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Effects of different combinations of gefitinib and irinotecan in lung cancer cell lines expressing wild or deletional EGFR

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Summary EGFR mutations are a major determinant of lung tumor response to gefitinib, an EGFR-specific tyrosine kinase inhibitor. Obtaining a response from lung tumors expressing wild-type EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase inhibitor irinotecan sulfate (CPT-11) is active against lung cancer. We examined the sensitivity of lung cancers expressing wild- or mutant-type EGFR to the combination of gefitinib and CPT-11. The in vitro effect of gefitinib and SN-38 (the active metabolite of CPT-11) was examined in seven lung cancer cell lines using the dye formation assay with a combination index. When administered concurrently, gefitinib and SN-38 had a synergistic effect in five of the seven cell lines expressing wild-type EGFR, whereas the combination was antagonistic in PC-9 cells and a PC-9 subline resistant to gefitinib and expressing deletional mutant EGFR (PC-9/ZD). When administered sequentially, treatment with SN-38 followed by gefitinib had remarkable synergistic effects in the PC-9 and PC-9/ZD cells. In an in vivo tumor-bearing model, this combination had a schedule-dependent synergistic effect in the PC-9 and PC-9/ZD cells. An immunohistochemical analysis of the tumors in mice treated with CPT-11 and gefitinib demonstrated that the number of Ki-67 positive tumor cells induced by CPT-11 treatment was decreased when CPT-11 was administered in combination with gefitinib. In conclusion, the sequential combination of CPT-11 and gefitinib is considered to be active against lung cancer.

1. Introduction

Lung cancer is one of the leading causes of cancer-related death, despite the use of conventional chemotherapy regi-

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mens. The epidermal growth factor receptor (EGFR) is frequently expressed in non-small cell lung cancer (NSCLC) and is correlated with a poor prognosis. Gefitinib ('Iressa') is an orally active, selective EGFR-tyrosine kinase inhibitor that blocks signal transduction pathways. Its clinical efficacy has been show in refractory NCSLC patients, but the survival benefit of this agent remains unclear. EGFR mutations have been identified in NSCLC, and lung cancers carrying the EGFR mutation have been reported to be hyperresponsive to gefitinib [1,2]. Mutant EGFR is a major determinant of lung tumor response to gefitinib, but the hyperresponsiveness of tumors expressing mutant EGFR has been observed in a small population. Now, obtaining a clinical benefit in lung tumors expressing wild-ype EGFR is a major obstacle. The combination of gefitinib and cytotoxic drugs is one strategy against lung cancers expressing wild-type EGFR. The DNA topoisomerase I inhibitor irinotecan (CPT-11) is a key drug in the treatment of patients with lung cancer and has been shown to prolong survival. SN-38 is the active metabolite of CPT-11 in vitro. The objective of this study was to determine the potential therapeutic utility of gefitinib when combined with CPT-11 therapy to lung cancer cell according to the treatment schedule and EGFR status.

Acquired resistance to gefitinib is also of clinical interest. Recently, Kobayashi et al. [3] reported that an EGFR mutation was related to the development of acquired resistance to gefitinib. We have established subclone PC-9/ZD cells that are resistant to gefitinib [4]. Our results suggested that another mechanism of resistance was active in PC-9/ZD cells. The effect of the combination of gefitinib and SN-38 in these PC-9/ZD cells was also examined.

2. Materials and methods

2.1. Drugs and chemicals

Gefitinib (*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy]quinazolin-4-amine) was provided by AstraZeneca (Cheshire, UK). Gefitinib was dissolved in dimethyl sulfoxide (DMSO) for the in vitro study. CPT-11 and SN-38 were obtained from Yakult Honsha (Tokyo, Japan) and were dissolved in dimethyl sulfoxide (DMSO) for both of the in vitro studies.

2.2. Cells and cultures

Human NSCLC cell lines PC-9, PC-7, and PC-14 derived from untreated patients with pulmonary adenocarcinoma were provided by Professor Y. Hayata, Tokyo Medical College. A small cell lung cancer cell line, H69, was established at the National Cancer Institute (Bethesda, MD, USA). The gefitinib-resistant subline, PC-9/ZD, was established from intrinsic hypersensitive cell PC-9 [5] in our laboratory [4]. A small cell lung cancer cell line, SBC-3, and an adenocarcinoma cell line, A549, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). All cell lines were maintained in RPMI1640 (Nikken Bio Med. Lab., Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin in an incubator at 37 °C and 100% humidity in 5% CO_2 and air, as described previously [6].

2.3. RT-PCR

Specific primers designed for EGFR CDS were used to detect the EGFR mRNA, as described elsewhere [1]. Sixteen first-strand cDNAs were synthesized from the cells' RNA using an RNA PCR Kit (TaKaRa Biomedicals, Ohtsu, Japan). After the reverse transcription of 1 μg of total RNA with Oligo(dT)-M4 adaptor primer, the whole mixture was used for PCR with two oligonucleotide primers (5′-AATGTGAGCAGAGGCAGGGA-3′ and 5′-GGCTTGGTTTGGAGCTTCTC-3). PCR was performed with an initial denaturation at 94 °C for 2 min and 25 cycles of amplification (denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 105 s).

2.4. Western blot analysis

The cultured cells were washed twice with ice-cold phosphate buffered saline (PBS), lysate in EBC buffer (50 mM Tris—HCl, pH 8.0; 120 mM NaCl; 0.5% Nonidet P-40; 100 mM NaF; 200 mM Na orthovanadate; and 10 mg/ml each of leupeptin, aprotinin and phenylmethylsulfonyl fluoride). The lysate was cleared by centrifugation at 20,000 × *g* for 5 min, and the protein concentration of the supernatant was measured using a BCA protein assay (Pierce, Rockford, IL, USA). For immunoblotting, 20 μg samples of protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membrane was then probed with rabbit polyclonal antibodies against EGFR, HER2/neu, Her3 and Her4 (Santa Cruz Biotech, Santa Cruz, CA, USA) and phospho-EGFR specific for Tyr 845, Tyr 1045, and Tyr 1068 (numbers 2231, 2235 and 2234; Cell Signaling, Beverly, MA, USA).

2.5. Growth-inhibition assay

We used the tetrazolium dye (3,(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT) assay to evaluate the cytotoxicity of various drug concentrations. After incubation for 72 h at 37 °C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well; the plates were then incubated for a further 4 h at 37 °C. After centrifuging the plates at 200 × *g* for 5 min, the medium was aspirated from each well and 180 μl of dimethylsulfoxide was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm using a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340; Bio-Metallics, Princeton, NJ, USA). Each experiment was performed in six replicate wells for each drug concentration and was independently performed three or four times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance, as calculated based on the survival curves. Percent survival was calculated as follows:

$$\frac{\text{Mean absorbance of six replicate wells containing drugs} - \text{mean absorbance of six replicate background wells}}{\text{mean absorbance of six replicate drug-free wells} - \text{mean absorbance of six replicate background wells}} \times 100.$$

2.6. Combined effect of gefitinib and SN-38 in vitro

After 24 h of incubation, gefitinib and SN-38 were added to each cell line according to one of the two combination schedules. For the concurrent schedule, gefitinib and SN-38 were added concurrently and were then incubated under the same conditions for 72 h. For the sequential schedule, gefitinib or SN-38 were added sequentially and were then incubated under the same conditions for 72 h. The combined effect of gefitinib and SN-38 on lung cancer cell growth was evaluated using a combination index (CI) [7]. The CI was produced using CalcuSym software (Biosoft, NY, USA). For any given drug combination, the CI represents the degree of synergy, additivity, or antagonism. CI was expressed in terms of fraction-affected (F_a) values, which represents the percentage of cells killed or inhibited by the drug. Using mutually exclusive ($\alpha = 0$) or mutually non-exclusive ($\alpha = 1$) isobologram equations, the F_a/CI plots for each cell line were constructed by computer analysis of the data generated from the median effect analysis. The CI values were interpreted as follows: <1.0 = synergism; 1.0 = additive; >1.0 = antagonism.

