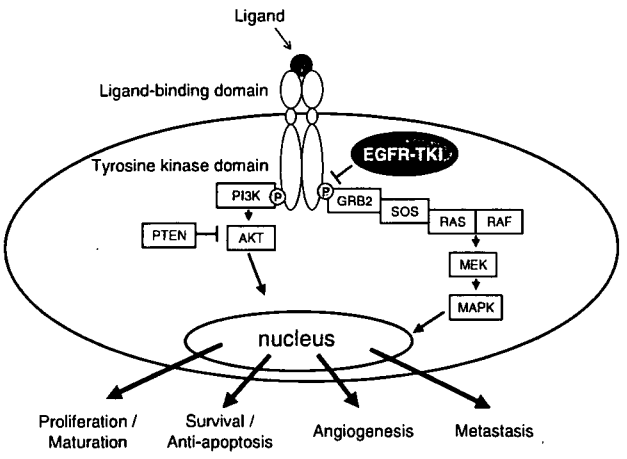

Figure 1 Epidermal growth factor receptor (EGFR) signal transduction, its biological consequences and EGFR tyrosine kinase inhibition (TKI).

the gefitinib and erlotinib will be discussed especially in terms of benefits and risks in clinical practice.

DEVELOPMENT

Two EGFR-TKIs, gefitinib and erlotinib, have been developed using similar procedures. Both drugs were well tolerated and had higher response rates in single agent phase I and phase II studies, but failed to indicate synergistic effects in phase III studies with combination of cytotoxic agents. Results of single agent phase III studies for patients with recurrent or resistant disease differed between the two drugs. Although erlotinib apparently prolonged survival, gefitinib did not.

Phase I trials

Four phase I trials of gefitinib were performed in 252 patients with a variety of solid tumours, including NSCLC, head and neck cancer and colorectal cancer.[9–12] Major adverse events were rash and diarrhoea. These events were generally mild and tolerable at doses not exceeding 600 mg/day and 700 mg/day came to be the maximal tolerated dose (MTD). Because doses of 150–800 mg/day were associated to tumour responses, 250 and 500 mg/day were selected for subsequent phase II trials. In the case of erlotinib, phase I trials identified a dose of 150 mg/day for further clinical development. Adverse events were similar to those of gefitinib and the incidence and severity of the adverse events generally increased as the dose increased.

Second-line phase II trials

Among single-agent phase II studies, one erlotinib and two gefitinib studies were conducted in patients with NSCLC. Two large scale multicentre phase II trials of gefitinib were performed; Iressa™ dose evaluation in advanced lung cancer (IDEAL)-1 in Japan, Europe and Australia,[13] and IDEAL-2 in North America.[14] Eligibility criteria of the IDEAL-1 included having failed only one prior platinum-containing regimen, whereas the IDEAL-2 criteria were to have failed a platinum-containing regimen and docetaxel. Patients were randomized to gefitinib 250 or 500 mg/day. In the IDEAL trials, response rates ranged from 9 to 19% and severe toxicities were relatively uncommon. Treatment related toxicities, that is, diarrhoea, rash, acne, dry skin, nausea and vomiting, were slightly more severe and more frequent on 500 mg/day than on 250 mg/day. Because no additional response was observed with 500 mg/day, gefitinib at a dose of 250 mg/day was approved in Japan and the USA for treatment of advanced NSCLC.

A randomized phase II study was also performed with erlotinib in patients with previously treated advanced NSCLC.[15] The study, a comparison with best supportive care, revealed 150 mg/day of the drug to produce a 12.3% of objective response rate in patients with previously treated advanced NSCLC. MST was 8.4 months and the 1-year survival rate was 40% with no grade 4 toxicity.

First-line combination phase III trials

In a preclinical study, EGFR-TKI showed an additive effect on antitumour activity with no toxicity increase when combined with cytotoxic agents.[16–18] On the basis of these data, four randomized trials were conducted with gefitinib; Iressa™ NSCLC trial assessing combination treatment (INTACT)-1, 2,[19,20] and with erlotinib; TALENT[21] and TRIBUTE,[22] in chemotherapy-naïve patients with advanced NSCLC to compare chemotherapy plus EGFR-TKIs to chemotherapy alone.

In the INTACT-1 and TALENT trials, the chemotherapy regimen consisted of cisplatin and gemcitabine. In the INTACT-2 and TRIBUTE trials, the chemotherapy regimen was carboplatin and paclitaxel. Unfortunately, none of those studies showed any definitive benefit of adding an EGFR-TKI to standard chemotherapy in patients with NSCLC. These trials failed to support the concept of synergistic preclinical studies and to show additive or synergistic effects when combined with platinum-based chemotherapy as a first-line treatment for NSCLC.

Second-line phase III trials

To investigate the survival benefit of EGFR-TKIs as single agents, two large scale placebo controlled phase III trials were conducted as second- or third-line treatment for the patients with NSCLC. Iressa™ survival evaluation in lung cancer (ISEL) trial was planned to compare gefitinib with a placebo with 1692 patients.[23] Although the results of the study showed a response in the gefitinib group, there was no survival prolongation effect with gefitinib. MST

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was 5.6 months in the gefitinib arm and 5.1 months in the placebo arm. On the other hand, the BR.21 trial investigated erlotinib as compared with a placebo.[24] Results of the trial included 731 patients showing a 9% of response rate in the erlotinib arm and less than 1% in the placebo arm. In terms of survival, 2-month prolongation of MST was achieved in the erlotinib arm, 6.7 months compared with 4.7 months in the placebo arm. Based on these data, erlotinib was approved in the USA and European countries.

Although study results were similar in phase I and phase II trials, only erlotinib, not gefitinib, produced a survival benefit compared with the placebo. The reason for the difference may be explained partly by the administered dose of gefitinib possibly being lower. In a phase I trial of gefitinib, a dose of 250 mg/day was less toxic and as effective as a dose of 500 mg/day but the MTD in the trial was 700 mg/day. On the other hand, a dose of 150 mg of erlotinib is nearly the MTD in a phase I trial. Another explanation is an issue of the difference in the response to previous treatment between participants in two trials. In terms of the best response, 18% of the patients in the gefitinib group had responded and 45% had progressed in the ISEL trial. By contrast, 38% of the patients in the erlotinib group had responded and 28% had progressed in BR.21 trial.

EGFR-TKI-INDUCED INTERSTITIAL LUNG DISEASE

Incidence

Although toxicities, like myelosuppression and vomiting, were not dose limiting for patients receiving gefitinib, a proportion of Japanese patients experienced ILD.[25–27] This type of adverse event has also been reported from Korea[28] and Taiwan.[29] In a large scale surveillance by the West Japan Thoracic Oncology Group (WJTOG), among 1976 patients, 70 patients (3.5%) were identified as having ILD after a panel review of 102 patients who were suspected by their physicians, and 31 patients (1.6%) who had died due to the event.[30] Another large scale post marketing surveillance conducted in Japan, an analysis involving 3322 patients, found that the incidence of ILD was 5.8%, and mortality due to ILD 2.3%.[31] Other smaller but detailed studies reported similar ratios of 5.4% and 4.5% for incidence, 3.6% and 2.4% for mortality.[32,33] Report of the incidence of ILD in first-line single agent treatment is limited. In a phase II study of chemo-naïve patients with advanced NSCLC, 4 of 37 patients (11%) died due to severe ILD.[34] In this study, only a CXR was required to exclude preceding interstitial pneumonia or pulmonary fibrosis.

