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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 ± 0.3 μM) and mutant EGFR (K_m for ATP = 2.5 ± 0.2 μM). There was no difference in K_m values between EGF-stimulated wild-type EGFR (K_m for ATP = 1.9 ± 0.1 μM) and deletion mutant EGFR (K_m for ATP = 2.2 ± 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 20 000 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,

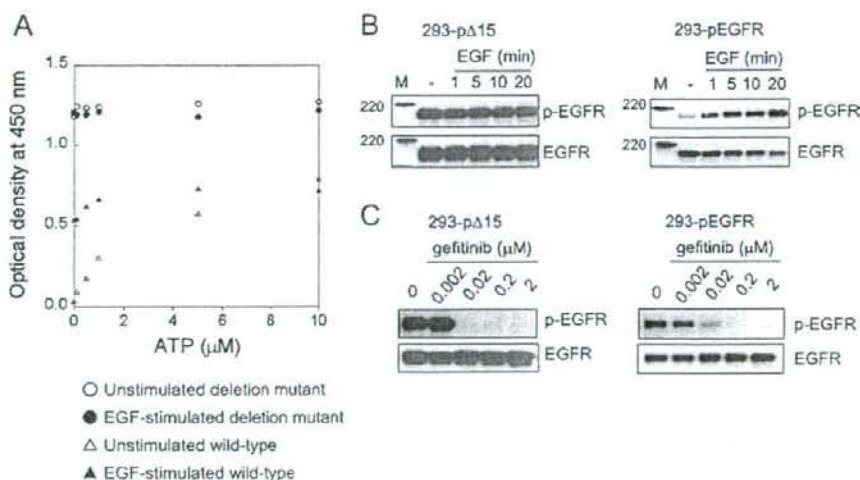

Figure 1 Autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR

(**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.

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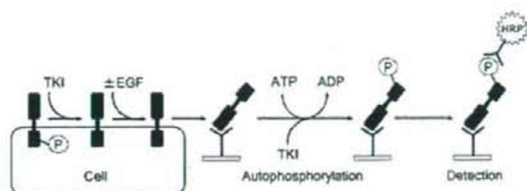

Figure 2 Schematic illustration of the cell-based autophosphorylation assay

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

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

Data analysis

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

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

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

RESULTS

Autophosphorylation of deletion mutant EGFR and wild-type EGFR

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

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

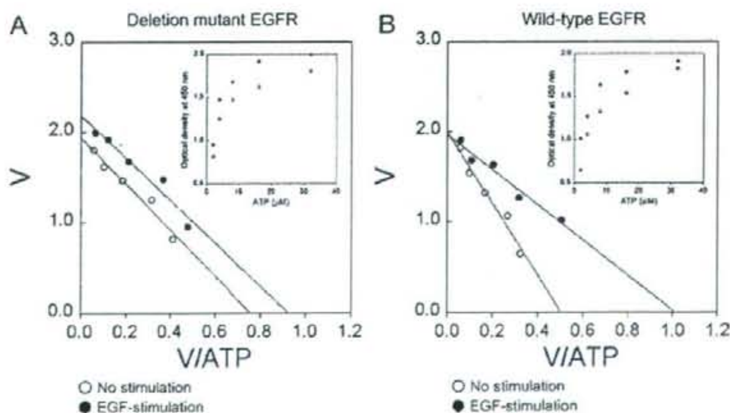

Figure 3 Autophosphorylation activities of deletion mutant EGFR and wild-type EGFR

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

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Table 1 Kinetic parameters for ATP

The autophosphorylation reaction was performed using the indicated enzyme and gefitinib (0.5–5 nM). The steady-state kinetic parameters for ATP were determined from the Eadie–Hofstee plot in Figure 5. Results are means ± S.D. for three independent duplicate experiments.

		K_m (μM)				V_{max} (μM · min^{-1})			
		Deletion mutant		Wild-type		Deletion mutant		Wild-type	
Gefitinib (nM)	EGF stimulation...	−	+	−	+	−	+	−	+
0		2.5 ± 0.2	2.2 ± 0.2	4.0 ± 0.3	1.9 ± 0.1	1.9 ± 0.1	2.1 ± 0.1	2.0 ± 0.0	1.9 ± 0.0
0.5		5.6 ± 0.5	5.7 ± 0.4	4.1 ± 0.4	2.3 ± 0.1	1.9 ± 0.1	1.9 ± 0.2	2.0 ± 0.1	1.9 ± 0.1
1		9.8 ± 2.8	10.9 ± 3.0	4.6 ± 1.2	2.5 ± 0.1	2.0 ± 0.1	1.9 ± 0.0	2.0 ± 0.2	1.8 ± 0.1
5		26.1 ± 5.4	30.2 ± 4.2	7.0 ± 2.3	4.9 ± 0.9	1.9 ± 0.1	1.8 ± 0.2	2.0 ± 0.1	1.8 ± 0.2