2.7. In vivo growth-inhibition assay

Experiments were performed in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals with experimental neoplasia (second edition). Fig. 2A shows the treatment schedule. For the in vivo experiments, the combined therapeutic effect of orally or intraperitoneally administered gefitinib and intravenously injected CPT-11 was evaluated according to a predetermined schedule. The dose of each drug was set based on the results of a preliminary experiment involving the administration of each drug alone. Ten days before administration, PC-9 and PC-9/ZD cells were injected subcutaneously into the backs of the mice. Six mice per group were injected with tumor cells. Tumor-bearing mice were given either gefitinib (40 mg/kg/day, p.o.) on days 2–6, CPT-11 (50 mg/kg/day, i.v.) on day 1, both, or a placebo (5%(w/v) glucose solution). Alternatively, tumor-bearing mice were given gefitinib on days 2–6 and CPT-1 on days 2. The diameters of the tumors were measured using calipers on days 1, 5, 8, 12, 15 and 20 to evaluate the effects of treatment, and tumor volume was determined using the following equation: tumor volume $ab^2/2$ (mm^3) (where a is the largest diameter of the tumor and b is the shortest diameter). Day 20 denotes the day on which the effects of the drugs were estimated, and day "0" denotes the first day of treatment. All mice were sacrificed on day 20 after their tumors had been measured.

2.8. Immunohistochemistry

The tumors were harvested from the mice at the time of sacrifice. For hematoxylin-eosin (HE) and anti-CD31 and Ki-67 staining, the resected tumors were fixed in zinc-buffered formalin (Shandon Lipshaw, Pittsburgh, PA) overnight at 4 °C. After paraffin embedding and sectioning at 6 μm, formalin-fixed sections were stained with Mayer's H&E (Richard Allen, Kalamazoo, MI, USA). For anti-Ki-67 and anti-CD31 immunohistochemistry, the slides were heated in a water bath at 95–99 °C in Target Retrieval Solution (DAKO, Carpinteria, CA, USA) for 20 min, followed by a 20-min cool-down period at room temperature. After heat retrieval, the sections were rinsed well in PBS and stained with rabbit antihuman Ki-67 antigen (DAKO N-series, ready to use) or rat antimouse CD-31 antibody (BD PharMingen, Tokyo, Japan) according to the manufacturer's instructions and then were lightly counterstained with Mayer's hematoxylin. The sections were finally stained with an in situ Death Detection POD Kit (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions.

TUNEL staining was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI, USA). Briefly, 6-μm cryostat sections were fixed in 4% paraformaldehyde for 10 min at room temperature and rinsed in PBS with 0.1% Triton X-100. The sections were then incubated in Equilibration Buffer for 5 min at room temperature followed by incubation in TUNEL Mix, prepared according to the manufacturer's instructions, for 1 h at 37 °C. After successive washes in PBS, the sections were coverslipped using an antifade reagent.

Microvessel density was determined by calculating the proportion of CD31-positive cells. The Proliferation Index was determined by Ki-67 immunostaining and calculating the population of Ki-67-positive cells in five fields at 200×. The Apoptosis Index, determined by TUNEL staining, was calculated from the population of TUNEL-positive cells in five fields at 200×. The apoptosis:proliferation ratio equals the apoptosis index/proliferation index × 100. At least 1000 tumor cell nuclei from the most evenly and distinctly labeled areas were examined in each examination.

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.

3. Results

3.1. Expression of Her-receptors and cellular sensitivity to gefitinib or SN-38 in lung cancer cell lines

The expression levels of EGFR in seven lung cancer cell lines were examined using RT-PCR with a primer set for exon 20 in EGFR. PC-14, SBC-3, H69, PC-7, and A549 cells showed a 570-bp-long PCR amplified product exhibiting wild-type EGFR mRNA (data not shown). On the other hand, a smaller PCR product was also detected in the PC-9 and PC-9/ZD cells, and this band was confirmed to be an in-frame 15-base deletion of exon 20 (E746_A750del).

We examined the protein levels of EGFR, Her2, Her3, and Her4 in the lung cell lines using immunoblotting. The quantitative data obtained by densitometorical analysis is summarized in Table 1. The protein levels of EGFR, Her2, and Her3 in the PC-9 cells were one- to four-fold higher than those in the other cell lines (PC-7, H69, PC-14, A549, and SBC-3).

Table 1 Comparison of Her family protein levels and gefitinib- and SN-38-induced growth inhibition

Cell lines	Relative expression[a]				Growth inhibition[b], $IC_{50} \pm$ S.D.	
	EGFR	Her2	Her3	Her4	Gefitinib (μM)	SN-38 (nM)
PC-9	2.8[c]	3.2	3.7	ND	0.047 ± 0.061	8.09 ± 1.9
PC-9/ZD	1.6[c]	2.6	3.8	ND	7.7 ± 0.5	38.9 ± 7.0
PC-14	1.5	2.8	1.1	ND	17.1 ± 0.8	42.1 ± 2.6
SBC-3	2.4	2.6	1.0	ND	19.9 ± 5.4	1.07 ± 0.1
A549	2.3	2.3	1.4	ND	30.2 ± 2.2	293 ± 64.5
H69	1.3	1.3	2.0	ND	56.5 ± 3.2	27.2 ± 4.1
PC-7	1.0	1.0	1.2	ND	68.8 ± 14.8	20.5 ± 8.2

The IC_{50} value (μM) of each drug was measured by MTT assay, as described in Section 2. Each value is the mean ± S.D. of three or four independent experiments.

[a] Protein expression levels were analyzed by Western blotting.

[b] Drug concentration responsible for 50% growth inhibition in MTT assay at 72 h, calculated data for at least three dependent experiments.

[c] 15-base deletion EGFR, ND: not determined.

3.2. Cellular sensitivity of lung cancer cells to gefitinib and SN-38

The growth inhibitory effect of gefitinib and SN-38 on lung cancer cells was examined using an MTT assay. The IC_{50} values of gefitinib for the cell lines ranged from 46 nM (PC-9 cells) to 68 μM (PC-7 cells). The PC-9/ZD cells were ~200-fold resistant to gefitinib, compared with the parental PC-9 cells. Cellular sensitivity to gefitinib and the expression levels of EGFR and Her2 were negatively correlated with the IC_{50} values of gefitinib (Table 1). The IC_{50} values of SN-38 for these cell lines ranged from 1 nM (SBC-3) to 300 nM (A549). The range of sensitivity to gefitinib was wider than that to SN-38. No correlation in cellular sensitivity to gefitinib and SN-38 was seen.