A Korean report indicated two of 65 patients (3%) to have ILD.[28] Another study in Taiwan, of patients with brain metastases reported four of 76 patients (6%) to have experienced non-lethal ILD.[29] Regional differences seem to exist because relatively higher ratios of pulmonary involvement have only been reported in East Asian countries. The incidence of ILD is reportedly only 1.0–1.1% in the USA or Europe.[19,20,35,36] The ISEL study conducted in both Europe and Asia was the only study in which a difference in incidence between ethnicities could be compared directly. East and South-east Asian patients tend to suffer more ILD in 3–4% of the patients although the frequency of ILD in all population was 1%. However, there were no differences in incidence between patients receiving gefitinib versus a placebo.[23]

Risk factor

Several Japanese studies have reported risk factors for ILD. In the WJTOG surveillance, the presence of idiopathic pulmonary fibrosis (IPF), male gender and history of smoking were independent predictive factors for developing ILD.[30] In other studies, multivariate analysis revealed that preceding IPF, poor performance status (PS), smoking history, prior history of irradiation or chemotherapy were independent risk factors for ILD.[31–33,37] In these studies, the most striking factor was pre-existing IPF, with a higher odds ratio. Factors associated with a poor prognosis have been analysed. These included a short interval from initiation of gefitinib treatment to the onset of ILD, an acute interstitial pneumonia (AIP) pattern on CT, the presence of preceding IPF, male gender and a poor PS.[30–33,37](Table 1)

Diagnosis and patterns

Most patients with EGFR-TKI-induced ILD experienced symptoms such as coughing, increasing

Table 1 Factors associated to EGFR-TKI-induced interstitial lung disease

	Risk factors for ILD	Factors associated to poor prognosis after ILD
Definitive	Preceding IPF	
Possible	Male gender	AIP patterns
	History of cigarette smoking	Preceding IPF
	Poor PS	Early onset of ILD
	Prior chemotherapy	Male gender
	Japanese/East Asian ethnicity	Squamous cell carcinoma

AIP, acute interstitial pneumonia; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; PS, performance status.

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Table 2 Predictive markers for response to EGFR-TKI

	Clinical	Molecular
Definitive	Female gender adenocarcinoma Lack of smoking history Japanese/East Asian	Positive EGFR mutation
Possible	Better PS No preceding IPF Skin eruption as adverse event	Increased EGFR gene copy number Positive p-Akt expression Negative K-ras mutation

EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; IPF, idiopathic pulmonary fibrosis; PS, performance status.

dyspnoea and fever. It is difficult to diagnose ILD and differentiate it from other respiratory conditions that produce similar symptoms, such as infections, and cancer progression. According to the surveillance, suspicion of ILD by the attending physician was refuted by an expert panel consisting of pulmonologists and radiologists in 15.7–31.4% of patients.[30,31,33,37] These findings indicate that a diagnosis of ILD might not always have been correct in other reports.

As well as other pulmonary infiltrative disease, it must be emphasized that accurate diagnosis before the start of the treatment is necessary. When any sign or symptom or CXR abnormality appears, CT, especially high-resolution CT (HRCT), is recommended to diagnose interstitial shadows on the CXR. Screening for respiratory tract infection is also essential, including culture and polymerase chain reaction examination for pneumocystis carinii and aspergillus, for example. Transbronchial biopsy or BAL may contribute to making a correct diagnosis, and may be the key to the mechanism of EGFR-TKI-induced ILD.

Some reports have tried to classify radiological patterns and clinical course.[31,33,36,38] There seem to be four patterns. About a half of the ILD patients showed non-specific ground-glass attenuation on CT or HRCT without lung volume loss on CXR.[33,36,38] This group and two other small groups, including cryptogenic organizing pneumonia-like pattern and acute eosinophilic pneumonia pattern, seemed to correlate with a fair prognosis or better response to steroid therapy.[38] The remaining one third of the patients showed AIP-like pattern with extensive bilateral ground-glass attenuation or airspace consolidation with traction bronchiectasis on CT and lung volume loss on CXR. The prognosis of AIP-like pattern patients were very poor with 75–100% dying early.[33,38] In some patients, the histopathology at autopsy revealed diffuse alveolar damage.[25,31] These findings may support the EGFR-TKI-induced ILD hypothesis that EGFR inhibitor suppresses lung injury repair and results in irreversible alveolar damage.

PROGNOSTIC FACTORS

According to the trials and surveillance of EGFR-TKIs used as first-line treatments or for refractory cases, as single agents or combined with other therapies, several factors have been proposed to predict response, long-term prognosis, or risk for ILD.

In summary, female gender, adenocarcinoma, lack of smoking history, being Japanese or another East Asian ethnic group are considered to be clinical factors predicting response. In addition, better PS and lack of preceding pulmonary fibrosis and skin rash while taking the drug could be predictive factors.[13,14,30,32,39] (Table 2)

Based on molecular analysis, being positive for mutations in EGFR exons 18–24, which encode the kinase domain of the protein, strongly predict response to EGFR-TKI, especially in the Asian population.[40–46] Other molecular factors include an increased EGFR gene copy number,[47–49] p-Akt expression[28,50] and lack of K-ras mutation,[51,52] but are still controversial. To clarify whether being positive for EGFR mutations correlates with better survival, some prospective studies are now underway.

In addition to these molecular factors associated with primary response or resistance, a second mutation T790M in exon 20 is reported in acquired resistance to EGFR-TKI.[53,54]

A numbers of research reports and practical experience from Asian countries support the favourable 'benefit to risk' balance of treating NSCLC patients. However, these results still lack survival advantages, and there is the problem of ILD, which appears to limit the use in patients with advanced NSCLC in taking EGFR-TKIs, even as second- or third-line treatments. It is necessary to assess benefit and risk individually before prescribing the drug and to give the patients adequate information to make an informed decision. Even in East Asian countries, gefitinib should be used only in clinical trials or for well-assessed patients.

SUMMARY

It remains unclear why gefitinib appears to produce a greater response in Asian patients than in patients from the rest of the world. Now, several genetic studies are starting to provide clarification of the mechanism underlying the differences in response and adverse events between ethnicities.

The benefits of using EGFR-TKI as the first-line treatment for NSCLC also remain unclear. There are

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no results confirming the superiority of EGFR-TKI even showing clinical or genetic prediction of a better response, because these trials failed to show better survival over standard combination regimens including platinum agents.

We continue to await the results of research which will demonstrate clinical benefits in terms of survival even in selected patients, and which may help us to identify patients who are most likely to benefit from treatment with EGFR-TKI. Such results would tell us when and to whom we should prescribe the best drug to treat NSCLC.

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Gefitinib Efficacy Associated with Multiple Expression of HER Family in Non-small Cell Lung Cancer

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Abstract. *The aim of this study was to compare the relationship between HER family expression and clinical response to gefitinib. Patients and Methods: Tissues from thirty-one non-small cell lung cancer (NSCLC) patients treated with a monotherapy of gefitinib were analyzed. Expressions of HER family in 31 tumors were examined by immunohistochemistry. Results: The total expressions were 21 for EGFR (68%), 24 for HER2 (77%), 17 for HER3 (55%) and 4 for HER4 (13%). Fourteen out of 31 (45%) demonstrated triple expression of EGFR and HER2, as well as HER3 or HER4. A significantly better response rate (RR) and time to progression (TTP) were observed for the group with the triple expression than for the other groups (RR 50 vs. 11%; $p<0.05$, median TTP 4.29 vs. 1.2 months; $p<0.05$). Conclusion: Mutliple expression of the HER family might be related with the clinical response to gefitinib and EGFR mutation status.*

The epidermal growth factor receptor (EGFR/HER1) is a promising target for anticancer therapy. Gefitinib (ZD 1839, Iressa; AstraZeneca, London, UK) is an orally active, selective EGFR-tyrosine kinase inhibitor (1), which showed promise in a recent clinical trial of non-small cell cancer (NSCLC) cases, in terms of rapid symptom improvements (2-7) and clinically meaningful benefit in some patients (5, 7). Thus, the selection of individuals who may demonstrate a response to gefitinib is important. The degree of EGFR expression seems not to directly determine the response, although gefitinib is considered to be a targeted therapy by virtue of its selective inhibition of EGFR tyrosine kinase (8-10). Recent reports show that specific missense and deletion mutations in the tyrosine kinase domain of the EGFR gene (11-13) are associated with EGFR tyrosine kinase inhibitor sensitivity. Although these EGFR mutations can account for almost all objective responses to tyrosine kinase inhibitors, the clinical benefit observed with these drugs and the survival benefit cannot be explained only by the presence of mutations.

