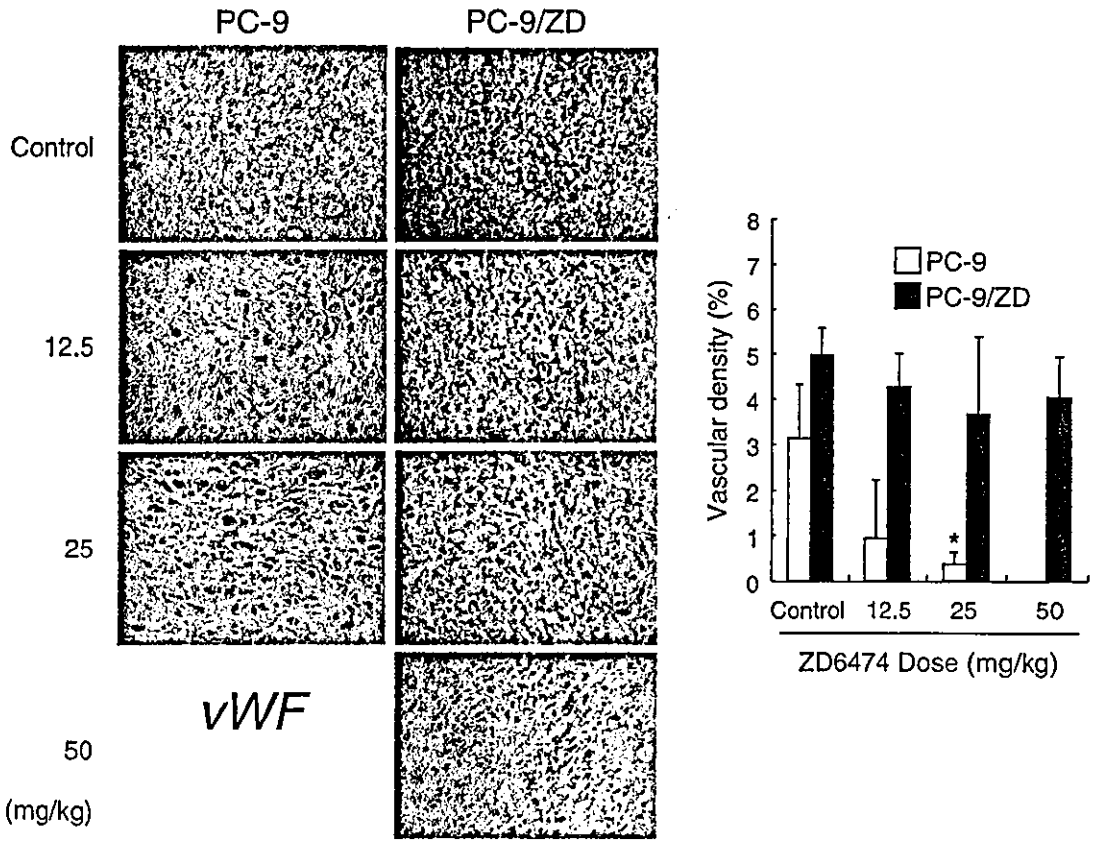

Fig. 5. Effect of ZD6474 on the vascular density of PC-9 and PC-9/ZD tumors stained *in vivo* with anti-vWF. Values are means±SD. Significant difference from the control by the Dunnett test (* $P<0.05$).

