▶▶▶ま と め

臨床応用されている分子標的薬剤は，確実に癌治療戦略を前進させるとともに新しい課題を提示しつつある．新しい分子標的を対象にした薬剤開発は現在活発に進められており，魅力的な研究分野である．肝癌や肝転移に対しても，インターフェロンや癌化学療法剤との併用をはじめ多くの治療法が進められている．肝癌の治療をさらに改善するために，肝癌や肝転移に関与する分子標的を探索し治療薬を開発するトランスレーショナル研究を活発化することは，肝癌治療へ大きな貢献が期待できると確信している．

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What phase III trials are needed to improve the treatment of advanced non-small-cell lung cancer?

Nagahiro Saijo

Platinum-based doublets are standard treatments for stage IV non-small-cell lung cancer (NSCLC). Several doublets that include new drugs improve survival, but no one regimen is clearly superior to the others, as previously discussed by Scagliotti[1] and Govindan[2] in *Nature Clinical Practice Oncology*.

Numerous molecular-target-based drugs have been introduced for the treatment of NSCLC, but can they replace or be used as an adjuvant to current therapy, and can they be combined with other chemotherapeutic agents, radiotherapy and/or surgery? We hypothesize that incorporation of novel molecular-target-based therapies into current treatment paradigms will improve outcomes. However, carefully designed clinical trials and translational science will be required to identify the subsets of patients likely to benefit. If these treatment strategies are to be used, we must first answer the following critical questions. First, will patients lacking the target still respond? It is still unclear why responses occur in those lacking the correct molecular target. Second, what expression levels of the target are sufficient for a response, and can we measure the target in a biologically relevant and/or technologically valid way? Third, does the agent inhibit the proposed target at the dose and schedule utilized? Fourth, is the target a critical driving force for cell growth in the tumor type in question?

Various molecular-target-based drugs for advanced NSCLC have been evaluated in randomized controlled trials, but the majority, including a matrix metalloproteinase inhibitor, a protein kinase C inhibitor, and trastuzumab, have yielded negative results.[3,4] Gefitinib (Iressa®) and erlotinib (Tarceva™) are orally available selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) that exhibit antitumor activity in patients with previously treated advanced NSCLC. However, both drugs failed to show additive or synergistic effects when combined with platinum-based chemotherapy as a first-line treatment for NSCLC. On 17 December 2004, AstraZeneca announced the preliminary results of their ISEL (Iressa® Survival Evaluation in Lung Cancer) study of 1,692 patients with advanced recurrent or refractory NSCLC. Unfortunately, gefitinib failed to prolong survival significantly compared with placebo (hazard ratio 0.89, $P=0.11$) in the overall patient population or among patients with adenocarcinoma (hazard ratio 0.83, $P=0.07$). A retrospective analysis of patients treated with gefitinib showed that tumor response was associated with distinct subgroups: women, patients with no history of smoking, patients with adenocarcinoma, and Japanese patients. Survival in the gefitinib group in the ISEL study was significantly higher for non-smokers ($P<0.01$) and Asians ($P<0.01$) than in the placebo group. The survival curves of the two treatment groups were the same for non-Asians. The results of similar randomized trials of erlotinib (the BR21 study) were presented at the American Society of Clinical Oncology meeting in 2004. Erlotinib significantly prolonged survival in patients with advanced, previously treated, refractory or recurrent NSCLC. The survival of non-smokers in the erlotinib group in the BR21 study was extremely good and contributed to the improvement in overall survival. The presence of an *EGFR* mutation has been demonstrated to be a strong predictor of a favorable response to EGFR-TKI. Mutations have recently been reported to be significantly more frequent in women, in patients with adenocarcinoma, and in those who had never smoked, and these findings are consistent with the clinical predictors of tumor response in patients treated with EGFR-TKI. Mitsudomi *et al.* reported that patients with *EGFR* mutations survived longer after the initiation of gefitinib treatment than those without mutations.[5] It can be concluded that translational studies are extremely important for the development of molecular-target-based drugs.

Numerous molecular-target-based drugs have been introduced for the treatment of NSCLC, but what is their place in current therapy?

N Saijo is an Advisory Board member of Nature Clinical Practice Oncology.

Competing interests
The author declared he has no competing interests.

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FROM THE ASCO-JSCO JOINT SYMPOSIUM

Nagahiro Saijo · Yuji Nimura

Summary of the ASCO–JSCO Joint Symposium

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The American Society of Clinical Oncology (ASCO) is now rapidly expanding as an international society for clinical oncology. The ASCO mission statement is as follows: "As a nonprofit organization, ASCO is dedicated to achieving its charitable mission outlined by the organization's founders in 1964. ASCO strongly supports all types of cancer research, but in particular, patient-oriented clinical research." To realize the ASCO mission statement, ASCO makes strategic plans, and the new strategic plan is titled "Cancer Prevention and New Control." Because there now are more than 20000 ASCO members, the choice of meeting places is limited. ASCO 2005 will be held in Orlando, Florida, USA, May 14–17, and ASCO 2006 will be held in Atlanta. Thereafter, all meetings are scheduled to be held in Chicago because of the number of flights to the city, hotel accommodations, and the size of the convention center. ASCO has many scientific activities in addition to the annual meeting. For example, the Gastrointestinal Council Symposium and the Multidisciplinary Prostate Cancer Program will be conducted in Miami and Orlando, respectively. In addition, the "Best of ASCO" meetings are scheduled not only in the United States but also in Japan as an advanced course organized by the Japanese Society of Medical Oncology (JSMO), to be held June 11 and 12, 2005. ASCO also publishes materials such as educational curricula and self-assessment tools.

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The ASCO-JSCO Joint Symposium was held in Kyoto, Japan, on October 29, 2004.