The HER family includes the following four distinct receptors: EGFR, HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB-4) and recent preclinical studies indicated that gefitinib causes reduced phosphorylation levels of not only EGFR, but also of HER2 and HER3 (14), inducing the formation of inactive EGFR/HER2 and EGFR/HER3 heterodimers (15). Based on these data, the co-expression profile of HER family receptors (especially the additional expression of HER2 and HER3) was hypothesized to play an important role in determining the efficacy of gefitinib in NSCLC cases. The relationship between the co-expression status of HER family members and gefitinib efficacy was evaluated with regard to response rate (RR), time to progression (TTP) and overall survival (OS).

Patients and Methods

Between September 2002 and January 2004, 31 advanced or recurrent NSCLC patients from whom tumor tissues were available, were treated with 250 mg of gefitinib monotherapy until disease progression at our institution. The medical records, pathology slides and imaging studies of these patients were retrospectively reviewed. The study was conducted after obtaining approval of the appropriate ethical review boards following recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

For all patients, archival paraffin blocks of transbrochial lung biopsy (TBLB) specimens taken at the time of initial diagnosis (n=14) or tumor tissue specimens obtained by surgical resection (n=17) were sectioned for staining with antibodies against EGFR, HER2, HER3 and HER4 using an EGFR pharmDx kit (DAKO), Hereceptest (DAKO), anti-HER3 (Chemicon) and anti-HER4 (Chemicon), respectively, with the Autostainer (DAKO).

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Key Words: NSCLC, gefitinib, HER2, tyrosine kinase inhibitor, molecular target agent, EGFR mutation.

The results of the immunostaining were reviewed by an experienced pathologist (Y.I.). For HER2 staining, only moderate to strong cell membrane-specific immunostaining was considered positive, whereas cytoplamic staining was also taken into account for the other receptors.

Genetic analysis of the *EGFR* gene was performed on the 17 frozen tumor specimens obtained by surgical resection. Genomic DNA was extracted from 1-2 mm^3 tumor specimens using REDExtract-N-AmpTM Tissue PCR Kit (Sigma) and the DNA was purified with a QIAmpDNA blood mini kit.

Genetic analysis of the *EGFR* gene was performed by PCR amplification of exons 18, 19 and 21. The following primers, specifically designed for this study, were used for PCR amplification: exon 18 (forward, 5'-AGGTGACCCTTGTCTCTGTGTTCT-3'; reverse, 3'-CACCSGACCATGAGAGGCCCTGCG-5'), exon 19 (forward, 5'-GA TCACTGGGCAGCATGTGGCACC-3'; reverse, 3'- TGGACCCCCAC ACAGCAAAGCAAAGCAGA-5'), exon 21 (forward, 5'-TTCCCATG ATGATCTGTC-3'; reverse, 3'-ATGCTGGCTGACCTAAA -5'). PCR was performed in a total volume of 20 μL, containing reaction buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 100 nmol/L each primer, 0.5 units AmpliTaq (Biosystems) and 4 μL genomic DNA. Thermal cycling conditions included 3 min at 94°C, followed by 35 cycles of 94°C for 20 sec, 68°C for 40 sec and 72°C for 3 min.

After completion of the PCR reaction, the products were denatured (5 min at 90°C), immediately cooled on ice and loaded onto a nondenaturing polyacrylamide gel. The concentration of acrylamide was 1%-14% gradient. Electrophoresis was carried out for 3 h at 0°C at 72V/cm. The shifted bands were removed from the gel, and the recovered DNA was amplified in duplicate and subjected to bidirectional dye-terminator sequencing using the same primers used for amplification. Sequencing fragments were detected by capillary electrophoresis. SSCP and sequencing were performed by Hitachi Hitechnology Co. (Tokyo, Japan).

All 31 patients were evaluated for responses to gefitinib using WHO criteria (16). TTP and OS were measured from the date of initial gefitinib treatment to the date of disease progression and to the date of death or last follow-up examination, respectively, and were estimated using the Kaplan-Meier method (17).

Since recent clinical studies suggested that females with no smoking history and an adenocarcinoma were positive predictors for gefitinib responses (5, 7, 18), these were evaluated as potential prognostic factors for gefitinib sensitivity. Age, gender, performance status, histology type, number of prior chemotherapy, prior platinum or docetaxel use, smoking history and HER family-expression were analyzed using the Fisher's exact test and the Students *t*-test. Differences in TTP and OS between the two groups were tested using the log-rank test. All statistical analyses were performed using SPSS Version 8 statistical software (SPSS, Inc., IL, USA).

Results

Patients characterization. Of the 31 patients, eleven (35%) were females and 74% of the patients had adenocarcinomas. Eleven patients (35%) had never smoked (Table I). The median age was 62 years (range, 51 to 77 years). Eight patients (25%) had no prior chemotherapy and the remainder had received platinum-based chemotherapy (Table I).

Immunohistochmical staining of HER family in tumors. Total positive staining included 21 for EGFR (68%), 24 for HER2

Table I. *Patient characteristics.*

Variable	No. of patients	%
Portial response		
yes	9	29
no	22	71
Gender		
male	20	65
female	11	35
Age, years		
median	62	
range	51 - 77	
Smoking history		
never	11	35
former/current	20	65
Histology		
adenocarcinoma	23	74
non-adenocarcinoma	8	25
Stage		
III	5	16
IV	12	39
recurrence after surgery	14	45
Performance status		
0,1	18	58
>2	13	42
No. of prior chemotherapy regimens		
0	8	25
1	14	45
2	7	23
3	2	6
Prior platinum		
yes	23	74
no	8	26
Prior docetaxel		
yes	7	23
no	24	77

Table II. *EGFR/HER2/HER3/HER4 expression status.*

Status	No. of patients	%
EGFR		
negative	10	32
positive	21	68
HER2		
negative	7	23
positive	24	77
HER3		
negative	14	45
positive	17	55
HER4		
negative	27	87
positive	4	13
Co-expesion		
no expression of EGFR	10	32
mono-expression of EGFR	4	13
double-expression of EGFR/HER2	3	10
triple-expression of EGFR/ HER2/HER3 or HER4 +	14	45

(77%), 17 for HER3 (55%) and 4 for HER4 (13%) (Table II). Fourteen out of 31 (45%) samples demonstrated triple expression of HER family members (13 were positive for EGFR/HER2/HER3 and 1 for EGFR/HER2/HER4). Three (10%) double-expressed EGFR /HER2, 4 (13%) mono-expressed EGFR and 10 (32%) exhibited no expression of EGFR (Table II).

Clinical response to therapy. The response to treatment was evaluated in all 31 patients. Nine partial responses (PR; 29%) were observed. The results of univariate analysis of the significance of potential prognostic factors for gefitinib sensitivity using Fisher's exact test and a *t*-test for age are provided in Table III. An objective response was observed in 6 out of 11 females and in 3 out of 20 males ($p<0.05$), 6 out of 11 non-smokers and 3 of 20 current or former smokers ($p<0.05$), 8 out of 23 adenocarcinoma and 1 out of 8 non-adenocarcinoma ($p=0.37$) cases (Table III).