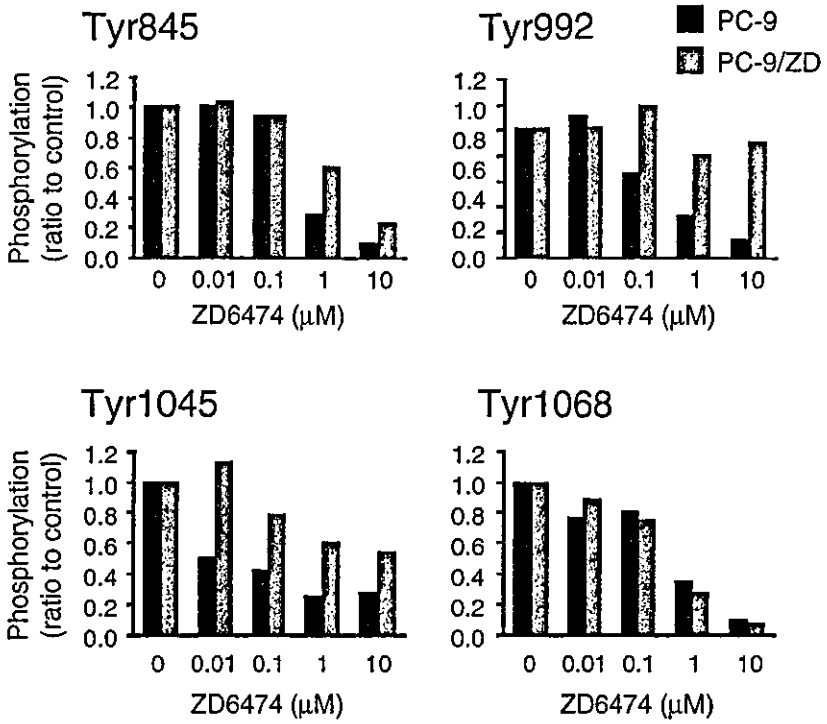

Fig. 6. Phosphorylation of EGFR tyrosine residues in PC-9 and PC-9/ZD cells after exposure to ZD6474.

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.

1. Ciardiello F, Caputo R, Damiano V, Troiani T, Vitagliano D, Carlomagno F, Veneziani BM, Fontanini G, Bianco AR, Tortora G. Antitumor effects of ZD6474, a small molecule vascular endothelial growth factor receptor tyrosine kinase inhibitor, with additional activity against epidermal growth factor receptor tyrosine kinase. *Clin Cancer Res* 2003; 9: 1546–56.
2. Ciardiello F, Bianco R, Caputo R, Damiano V, Troiani T, Melisi D, De Vita F, De Placido S, Bianco AR, Tortora G. Antitumor activity of ZD6474, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in human cancer cells with acquired resistance to antiepidermal growth factor receptor therapy. *Clin Cancer Res* 2004; 10: 784–93.
3. Minami H, Ebi H, Tahara M, Sasaki Y, Yamamoto N, Yamada Y, Tamura T, Saijo N. A phase I study of an oral VEGF receptor tyrosine kinase inhibitor ZD6474, in Japanese patients with solid tumors. *Proc Am Soc Clin Oncol* 2003; 22: abstr 778.
4. Hurwitz H, Holden SN, Eckhardt SG, Rosenthal M, de Boer R, Rischin D, Green M, Basser R. Clinical evaluation of ZD6474, an orally active inhibitor of VEGF signaling, in patients with solid tumors. *Proc Am Soc Clin Oncol* 2002; 22: abstr 325.
5. Ciardiello F, Caputo R, Bianco R, Damiano V, Pomatico G, De Placido S, Bianco AR, Tortora G. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000; 6: 2053–63.
6. Moasser MM, Basso A, Averbuch SD, Rosen N. The tyrosine kinase inhibi-