3.3. In vitro combined effect of gefitinib and SN-38 on lung cancer cell lines

To evaluate the potential combined effect of gefitinib and SN-38, the combination index was determined using an MTT assay. The combined effects of gefitinib and SN-38 under the concurrent schedule are shown in Fig. 1. CI values of <1, >1, and 1 indicate a supra-additive effect (synergism), an antagonistic effect, and an additive effect, respectively. An additive to supra-additive growth-inhibitory effect was observed for all doses of gefitinib and SN-38 tested in cell lines expressing wild-type EGFR. On the other hand, a high CI index was observed in PC-9 cells and PC-9/ZD cells expressing mutant EGFR over a wide range of inhibition levels. These results suggest that gefitinib and SN-38 are synergistic in lung cancer cells expressing wild-type EGFR but not in cell lines expressing mutant EGFR in vitro.

3.4. Schedule-dependent synergy of gefitinib and SN-38 in lung cancer cells

Next, we examined the schedule dependency of the combined effects of gefitinib and SN-38 in the cell lines. The five cell lines expressing wild-type EGFR showed synergistic (PC-14, H69, and A549 cells) or additive effects (SBC-3 and PC-7 cells) for all three schedules: concurrent administration, SN-38 followed by gefitinib administration, and gefitinib followed by SN-38 administration (Fig. 1A). In the PC-9 cells, concurrent administration and gefitinib followed by SN-38 administration were antagonistic, but SN-38 followed by gefitinib administration was synergistic (Fig. 1B). In the PC-9/ZD cells, concurrent administration was antagonistic, but sequential administration was synergistic. These schedule-dependent combined effects were observed in the cells expressing mutant EGFR.

3.5. Combined effects of gefitinib and SN-38 in vivo

To estimate the schedule-dependent effects in vivo, nude mice bearing tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules (Fig. 2A). Mice bearing PC-14 tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. CPT-11 (50 mg/kg) alone potentially reduced the tumor size, and the combination of gefitinib and CPT-11 was synergistic. In particular, the administration of CPT-11 followed by gefitinib cured the mice bearing PC-14 cells (Fig. 2B).

Mice bearing PC-9 or PC-9/ZD tumors were treated with gefitinib and CPT-11 according to sequential or concurrent schedules. Gefitinib (40 mg/kg) alone potentially reduced the PC-9 tumors, and CPT-11 (50 mg/kg) followed by gefitinib administration reduced the tumor size of PC-9 xenografts more dramatically (gefitinib alone: $P = 0.012$, sequential combination: $P = 0.005$) (Fig. 2B). On the other hand, the concurrent schedule produced an antagonistic effect. Body weight loss was not observed in any of the mice treated according to the above schedules (Fig. 2C). CPT-11 followed by gefitinib administration is a potentially beneficial schedule against PC-9 and PC-9/ZD cells expressing mutational EGFR. The results of these in vivo experiments were consistent with those of the in vitro studies.

To elucidate the synergistic mechanisms of CPT-11 and gefitinib in vivo, tumor samples of the PC-9 and PC-9/ZD

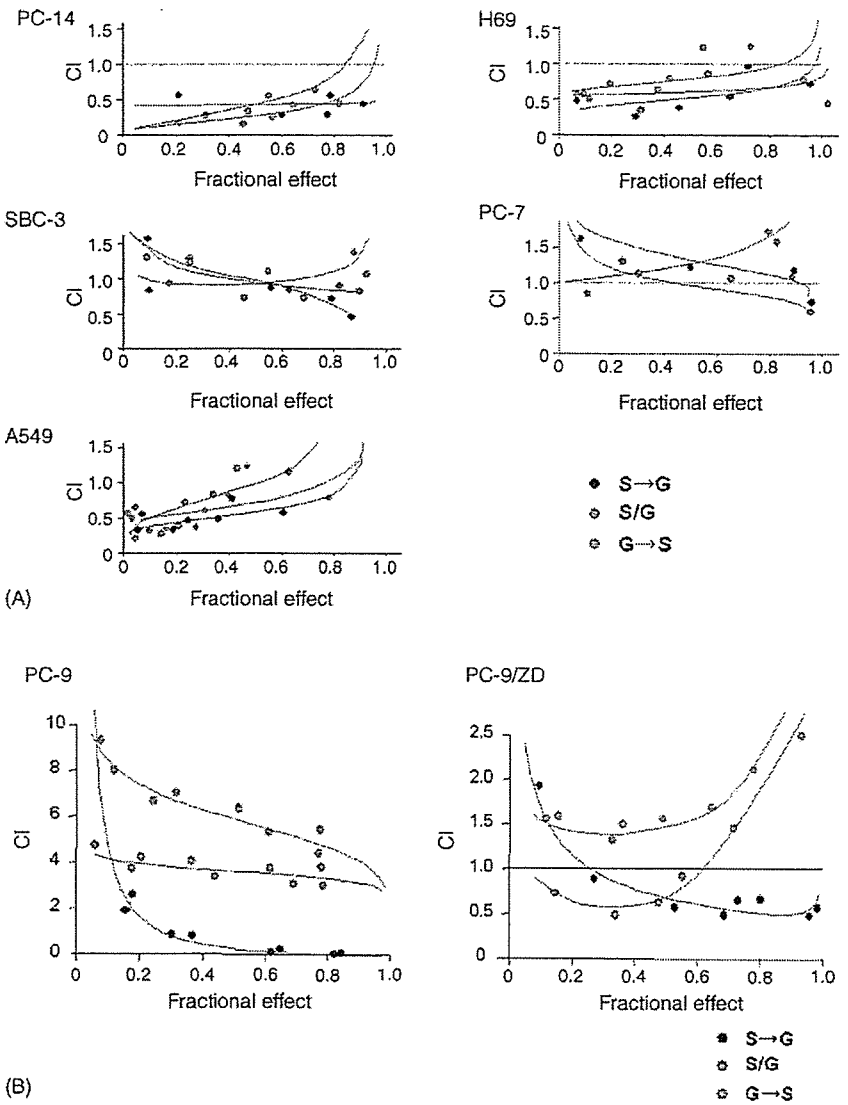

Fig. 1 Combination index (CI) plots of interactions between gefitinib and SN-38 in lung cancer cell lines. Each cell line was treated with gefitinib and SN-38, either alone or in combination at a fixed molar ratio. (A) (PC-14) gefitinib: SN-38 = 425:1; (SBC-3) 20000:1; (A549) 100:1; (H69) 2000:1; (PC-7) 3500:1. (B) (PC-9) gefitinib: SN-38 = 6:1; (PC-9ZD) 175:1. Treatment schedule: (1) SN-38 was applied first and gefitinib was applied 12 h later, followed by incubation in medium for 72 h (blue). (2) SN-8 and gefitinib were applied concurrently, followed by incubation in medium for 72 h (red). (3) Gefitinib was applied first and SN-38 was applied 12 h later, followed by incubation in medium for 72 h (green). S → G: sequential combination (SN-38 followed by gefitinib); C/G: concurrent combination; G → S: sequential combination (gefitinib followed by SN-38).

cells were stained with anti-Ki-67, anti-CD31 and the TUNEL assay (Fig. 3A and B). A reduction in tumor cell proliferation (Ki-67 staining), a reduction in tumor vasculature (CD31 staining), and an increase in tumor apoptosis (TUNEL staining) were observed in tumors treated with gefitinib alone or gefitinib and CPT-11. The administration of CPT-11 alone increased the number of Ki-67 positive tumor cells. In the PC-9 tumors, sequential treatment resulted in a 2.7-fold increase in tumor cell apoptosis and a 1.9-fold decrease in vessel staining, compared with the results obtained in tumors treated concurrently. The ratio of apoptosis:proliferation increased 1.7-fold in sequentially treated tumors compared with tumors treated with both drugs concurrently. Quantitative analysis of tumor cell proliferation and apoptosis showed a significant difference between the effects of the concurrent and sequential schedules ($P < 0.001$), but not between concurrent and gefitinib-alone ($P > 0.01$ for all comparisons, Fig. 3C). No significant difference in CD31-positive cells was observed between the control and gefitinib-alone treatments, suggesting that gefitinib exerts no remarkable anti-angiogenetic effects ($P > 0.01$, Fig. 3C). Similar findings were observed in PC-9/ZD tumors. These findings suggest that the antitumor activity of sequential treatment using gefitinib and CPT-11 is mediated by an increase in tumor cell apoptosis, compared with concurrent treatment.