No correlation was found between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2 /HER3 or HER4 and the remainder (50% *vs.* 11%, $p=0.043$) (Table III). There was also a significant difference in TTP between these groups (TTP; 4.3 *vs.* 1.2 months, $p=0.0449$, Figure 1A). Median OS time of the group with triple expression was longer time than that of the others remainder, but was not significant (15.3 *vs.* 6.7 months, $p=0.099$, Figure 1B).

EGFR mutations. The genomic status of the TK domain of the *EGFR* gene was evaluated in 17 frozen primary NSCLC tumor specimens. Exons 18, 19 and 21 were subjected to mutational analysis. PCR amplification followed by SSCP analysis was used since SSCP analysis is more sensitive than direct sequencing (19). A total of 6 of the shifted bands were found and were directly sequenced. Four mutations were located in exon 19, 1 in exon 21, and 1 in exon 18. Of the 6 mutations identified, 4 were in frame deletions in exon 19, and 2 were aminoacidic substitutions in exons 21 and 18. The deletions "E746-A750 del", "L747-S752" and "L747-S752 del, P753S" in exon 19, the leucine to arginine mutation (L858R) in exon 21 and "E709D, T710del" in exon 18 were found.

EGFR mutations were more frequently found in adenocarcinomas than non-adenocarcinomas (6 out of 12 adenocarcinomas and 0 out of 5 non-adenocarcinoma;). There were no obvious differences in *EGFR* mutation status with gender or smoking history (3/6 females and 3/11 males; 3/5 non-smokers and 3/12 current or former smokers). The clinical responses to gefitinb in 6 cases with *EGFR* mutations were 3 partial response (PR) and 3 stable disease. No PR was observed in the 11 cases without *EGFR* mutations.

In addition, all 6 cases with *EGFR* mutations showed triple expression of EGFR/HER2/HER3 or HER4 and no *EGFR* mutation was detected in non-triple expression cases; $p=0.035$.

Table III. *Univariate analysis of features associated with sensitivity to genitinib.*

Variable	Response (n=9)	No response (n=22)	*P*
	No. of patients	No. of patients	
Gender			
male	3	17	0.037
female	6	5	
Age, years			
median	62	63	0.97
range	53 - 74	51 - 77	
Histology			
adenocarcinoma	8	15	0.37
non-adenocarcinoma	1	7	
No. of prior chemotherapy regimens			
0	1	7	0.37
>1	8	15	
Prior platinum			
yes	8	15	0.38
no	1	7	
Prior docetaxel			
yes	1	6	0.64
no	8	16	
Performance status			
0,1	5	13	0.99
>2	4	9	
Smoking history			
never	6	5	0.037
former/current	3	17	
HER family expression status			
EGFR			
negative	1	9	
positive	8	13	0.21
HER2			
negative	1	6	0.64
positive	8	16	
HER3			
negative	3	11	0.46
positive	6	11	
HER4			
negative	7	20	0.56
positive	2	2	
Co-expression of HER family			
triple expression of EGFR/ HER2 /HER3 or HER4+	7	7	0.043
Other	2	15	

Discussion

We studied the correlation between HER family expression status and sensitivity to gefitinib monotherapy in patients with advanced NSCLC and we showed that sensitivity to gefitinib is related to the triple expression of EGFR, HER2 and HER3 or HER4.

Although inhibition of EGFR tyrosine kinase is considered an essential mode of action of gefitinib, previous studies

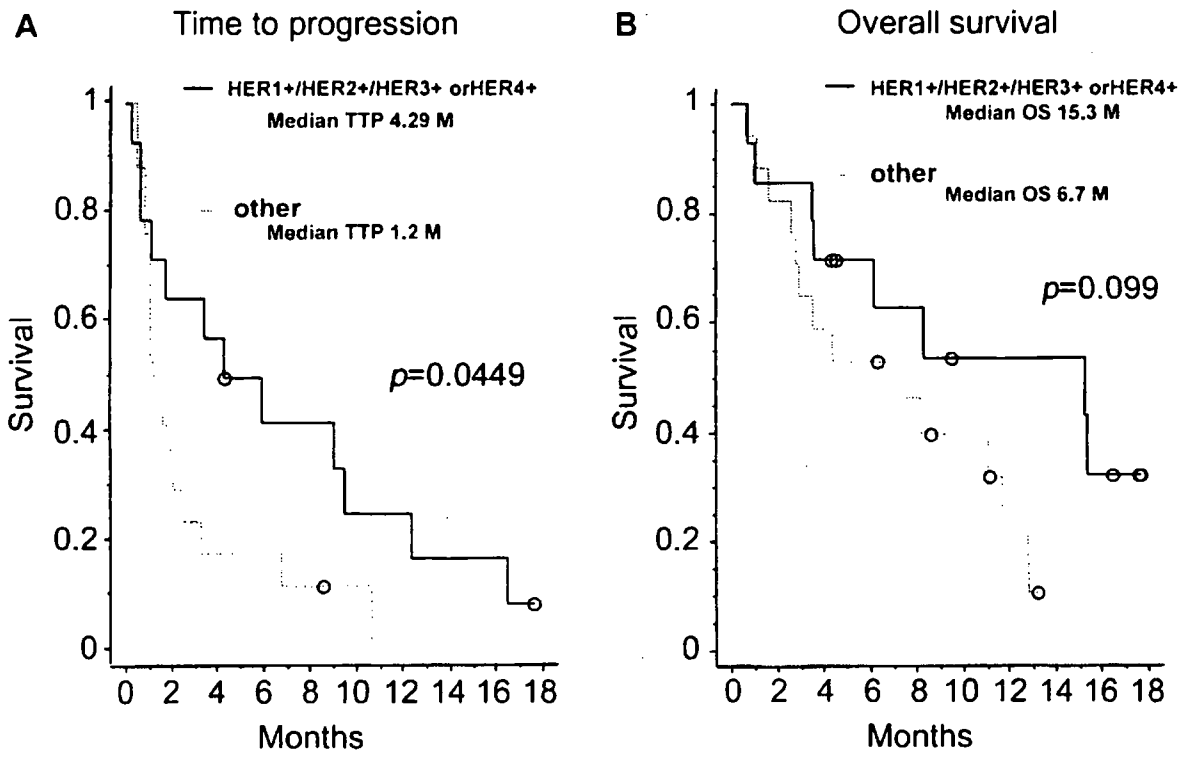

Figure 1. *(A) Time to progression (TTP) and triple expression of HER family (EGFR1+/HER2+/HER3+ or /HER4+); (B) Overall survival (OS) and triple expression of HER family (EGFR+/HER2+/HER3+ or /HER4+).*

indicated that expression of EGFR does not itself determine sensitivity to gefitinib therapy (8-10, 14, 20, 21). We also found no correlation between the EGFR-expression status and gefitinib efficacy. However, there was a significant difference in the gefitinib response between the group with triple expression of EGFR/HER2/HER3 or HER4 and the remainder (50% *vs.* 11%, $p=0.0439$). There was also significant difference in TTP between these groups (4.3 *vs.* 1.2 months, respectively; $p=0.0449$). The results are, thus, in line with those of several recent studies which indicated that all of the HER family members are targeted by gefitinib. Preclinical studies indicate that heterodimer formation is a factor impacting on sensitivity. Gefitinib causes reduced basal phosphorylation of EGFR/HER2, EGFR/HER3 and HER2/HER3 and this might correlate with the antitumor activity of this agent (14, 15, 22).