The *Journal of Clinical Oncology* (JCO), published by ASCO, is widely read and has an impact factor above 10. In 2005, publication of review article issues began. Membership in ASCO grew from 66 in 1964 to 21837 at the end of 2003. Some 23000 investigators attend the annual meeting every year. Members' board certifications show more than half in medical oncology, with 15% in hematology/oncology, followed by pediatric oncology, radiation oncology, and others. The distribution is similar to that of the Japanese Society of Medical Oncology (JSMO) and is quite different from that of the Japanese Society of Clinical Oncology (JSCO). Domestic membership is 73% and international membership is 27%. By world region, about 50% of the international members are in Europe, 19% are from Asia, 8% from Latin America, with Canada and Mexico accounting for 15%. After the United States, the top 10 countries for ASCO membership are Japan (No. 1 at 591), followed by Canada, Germany, Italy, France, the United Kingdom, Spain, Brazil, and Switzerland. Thanks to the efforts of the International Committee (Nagahiro Saijo, chairman) of JSCO, reciprocal membership application became available to JSCO members, making it possible to avoid complicated application procedures to become an ASCO member. JSCO members are encouraged to use this system and to apply for active membership in ASCO.

The ASCO International Affairs Committee organizes various joint symposiums and workshops. For example, with FRASCA, ASCO held a joint international symposium in 2003 and an Australia/Asia–Pacific clinical research development workshop in 2004. Every year ASCO and AACR have a joint workshop on clinical trials in Vail, Colorado, USA. Japanese oncologists, in addition to facing language barriers, still do not have enough scientific knowledge to attend this meeting. ASCO and the European Society of Medical Oncology (ESMO) approved the core curriculum of medical oncology, which has been published in the *Journal of Clinical Oncology* and the *Annals of Oncology*. The JSMO has almost completed the translation of the curriculum, which will be accredited by ASCO. JSMO provides educational seminars twice a year based on that

Table 1. Japanese contribution to ASCO

1. Description	Total
International members	27%
US members	73%
Total ASCO members	21800
2. Number of international members by country	
Japan 591, Canada 575, Germany 444, Italy 423, France 337, UK 289	
3. Japanese ASCO presentation	
Original papers (oral, poster discussion, poster)	
1994, 17; 1997, 37; 2000, 64; 2003, 85; 2004, 92	
Participants in poster discussions – N. Saijo (2003)	
Educational sessions	
International symposium	N. Saijo (2002), M. Sasako (2003)
Meet the professor	N. Saijo (2003), M. Tsuboi (2004)
Educational symposium	H. Wada (2005)
4. Committee member in ASCO	
International affairs committee (Director: Paula T. Rieger)	
2001–2003	Nagahiro Saijo
2003–2005	Yasuhiro Fujiwara
2004–2006	Masahiro Fukuoka
5. Endorsement for	
ASCO–JSCO joint symposium (2002, 2003, 2004, 2005)	
ASCO–JSMO joint symposium (2004, 2005, 2006)	
6. ASCO Board member	Nagahiro Saijo (2004–2007)

curriculum. ASCO has established a new international seat on the ASCO Board of Directors, and I (Nagahiro Saijo) was elected as a 2004–2007 ASCO Board member, as was Dr. José Baselga from Spain for 2003–2006.

Japan is the top country in terms of the number of active international members of ASCO (Table 1). The number of Japanese attendees at the ASCO annual meeting increased to 900 in 2004. The acceptance ratio for Japanese abstracts is improving; for posters, poster discussions, and oral presentations it is nearly 50%, and the numbers of presented abstracts are about 80–90 every year. The ASCO–JSCO joint symposium started in 2002 in Tokyo, followed by the one in Sapporo last year. Because we could not attract a large enough audience for those meetings, the program for this year shifted to topics of surgery. Dr. Nimura, a moderator of the symposium, and Drs. Kato, Sasako, and Blumgart are surgeons; Dr. Ajani and I are medical oncologists.

Dr. Kato, a professor of Tokyo Medical College, spoke on adjuvant chemotherapy of early-stage lung cancer. Uracil-tegafur (UFT) is a chemotherapy drug that most American oncologists do not recognize because it has not been approved for use in the United States, although limited numbers of clinical trials of the drug have been conducted against gastrointestinal tumors there. UFT has been widely used in Japan against various tumor types and has been approved for use in many countries. Uracil-tegafur, a prodrug of 5FU, is one of the oral fluorinated pyrimidine drugs that has been synthesized mainly by pharmaceutical companies in Japan. The main purpose of oral fluorinated pyrimidine is to improve the delivery of low-dose 5FU over time, mimicking a continuous infusion of 5FU. The beneficial effect of tegafur is believed to derive from its slow conversion to 5FU through the cytochrome P450 pathway. The released 5FU from the prodrug tegafur competes with uracil for catabolism by the rate-limiting enzyme dihydropyrimidine dehydrogenase. The presence of excess uracil is believed to decrease the degradation of 5FU, maintaining a continuous drug level. Although it has been widely used in various diseases, there have been no large confirmatory randomized controlled trials. In the treatment of non-small cell lung cancer (NSCLC), a small phase II study showed that the response rate of UFT was less than 10%. Surgeons in Japan still prefer to use it after surgery, however, because of its mild adverse effect and because of oral administration. In a previous preliminary phase III trial of adjuvant chemotherapy after resection of NSCLC, UFT taken orally was shown to prolong survival, especially in pathological stage I adenocarcinoma. Based on these data, the Taiho Pharmaceutical Company organized the Japan Lung Cancer Research Group on Postsurgical Adjuvant Chemotherapy and conducted a randomized controlled trial against pathological stage I adenocarcinoma. Patients were randomly assigned to UFT (250mg) for 2 years or to no treatment. From January 1994 through March 1997, 999 patients were enrolled. Twenty patients were found to be ineligible and were excluded from the analysis after randomization, 491 patients were assigned to receive UFT, and 488 were assigned to observation. The median duration of follow-up for surviving patients was 73 months. The difference in overall survival between the two groups was statistically significant in favor of the UFT group ($P = 0.04$ by stratified log-rank test). Grade 3 toxic effects occurred in 10 of the 482 patients (2%) who received UFT.

So far, six randomized trials, including the present one, have been conducted that compare surgery alone with adjuvant UFT chemotherapy. Among them, three trials have shown a survival benefit from treatment with UFT. A meta-analysis of those six trials showed that adjuvant chemotherapy with UFT improved overall survival (hazard ratio for death, 0.77; 95% confidence interval, 0.63–0.94; $P =$

0.01). It is unclear whether patients with stage II or stage III disease benefit from treatment with UFT and whether treatment for 1 year is equivalent to treatment for 2 years.