4. Discussion

The EGFR-targeting drug gefitinib has been approved in many countries for the treatment of NSCLC patients who have previously received chemotherapy. Previous preclinical models have demonstrated the synergistic effects of gefitinib and platinums or taxanes [8,9]. However, no significant difference in survival was demonstrated in two randomized placebo-controlled phase II trials examining over 2000 previously untreated patients with NSCLC. In these trials, gefitinib was given in combination with paclitaxel and carboplatin or with gemcitabine and cisplatin [10,11]. Different administration schedules for gefitinib and cytotoxic agents may be necessary for select populations.

EGFR gene mutations have been demonstrated in NSCLC, and patients with lung cancers expressing mutant EGFR are strongly suspected to be hypersensitive to gefitinib alone. An in-frame short deletion in exon 19 of EGFR is strongly related to hyperresponsiveness to gefitinib and other tyrosine kinase inhibitors [12,13]. Cells expressing this deletional EGFR mutation are hypersenstivie to EGFR-targeted tyrosine kinase inhibitors [5]. On the other hand, the treat-

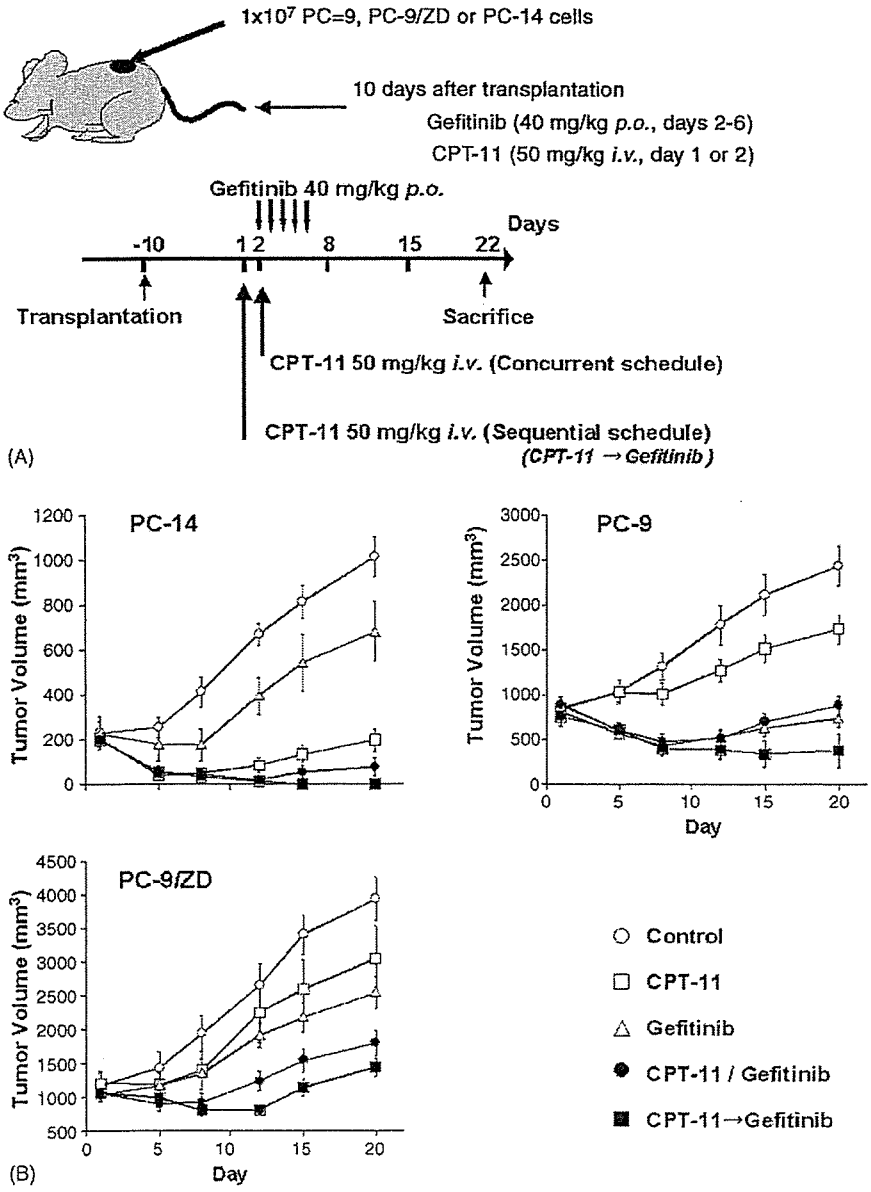

Fig. 2 Dose-dependent effects of combination therapy in PC9 and PC9/ZD cells in vivo. (A) Treatment schedule; (B) significant tumor growth-inhibition was observed in mice treated with the combination of gefitinib and CPT-11. Mice were allocated to five groups (6 mice/group) (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: gefitinib 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). (C) Treatment-related body weight loss in mice treated with gefitinib and/or SN-38. (○: 5% (w/v) glucose solution; □: CPT-11 50 mg/kg; △: ZD1839 40 mg/kg; ■: ZD1839 40 mg/kg + CPT-11 50 mg/kg concurrently; ●: CPT-11 50 mg/kg followed by ZD1839 40 mg/kg). Bars: ±S.D.

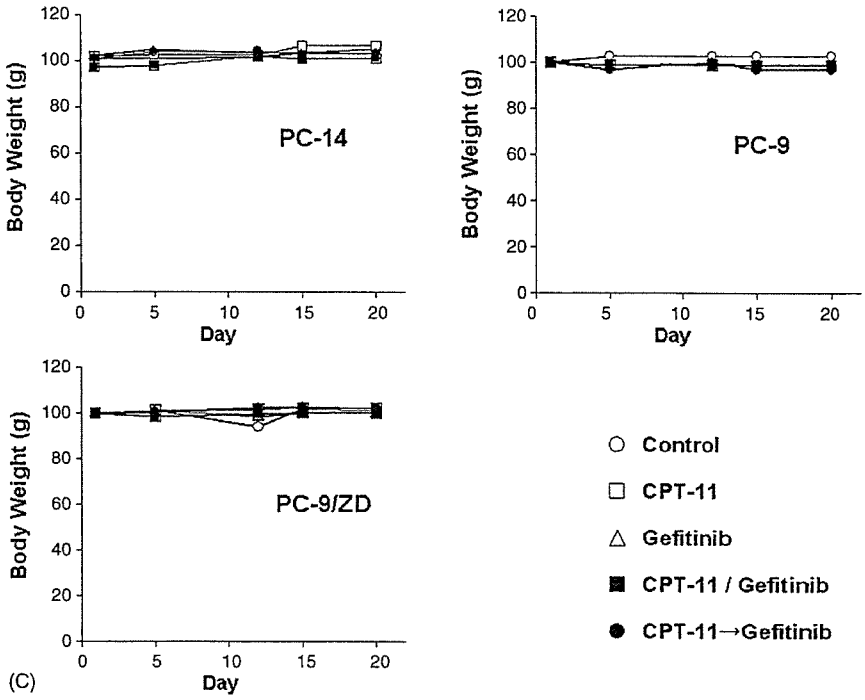

Fig. 2 (*Continued*).

ment of lung cancers expressing wild-type EGFR is a major obstacle. Combined therapies are still considered to be a major strategy against lung cancer expressing wild-type EGFR. Our previous preclinical study demonstrated that gefitinib and CPT-11 have synergistic effects in colorectal cancer cell lines [14]. Here, we reevaluated the combined effects of gefitinib and cytotoxic agents based on the status of EGFR mutations in lung cancer.