Cappuzzo *et al.* reported no correlation between co-expression of EGFR and HER2 in NSCLC patients and the results of treatment with gefitinib with regard to RR, TTP and OS (8). However, these authors did not evaluate the expressions of HER3 and HER4, and HER3 positive rate which is relatively high (>50%) in this study. This might be critical for sensitivity to gefitinib. In addition, the RR in our study was relatively high compared with their value (29% *vs.* 15.9%, respectively) and an ethnic difference may account for this difference.

Sensitivity to gefitinib appears to be greatly influenced by the presence of activating mutations within the kinase domains (11-13) and the mutations were more frequent in tumors from Japanese and other East Asian patients (23-25). *EGFR* gene mutations in exons 18, 19, 21 were analyzed by SSCP in this study and a comparable frequency of *EGFR* mutations was detected by the SSCP methods in a previous report (6 out of 17 cases; 35%). *EGFR* mutations were detected in cases with triple expression of EGFR/HER2 /HER3 or HER4, but no mutation was found in cases without triple expression of HER family receptors. Although these *EGFR* mutations can account for almost all objective responses obtained with gefitinib, the clinical benefits, such as long stable disease, cannot be explained only by the presence of mutations.

Recently, Hirata *et al.* reported that NSCLC cells trasfected with the *HER2* gene (LK2/HER2) were approximately 5-fold more sensitive to gefitinib than LK2/mock cells and cell survival and death were dependent on HER2/HER3 signaling. However, the sensitivity was about 20-fold lower in the LK2/HER2 cells than in the PC9 cells, which harbor in-frame deletion mutation of *EGFR* (E746-A750) in exon 19 (22). These results support our findings that multiple expressions of HER family members may contribute to gefitinib efficacy and multiple expressions of HER family members may play more important roles in cases without *EGFR* mutations than in case with mutations of this gene.

This small pilot study is not enough to conclude that triple expression of HER family members is strong predictive factor for response to gefitinb. Further large-scale prospective trials are necessary to confirm these results.

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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,

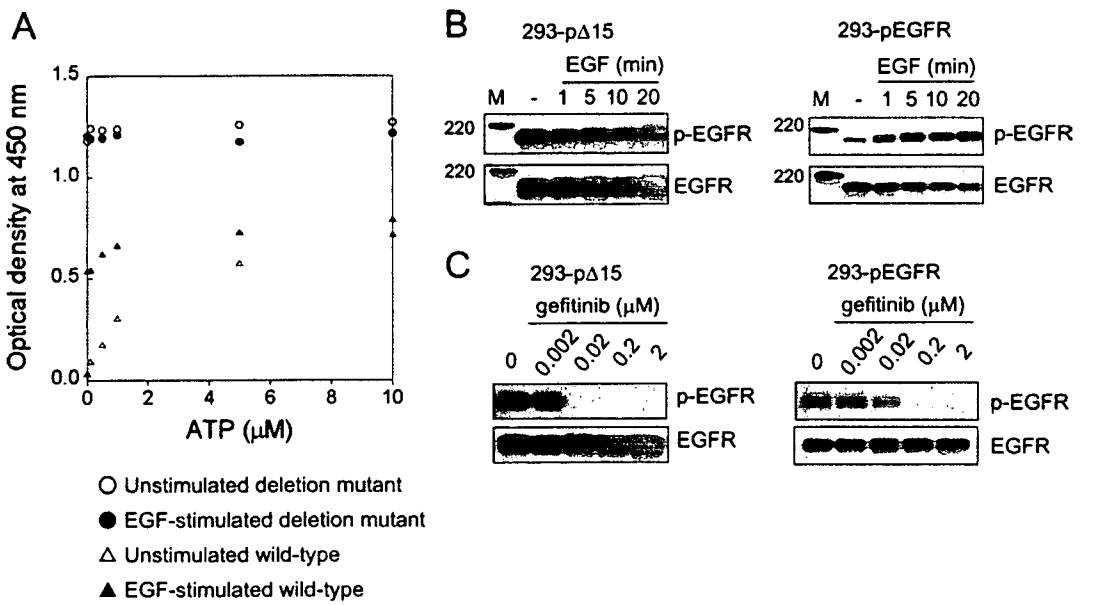

Figure 1 Autophosphorylation reactions of deletion mutant EGFR and wild-type EGFR

(**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 (O) 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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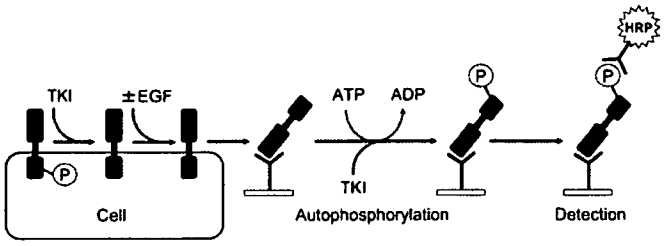

Figure 2 Schematic illustration of the cell-based autophosphorylation assay

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

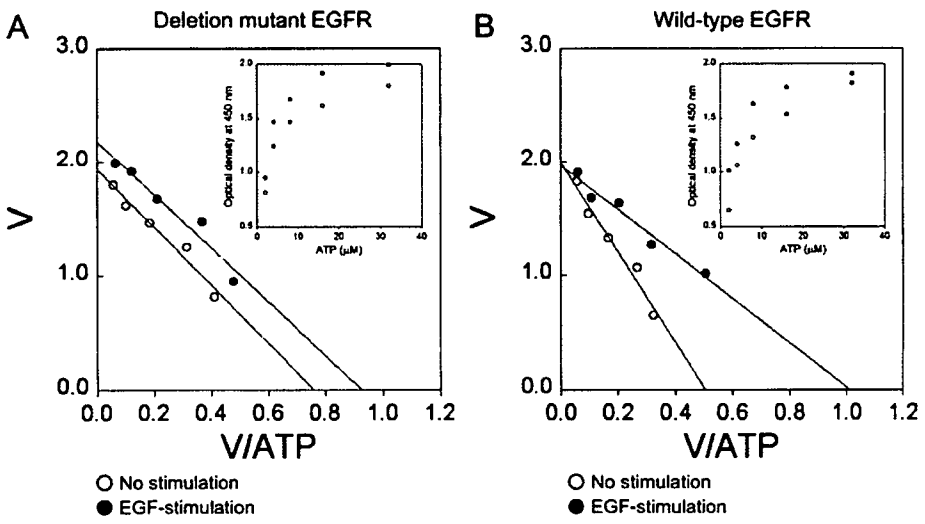

Figure 3 Autophosphorylation activities of deletion mutant EGFR and wild-type EGFR

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 (O) 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