tor ZD1839 ("Iressa") inhibits HER2-driven signaling and suppresses the growth of HER2-overexpressing tumor cells. *Cancer Res* 2001; **61**: 7184–8.
7. Koizumi F, Kanzawa F, Ueda Y, Koh Y, Tsukiyama S, Taguchi F, Tamura T, Saijo N, Nishio K. Synergistic interaction between the EGFR tyrosine kinase inhibitor gefitinib ("Iressa") and the DNA topoisomerase I inhibitor CPT-11 (irinotecan) in human colorectal cancer cells. *Int J Cancer* 2004; **108**: 464–72.
8. Koizumi F, Taguchi F, Shimoyama T, Saijo N, Nishio K. Mechanism of resistance to epidermal growth factor receptor inhibitor ZD1839: a role for inhibiting phosphorylation of EGFR at Tyr1068. *Am Assoc Cancer Res* 2003: abstr 1001.
9. Kawamura-Akiyama Y, Kusaba H, Kanzawa F, Tamura T, Saijo N, Nishio K, Arioka H, Ishida T, Fukumoto H, Kurokawa H, Sata M, Ohata M, Morikage T, Ohmori T, Fujiwara Y, Takeda Y. Non-cross resistance of ZD0473 in acquired cisplatin-resistant lung cancer cell lines. *Lung Cancer* 2002; **38**: 43–50.
10. Nishio K, Arioka H, Ishida T, Fukumoto H, Kurokawa H, Sata M, Ohata M, Saijo N, Morikage T, Ohmori T, Fujiwara Y, Takeda Y. Enhanced interaction between tubulin and microtubule-associated protein 2 via inhibition of MAP kinase and CDC2 kinase by paclitaxel. *Int J Cancer* 1995; **63**: 688–93.
11. Naruse I, Ohmori T, Ao Y, Fukumoto H, Kuroki T, Mori M, Saijo N, Nishio K. Antitumor activity of the selective epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) Iressa (ZD1839) in an EGFR-expressing multidrug-resistant cell line *in vitro* and *in vivo*. *Int J Cancer* 2002; **98**: 310–5.
12. Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, Kuwano M. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol Cancer Ther* 2004; **3**: 465–72.
13. Wedge SR, Ogilvie DJ, Dukes M, Kendrew J, Chester R, Jackson JA, Boffey SJ, Valentine PJ, Curwen JO, Musgrove HL, Graham GA, Hughes GD, Thomas AP, Stokes ES, Curry B, Richmond GH, Wadsworth PF, Bigley AL, Hennequin LF. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. *Cancer Res* 2002; **62**: 4645–55.
14. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. *J Natl Cancer Inst* 2002; **94**: 883–93.
15. Holbrook MR, O'Donnell JB Jr, Slakey LL, Gross DJ, Bishayee A, Beguinot L, Bishayee S. Epidermal growth factor receptor internalization rate is regulated by negative charges near the SH2 binding site Tyr992. *Biochemistry* 1999; **38**: 9348–56.
16. Bishayee A, Beguinot L, Bishayee S. Phosphorylation of tyrosine 992, 1068, and 1086 is required for conformational change of the human epidermal growth factor receptor c-terminal tail. *Mol Biol Cell* 1999; **10**: 525–36.
17. Nogami M, Yamazaki M, Watanabe H, Okabayashi Y, Kido Y, Kasuga M, Sasaki T, Maehama T, Kanaho Y, Holbrook MR, O'Donnell JB Jr, Slakey LL, Gross DJ, Bishayee A, Beguinot L, Bishayee S. Requirement of autophosphorylated tyrosine 992 of EGF receptor and its docking protein phospholipase C gamma 1 for membrane ruffle formation. *FEBS Lett* 2003; **536**: 71–6.
18. Ravid T, Sweeney C, Gee P, Carraway KL 3rd, Goldkorn T. Epidermal growth factor receptor activation under oxidative stress fails to promote c-Cbl mediated down-regulation. *J Biol Chem* 2002; **277**: 31214–9.

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

www.bjcancer.com

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 4.0×10^9 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 10.0×10^9 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.

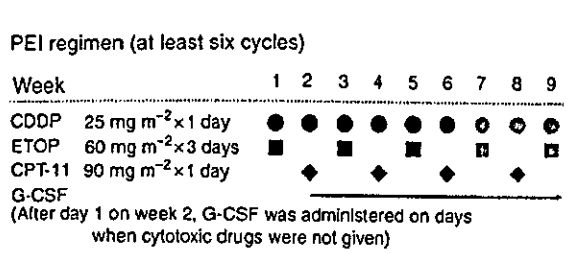

Figure 1 Treatment schedule.

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.

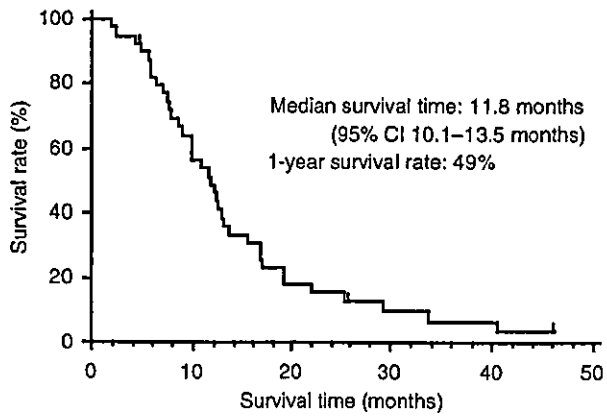

Figure 2 Overall survival (n = 40).

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.