We demonstrated that gefitinib and SN-38, the active form of CPT-11, have synergistic or additive effects in lung cancer cells expressing wild-type EGFR. The combination of gefitinib and CPT-11 may be useful against lung cancers expressing wild-type EGFR. On the other hand, this combination had antagonistic effects in PC-9 cells expressing mutant EGFR, even though PC-9 cells are basically hypersensitive to gefitinib alone.

The concurrent administration of gefitinib and SN-38 also had an antagonistic effect in the PC-9/ZD cells. The PC-9/ZD cells developed an acquired resistance to gefitinib after exposure to gefitinib in vitro. New treatment strategies for patients who are refractory to gefitinib treatment are clinically needed. We demonstrated that the sequential administration of SN-38 (CPT-11) and gefitinib improved the combined effects in PC-9/ZD cells both in vitro and in vivo.

The above results led us to propose a combined gefitinib and CPT-11 treatment strategy based on the EGFR mutation status of lung cancers: (1) combined treatment according to any schedule for lung cancers expressing wild-type EGFR, (2) gefitinib treatment alone for lung cancers expressing mutant EGFR, and (3) the sequential administration of gefitinib and CPT-11 for patients who are refractory to gefitinib treatment. Based on the above preclinical evidence, we are preparing to begin a clinical phase II trial for combined gefitinib and CPT-11 treatment in Japan.

We previously demonstrated that CPT-11 and gefitinib have a synergistic effect against colorectal cancer [14]. EGFR mutations are rarely observed in colorectal cancer cells [15]. Therefore, the combined effects of these agents against colorectal cancers were consistent with those against the lung cancers expressing wild-type EGFR in this study.

Different combined effects were observed for the concurrent and sequential schedules in vitro and in vivo. While the mechanisms responsible for the combined effects remain unclear, cell cycle distributions might explain some of the differences. In cells treated according to the sequential gefitinib followed by SN-38 (CPT-11) treatment schedule, treatment with gefitinib resulted in an increase in the G0—G1 phase and a decrease in the S phase populations (data not shown). The decreased S phase population was not sensitive to CPT-11 [16]. Thus, the antagonistic effects of the sequential administration of gefitinib followed by CPT-11 (SN-38) could be explained by this mechanism. On the other hand, in cells treated according to the sequential SN-38 followed by gefitinib treatment schedule, SN-38 treatment induced an increase in the S phase population. If the S phase population is sensitive to gefitinib, this might explain the synergistic effects of this sequential schedule [17]. An increase in EGFR phosphorylation induced by CPT-11 is another previously reported possible mechanism responsible for this synergistic action [14].

In conclusion, we demonstrated the different effect on lung cancer cell expressing mutant EGFR according to the

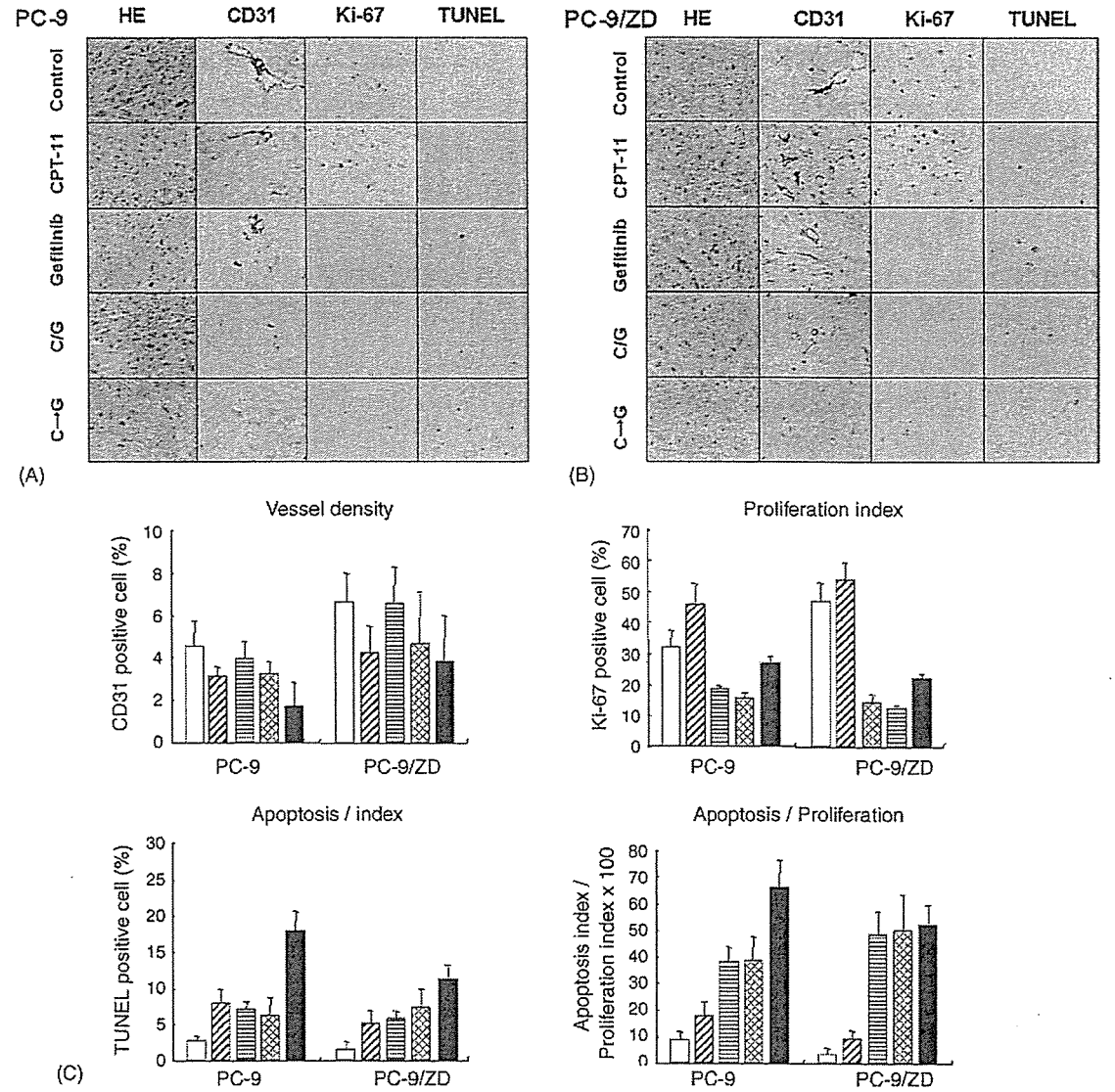

Fig. 3 (A) Historical examination of PC-9 tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C/G: concurrent combination, C→G: sequential combination. (B) Historical examination of PC-9ZD tumor xenografts (day 22) stained with H&E, anti-CD31 vessel staining, TUNEL staining (magnification: 400×) and anti-Ki-67 nuclear antigen (magnification: 200×). The number of Ki-67-positive cells increased with the administration of CPT-11. The number of Ki-67-positive cells decreased with the gefitinib-alone and combination treatments. C→G: sequential combination; C/G: concurrent combination. (C) Quantitation of CD31 vessel staining, Ki-67 proliferation index, apoptosis index, and apoptosis: proliferation ratio. The columns represent the mean population of positive cells in five fields. Bars: ±S.D. Tumors from mice treated with vehicle (white), CPT-11 (diagonal hatched), Gefitinib (horizontal hatched), concurrent combination of CPT-11 plus Gefitinib (cross-hatched), or sequential combination of CPT-11 plus Gefitinib (cross-hatched).

combination schedule of gefitinib and CPT-11. The sequential combined treatment also active against lung cancer cell expressing wild-type EGFR.