In addition, Dr. Kato briefly presented data on adjuvant chemotherapy with platinum-based regimens that had been presented at ASCO 2004.

Dr. Ajani, professor of Medicine at the M.D. Anderson Cancer Center, spoke on "Current advances in the treatment of unresectable gastric and gastroesophageal adenocarcinoma." He touched first on ethnic differences in metabolism of fluorinated pyrimidines. S-1 contains ftorafur, which is converted by the cytochrome P450. CYP2A6 is responsible for the conversion from ftorafur to 5FU. It has been discovered that CYP2A6 polymorphism makes the enzyme very efficacious in Caucasians. For the same dose of S-1, accumulation of 5FU is higher in Caucasians than in Japanese, resulting in high frequency and high grade of toxicities. The recommended dose of S-1 in the Japanese population is 35–40 mg/m^2 twice daily, whereas that in Caucasians is 25 mg/m^2 if combined with cisplatin. It is quite important to determine the correct dose of S-1 for Caucasians.

Pharmacokinetic and pharmacodynamic analysis showed a clear relationship between the AUC of 5FU and grade 1 frequency of any dose-limiting toxicity. The recommended dose for Caucasians was 25 mg/m^2, twice daily, S-1 and 75 mg/m^2 cisplatin, a combination that showed a high response rate in gastrointestinal carcinoma.

Dr. Ajani presented recent results of a docetaxel-containing regimen in gastric cancer. In phase III of V325, all 463 patients have been enrolled. A planned interim analysis was carried out when 162 TTP (time-to-tumor-progression) events occurred. By this time 232 patients have been accrued. The following results of an interim analysis were presented at the proceedings of ASCLO in June 2003. All patients had advanced, untreated gastric cancer. Patients with potentially resectable primary cancer were not eligible for the study. Patients were stratified according to the level of weight loss, presence or absence of liver and peritoneal metastases, presence or absence of the primary carcinoma, and by center. Once patients signed an informed consent, they were registered and randomized to receive either DCF or CF. The doses and schedule of the DCF arm were: docetaxel 75 mg/m^2 on day 1, cisplatin 75 mg/m^2 on day 1, and 5-fluorouracil 750 mg/m^2 per day as continuous infusion on days 1–5 repeated every 3 weeks. The doses and schedule for the CF arm were: cisplatin 100 mg/m^2 on day 1 and 5-fluorouracil 1000 mg/m^2 per day as continuous infusion on days 1–5, given every 4 weeks. Even though the two regimens had different cycles, the response assessments were synchronized. This removed the bias in TTP assessments. All responses were independently reviewed and confirmed. TTP was the primary endpoint, and overall survival (OS) of the patients was the main secondary endpoint. Currently, results on 232 patients (115/117 in DCF/CF) are available, constituting the results of a planned interim analysis. The median age was 54 years, and 98% of the patients had metastatic cancer. The median administered dose intensity calculated by dose/week basis for 5-fluorouracil and cisplatin was the same for DCF and CF. The TTP was statistically superior ($P = 0.0008$) for DCF (5.2 months compared with 3.7 months for CF). This meant that patients receiving DCF had a 70% lower chance of having cancer progression than those receiving CF. The median survival time was longer for patients receiving DCF (10.2 months) than those receiving CF (8.5 months) ($P = 0.0064$). This meant that patients receiving DCF had a 50% lower risk of death than those receiving CF during the study. This P value did not cross the preset boundary at the interim analysis, but the conditional probability of DCF having a statistically median survival time superior to CF is 99.4%. The response rate was 39% for DCF and 23% for CF. This difference is statistically superior ($P = 0.012$). DCF can result in bone marrow suppression and increased risk of infection. Thus, neutropenic fever and the neutropenic infection rate, as expected, were higher from DCF than from CF. DCF can also cause diarrhea and mucositis. Careful patient selection is highly recommended. In addition, aggressive management of the side effects of DCF is essential. DCF should now be offered to all patients with advanced gastric or gastroesophageal junction cancer who are in good general condition. Further development of this regimen is also warranted. The V325 study was sponsored by Aventis. Recent data from Roth et al. (ASCO noncolorectal GI presentation in 2004) also demonstrated that the combination of docetaxel, cisplatin, and 5-flurouracil had a higher response rate and longer time-to-progression than docetaxel plus cisplatin, or epirubicin, cisplatin, and 5-flurouracil. The SAKK group has now decided to compare docetaxel, cisplatin, and 5-fluorouracil (as the experimental arm) with epirubicin, cisplatin, and 5-fluorouracil ("ECF" as a reference regimen). Thus two separate studies seem to establish the value of docetaxel in patients with advanced gastric or gastroesophageal adenocarcinoma.

Dr. Sasako, chief of surgery, National Cancer Center Hospital, presented results of surgical procedures in operable stomach cancer. In many solid tumors, surgery remains the major part of the treatment with curative intent. To establish a better standard treatment, many clinical trials have been carried out on multidisciplinary treatments, including surgery, and some on purely surgical procedures. Unlike drug treatment, the results of surgery are often hampered by the heterogeneity in the quality of treatment. The results of surgery are affected by the surgeons' skill, experience (learning curve), and personal preference. Experience includes not only the quality of surgery but also that of postoperative care. A Dutch trial on D2 dissection for gastric cancer provided a good example by showing the difficulty and importance of quality control of surgery and postoperative care. In this trial, more than 28% of patients who developed major complications died, whereas death occurred in only 9% of such patients in a Japanese specialist center, most likely due to lack of knowledge and experience of managing complications in participating hospitals. It seems that the hospital volume per year, while it was as small as 1.0 on average, was insufficient for carrying out D2 dissection safely. The impact of a significantly larger proportion of treatment-related deaths after D2 dissection was

too large to be redeemed by the treatment effect in the long term. This was also the case in two clinical trials on esophageal cancer in France and Germany reported in the 2003 ASCO meeting.