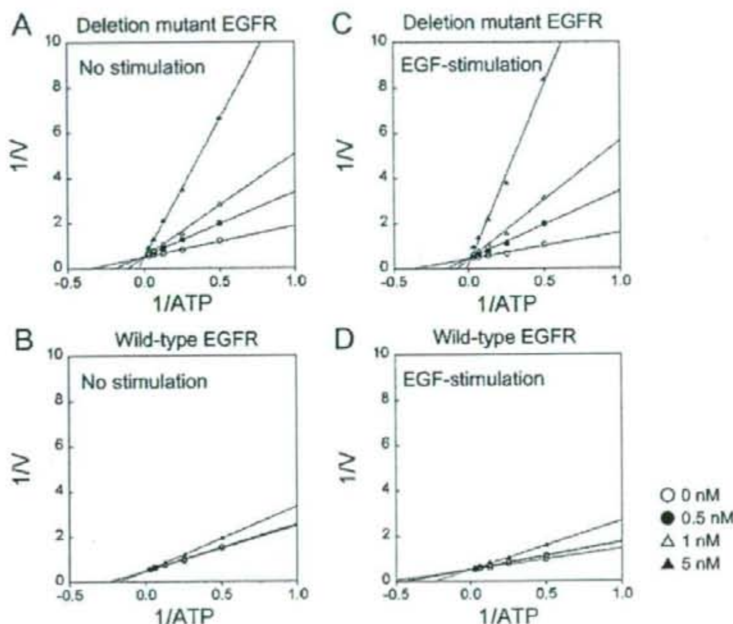

Figure 4 Mechanism of inhibition of deletion mutant EGFR by gefitinib

Autophosphorylation of unstimulated deletion mutant (**A**), unstimulated wild-type (**B**), EGF-stimulated deletion mutant (**C**) and EGF-stimulated wild-type (**D**) EGFR was measured with or without gefitinib at concentrations of 0 (○), 0.5 (●), 1 (△) and 5 (▲) nM. Reciprocal velocity against reciprocal ATP concentrations (0.5–32 μM) were plotted. Data are representative of at least three independent experiments.

low level of EGF-independent basal phosphorylation, whereas autophosphorylation using EGF-stimulated EGFR represents EGF-induced phosphorylation.

Kinetic parameters of autophosphorylation

The deletion mutant EGFR is constitutively phosphorylated under unstimulated conditions. Measuring the autophosphorylation activity of deletion mutant EGFR requires unphosphorylated tyrosine residues of EGFR. An autophosphorylation assay was reconstructed to determine the kinetic parameters of deletion mutant EGFR. The method is summarized in Figure 2. The concentrations of gefitinib used (2 μM) completely inhibited phosphorylation of both the deletion mutant and wild-type EGFR, as demonstrated by immunoblot analysis (Figure 1C). We performed autophosphorylation assays with various amounts of EGFR (results not shown). In our autophosphorylation assay, a constant amount of EGFR (130 ng/well) was adopted to measure its autophosphorylation, because this amount of EGFR was found to be appropriate for detecting changes in the absorbance of both wild-type and deletion mutant EGFR. The autophosphorylation of deletion mutant EGFR and wild-type EGFR was analysed by comparison with unstimulated and EGF-stimulated EGFR (Figure 3). The higher phosphorylation of deletion mutant EGFR shown in Figure 1(A) was lowered by using gefitinib-treated lysates, while the autophosphorylation reaction was initiated by addition of ATP. The ATP-dependent autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR in crude cellular extracts were monitored (Figure 3, insets). The data were transformed into an Eadie–Hofstee plot, and the kinetic parameters were determined as apparent K_m and V_{max} values for ATP (Figure 3 and Table 1). Under unstimulated conditions,