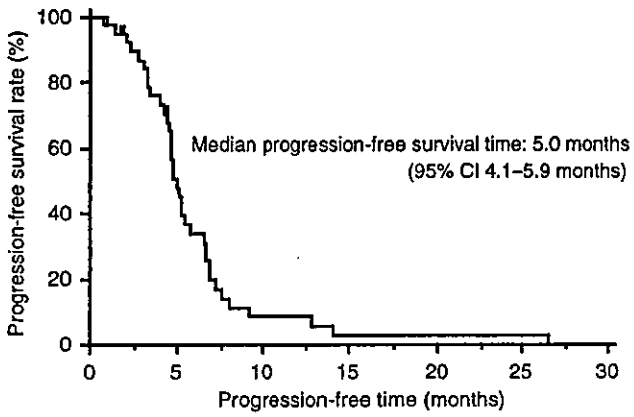

Figure 3 Progression-free survival ($n=40$).

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.

REFERENCES

Aisner J (1996) Extensive-disease small-cell lung cancer: the thrill of victory; the agony of defeat. *J Clin Oncol* 14: 658–665

Albain KS, Crowley JJ, Hutchins L, Gandara D, O'Bryan RM, Von Hoff DD, Griffin B, Livingston RB (1993) Predictors of survival following relapse or progression of small cell lung cancer. Southwest Oncology Group Study 8605 report and analysis of recurrent disease data base. *Cancer* 72: 1184–1191

Ardizzoni A, Hansen H, Dombernowsky P, Gamucci T, Kaplan S, Postmus P, Giaccone G, Schaefer B, Wanders J, Verweij J (1997) Topotecan, a new active drug in the second-line treatment of small-cell lung cancer: a

phase II study in patients with refractory and sensitive disease. The European Organization for Research and Treatment of Cancer Early Clinical Studies Group and New Drug Development Office, and the Lung Cancer Cooperative Group. *J Clin Oncol* 15: 2090–2096

Domine M, Larriba J, Morales S, Gomez R, Isla D, Terrasa S, Giner V, Giron C, Andrade J, Maestu I, Lobo F, Diaz F (2001) Gemcitabine and paclitaxel as second line treatment in small cell lung cancer. A multicentric phase II study. *Proc Am Soc Clin Oncol* 20: 317a

Ebi N, Kubota K, Nishiwaki Y, Hojo F, Matsumoto T, Kakinuma R, Ohmatsu H, Sekine I, Yokosaki M, Gotoh K, Yamamoto H, Kodama T (1997) Second-line chemotherapy for relapsed small cell lung cancer. *Jpn J Clin Oncol* 27: 166–169

Einhorn LH, Pennington K, McClean J (1990) Phase II trial of daily oral VP-16 in refractory small cell lung cancer: a Hoosier Oncology Group study. *Semin Oncol* 17: 32–35

Evans WK, Feld R, Osoba D, Shepherd FA, Dill J, Deboer G (1984) VP-16 alone and in combination with cisplatin in previously treated patients with small cell lung cancer. *Cancer* 53: 1461–1466

Evans WK, Osoba D, Feld R, Shepherd FA, Bazos MJ, DeBoer G (1985) Etoposide (VP-16) and cisplatin: an effective treatment for relapse in small-cell lung cancer. *J Clin Oncol* 3: 65–71

Faylona EA, Loehrer PJ, Ansari R, Sandler AB, Gonin R, Einhorn LH (1995) Phase II study of daily oral etoposide plus ifosfamide plus cisplatin for previously treated recurrent small-cell lung cancer: a Hoosier Oncology Group Trial. *J Clin Oncol* 13: 1209–1214

Fukuoka M, Furuse K, Saijo N, Nishiwaki Y, Ikegami H, Tamura T, Shimoyama M, Suemasu K (1991) Randomized trial of cyclophosphamide, doxorubicin, and vincristine *versus* cisplatin and etoposide *versus* alternation of these regimens in small-cell lung cancer. *J Natl Cancer Inst* 83: 855–861

Furuse K, Fukuoka M, Nishiwaki Y, Kurita Y, Watanabe K, Noda K, Ariyoshi Y, Tamura T, Saijo N (1998) Phase III study of intensive weekly chemotherapy with recombinant human granulocyte colony-stimulating factor *versus* standard chemotherapy in extensive-disease small-cell lung cancer. The Japan Clinical Oncology Group. *J Clin Oncol* 16: 2126–2132