In the IT-0116 trial on adjuvant treatment of gastric cancer, adjuvant chemoradiotherapy (CRT) after curative surgery was shown to improve the survival of patients with gastric cancer. In this trial, 50% of patients underwent D0 dissection, 40% had D1, and only 10% had D2, in spite of the description of the protocol. Therefore, the results of this trial suggest that adjuvant CRT is effective for those who underwent limited surgery and for whom limited surgery is not a sufficient treatment for curable gastric cancer. From the large database of lymph node metastasis in Japanese patients, limited surgery theoretically often leaves metastatic nodes unresected, thus leading to recurrence. An in-depth analysis of this trial showed that surgical under treatment was an independent prognostic factor. This trial clearly showed that the effects of adjuvant treatment can differ depending on the type of surgery. To evaluate the efficacy of adjuvant treatment, the type of surgery should be defined in the protocol, and strict quality control of surgery is mandatory. Through the experience of planning and carrying out clinical trials on surgical treatment of malignant diseases inside and outside of Japan, the key issues in surgical trials on cancer treatment were discussed.

Dr. Blumgart, professor of surgery, Cornell University Medical College, spoke on "Surgical advances in hepatobiliary cancer." He focused his talk on hepatic resection. Hepatectomy has a long history, starting with a record of 1801 liver resections. Compared with results in the early twentieth century, blood loss has significantly decreased to about 500ml, segmental resections have been developed, and the transfusion rate and operating time were down at the beginning of the twenty-first century. Even if the tumor is large and hepatocellular cancer invades a major vessel, the 5-year survival rate was 37% in 412 patients treated from 1991 to 1998 at Memorial Sloan-Kettering Cancer Center (MSKCC). Tumor size is closely related to patient prognosis.

Dr. Blumgart mentioned the indications for liver transplantation after partial hepatectomy. The objective was to determine the survival and recurrence pattern of the partial hepatectomy for patients with hepatocellular carcinoma (HCC) who have been selected for transplantation. In MSKCC, among 611 cases, 180 were resectable but only 36 (20%) met the Milan Criteria. The operative mortality of these 36 patients receiving partial hepatectomy with transplantation was 2.8%. In 20 recurrent cases, the 5-year survival rate was 57%, and for 14 no-recurrence patients, it was 93%. From these results, partial hepatectomy for patients otherwise eligible for transplant can be performed with reasonable morbidity and mortality.

Hepatic resection for metastatic colorectal cancer was not justified in the early 1950s because metastases are nearly always multiple. Although there is no randomized controlled trial to solve the problem of this issue, retrospective analysis demonstrates that resected cases showed a high survival rate compared with nonresected cases (38% vs 0%). At MSKCC, 1001 resections were conducted for metastatic hepatic carcinomas, and the number of 5-year survivors reached 136. Perioperative mortality was 2.8%, the 5-year survival rate 39%, and the 10-year survival rate 23%. Five clinical risk factors were identified by multivariate analysis: (1) node positive primary, (2) disease-free interval less than 12 months, (3) more than one tumor, (4) tumor size more than 5cm, and (5) CEA greater than 200ng/ml. These factors are important for patient selection and stratification in clinical trials.

Although the majority of the symposium topics concentrated on surgery, including lung cancer, gastrointestinal cancers, and hepatobiliary cancer, the peak number of attendees was less than 200; by the end of symposium it was less than 50. ASCO and JSCO were disappointed again with their joint scientific symposium. In the JSMO meeting it is possible for us to attract audiences of 700–1000. In 2005, JSCO will organize a symposium on the topic of "The Role of Board-Certified Medical Oncologists."

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Establishment of a human non-small cell lung cancer cell line resistant to gefitinib

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The epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitor gefitinib (Iressa®, ZD1839) has shown promising activity preclinically and clinically. Because comparative investigations of drug-resistant sublines with their parental cells are useful approaches to identifying the mechanism of gefitinib resistance and select factors that determine sensitivity to gefitinib, we established a human non-small cell lung carcinoma subline (PC-9/ZD) that is resistant to gefitinib. PC-9/ZD cells are ~180-fold more resistant to gefitinib than their parental PC-9 cells and PC-9/ZD cells do not exhibit cross-resistance to conventional anticancer agents or other tyrosine kinase inhibitors, except AG-1478, a specific inhibitor of EGFR. PC-9/ZD cells also display significant resistance to gefitinib in a tumor-bearing animal model. To elucidate the mechanism of resistance, we characterized PC-9/ZD cells. The basal level of EGFR in PC-9 and PC-9/ZD cells was comparable. A deletion mutation was identified within the kinase domain of *EGFR* in both PC-9 and PC-9/ZD, but no difference in the sequence of *EGFR* cDNA was detected in either cell line. Increased EGFR/HER2 (and EGFR/HER3) heterodimer formations were demonstrated in PC-9/ZD cells by chemical cross-linking and immunoprecipitation analysis in cells unexposed to gefitinib. Exposure to gefitinib increased heterodimer formation in PC-9 cells, but not in PC-9/ZD cells. Gefitinib inhibits EGFR autophosphorylation in a dose-dependent manner in PC-9 cells but not in PC-9/ZD cells. A marked difference in inhibition of site-specific phosphorylation of EGFR was observed at Tyr1068 compared to other tyrosine residues (Tyr845, 992 and 1045). To elucidate the downstream signaling in the PC9/ZD cellular machinery, complex formation between EGFR and its adaptor proteins GRB2, SOS, and Shc was examined. A marked reduction in the GRB2-EGFR complex and absence of SOS-EGFR were observed in PC-9/ZD cells, even though the protein levels of GRB2 and SOS in PC-9 and PC-9/ZD cells were comparable. Expression of phosphorylated AKT was increased in PC-9 cells and inhibited by 0.02 μM gefitinib. But the inhibition was not significant in PC-9/ZD cells. These results suggest that alterations of adaptor-protein-mediated signal transduction from EGFR to AKT is a possible mechanism of the resistance to gefitinib in PC-9/ZD cells. These phenotypes including EGFR–SOS complex and heterodimer formation of HER family members are potential biomarkers for predicting resistance to gefitinib.