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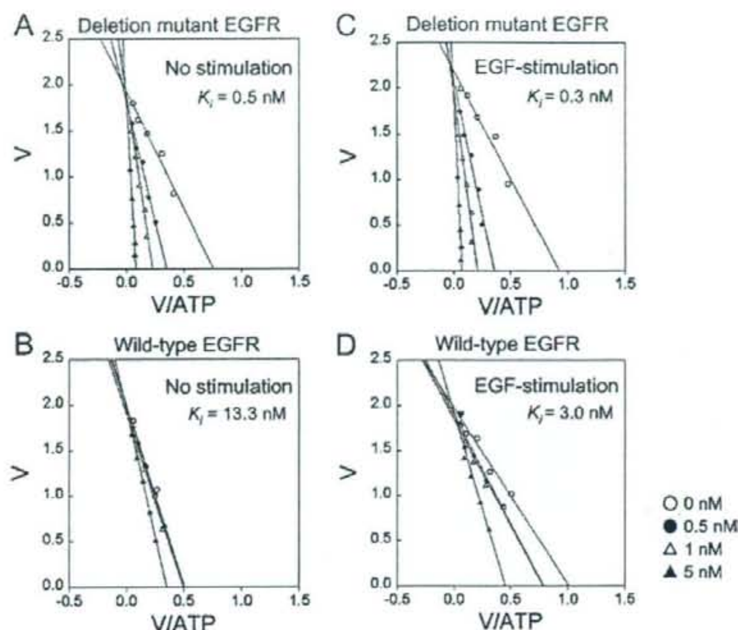

Figure 5 Inhibition constant of gefitinib for autophosphorylation activity of deletion mutant EGFR

The same dataset as shown in Figure 4 was fitted to an Eadie–Hofstee plot, and kinetic parameters from this fit are summarized in Table 1. Shown are the results for the unstimulated (**A**) and EGF-stimulated (**C**) deletion mutant EGFR and unstimulated (**B**) and EGF-stimulated (**D**) wild-type EGFR in response to ATP with or without gefitinib at concentrations of 0 (○), 0.5 (●), 1 (△) and 5 (▲) nM. Results are representative of at least three independent experiments.

differences in activities were seen between unstimulated wild-type (K_m for ATP = 4.0 ± 0.3 μM) and deletion mutant EGFR (K_m for ATP = 2.5 ± 0.2 μM). Under EGF-stimulated conditions, there was no difference in K_m values between EGF-stimulated wild-type EGFR (K_m for ATP = 1.9 ± 0.1 μM) and deletion mutant EGFR (K_m for ATP = 2.2 ± 0.2 μM). The V_{max} values of wild-type EGFR and deletion mutant EGFR were equal under both conditions. These results suggest that the wild-type EGFR is conformationally activated by EGF stimulation, and that the mutant EGFR is active without ligand stimulation.

Gefitinib inhibits autophosphorylation of deletion mutant EGFR

We examined the inhibitory effect of gefitinib (0.5, 1 and 5 nM) on the autophosphorylation of deletion mutant EGFR in comparison with wild-type EGFR under unstimulated and EGF-stimulated conditions. The data were transformed into a Lineweaver–Burk plot for estimation of the mode of inhibition (Figure 4). Lineweaver–Burk plot analysis showed that gefitinib competitively inhibited the autophosphorylation of deletion mutant EGFR as well as that of wild-type EGFR. The data were transformed into an Eadie–Hofstee plot for determination of kinetic parameters (Figure 5). Eadie–Hofstee plot analysis revealed the apparent K_m and V_{max} values for ATP in the presence of various gefitinib concentrations, and the kinetic parameters are summarized in Table 1. The K_i for deletion mutant EGFR and wild-type EGFR was calculated using eqn 1 (see the Materials and methods section). The K_i value of gefitinib for deletion mutant EGFR (K_i for gefitinib = 0.5 ± 0.1 nM) was 26-fold lower than that for wild-type EGFR (K_i for gefitinib = 13.3 ± 5.1 nM) under unstimulated conditions (Figure 5). Under EGF-stimulated conditions, the K_i value of gefitinib for deletion mutant EGFR (0.3 ± 0.1 nM) was 10-fold lower than that for wild-type EGFR (3.0 ± 0.6 nM) (Figure 5). Based on these comparative studies, we concluded that gefitinib binds deletion mutant EGFR more strongly than wild-type EGFR. In addition, we calculated the inhibitory effect of gefitinib for both types of EGFR in the presence of 2 μM ATP (Figure 6). Relatively strong inhibitory activity was detected for deletion mutant EGFR as compared with wild-type EGFR. These results suggest that gefitinib had a high affinity (low K_i value) for deletion mutant EGFR compared with wild-type EGFR.