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Common arm analysis: One approach to develop the basis for global standardization in clinical trials of non-small cell lung cancer

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Summary The global development of new anticancer treatments is desirable. However, whether results of clinical trials performed in one population can be fully extrapolated to another population remains in question. We retrospectively compared "common arms" of platinum-based doublet phase III trials among Japanese, European, and American patients with non-small cell lung cancer to develop the basis for global standardization in clinical trials. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors including socioeconomic factors, medical service background, and patient selection process for clinical trials may be consistent between geographically different oncology groups. The doses of docetaxel, gemcitabine, and vinorelbine were lower in Japanese studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations in these countries. Response rates seemed to be distributed randomly from one study to another, whereas patient survival might be better in Japanese studies. In conclusion, geographical differences in the dose of anticancer agents, response, survival and toxicity of lung cancer chemotherapy were actually observed. However, extrapolation of clinical data obtained in one country to another population and global clinical trials were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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1. Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and the incidence lung caner is increasing globally at a rate of 0.5% per year [1]. Lung cancer currently claims more than 55 000 lives annually in Japan, and this figure is projected to double during the next three decades due to the aging of the Japanese population [2]. Non-small cell lung cancer (NSCLC) comprises 80% of all lung cancers, and more than half of the patients with this disease are found to have developed distant metastases or pleural effusion at the time of the initial diagnosis. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely livelong [3].

The development of new anticancer agents and chemotherapeutic regimens are among the urgent tasks for medical oncologists who are involved in the treatment of lung cancer. Since it is time- and money-consuming work, the development of new agents and regimens is desirable on a global scale. Under the present situation in Japan, in that we are considerably behind with the development of new anticancer agents, it is worth evaluating the possibility that the results of clinical trials held outside Japan could be used for approval of these agents by the Japanese authorities. However, whether the results of clinical trials performed in one population can be fully extrapolated to another population remains in question due to the potential differences in trial designs, study-specific criteria, patient demographics, and population-related pharmacogenomics. According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline E5, Ethnic Factors in the Acceptability of Foreign Clinical Data, the impact of genetic and physiologic (intrinsic) factors and cultural and environmental (extrinsic) factors upon the efficacy and safety of anticancer agents at a particular dosage and dose regimen must be assessed for the application of new agent approval [4].

One approach to develop the basis for global standardization in clinical trials of anti-NSCLC agents is a planned comparative analysis of a "common arm" with similar eligibility, staging, response and toxicity criteria of prospectively designed and conducted separate phase III trials for the treatment of advanced NSCLC, although this approach may have potential limitation in comparability [5]. In this review we retrospectively compared the outcome of phase III trials conducted in Japan, Europe, and USA for chemotherapy doublet regimens using a platinum and a third-generation cytotoxic agent, including paclitaxel, docetaxel, gemcitabine, and vinorelbine.

2. Methods

Combinations of paclitaxel and carboplatin, docetaxel and cisplatin, gemcitabine and cisplatin, and vinorelbine and cisplatin were evaluated in patients with advanced NSCLC as the post-marketing sponsored phase III trials in Japan [6,7]. Phase III trials evaluating these regimens conducted outside Japan were identified by Medline searches. The selection criteria of phase III trials for this analysis were (1) first-line treatment for stage IIIB or IV NSCLC; (2) not intended for a special cohort of patients such as the elderly or those with poor performance status; (3) each arm included more than 120 patients; (4) tumor response was evaluated according to the World Health Organization (WHO) criteria, modified WHO criteria such as Eastern Cooperative Oncology Group (ECOG) criteria and Southwest Oncology Group (SWOG) criteria, or response evaluation criteria in solid tumors (RECIST) criteria; (5) toxicity was evaluated according to the WHO criteria or the National Cancer Institute-Common Toxicity Criteria (NCI-CTC). The dose and schedule of anticancer agents, patient demographics, treatment delivery, tumor response, patient survival, and toxicity were compared between common arms in separate phase III trials. To assess the influence of demographic variables on tumor response and survival, multiple linear regression analysis was performed as previously described [8].

3. Results

3.1. Taxane and platinum

The schedule was identical between the studies in both paclitaxel and carboplatin, and docetaxel and cisplatin combinations (Tables 1 and 2). The dose of paclitaxel ranged from 175 to 225 mg/m^2 without ethnic tendency. The dose of docetaxel was set to be 20% lower in a Japanese study [7] than that of USA studies [9,10]. This difference was mainly attributable to differences in the criteria of the maximum tolerated dose in phase I studies of docetaxel between Japan and the USA. Patient demographics were very similar among these studies. Response rates (RRs) in the combination of paclitaxel and carboplatin varied widely from 17% to 46%, and median survival time (MST) from 7.8 to 12.3 months. The RR and MST in Japanese and Greek studies appeared to be better than those in ECOG study, but did not differ from those in other American studies. A multiple linear regression analysis failed to show correlation between demographic variables and the RR or MST. In the docetaxel and cisplatin combination, the RR and survival in the Japanese study appeared to be better that those in the ECOG study [9], but similar to those in the other USA study [10].

Among paclitaxel and carboplatin studies, the incidence of grade 3-4 neutropenia and febrile neutropenia was higher in the Japanese study than in the other studies. The toxicity profile of the docetaxel and cisplatin combination was identical among all studies.

3.2. Gemcitabine and cisplatin

The dose of gemcitabine per one course was smaller in the Japanese study than in other studies outside Japan (Table 3). The RR in ECOG study was lower than that in European studies, while the MST of 14.8 months and 1-year survival rate of 60% in the Japanese study seemed higher than those in the other studies [6]. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.