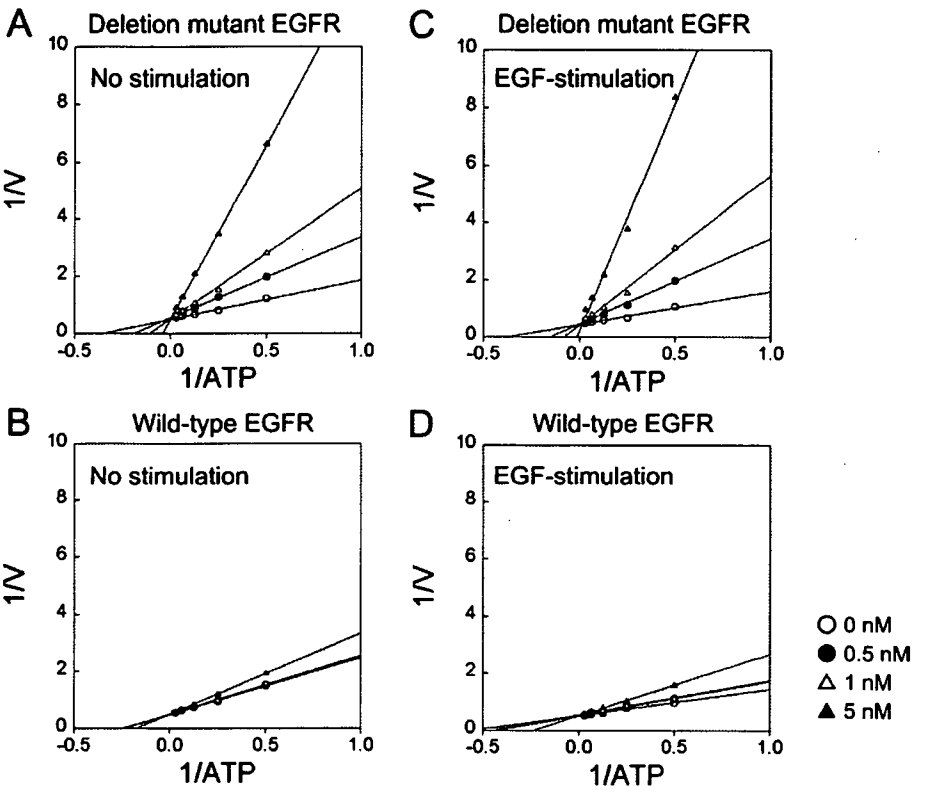

Figure 4 Mechanism of inhibition of deletion mutant EGFR by gefitinib

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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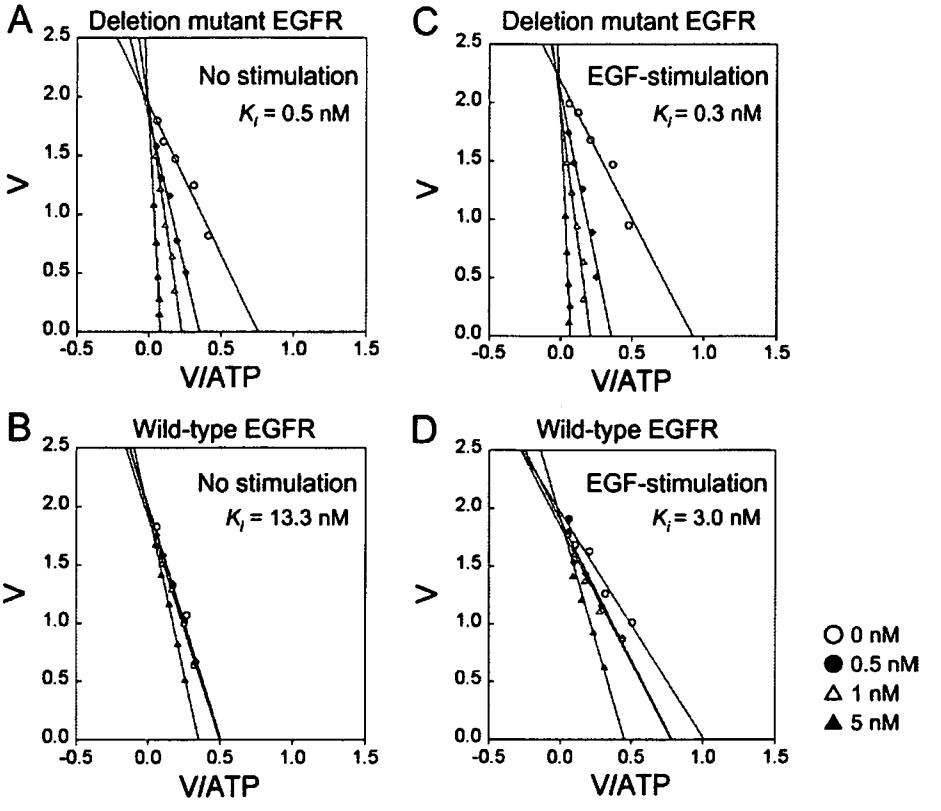

Figure 5 Inhibition constant of gefitinib for autophosphorylation activity of deletion mutant EGFR

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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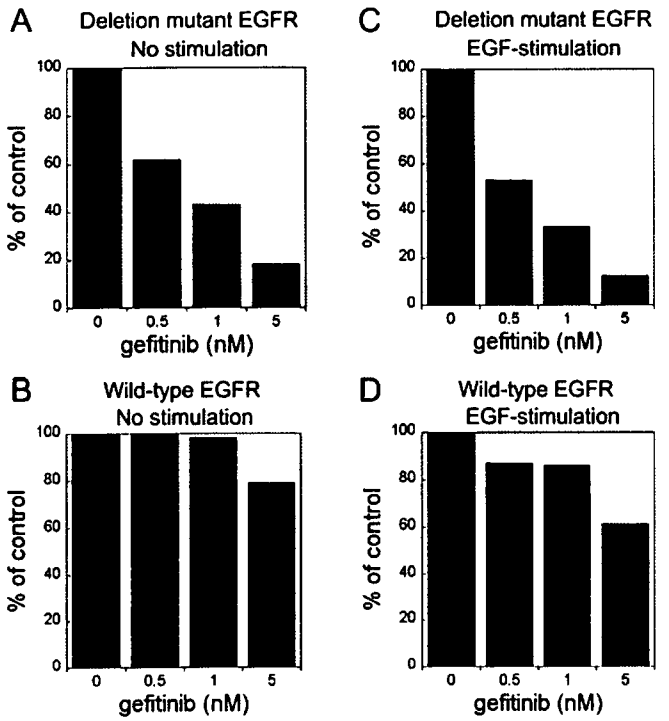

Figure 6 Effects of gefitinib on autophosphorylation of deletion mutant EGFR

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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A Photon Counting Technique for Quantitatively Evaluating Progression of Peritoneal Tumor Dissemination

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Abstract

We recently established a mouse model of peritoneal dissemination of human gastric carcinoma, including the formation of ascites, by orthotopic transplantation of cultured gastric carcinoma cells. To clarify the processes of expansion of the tumors in this model, nude mice were sacrificed and autopsied at different points of time after the orthotopic transplantation of the cancer cells for macroscopic and histopathologic examination of the tumors. The cancer cells grew actively in the gastric submucosa and invaded the deeper layers to reach the serosal plane. The tumor cells then underwent exfoliation and became free followed by the formation of metastatic lesions initially in the greater omentum and subsequent colonization and proliferation of the tumors on the peritoneum. Although this model allowed the detection of even minute metastases, it was not satisfactory from the viewpoint of quantitative and objective evaluation. To resolve these problems, we introduced a luciferase gene into this tumor cell line with a high metastasizing potential and carried out *in vivo* photon counting analysis. This photon counting technique was found to allow objective and quantitative evaluation of the progression of peritoneal dissemination on a real-time basis. This animal metastatic model is useful for monitoring the responses of tumors to anticancer agents. (Cancer Res 2006; 66(15): 7532-9)

Introduction

Tumor dissemination and ascites are the two major features of cancerous peritonitis. Of the various manifestations of the progression of cancer affecting the i.p. organs (gastric, hepatic, ovarian, and other cancers), cancerous peritonitis is the most closely associated with poor operative results (1–6). In particular, scirrhous gastric cancer (diffusely infiltrative carcinoma or Borrmann's type IV carcinoma or the linitis plastica type) is a high-grade gastric cancer that is difficult to detect in the early stages and is often complicated by peritoneal dissemination (7–9). Although peritoneal dissemination is an important subject, very few experimental studies have been conducted to characterize its occurrence. In general, most of the experimental models of peritoneal dissemination from gastric cancer established to date have involved direct i.p. implantation of cancer cells (10–12). Although these conventional models may allow limited examination of the later stages of peritoneal dissemination, they cannot be expected to allow reasonable evaluation of its early stages. It is well known that implanting human tumor fragments and tumor cells orthotopically into the corresponding organs of nude mice results in much higher metastatic rates (13, 14). However, only one orthotopic implantation model, scirrhous carcinoma of the stomach, has been reported (15). We recently established two scirrhous gastric carcinoma-derived tumor cell lines capable of spontaneous metastasis following ectopic implantation (16). We repeated cycles of orthotopic transplantation of these tumor cell lines, collected cancer cells from the ascitic fluid formed as a result of cancerous peritonitis, and used the collected cells for further cycles of orthotopic transplantation. In this way, we isolated cell lines (44As3, 58As1, and 58As9) with high metastasizing potential and stable metastatic characteristics (17). When these cells were implanted orthotopically into the animals, bloody ascites formed within 3 to 5 weeks, resulting in the death of the animals.