DISCUSSION

Wild-type EGFR is unphosphorylated, being in an inactive form, under unstimulated conditions. The binding of ligands to the extracellular domain of EGFR induces dimerization and phosphorylation of the receptor into the active form [13]. The kinetic parameters of wild-type EGFR in our autophosphorylation assay are consistent with those of previous reports [14,15]. Crystallographic analysis has shown that the structure of the EGFR kinase domain after forming a complex with erlotinib exhibits a conformation consistent with the active form of protein kinases [16,17]. Previously, we reported that the deletion mutant EGFR was dimerized and phosphorylated constitutively without ligand stimulation, suggesting an active conformation [9]. We analysed the enzymatic properties of the deletion mutant EGFR, and

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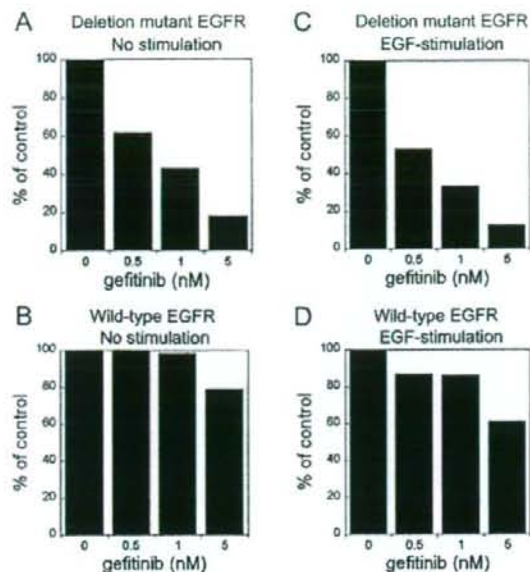

Figure 6 Effects of gefitinib on autophosphorylation of deletion mutant EGFR

The percentage of absorbance compared with the control under conditions of 2 μM ATP was calculated using the same dataset as shown in Figure 4 at a concentration of 2 μM ATP. The results shown are for unstimulated (**A**) and EGF-stimulated (**C**) deletion mutant EGFR and unstimulated (**B**) and EGF-stimulated (**D**) wild-type EGFR in response to ATP with or without gefitinib. Results are representative of at least three independent experiments.

determined the K_i value of gefitinib for deletion mutant EGFR. The inhibition constant of gefitinib for wild-type EGFR was similar to the value reported by Wakeling et al. [18]. We showed that the K_i value of gefitinib for deletion mutant EGFR was much lower than that for wild-type EGFR. The evidence of the decreased K_i value of gefitinib for deletion mutant EGFR means that gefitinib binds deletion mutant EGFR more strongly than wild-type EGFR. The high-affinity interaction between deletion mutant EGFR and gefitinib may be attributable to structural differences between deletion mutant EGFR and wild-type EGFR.

Our conclusion does not contradict the previous report by Stamos et al. [16] on a similar EGFR-targeted tyrosine kinase inhibitor, erlotinib, which binds to the active form of EGFR [14]. This result differs from that reported elsewhere: Fabian et al. [19] reported that there were no differences in the binding affinity of EGFR-targeted tyrosine kinase inhibitors between wild-type EGFR and mutant EGFR, including the deletion mutation. They constructed and expressed the kinase domain of EGFR on a bacteriophage surface, followed by interaction with immobilized inhibitors using biotin–avidin systems. Conversely, in our experiments, we performed autophosphorylation assays with EGFR extracted from 293-pΔ15 and the 293-pEGFR cells overexpressing deletion mutant and wild-type EGFR respectively. We consider our cell-based autophosphorylation assay results to reflect the native state of deletion mutant EGFR and to possibly explain the hypersensitivity of mutant-expressing cells to gefitinib.

We demonstrated that the deletion mutant actually binds gefitinib more strongly than wild-type EGFR. This is likely to be the mechanism of action of other tyrosine kinase inhibitors such as erlotinib, ZD6474 [dual inhibitor targeted to VEGFR2 (vascular endothelial growth factor receptor 2)/KDR (kinase insert domain-containing receptor) and EGFR] and other possible multi-targeted tyrosine kinase inhibitors. Indeed, EGFR-specific tyrosine kinase inhibitors AG1478 and erlotinib, as well as ZD6474, as described in our previous report [7] showed different growth-inhibitory activities against HEK-293 transfected with deletion mutant EGFR (results not shown). Thus it is likely that these (ATP competitive) tyrosine kinase inhibitors have different binding property effects on wild-type and deletion mutant EGFR to those of gefitinib.