Table 1 The combination of carboplatin and paclitaxel

Characteristics	Japan [6]	Greece [13]	Greece [14]	EU [18]	ECOG [19]	SWOG [19]	SWOG [5]	USA [20]	USA [12]
Chemotherapy dose									
CBDCA (AUC)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)	6 (day 1)
PTX (mg/m^2)	200 (day 1)	175 (day 1)	200 (day 1)	200 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)	225 (day 1)
Demographics (% not specified)									
No. of patients	145	185	252	309	290	206	182	190	345
Age (median) (range)	63 (33–74)	65 (30–83)	63 (31–81)	58 (27–76)	63 (30–85)	62 (26–80)	63 (28–80)	62 (28–80)	63 (31–85)
Female	32	14	13	17	38	30	37	34	39
PS 0–1	100	80	86	83	95	100	100	NA	91
Stage IV	81	49	62	68	86	88	87	77	78
Non-squamous	79	63	69	63	NA	NA	82	NA	81
Treatment delivery and efficacy (% not specified)									
Cycles (median)	3	NA	NA	4	4	NA	4	NA	6
Response rate (95% CI)	32 (25–40)	46 (39–53)	28 (22–34)	23 (20–30)	17 (13–21)	25 (19–31)	34 (27–41)	23 (17–29)	29 (24–34)
MST (month) (95% CI (month))	12.3 (NA)	11.0 (10–12)	10.4 (8.8–12)	8.2 (7.4–9.6)	8.1 (7.0–9.5)	8.6 (7.2–10.7)	9.0 (NA)	7.8 (NA)	9.9 (NA)
1-year survival (%)	51	43	42	32	34	38	37	32	42
Grade 3-4 toxicity (%)									
Neutropenia	88	14	15	51	63	57	NA	65	6
Febrile neutropenia	16	9	0	4	4	2	3	NA	NA
Thrombocytopenia	11	2	2	2	10	10	8	8	NA
Neuropathy	5	26	8	9	10	13	16	5	1

Table 2 The combination of cisplatin and docetaxel

Characteristics	Japan [7]	ECOG [9]	USA [10]
Chemotherapy dose			
CDDP (mg/m^2)	80 (day 1)	75 (day 1)	75 (day 1)
DTX (mg/m^2)	60 (day 1)	75 (day 1)	75 (day 1)
Demographics (% not specified)			
No. of patients	151	289	408
Age (median) (range)	63 (30–74)	63 (34–84)	61 (30–81)
Female	36	37	28
PS 0–1	96	94	96
Stage IV	100	86	67
Non-squamous	89	NA	68
Treatment delivery and efficacy (% not specified)			
Cycles (median)	3	4	5
Response rate (95% CI)	37 (29–45)	17 (12–21)	32 (27–36)
MST (month) (95% CI (month))	11.3 (NA)	7.4 (6.6–8.8)	11.3 (10.1–12.4)
1-year survival	48	31	46
Grade 3-4 toxicity (%)			
Neutropenia	74	69	75
Febrile neutropenia	2	11	5
Thrombocytopenia	1	3	3
Neuropathy	0	5	4

The toxicity was similar among many studies except for the gemcitabine and cisplatin arm of the Iressa NSCLC Trial Assessing Combination Treatment (INTACT) study [11], where the incidence of grade 3-4 neutropenia and thrombocytopenia was reported to be about one tenth of that in other studies (Table 3).

3.3. Vinorelbine and cisplatin

The dose of vinorelbine per one course was also smaller in the Japanese study than in other studies outside Japan (Table 4). The RR in the Greek study was higher than that in an American study. There was no difference in survival for this combination among all studies. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.

Grade 3-4 neutropenia was less common in the Greek study than in other studies, but the frequency of febrile neutropenia in that study was intermediate among studies.

4. Discussion

This study showed that geographical differences in the outcome of lung cancer chemotherapy may be present. However, extrapolation of clinical data in a country to another population and global clinical trials were considered possible with adequate considerations as discussed below.

The dose of third-generation cytotoxic agents was smaller in Japanese studies than in European and American studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations of these countries. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors may be comparable and consistent between geographically different oncology groups. These factors include socioeconomic factors, medical service background, and patient selection process for clinical trials.

RRs in phase III studies including third-generation cytotoxic agents seemed to be distributed randomly from one study to another, whereas patient survival might have been better in Japanese studies. The Japanese phase III trials were performed in academic institutes, including university-affiliated hospitals, cancer center hospitals, and central city general hospitals. Thus, the distribution of patients selected for Japanese phase III trials may be skewed, in that they were in good general condition, although established prognostic factors in patients with NSCLC were almost identical among Japanese and non-Japanese studies. In addition, better survival among Japanese patients may be attributable to true ethnic differences. One possibility is the relatively high frequency of non-squamous histology in Japanese studies, but the reason is largely unknown.

The severity and frequency of common toxicity were comparable in all these phase III studies with a few exceptions. The incidence of grade 3-4 neutropenia was only 5–6% in the carboplatin and paclitaxel arm of the INTACT2 study [12] and in the cisplatin and gemcitabine arm of the INTACT1 study [11], both of which were sponsored by one pharmaceutical company. Similarly, the incidence of neutropenia was lower in Greek studies [13–15] than in other studies. These differences in the incidence of toxicity may be associated with the frequency of monitoring, including patient hospital visits and blood cell count and chemistry evaluation.

Table 3 The combination of cisplatin and gemcitabine

Characteristics	Japan [6]	Italy [21]	Spain [22]	EORTC [23]	EU [11]	ECOG [9]	EU + USA [24]
Chemotherapy dose							
CDDP (mg/m^2)	80 (day 1)	100 (day 2)	100 (day 1)	80 (day 1)	80 (day 1)	100 (day 1)	100 (day 1)
GEM (mg/m^2)	1000 (day 1, 8)	1000 (day 1, 8, 15)	1250 (day 1, 8)	1250 (day 1, 8)	1250 (day 1, 8)	1000 (day 1, 8, 15)	1000 (day 1, 8, 15)
Demographics (% not specified)							
No. of patients	146	155	182	160	363	288	260
Age (median) (range)	61 (34–74)	62 (28–76)	59 (33–75)	57 (28–75)	61 (33–81)	64 (32–87)	62 (36–88)
Female	33	37	12	29	28	37	30
PS 0–1	100	93	85	89	90	95	80
Stage IV	81	79	77	79	69	86	67
Non-squamous	81	68	55	74	71	NA	70
Treatment delivery and efficacy (% not specified)							
Cycles (median)	3	NA	4	5	6	3	4
Response rate (95% CI)	30 (23–38)	38 (30–46)	42 (35–50)	37 (29–45)	47 (42–53)	22 (17–27)	30 (25–36)
MST (month) (95% CI (month))	14.8 (NA)	8.6 (NA)	9.3 (8.1–10.5)	8.9 (7.8–10.5)	10.9 (NA)	8.1 (7.2–9.4)	9.1 (8.3–10.6)
1-year survival	60	33	38	33	44	36	39
Grade 3-4 toxicity (%)							
Neutropenia	63	40	32	43	5	63	57
Febrile neutropenia	2	1	4	3	NA	4	5
Thrombocytopenia	35	64	19	36	6	50	50

Table 4 The combination of cisplatin and vinorelbine

Characteristics	Japan [6]	Greece [15]	France [25]	EU [26]	SWOG [19]	USA [10]
Chemotherapy dose						
CDDP (mg/m^2)	80 (day 1)	80 (day 8)	100 (day 1)	120 (day 1)	100 (day 1)	100 (day 1)
VNR (mg/m^2)	25 (day 1, 8)	30 (day 1, 8)	30 (day 1, 8, 15, 22)	30 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)	25 (day 1, 8, 15, 22)
Demographics (% not specified)						
No. of patients	145	204	156	206	202	404
Age (median) (range)	61 (28–74)	64 (46–75)	57 (39–74)	59 (NA)	61 (32–83)	61 (35–80)
Female	30	25	21	12	33	25
PS 0–1	100	90	92	80	100	96
Stage IV	83	64	86	59	89	67
Non-squamous	81	54	76	44	NA	65
Treatment delivery and efficacy (% not specified)						
Cycles (median)	3	4	4	3	NA	4
Response rate (95% CI)	33 (25–41)	39 (33–46)	36 (28–43)	28 (22–34)	28 (22–34)	25 (20–29)
MST (month) (95% CI (month))	11.4 (NA)	9.7 (8.3–11.2)	9.6 (8.1–12.2)	9.3 (NA)	8.1 (6.7–9.6)	10.1 (9.2–11.3)
1-year survival	48	41	42	37	36	41
Grade 3-4 toxicity (%)						
Neutropenia	88	37	83	79	76	79
Febrile neutropenia	18	11	22	4	1	5
Thrombocytopenia	1	6	3	3	4	4

Anticancer agents are considered to be sensitive to ethnic factors, because of a steep pharmacodynamic curve for both efficacy and safety, a narrow therapeutic dose range, non-linear pharmacokinetics, their metabolic enzymes with the potential for drug-drug interaction, and these enzymes with the potential for ethnically variable activity caused by genetic polymorphism. Thus, bridging studies using pharmacologic endpoints are extremely important to apply efficacy, safety, and dose data from one place to another [16]. These pharmacologic studies can be incorporated into phase I trials and, when it is necessary, phase II trials. Furthermore, the current study suggests that, once the pharmacological property and recommended dose of a new cytotoxic agent are established in one country, the outcome of randomized controlled trials developed in other countries can be extrapolated to the population.