Giaccone G, Donadio M, Bonardi G, Testore F, Calciati A (1988) Teniposide in the treatment of small-cell lung cancer: the influence of prior chemotherapy. *J Clin Oncol* 6: 1264–1270

Giaccone G, Ferrati P, Donadio M, Testore F, Calciati A (1987) Reinduction chemotherapy in small cell lung cancer. *Eur J Cancer Clin Oncol* 23: 1697–1699

Glisson BS (2003) Recurrent small cell lung cancer: update. *Semin Oncol* 30: 72–78

Gridelli C, Contegiacomo A, Lauria R, Gentile M, Airoma G, De Placido S, Perrone F, Ferrante G, Bianco AR (1991) Salvage chemotherapy with CCNU and methotrexate for small cell lung cancer resistant to CAV/PE alternating chemotherapy. *Tumori* 77: 506–510

Groen HJ, Fokkema E, Biesma B, Kwa B, van Putten JW, Postmus PE, Smit EF (1999) Paclitaxel and carboplatin in the treatment of small-cell lung cancer patients resistant to cyclophosphamide, doxorubicin, and etoposide: a non-cross-resistant schedule. *J Clin Oncol* 17: 927–932

Hansen HH (1992) Management of small-cell cancer of the lung. *Lancet* 339: 846–849

Ihde DC (1992) Chemotherapy of lung cancer. *N Engl J Med* 327: 1434–1441

Johnson DH, Greco FA, Strupp J, Hande KR, Hainsworth JD (1990) Prolonged administration of oral etoposide in patients with relapsed or refractory small-cell lung cancer: a phase II trial. *J Clin Oncol* 8: 1613–1617

Kaplan E, Meier P (1958) Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53: 457–481

Kosmas C, Tsavaris NB, Malamos NA, Vadiaka M, Koufos C (2001) Phase II study of paclitaxel, ifosfamide, and cisplatin as second-line treatment in relapsed small-cell lung cancer. *J Clin Oncol* 19: 119–126

Kubota K, Nishiwaki Y, Kakinuma R, Hojo F, Matsumoto T, Ohmatsu H, Sekine I, Yokozaki M, Goto K, Ebi N, Kodama T (1997) Dose-intensive weekly chemotherapy for treatment of relapsed small-cell lung cancer. *J Clin Oncol* 15: 292–296

Kudoh S, Fujiwara Y, Takada Y, Yamamoto H, Kinoshita A, Ariyoshi Y, Furuse K, Fukuoka M (1998) Phase II study of irinotecan combined with cisplatin in patients with previously untreated small-cell lung cancer. West Japan Lung Cancer Group. *J Clin Oncol* 16: 1068–1074

Le Chevalier T, Ibrahim N, Chomy P, Riviere A, Monnier A, Magherini E, Pujol J (1997) A phase II study of irinotecan in patients with small cell lung cancer progressing after initial response to first-line chemotherapy. *Proc Am Soc Clin Oncol* 16: 450a

Masuda N, Fukuoka M, Kusunoki Y, Matsui K, Takifuji N, Kudoh S, Negoro S, Nishioka M, Nakagawa K, Takada M (1992) CPT-11: a new derivative of camptothecin for the treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 10: 1225–1229

Masuda N, Fukuoka M, Matsui K, Negoro S, Takada M, Sakai N, Ryu S, Takifuji N, Ito K, Kudoh S, Kusunoki Y (1990) Evaluation of high-dose etoposide combined with cisplatin for treating relapsed small cell lung cancer. *Cancer* 65: 2635–2640

Masuda N, Matsui K, Negoro S, Takifuji N, Takeda K, Yana T, Kobayashi M, Hirashima T, Kusunoki Y, Ushijima S, Kawase I, Tada T, Sawaguchi H, Fukuoka M (1998) Combination of irinotecan and etoposide for treatment of refractory or relapsed small-cell lung cancer. *J Clin Oncol* 16: 3329–3334

Mavroudis D, Papadakis E, Veslemes M, Tsiafaki X, Stavrakakis J, Kouroussis C, Kakolyris S, Bania E, Jordanoglou J, Agelidou M, Vlachonicolis J, Georgoulias V (2001) A multicenter randomized clinical trial comparing paclitaxel–cisplatin–etoposide *versus* cisplatin–etoposide as first-line treatment in patients with small-cell lung cancer. *Ann Oncol* 12: 463–470