In the present study, we focused on the enzymatic properties of in-frame deletion mutant EGFR (delE746–A750). The inhibition of receptor autophosphorylation in deletion mutant EGFR by gefitinib was much greater than that in wild-type EGFR. Next, it is necessary to examine the kinetic properties of other types of EGFR mutants, especially L858R, and these findings may pave the way for the discovery of different kinase inhibitors with different inhibition profiles for EGFR.

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Original Articles

Pharmacokinetics and Pharmacodynamics of Weekly Epoetin Beta in Lung Cancer Patients

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Background: To assess the pharmacokinetic profile and time-course of trough concentrations and hemoglobin levels associated with subcutaneous weekly administration of epoetin beta in lung cancer patients with chemotherapy-induced anemia.
Methods: Epoetin beta was subcutaneously administered to 15 anemic lung cancer patients once weekly for 8 weeks at doses of 9000, 18 000 and 36 000 IU. Pharmacokinetic parameters (C_{max}, AUC_{inf} and $T_{1/2}$) were determined after the first single dose administration on a model-independent basis, and the relationship between the dose and these parameters was examined for linearity.
Results: Weekly administration of epoetin beta at 9000, 18 000 and 36 000 IU produced C_{max} values of 308 ± 117 (mean ± standard deviation), 678 ± 86.7 and 1316 ± 766 mIU/ml, and AUC_{inf} values of 15 300 ± 9524, 54 574 ± 16 265 and 88 501 ± 55 687 hr mIU/ml, respectively, showing dose-proportional increases. Trough concentrations tended to increase in the presence of severe bone marrow suppression induced by chemotherapy or other factors. Extremely high values were seen in three patients, but there was no apparent trend toward an increase with repeated doses. After 8 weeks' administration at 9000, 18 000 and 36 000 IU, hemoglobin levels were changed by −0.37 ± 1.26, 2.15 ± 1.36 and 2.82 ± 2.17 g/dl, respectively.
Conclusions: Epoetin beta exhibited linear pharmacokinetics when administered to anemic cancer patients at weekly doses of 9000–36 000 IU and did not cause drug accumulation. Hemoglobin levels increased with weekly doses of 18 000 or 36 000 IU.

Key words: anemia – epoetin beta – pharmacokinetics

INTRODUCTION

Cancer patients receiving multicycle chemotherapy and radiotherapy frequently develop anemia, with one clinical study reporting that hemoglobin levels fell to 8–12 g/dl in 75% of patients undergoing these therapies (1). Among patients undergoing chemotherapy, anemia with hemoglobin levels of <8.0 g/dl reportedly occurs in 50–60% of ovarian cancer, lung cancer, non-Hodgkin's malignant lymphoma or multiple myeloma patients (2).

The etiology of chemotherapy-induced anemia includes the following: myelosuppression of chemotherapy or radiotherapy, reduced production of the bone-marrow-stimulating hormone erythropoietin (EPO), diminished bone marrow response to EPO and cancer cell-induced immune system activation resulting in reduced iron availability (3).

EPO, a hematopoietic hormone mainly produced in the kidneys, acts on erythroblastic precursor cells to promote differentiation and proliferation of erythrocytes and disappears in the bone marrow and spleen. Epoetin beta is a human EPO preparation that is mass-produced by recombinant gene technology and is commonly used in treatment of patients with renal failure-induced anemia. In Europe and the United States, it has already been approved and has also been administered to cancer patients with anemia with demonstrated effects in reducing required blood transfusion volumes, elevating hemoglobin concentrations and improving quality of life (QOL) (4,5). Furthermore, in the US, the American Society of

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Hematology and the American Society of Clinical Oncology jointly issued clinical practice guidelines in 2002 for the use of EPO preparations (6). Thus, the general use of epoetin in anemic cancer patients has been advocated. Meanwhile, in Japan, EPO preparation has not been approved for cancer patients with anemia, but clinical trials are now in progress.

Despite the increasing usage of epoetin, its pharmacokinetics have not been adequately investigated at high, once-weekly doses of 30 000 or 40 000 IU that are typically administered subcutaneously to cancer patients with anemia (7). To the best of our knowledge, the literature contains no pharmacokinetic data for epoetin beta in patients with cancer-related or chemotherapy-induced anemia, and the effect of the chemotherapy on serum EPO concentrations was not clear. We therefore studied the pharmacokinetic profile and time-course of trough concentrations and hemoglobin levels associated with subcutaneous weekly administration of epoetin beta in lung cancer patients with chemotherapy-induced anemia.