Key words: resistance; gefitinib; EGFR; Grb2; SOS; non-small cell lung cancer

Chemotherapy has played a central role in the treatment of patients with inoperable NSCLC for over 30 years, although its efficacy seems to be of very limited value.[1,2] Human solid tumors, including lung cancer, glioblastoma, breast cancer, prostate cancer, gastric cancer, ovarian cancer, cervical cancer and head and neck cancer, express epidermal growth factor receptor (EGFR) frequently, and elevated EGFR levels are related to disease progression, survival, stage and response to therapy.[2–10] The therapies directed at blocking EGFR function are attractive.

Interest in target-based therapy has been growing ever since the clinical efficacy of STI-571 was first demonstrated,[11–13] and small molecules and monoclonal antibodies that block activation of the EGFR and HER2 have been developed over the past few decades. The leading small-molecule EGFR tyrosine-kinase inhibitor, gefitinib (Iressa®, ZD1839), has shown excellent antitumor activity in a series of Phase I and II studies,[14,15] and Phase II international multicenter trials (Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL) 1 and 2) yield an overall RR of 11.8–18.4% and overall disease control rate of 42.2–54.4% (gefitinib 250 mg/day) in patients with advanced non-small cell lung cancer (NSCLC) who had undergo at least 2 previous treatments with chemotherapy. INTACT 1 and 2 ('Iressa' NSCLC Trials Assessing Combination Therapy) have demonstrated that gefitinib does not provide improvement in survival when added to standard first line platinum-based chemotherapy *vs.* chemotherapy alone in advanced NSCLC.[16,17] Two small retrospective studies reported recently that activating mutation of *EGFR* correlate with sensitivity and clinical response to gefitinib and erlotinib.[18–20] Although information of *EGFR* mutation may enable to identify the subgroup of patients with NSCLC who will respond to gefitinib and erlotinib, it would be expected that acquired resistance would develop in such patients after treatment. The problem of acquired resistance to gefitinib might be growing, but there has been no preclinical research about the mechanism of developing resistance to gefitinib. We established resistant subline using PC-9 that is highly sensitive to gefitinib.

Establishment of drug-resistant sublines and comparative investigations with their parental cells to identify their molecular, biological and biochemical properties are useful approaches to elucidating the mechanism of the drug's action. Our study describes the establishment of a gefitinib-resistant cell line and its characterization at the cellular and subcellular levels. The PC-9/ZD cell line is the first human NSCLC cell line resistant to gefitinib ever reported. PC-9 is a lung adenocarcinoma cell line that is highly sensitive to gefitinib at its IC_{50}-value of 0.039 μM, but the PC-9/ZD subline, which has a level of EGFR expression comparable to that of PC-9 cells, is specifically resistant to gefitinib. Thus, PC-9 and PC-9/ZD cells will provide useful information about the mechanism of developing resistance to gefitinib and molecules as surrogate markers for predicting chemosensitivity to gefitinib.

Material and methods

Drugs and cells

Gefitinib(*N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy] quinazolin-4-amine) was supplied by AstraZeneca Pharmaceuticals (Cheshire, UK). AG-1478, AG-825, K252a, staurosporin, genistein, RG-14620 and Lavendustin A were purchased from Funakoshi Co. Ltd (Tokyo, Japan).

NSCLC cell line PC-9 (derived from a patient with adenocarcinoma untreated previously) was provided by Prof. Hayata of Tokyo Medical University (Tokyo, Japan).[21] PC-9 and PC-9/ZD cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS (GIBCO-BRL, Grand Island, NY), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; GIBCO-BRL) in a humidified atmosphere of 5%

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CO_2 at 37°C. Gefitinib-resistant PC-9/ZD cells were selected from a subculture that had acquired resistance to gefitinib using the following procedure. Cultured PC-9 cells were exposed to 2.5 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 24 hr and then washed and cultured in medium containing 0.2 μM gefitinib for 7 days. After exposure to gefitinib, they were washed and cultured in drug-free medium for 14 days. When variable cells had increased, they were seeded in medium containing 0.3–0.5 μM of gefitinib on 96-well cultured plates for subcloning. After 21–28 days, the colonies were harvested and a single clone was obtained. The subcloned cells exhibited an 182-fold increase in resistance to the growth-inhibitory effect of gefitinib as determined by MTT assay, and the resistant phenotype has been stable for at least 6 months under drug-free conditions.

In vitro *growth-inhibition assay*

The growth-inhibitory effects of cisplatin, carboplatin, adriamycin, irinotecan, gemcitabine, vindesine, paclitaxel, genistein, K252a, staurosporin, AG-825, AG-1478, Tyrophostin 51, RG-14620, Lavendustin A and gefitinib in PC-9 and PC-9/ZD cells were examined by using a 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay.[22] A 180 μl volume of an exponentially growing cell suspension (6×10^3 cells/ml) was seeded into a 96-well microtiter plate, and 20 μl of various concentrations of each drug was added. After incubation for 72 hr at 37°C, 20 μl of MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 hr at 37°C. After centrifuging the plates at 200*g* for 5 min, the medium was aspirated from each well and 180 μl of DMSO was added to each well to dissolve the formazan. Optical density was measured at 562 and 630 nm with a Delta Soft ELISA analysis program interfaced with a Bio-Tek Microplate Reader (EL-340, Bio-Metallics, Princeton, NJ). Each experiment was carried out in 6 replicate wells for each drug concentration and carried out independently 3 or 4 times. The IC_{50}-value was defined as the concentration needed for a 50% reduction in the absorbance calculated based on the survival curves. Percent survival was calculated as: (mean absorbance of 6 replicate wells containing drugs − mean absorbance of six replicate background wells)/(mean absorbance of 6 replicate drug-free wells − mean absorbance of 6 replicate background wells) × 100.