As stated above, conventionally, progression of peritoneal dissemination has been analyzed by implanting cancer cells directly into the peritoneal cavity followed by sacrifice and autopsy of the animals at certain points of time after implantation and, finally, measurement of the number and weight of the tumor nodules in the sacrificed animals (18–20). Evaluation of the efficacy of anticancer agents was also hampered by this limitation (21–25). Evaluation using these methods may be affected by subjective factors and, therefore, unsatisfactory from the viewpoint of quantitative or objective evaluation. In order for our animal model of peritoneal dissemination to be applied universally as a drug evaluation system, we needed to establish a method for quantitative observation and objective evaluation of the relevant variables.

Recent progress in the optical imaging of cancers in animal models presents many potential advantages for recreating the disease process, disease detection, screening, diagnosis, drug development, and treatment evaluation. Fluorescence-based imaging (26–35) and photon counting analysis (36–43) modalities are well developed and allow specific, highly sensitive and quantitative measurements of a wide range of tumor-related variables in mice. Herein, we have shown that photon counting technique is an effective technology in living mice.

Materials and Methods

Established highly metastatic cell lines and culture. 44As3, highly peritoneal metastatic cell line, and parent HSC-44PE, human scirrhous gastric carcinoma-derived cell line, were previously reported (16, 17). When the subclones isolated by repeated s.c. injection of HSC-44PE cells were implanted orthotopically, they spread to the greater omentum, mesenterium, etc. and caused the formation of bloody ascites in a few animals (16). We repeated cycles of isolation of ascitic tumor cells and orthotopic inoculation of these cells, in turn, into animals to isolate highly metastatic

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44As3 cell lines, having a strong capability of inducing the formation of ascites (17).

The cell lines were maintained in RPMI 1640 supplemented with 10% FCS (Sigma Chemical, St. Louis, MO), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Immuno-Biological Laboratories, Takasaki, Japan) in a 5% CO_2 and 95% air atmosphere at 37°C (17).

***In vivo* photon counting analysis.** 44As3 and HSC-44PE cells were transfected with a complex of 4 μg pEGF-PLuc plasmid DNA (Clontech, Palo Alto, CA) and 24 μL GeneJammer reagent (Stratagene, Cloning Systems, La Jolla, CA) in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (400 μg/mL; Invitrogen, Carlsbad, CA), and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system (Xenogen, Alameda, CA). Clones expressing the luciferase gene were named 44As3Luc and HSC44Luc.

Orthotopic implantation of 1×10^6 44As3Luc and HSC44Luc cells was conducted in 6-week-old female BALB/c-*nu/nu* mice (day 0) as described previously (17). *In vivo* photon counting analysis was conducted on a cryogenically cooled IVIS system using Living Image acquisition and analysis software (Xenogen) as described previously (39).

Animal protocols were approved by the committee for Ethics of Animal Experimentation and were in accordance with the Guideline for Animal Experiments in the National Cancer Center. Mice were purchased from CLEA Japan (Tokyo, Japan). The mice were maintained under specific pathogen-free conditions and provided with sterile food, water, and cages. Ambient light was controlled to provide regular cycles of 12 hours of light and 12 hours of darkness.

Therapeutic study with irinotecan (CPT-11). The experimental mice were divided into a control group that received vehicle alone (saline) and experimental groups that received i.v. inoculation of 200 mg/kg/mouse of CPT-11, a clinically active topoisomerase I inhibitor, a level that has been reported to be highly effective in tumor growth (17). On days 3, 7, and 11, tumor-bearing mice received an i.v. injection of CPT-11. The additional injection of CPT-11 was done on days 28, 31, and 35. CPT-11 was purchased from Yakult Honsha (Tokyo, Japan) and dissolved in saline before being injected.

Statistical analysis. All data were analyzed by using the unpaired *t* test and expressed as the mean ± SE. A $P < 0.05$ was considered statistically significant.

Results

Animal model of peritoneal dissemination. The highly metastatic peritoneal cell line used in this study (44As3) was isolated by repeated cycles of orthotopic implantation of HSC-44PE cells and collection of the ascitic tumor cells as described in Materials and Methods (16, 17). As shown in Table 1 and Fig. 1, the tumor formed by this cell line was characterized by a propensity for early peritoneal dissemination and was frequently associated with the formation of ascites and the animals became moribund ~35 days after implantation. On the other hand, the graft cell survival after implantation of the parent cell line (HSC-44PE) was 67% and moribund animals were not seen until ~90 days after implantation, although no ascites formation was observed.

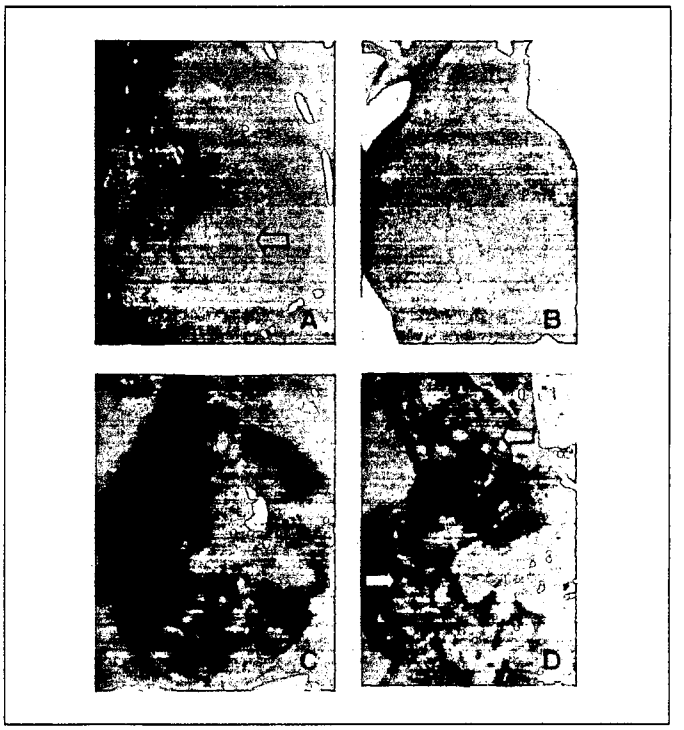

Figure 1. Macroscopic appearance of the peritoneal disseminations after orthotopic implantation of 44As3 cells. *A, green arrow,* orthotopic implantation of the cells in the stomach of nude mice was followed by tumor formation 3 weeks later. *B* and *C,* carcinomatous peritonitis was observed 5 weeks after orthotopic implantation of the cells. Abdominal distension because of bloody ascites was evident. *D,* peritoneal dissemination was recognized from the innumerable whitish nodules visualized in the abdominal cavity, mesenterium (*yellow arrow*), omentum, parietal peritoneum, and diaphragm (*green arrow*).