PATIENTS AND METHODS

PATIENTS

Inclusion criteria were as follows: (i) histological or cytological confirmation of lung cancer diagnosis; (ii) treated with cyclic chemotherapy; (iii) aged between 20 and 79 years; (iv) life expectancy of at least 2 months; (v) anemia (hemoglobin level of $\leq$11.0 g/dl) considered to be primarily chemotherapy-induced; and (vi) adequate renal and hepatic function.

Exclusion criteria included (i) iron deficiency (Mean corpuscular volume $\leq 80 \mu m^3$ or iron saturation [{Fe/(Fe+ Unsaturated iron-binding capacity)} × 100] $\leq$ 15.0%); (ii) blood cell transfusion in the 4 weeks prior to the study; (iii) rHuEPO therapy in the 4 weeks prior to the study; (iv) documented hemorrhagic lesion; (v) pregnancy, breastfeeding or not using adequate birth control measures; (vi) history of myocardial, pulmonary or cerebral infarction, serious drug allergy, uncontrolled hypertension, hypersensitivity to any EPO preparation, any serious complication; and (vii) a primary hematologic disorder as the cause of the present anemia.

The protocol was approved by the institutional review board of the National Cancer Center Hospital, and written informed consent was obtained from all patients who participated in the study.

STUDY DESIGN

This was an open-label, single-arm, dose-escalation study. Patients were assigned sequentially to one of three groups, receiving epoetin beta at either 9000, 18 000 or 36 000 IU per patient. This was administered by weekly subcutaneous injection for 8 weeks. If the patient's hemoglobin level recovered to 14 g/dl or higher, the treatment was stopped. Chemotherapy and radiotherapy were not performed from 7 days prior to until 4 days following the initial dose, and blood transfusion was not performed until 4 days after the initial dose. Oral iron supplementation (200 mg of ferrous sulfate) was administered daily. Blood samples for detection of epoetin beta antibody were collected before the first administration and 7 days after the last administration. Patients were followed for 1 week after the end of drug administration. Granulocyte colony-stimulating factor administration was allowed to the patients whose neutrophils count was <500 per cubic millimeter or those with neutropenic fever whose neutrophils count was <1000 per cubic millimeter.

SERUM ASSAY

To determine the pharmacokinetic parameters, blood samples were collected immediately prior to and 6, 10, 24, 34, 48, 72, 96 and 168 h after the initial dose of epoetin beta. To investigate the time-course of trough concentrations, samples were also collected immediately prior to the administration of each dose.

Blood samples were allowed to stand at room temperature for ~30 min and then centrifuged at 4°C and 3000 rpm for ~10 min to separate the serum. The resulting serum was stored frozen at below −20°C until used for measurement of serum EPO concentrations.

Serum EPO concentrations were measured by the RIA method developed and validated by Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. Validation of this assay revealed the following: quantification range, 6–384 mIU/ml; intra-assay precision (repeatability) and accuracy of 2.7–6.3% and −22.1 to −5.5%, respectively; and inter-assay precision (reproducibility) and accuracy of 2.4–7.6% and −18.1–3.0%, respectively. If the assayed value exceeded the upper limit of the quantification range (378 mIU/ml), the sample was diluted for re-measurement.

PHARMACOKINETIC ANALYSIS

Since EPO is an endogenous substance, measurements of serum EPO concentration following the first administration were baseline corrected to account for the presence of endogenous EPO. The corrected values were then used to determine descriptive statistics for drug concentration at each blood sampling time-point and the pharmacokinetic parameters.

The following pharmacokinetic parameters were determined after the initial dose by using WinNonlin Pro v.3.3 (Pharsight Corporation, Mountain View, CA) in a model-independent manner: C_{max}, AUC_{inf} and $T_{1/2}$.

C_{max} was observed values. AUC_{inf} was calculated by the trapezoidal method with infinite extrapolation by dividing the last plasma concentration by the elimination rate constant (K_{el}). $T_{1/2}$ was calculated as $0.693/K_{el}$.

Trough concentrations were not baseline corrected.

PHARMACODYNAMIC ANALYSIS

Hemoglobin levels and platelet counts were assessed weekly.

Statistical Analysis

All statistical analyses were performed using SAS v. 8.2 (SAS Institute, Cary, NC). Descriptive statistics were not calculated if they were to be based on available data from less than half the subjects.