In vivo *growth-inhibition assays*

Experiments were carried out in accordance with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the welfare of animals in experimental neoplasia (2nd ed.). Female BALB/c nude mice, 6-weeks-old, were purchased from Japan Charles River Co. Ltd (Atsugi, Japan). All mice were maintained in our laboratory under specific-pathogen-free conditions. *In vivo* experiments were scheduled to evaluate the effect of oral administration of gefitinib on pre-existing tumors. Ten days before administration, 5×10^6 PC-9 or PC-9/ZD cells were injected subcutaneously (s.c.) into the back of the mice, and gefitinib (12.5, 25 or 50 mg/kg, p.o.) was administered to the mice on Days 1–21. Tumor diameter was measured with calipers on Days 1, 4, 8, 11, 14, 19 and 22 to evaluate the effect of treatment, and tumor volume was determined by using the following equation: tumor volume = $ab^2/2$ (mm^3) (where *a* is the longest diameter of the tumor and *b* is the shortest diameter). Day "x" denotes the day on which the effect of the drugs was estimated, and Day "1" denotes the first day of treatment. All mice were sacrificed on Day 22, after measuring their tumors. We considered absence of a tumor mass on Day 22 to indicate a cure. Differences in tumor sizes between the treatment groups and control group at Day 22 were analyzed by the unpaired *t*-test. A *p*-value of <0.05 was considered statistically significant.

cDNA expression array

The gene expression profile of PC-9/ZD was assessed with an Atlas Nylon cDNA Expression Array (BD Bioscience Clontech, Palo Alto, CA). Total RNA was extracted by a single-step guanidinium thiocyanate procedure (ISOGEN, Nippon Gene, Tokyo, Japan). An Atlas Pure Total RNA Labeling System was used to isolate RNA and label probes. The materials provided with the kit were used, and the manufacturer's instructions were followed for all steps. Briefly, streptavidin-coated magnetic beads and biotinylated oligo(dT) were used to isolate poly A RNA from 50 μg of total RNA and the RNA obtained was converted into ^{32}P-labeled first-strand cDNA with MMLV reverse transcriptase. The ^{32}P-labeled cDNA fraction was purified on NucleoSpin columns and was added to the membrane on which fragments of 777 genes were spotted. Hybridization was allowed to proceed overnight at 68°C. After washing, the radiolabeled spots were visualized and quantified by BAS-2000II and Array Gauge 1.1 (Fuji Film Co., Ltd., Tokyo, Japan). The data were adjusted for the total density level of each membrane.

Quantitative real-time RT-PCR analysis

Total RNAs extracted from PC-9 cells and PC-9/ZD cells (1×10^6 cells each) were incubated with DNase I (Invitrogen, Carlsbad, CA) for 30 min. First-strand cDNA synthesis was carried out on 1 μg of RNA in 10 μl of a reaction mixture with 50 pmol of Random hexamers and 50 U of M-MLV RTase. Oligonucleotide primers for human *EGFR* were obtained from Takara (HA003051, Takara Bio Co., Tokyo, Japan). For PCR calibration, we generated a calibrator dilution series for *EGFR* cDNA in pUSEamp vector (Upstate, Charlottesville, VA) ranging from 10^8–10^2 copies/1 μl. A total of 2 μl of reverse transcriptase products was used for PCR amplification using Smart Cycler system (Takara) according to manufacturer's instructions. Absolute copy numbers were calculated back to the initial cell numbers, which were set into the RNA extraction. As a result we obtained copies/cell:ratio representing the average *EGFR* RNA amount per cell.

Immunoprecipitation and immunoblotting

The cultured cells were washed twice with ice-cold PBS, and lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 100 mM NaF, 200 mM Na orthovanadate, and 10 mg/ml each of leupeptin, aprotinin, pepstatin A and phenylmethylsulphonyl fluoride). The lysate was cleared by centrifugation at 15,000 r.p.m. for 10 min, and the protein concentration of the supernatant was measured by BCA protein assay (Pierce, Rockford, IL). The membrane was probed with antibody against EGFR (1005; Santa Cruz, Santa Cruz, CA), HER2/neu (c-18; Santa Cruz), HER3 (c-17; Santa Cruz), HER4 (c-18; Santa Cruz), PI3K (4; BD), Grb2 (81; BD), SOS1/2 (D-21; Santa Cruz), Shc (30; BD, San Jose, CA), PTEN (9552; Cell Signaling, Beverly, MA), AKT (9272; Cell Signaling), phospho-EGFR specific for Tyr 845, Tyr 992, Tyr 1045, and Tyr 1068 (2231, 2235, 2237, 2234; Cell Signaling), phospho-AKT (Ser473) (9271; Cell Signaling), phospho-Erk (9106; Cell Signaling), and phospho-Tyr (PY-20; BD) as the first antibody, and then with by horseradish-peroxidase-conjugated secondary antibody. The bands were visualized by enhanced chemiluminescence (ECL Western Blotting Detection Kit, Amersham, Piscataway, NJ). For Immunoprecipitation, 5×10^6 cells were washed, lysed in EBC buffer, and centrifuged, and the supernatants obtained (1,500 μg) were incubated at 4°C with the anti-EGFR (1005), -HER2 (c-18), and -HER3 (c-17) Ab overnight. The immunocomplexes were absorbed onto protein A/G-Sepharose beads, washed 5 times with lysate buffer, denatured, and subjected to electrophoresis on a 7.5% polyacrylamide gel.

Analysis of the genes of the HER families by direct sequencing

Total RNAs were extracted from PC-9 and PC-9/ZD cells with ISOGEN (Nippon Gene) according to manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA by using 400 U of SuperScript II (Invitrogen, Carlsbad, CA). After reverse transcription with oligo (dT) primer (Invitrogen) or random primer (Invitrogen), the first-strand cDNA was amplified by PCR by using specific primers for *EGFR*, *HER2* and *HER3*. The

reaction mixture (50 µl) contained 1.25 U AmpliTaq DNA polymerase (Applied Biosystem, Foster City, CA), and amplification was carried out by 30 cycles of denaturation (95°C, 30 sec), annealing (55–59°C, 30 sec), and extension (72°C, 30 sec) with a GeneAmp PCR System 9600 (Applied Biosystem). After amplification, 5 µl of the RT-PCR products was subjected to electrophoretic analysis on a 2% agarose gel with ethidium bromide. DNA sequencing of the PCR products was carried out by the dideoxy chain termination method using the ABI PRISM 310 Genetic Analyzer (Applied Biosystem).