Mori K, Kubota K, Nishiwaki Y, Sugiura T, Noda K, Kawahara M, Negoro S, Watanabe K, Yokoyama A, Nakamura S, Fukuda H, Tamura T, Saijo N (2002) Updated results of a pilot study of etoposide and cisplatin plus concurrent accelerated hyperfractionated thoracic radiotherapy followed by three cycles of irinotecan and cisplatin for the treatment of limited-stage small cell lung cancer: Japan Clinical Oncology Group (JCOG9903). *Proc Am Soc Clin Oncol* 21: 294a

Murray N, Livingston RB, Shepherd FA, James K, Zee B, Langleben A, Kraut M, Bearden J, Goodwin JW, Grafton C, Turrisi A, Walde D, Croft H, Osoba D, Ottaway J, Gandara D (1999) Randomized study of CODE *versus* alternating CAV/EP for extensive-stage small-cell lung cancer: an Intergroup Study of the National Cancer Institute of Canada Clinical Trials Group and the Southwest Oncology Group. *J Clin Oncol* 17: 2300–2308

Murray N, Shah A, Osoba D, Page R, Karsai H, Grafton C, Goddard K, Fairey R, Voss N (1991) Intensive weekly chemotherapy for the treatment of extensive-stage small-cell lung cancer. *J Clin Oncol* 9: 1632–1638

Nakanishi Y, Takayama K, Takano K, Inoue K, Osaki S, Wataya H, Takaki Y, Minami T, Kawasaki M, Hara N (1999) Second-line chemotherapy with weekly cisplatin and irinotecan in patients with refractory lung cancer. *Am J Clin Oncol* 22: 399–402

Niell HB, Herndon JE, Miller AA, Watson DM, Sandler A, Kelly K, Marks R, Green MR (2002) Randomized phase III intergroup trial (CALGB 9732) of etoposide (VP-16) and cisplatin (DDP) with or without paclitaxel (TAX) and G-CSF in patients with extensive stage small cell lung cancer (ED-SCLC). *Proc Am Soc Clin Oncol* 21: 293a

Noda K, Nishiwaki Y, Kawahara M, Negoro S, Sugiura T, Yokoyama A, Fukuoka M, Mori K, Watanabe K, Tamura T, Yamamoto S, Saijo N (2002) Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer. *N Engl J Med* 346: 85–91

Porter III LL, Johnson DH, Hainsworth JD, Hande KR, Greco FA (1985) Cisplatin and etoposide combination chemotherapy for refractory small cell carcinoma of the lung. *Cancer Treat Rep* 69: 479–481

Postmus PE, Berendsen HH, van Zandwijk N, Splinter TA, Burghouts JT, Bakker W (1987) Retreatment with the induction regimen in small cell lung cancer relapsing after an initial response to short term chemotherapy. *Eur J Cancer Clin Oncol* 23: 1409–1411

Roth BJ, Johnson DH, Einhorn LH, Schacter LP, Cherng NC, Cohen HJ, Crawford J, Randolph JA, Goodlow JL, Broun GO, Omura GA, Greco FA (1992) Randomized study of cyclophosphamide, doxorubicin, and vincristine *versus* etoposide and cisplatin *versus* alternation of these two regimens in extensive small-cell lung cancer: a phase III trial of the Southeastern Cancer Study Group. *J Clin Oncol* 10: 282–291

Sculier JP, Klastersky J, Libert P, Ravez P, Brohee D, Vandermoten G, Michel J, Thiriaux J, Bureau G, Schmerber J, Sergysels R, Coune A (1990) Cyclophosphamide, doxorubicin and vincristine with amphotericin B in sonicated liposomes as salvage therapy for small cell lung cancer. *Eur J Cancer* 26: 919–921

Sekine I, Nishiwaki Y, Kakinuma R, Kubota K, Hojo F, Matsumoto T, Ohmatsu H, Goto K, Kodama T, Eguchi K, Shinkai T, Tamura T, Ohe Y, Kunitoh H, Yoshimura K, Saijo N (2003) Phase I/II trial of weekly cisplatin, etoposide, and irinotecan chemotherapy for metastatic lung cancer: JCOG 9507. *Br J Cancer* 88: 808–813