Anatomic, histopathologic, and ultrastructural analysis of the progression of peritoneal dissemination. To analyze the process of progression of peritoneal dissemination, 44As3 cells (1×10^6) were implanted orthotopically into the gastric wall of nude mice. Every 7 days after transplantation, five animals were

Table 1. Comparison of the survival and metastatic behavior of animals following orthotopic implantation of the highly metastatic and the parent cell lines

Cell line	Survival days	Tumor formation*	Ascites†	Disseminated metastasis				Lymph node	Liver	Pancreas‡	Kidney‡
				Omentum	Mesenterium	Peritoneum	Diaphragm				
44As3	35 ± 15 (22-65)	15/15 (100%)	14/15 (93%)	15/15	15/15	15/15	9/15	15/15	10/15	6/15	1/15
HSC-44PE	135 ± 48 (90-200)	10/15 (67%)	0/10 (0%)	5/10	3/10	3/10	0/10	5/10	0/10	0/10	0/10

*Mice were sacrificed 200 days after the orthotopic implantation. Data are the number of mice bearing metastases at the site/total number of mice bearing tumor.
†Ascites formation: >0.5 mL of ascitic fluid.
‡Micrometastases.

Table 2. Detection of metastasis and peritoneal dissemination after the orthotopic implantation of 44As3 cells

Days	Stomach	Ascites*	Disseminated metastasis				Lymph node	Liver	Pancreas†	Kidney†
			Omentum	Mesenterium	Peritoneum	Diaphragm				
7	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
14	5/5	0/5	3/5	0/5	0/5	0/5	1/5†	0/5	1/5	0/5
21	5/5	1/5	5/5	3/5	3/5	0/5	2/5	1/5	1/5	0/5
28	5/5	3/5	5/5	5/5	5/5	2/5	5/5	1/5	2/5	0/5
35	5/5	5/5	5/5	5/5	5/5	3/5	5/5	2/5	2/5	1/5

*Ascites formation: >0.5 mL of ascitic fluid.
†Micrometastases.

sacrificed and subjected to postmortem examination for macroscopic, histopathologic, and ultrastructural analyses (Table 2; Fig. 2). The metastatic cells (44As3) proliferated actively in the submucous tissue of the stomach (Fig. 2*A*) and began to infiltrate in the lymphatics on the 7th day. During the 2nd week following transplantation, the tumor grew more rapidly within the gastric wall, with invasion of the muscularis propria and the subserosal tissue (Fig. 2*B*). In some mice showing rapid growth of the tumor, the cancer cells broke through the serosa to become exfoliated and freed (Fig. 2*C*). These exfoliated and freed cancer cells could be visualized under the scanning electron microscope (Fig. 2*D* and *E*). Peritoneal dissemination began to be noted in the 2nd week, with cells on the greater omentum (Table 2). Micrometastases to the lymph nodes and pancreas were also noted, although not frequently. By the 3rd week, the foci of metastasis were noted in the greater omentum, mesenterium, and peritoneum. Scanning electron microscopy revealed the proliferation of the cancer cells (e.g., those colonizing the mesenterium) with the formation of larger cell clusters (data not shown). In the peritoneum, colonization of the freed cancer cells and their interaction with

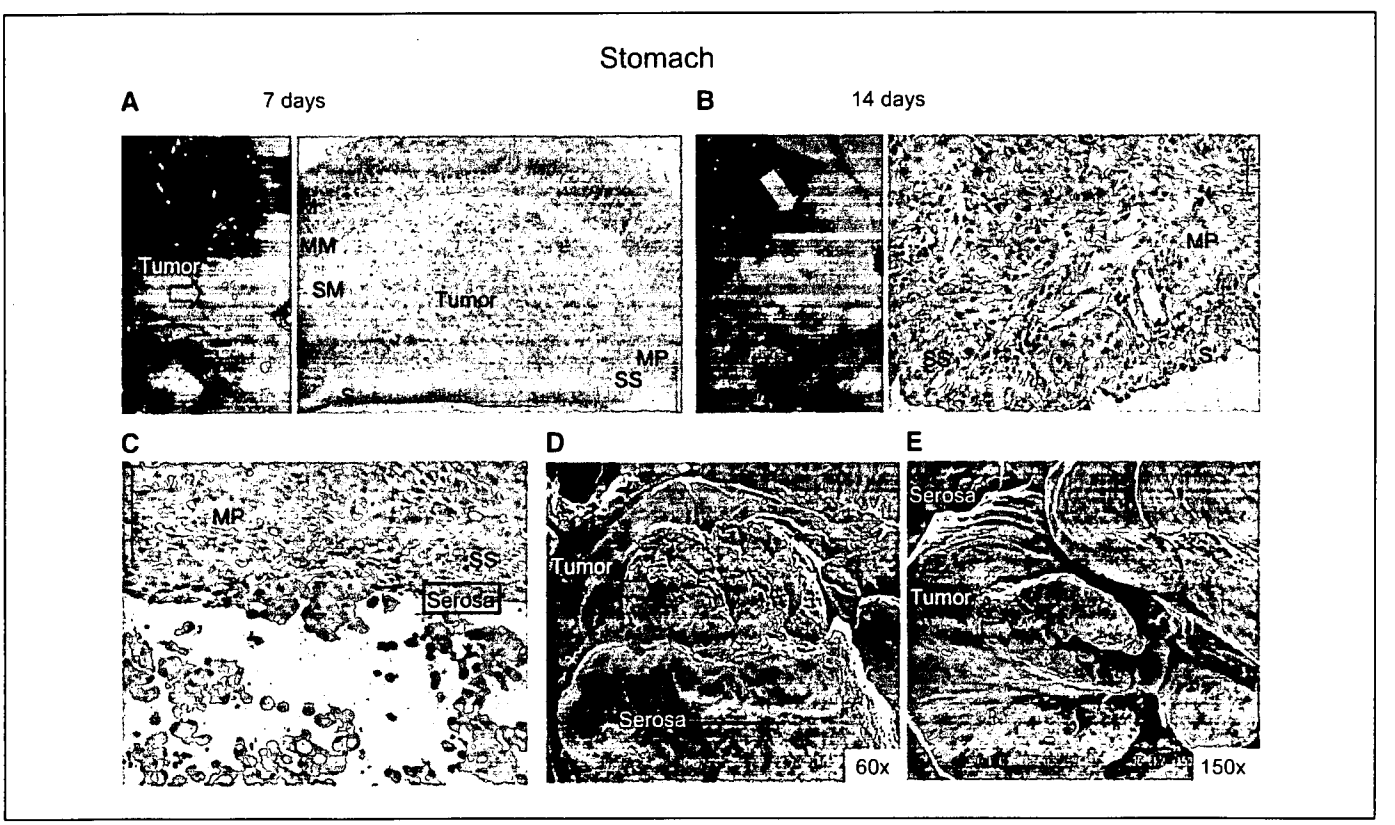

Figure 2. Macroscopic and microscopic appearance of the tumor growth of stomach of nude mice after orthotopic implantation of 44As3 cells. *A, green arrow,* orthotopic implantation of 44As3 cells in the stomach of nude mice was followed by tumor formation 7 days later. Actively proliferating 44As3 cells in the gastric submucosa (H&E). *M,* mucosa; *MM,* muscularis mucosae; *SM,* submucosa; *MP,* muscularis propria; *SS,* subserosa; *S,* serosa. *B,* tumor invasion of the muscularis propria and subserosal tissue (H&E). *C,* note 44As3 cells breaking through the serosa and becoming exfoliated and free (H&E). *D* and *E,* visualization of cancer cells breaking through the serosa and becoming exfoliated and free. Mice were sacrificed, and the tissues were examined for metastasis in various organs and processed for histologic examination as described (47, 48). Scanning electron microscopic examination was done according to standard procedures (49).