Chemical cross-linking

Chemical cross-linking in intact cells was carried out as described previously.[23] In brief, after 6 hr exposure to 0.2 µM gefitinib, cells were washed with PBS and incubated for 25 min at 4°C in PBS containing 1.5 mM of the nonpermeable cross-linker bis (sulfosuccinimidyl) substrate (Pierce, Rockford, IL). The reaction was terminated by adding 250 mM glycine for 5 min while rocking. Cells were washed in EBC buffer and 20 µg of protein was resolved by 5–10% gradient SDS-PAGE, and then immunoblot analyzed for EGFR, HER2, HER3 and P-Tyr.

Results

Sensitivity of PC-9/ZD cells to cytotoxic agents and tyrosine kinase inhibitors

No significant difference between PC-9 and PC-9/ZD cells was observed in *in vitro* cell growth (doubling time of 20.3 hr and 21.4 hr, respectively) and microscopic morphology. Figure 1 shows the growth-inhibitory effect of gefitinib on the parent PC-9 cell line and its resistant subline, PC-9/ZD. The IC_{50}-value of gefitinib in PC-9 cells was 0.039 µM, as compared to 7.1 µM in PC-9/ZD cells (182-fold resistance). PC-9/ZD cells exhibited no cross-resistance to other conventional anticancer agents, including cisplatin, carboplatin, adriamycin, vindesine, paclitaxel and irinotecan. We also examined the growth-inhibitory effect of the EGFR tyrosine kinase inhibitors AG-1478, RG-14620 and Lavendustin A and other tyrosine kinase inhibitors in PC-9 and PC-9/ZD cells. PC-9/ZD cells show cross-resistance to AG1478, but not to all of the tyrosine kinase inhibitors (Tables I, II). It is likely that PC-9/ZD would also be resistant to EGFR-targeted quinazoline derivatives including gefitinib and erlotinib.[20]

PC-9/ZD cells show significant resistance to gefitinib in an in vivo *model*

To ascertain whether the resistance of PC-9/ZD occurs *in vivo*, we investigated the growth-inhibitory effect of gefitinib on PC-9 cells and PC-9/ZD cells in a xenotransplanted model. There was no significant difference in the size of the of PC-9 and PC-9/ZD cell tumor masses in nude mice before the start of gefitinib injection. Figure 2 shows the growth-inhibition curve of PC-9 (Fig. 2*a*) and PC-9/ZD (Fig. 2*b*) cells *in vivo* during the observation period. The PC-9 tumor masses decreased markedly in volume at all doses of gefitinib. In the 50 mg/kg/day p.o. group, the PC-9 masses were eradicated in all mice and did not regrow within the observation period. Growth of the PC-9/ZD masses, on the other hand, was inhibited by gefitinib administration in a dose-dependent manner, but significant tumor reduction was observed only in the 25 and 50 mg/kg/day groups, and the PC-9/ZD masses were not eradicated even in 50 mg/kg/day group. These results clearly demonstrate the significant *in vivo* resistance of PC-9/ZD cells to gefitinib.

Expression of HER family members and related molecules in PC-9 and PC-9/ZD cells

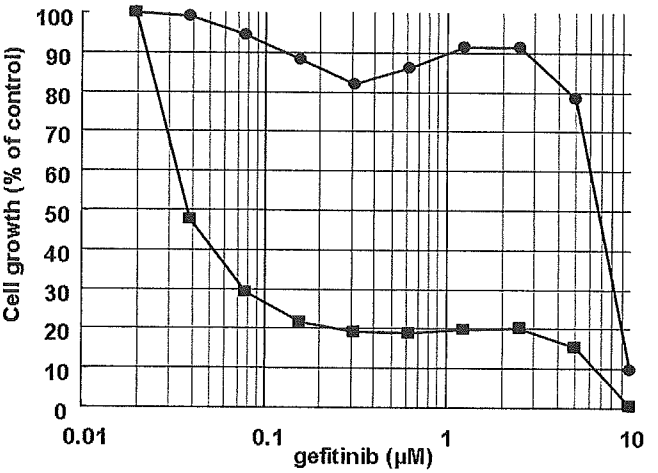
We examined the gene expression and protein levels of HER family members and related molecules by cDNA expression array (followed by confirmation using RT-PCR, data not shown) and immunoblotting. The ratios of the protein expression levels of PC-9 cells to PC-9/ZD cells almost paralleled the expression levels of their genes (Fig. 3*a*). The basal level of EGFR was comparable or slightly higher in PC-9/ZD cells (Fig. 3*a,b*), whereas the HER3 and AKT levels were lower in resistant cells.

We carried out quantitative RT-PCR to measure the copy numbers of *EGFR*. Estimated transcript levels of *EGFR* were 786.3 and 712.1 copies/cell for PC-9 cells and PC-9/ZD cells, respectively (Fig. 3*d*). Relative ratio of *EGFR* expression levels in PC-9 cells and PC-9/ZD cells is 1.104. Microarray analysis using CodeLink Bioarray (Amersham Bio, Piscataway, NJ) confirmed equivalent gene expression of *EGFR* with ratio of 1.002 between PC-9 and PC-9/ZD cells (data not shown).

Expression of PI3K, Grb2, SOS, and Shc, the adaptor proteins of EGFR, and PTEN was almost the same in PC-9 and PC-9/ZD cells, and no change in the protein levels was observed after exposure to gefitinib (data not shown). The relative densitometric units of each protein are shown in Figure 3*c*. These results suggest that the difference in protein levels of EGFR, HER2, and related proteins can not explain the high resistance of PC-9/ZD cells to gefitinib.

Sequence of HER family member in PC-9/ZD cells

Several reports suggest that the resistance to receptor tyrosine kinase inhibitor STI-571 is partially due to mutations in the



	PC-9	PC-9/ZD
IC_{50} value (µM)	0.039± 0.002	7.1± 0.06
Doubling time (hr)	20.3	21.0

FIGURE 1 – Growth-inhibitory effect of gefitinib on PC-9 and PC-9/ZD cells determined by MTT assay. The cells were exposed to the concentrations of gefitinib indicated for 72 hr. The growth–inhibition curves of PC-9 (■) and PC-9/ZD (●) are shown. Doubling time was determined by MTT assay.