We defined ethnic populations in the current study according to the country where the study was performed. However, patients enrolled into multicenter European and North American studies may include patients with a diverse ethnicity. It would be greatly interesting to see RR, MST and toxicity in subgroups of patients with different ethnicity in those trials, although there has been no such data published.

Randomization of patients in a trial guarantees the comparability between treatment arms within the trial, but not between treatment arms in different trials. Thus, it is impossible to compare the outcome of different trials exactly. Nevertheless, we frequently refer to the outcome of trials performed outside Japan and they furnish us with much information. To compensate this limitation, we tried to compare patient characteristics between trials, but other factors including the frequency of monitoring may also affect the outcome greatly. The number of combination regimens evaluated in this study is insufficient, but no large scale Japanese trials of other combination regimens have been available so far.

This study failed to demonstrate whether this approach to clinical trial analysis was really helpful. For future clinical trials, consistency in monitoring, as well as the use of the common toxicity and response criteria, is important to keep comparability between trials. A meta-analysis using individual patient data may be more useful than a subgroup analysis within a trial to compare the outcomes between ethnic subgroups with adequate statistical power.

A phase II study of gefitinib in patients with advanced NSCLC who had previously received one or two chemotherapy regimens was conducted in cooperation with 43 hospitals across Europe, Australia, South Africa, and Japan. The population was prospectively stratified into Japanese and non-Japanese patients to investigate whether there were any differences between the two patient populations with respect to efficacy [17]. This study clearly showed that a global study of NSCLC using the same protocol was completed, and this global strategy was an effective method to speed up the development of a new anticancer agent in Japan. In addition, the stratification by the county or ethnicity is important in a global study of an investigational new drug to investigate geographical differences in efficacy and toxicity.

In conclusion, the dose of anticancer agents, RR, survival and toxicity of lung cancer chemotherapy showed some differences among Japanese, European, and USA studies. However, extrapolation of clinical data in a country to another population and global clinical trials including many countries were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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

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

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

INTRODUCTION

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

MATERIALS AND METHODS

Reagents

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

Cell culture

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

Plasmid construction and transfection

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

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

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Immunoblotting

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

Determination of ligand secretion by ELISA

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

Preparation of cell lysates for EGFR autophosphorylation

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

Autophosphorylation assay

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

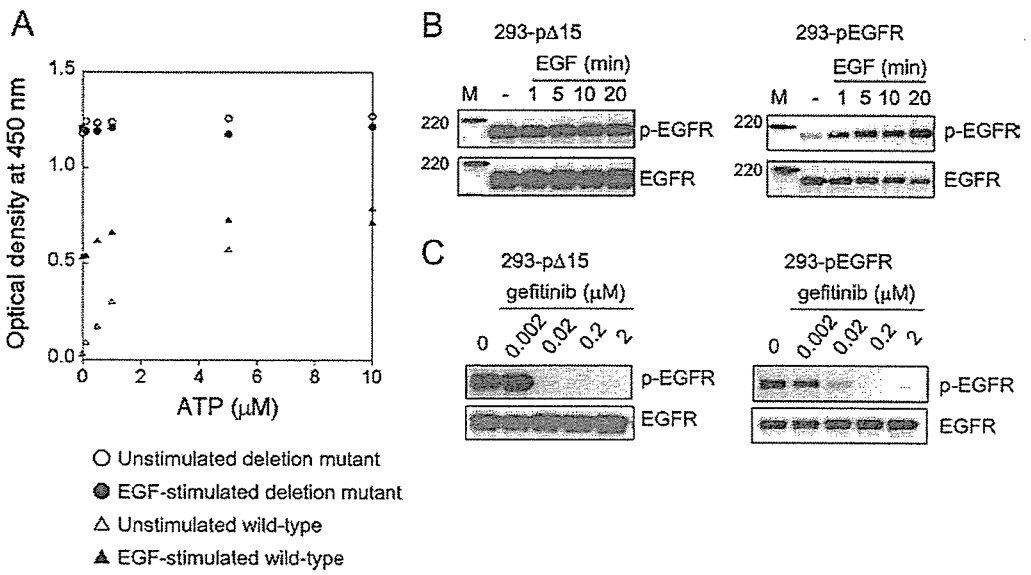

Figure 1 Autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR

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

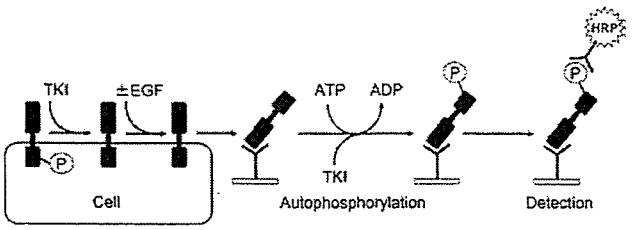

Figure 2 Schematic illustration of the cell-based autophosphorylation assay

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

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

Data analysis

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

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

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

RESULTS

Autophosphorylation of deletion mutant EGFR and wild-type EGFR

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

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

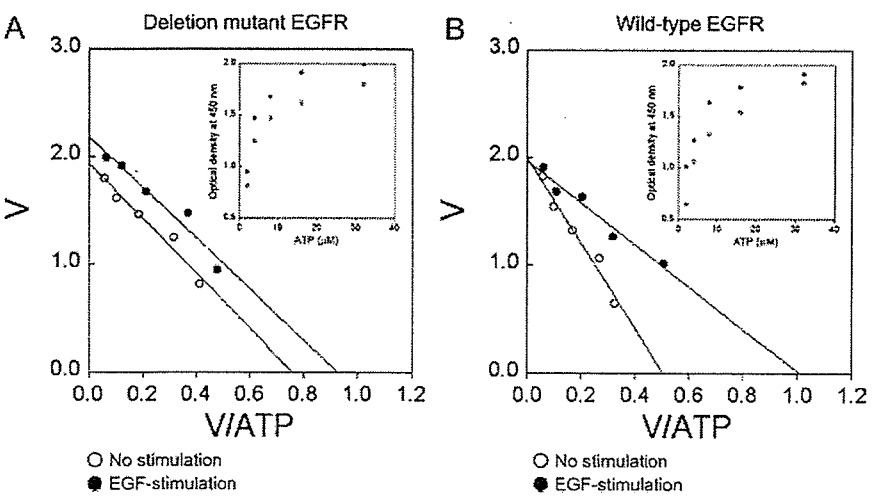

Figure 3 Autophosphorylation activities of deletion mutant EGFR and wild-type EGFR

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