Analyses of dose linearity were performed for C_{max} and AUC_{inf}. Each analysis used the power model: $\log y = \alpha + \beta \cdot \log$ dose, where β is the slope and y represents the pharmacokinetic parameter. Fitting a linear relationship between log y and log dose is an extension of the analysis of variance model. The key feature of the power model is the assumption of linearity between the log-transformed values of parameters and doses. The 95% confidential interval (CI) of the slope of the log-transformed parameters plotted against log dose was estimated, and dose-proportionality was concluded to be present if the 95% CI contained a slope with a value of 1.

RESULTS

Patients' Characteristics

Fifteen patients were enrolled in the study. Their characteristics are shown in Table 1. Participants were 8 men and 7 women, aged 30–78 years (median age, 69.0 years), who were being treated with chemotherapy (containing platinum in 12 cases). Four patients received prior radiation therapy (brain radiation in four cases and thoracic radiation in three cases). Ten patients had small cell carcinoma, four had adenocarcinoma and one had large cell carcinoma. Doses of 9000, 18 000 and 36 000 IU were administered to 3, 6 and 6 patients, respectively. Data from all 15 patients were included for evaluation of pharmacokinetic analysis and hemoglobin response. In all patients, the hemoglobin levels at the time of registration were <11.0 g/dl. Five patients discontinued this study for the following reasons: recovery of hemoglobin level to 14 g/dl or higher, $n = 1$ (36 000 IU); adverse effects (rotary vertigo), $n = 1$ (36 000 IU); withdrawal of consent, $n = 1$ (9000 IU); and disease progression, $n = 2$ (18 000 IU, 36 000 IU).

Pharmacokinetics Analysis

The mean baseline serum EPO concentration across all patients was 77.3 mIU/ml, with a median value of 59.9 mIU/ml, a minimum of 23.6 mIU/ml and a maximum of 301 mIU/ml. The 9000 IU group showed the highest mean, attributable to an extremely high value of 301 mIU/ml in one patient (Table 1).

The time-courses of the mean serum drug concentrations by dose group are shown in Fig. 1, and a summary of the pharmacokinetic parameters are given in Table 2.

The power model gave 95% CI of the slope (β) of the C_{max}-dose and AUC_{inf}-dose curves of 0.551–1.388 and 0.532–1.753, respectively, both including '1'.

Table 1. Patients' characteristics

Characteristic	Item	Total	9000 IU	18 000 IU	36 000 IU
Sex	Male	8	1	3	4
	Female	7	2	3	2
Histology	Small cell	10	3	4	3
	Large cell	1	0	1	0
	Adenocarcinoma	4	0	1	3
ECOG* performance status	0	3	1	1	1
	1	12	2	5	5
Prior chemotherapy	None	2	0	1	1
	Non plantinum based	1	0	0	1
	Plantinum based	12	3	5	4
Age (years)	Median	69.0	78.0	69.5	68.0
	Range	30–78	53–78	54–75	30–71
Hemoglobin** (g/dl)	Mean	9.4	9.1	9.2	9.8
	Range	6.8–11.4	6.8–11	7.5–10.3	7.1–11.4
Serum Fe (μg/dl)	Mean	76.8	111.3	69.5	66.8
	Range	17–154	45–154	37–148	17–106
Serum ferritin (ng/ml)	Mean	371.9	533.8	254.8	408.0
	Range	68.3–786	68.3–786	99.7–509.7	79.6–608.8
Serum endogenous erythropoietin (mIU/ml)	Mean	77.3	122.7	70.9	60.1
	Range	23.6–301	26.9–301	23.6–158	41.5–74.1

*Eastern Cooperative Oncology Group.
**The hemoglobin levels show the values just before the first administration of erythropoietin.

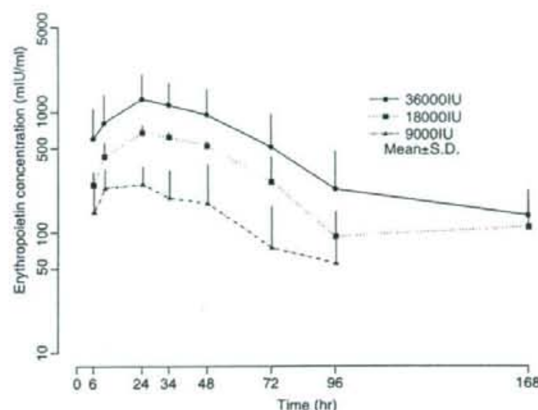

Figure 1. Time-course of mean serum drug concentrations of erythropoietin in each dose group following first dose. The mean drug concentrations for each group changed in a parallel manner up to 96 h.

Table 2. Summary of descriptive statistics for pharmacokinetic parameters of erythropoietin following the first dose

PK parameter	Unit	9000 IU	18 000 IU	36 000 IU
		$n = 3$ (Mean ± SD)	$n = 6$ (Mean ± SD)	$n = 6$ (Mean ± SD)
C_{max}	mIU/ml	308 ± 117	678 ± 86.7	1316 ± 766
AUC_{inf}	hr·mIU/ml	15 300 ± 9524	54 574 ± 16 265	88 501 ± 55 687
$T_{1/2}$	hr	24.5 ± 18.1	43.6 ± 22.0	30.4 ± 22.1

C_{max} and AUC_{inf} increased in an almost dose-proportional manner, whereas $T_{1/2}$ was constant.

Trough Concentrations

Time-courses of trough concentrations are shown by dose group in Fig. 2. Considerable variations in trough concentration occurred over the 8 week period. EPO concentration did not increase with repeated doses of epoetin beta, suggesting that drug accumulation did not occur. In some patients, trough concentrations were extremely high after chemotherapy (Fig. 3).

Relationship of Trough Concentration with Bone Marrow Suppression

Time-courses of trough concentrations, hemoglobin levels and platelet counts in the three patients with markedly elevated trough concentrations are shown in Fig. 3. In these patients, hemoglobin level and platelet count fell during the period in which trough concentration increased rapidly.

Pharmacodynamics Results

The time-course of mean hemoglobin levels is shown in Fig. 4. Hemoglobin levels were unchanged at a dose of 9000 IU, but tended to increase at doses of 18 000 and 36 000 IU. At 8 weeks, the change of hemoglobin levels from baseline was −0.37 ± 1.26 g/dl in the 9000 IU group, 2.15 ± 1.36 g/dl in the 18 000 IU group and 2.82 ± 2.17 g/dl in the 36 000 IU group. One patient receiving 9000 IU and two patients receiving 18 000 IU underwent blood cell transfusion. Only one patient (who received 36 000 IU weekly) exceeded predetermined threshold levels of hemoglobin for discontinuation of the study.

Safety

Once-weekly dosing of epoetin beta was well tolerated in all study patients, with no life-threatening toxic effects occurring during the trial. Leucopenia was the most frequent adverse event (13 of 15), followed by nausea (9 of 15). Other frequent adverse events were anorexia (7 of 15), diarrhea (7 of 15), thrombocytopenia (6 of 15), alopecia (5 of 15), fatigue (5 of 15), constipation (4 of 15), elevated serum lactate dehydrogenase (4 of 15), insomnia (3 of 15), dizziness (3 of 15), vomiting (3 of 15), back pain (3 of 15) and elevated aspartate aminotransferase (3 of 15). These adverse events are typical for this patient population receiving chemotherapy, and none occurred in an epoetin dose-dependent manner. Adverse events possibly associated with epoetin beta occurred in six patients, and these events were manageable. These adverse events consisted of grade 3 hypertension and vertigo, grade 2 increased bilirubin, constipation and hyperkalemia and grade 1 headache, nausea, vomiting, insomnia, diarrhea, mouth dryness, fatigue, neck pain, rash, hyperventilation, cardiomegaly, hyperkalemia, hyponatremia, increased phosphorus and increased asparatate aminotransferase. Only one patient in the 9000 IU cohort showed grade 3 hypertension from the 7th day of the first administration to the 65th day. One serious adverse event (rotary vertigo) occurred in a patient (a 31-year-old woman); it remitted after around 2 weeks and resolved after 5 weeks. This event was considered by the investigator to be related to epoetin beta, and the patient therefore discontinued the study. No antibodies to epoetin beta were detected.

DISCUSSION

Serum EPO levels are reported to be higher in cancer patients than in healthy adults (8). The results of this study were in accordance with this, showing higher baseline serum EPO concentrations in patients than in healthy adults (8.40 ± 3.82, 8.62 ± 5.83 mIU/ml) (9) or renal anemia patients (23.05 ± 16.63 mIU/ml) (10). In addition, serum EPO concentrations in cancer patients exhibited wide variation, from typical levels in healthy adults to extremely high levels. Overall, this suggests that the predose endogenous EPO exhibited high mean serum levels and wide individual differences in cancer patients with anemia.

In the present study, we have investigated the pharmacokinetic characteristics of epoetin beta after the initial dose of 9000, 18 000 and 36 000 IU and have studied the time-course