TABLE I – CHEMOSENSITIVITY TO OTHER ANTICANCER DRUGS

Drug	IC_{50} values (µM)[1]		RR[2] 1.6
	PC-9	PC-9/ZD	
Cisplatin	1.9 ± 0.7	3.1 ± 1.5	2.0
Carboplatin	25 ± 21	49 ± 23	1.3
Adriamycin	0.16 ± 0.13	0.20 ± 0.15	2.2
Irinotecan	15 ± 10	32 ± 11	1.5
Etoposide	4.5 ± 1.5	6.6 ± 1.3	1.5
Gemcitabine	18 ± 1.5	27 ± 1.5	0.7
Vindesine	0.0046 ± 0.0004	0.0032 ± 0.0009	1.2
Paclitaxel	0.0041 ± 0.0011	0.0048 ± 0.0004	1.6

[1]As assessed by MTT assay in PC-9 and PC-9/ZD cells. Values are the mean ± SD of >3 independent experiments.–[2]Relative resistance value (IC_{50} of resistant cells/IC_{50} of parental cells).

TABLE II – CHEMOSENSITIVITY TO PROTEIN KINASE INHIBITORS[1]

Inhibitor	Target	IC_{50} values (μM)		RR[2]
		PC-9	PC-9/ZD	
AG-1478	EGFR	0.052 ± 0.02	6.0 ± 0.8	117
RG-14620	EGFR	13 ± 1.0	13 ± 2.5	1.0
Lavendustin A	EGFR	20 ± 4.6	27 ± 2.6	1.3
Genistein	TK	18 ± 1.5	27 ± 1.5	1.5
K252a	PKC	0.47 ± 0.17	0.63 ± 0.04	1.3
Staurosporin	PKC	0.0036 ± 0.0019	0.004 ± 0.0014	1.1
AG-825	HER2	>50	>50	

[1]Assessed by MTT assay in PC-9 and PC-9/ZD cells. Values are the mean ± SD of >3 independent experiments.–[2]Relative resistance value (IC_{50} of resistant cells/IC_{50} of parental cells).

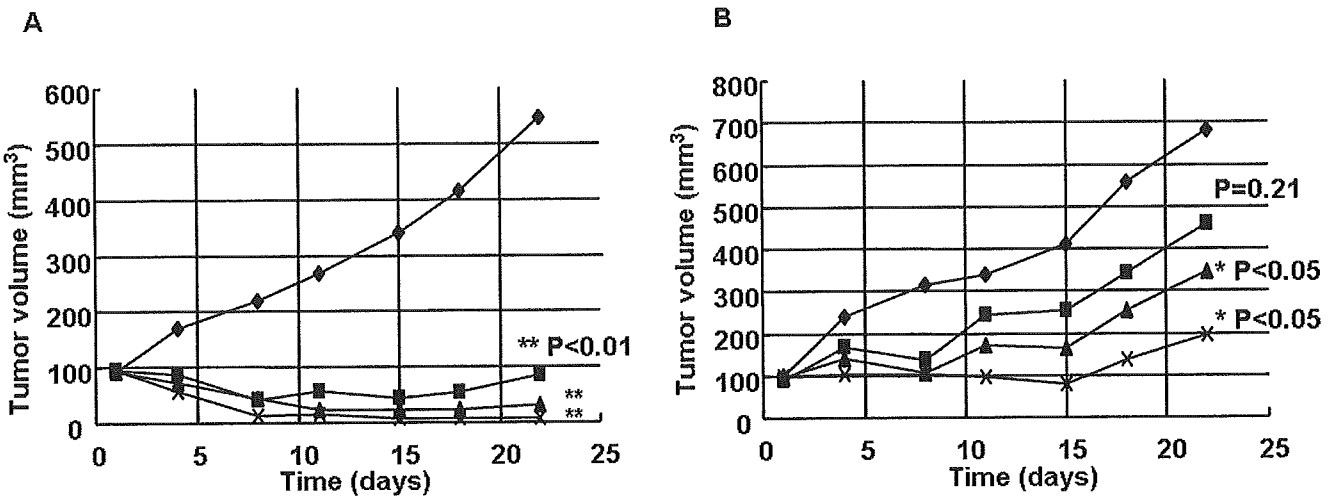


FIGURE 2 – Growth-inhibitory effect of gefitinib on PC-9 and PC-9/ZD cells xenotransplanted into nude mice. Ten days before gefitinib administration, 5×10^6 PC-9 (*a*) or PC-9/ZD (*b*) cells were injected s.c. into the back of mice. The mice were divided into 4 groups (◆, control group; ■, 12.5 mg/Kg group; ▲, 25 mg/Kg group; ×, 50 mg/Kg group). Gefitinib was administered p.o. to the tumor-inoculated mice on Days 1–21. Each group consisted of 6 mice. The statistical analysis was carried out by using the unpaired *t*-test.

ATP-binding site of the Bcr-Abl, the target of the drug.[24–27] We analyzed the sequences of the cDNAs of *EGFR*, *HER2*, and *HER3*, but found no differences in their sequences between PC-9 and PC-9/ZD cells. We did detect a deleted position of *EGFR* in both cell lines that results in deletion of 5 amino acids (Glu722, Leu723, Arg724, Glu725, and Ala726) (Fig. 4). Our findings indicate that the deletion does not directly contribute to the cellular resistance.

Inhibitory effect of gefitinib on autophosphorylation of EGFR in PC-9/ZD cells

Phosphorylation of EGFR is necessary for EGFR-mediated intracellular signaling. Although the EGFR phosphorylation levels of tumors were thought to be correlated with sensitivity to gefitinib, the basal level of phosphorylated EGFR in PC-9 and PC-9/ZD cells is almost the same. Gefitinib inhibited EGFR autophosphorylation in a dose-dependent manner and completely inhibited its phosphorylation at 0.2–2 μM in PC-9 cells (Fig. 5*a*), but its inhibitory effect on autophosphorylation of EGFR in PC-9/ZD cells was less than in PC-9 cells (Fig. 5*a*). Because each phosphorylation site of EGFR has a different role in the activation of downstream signaling molecules, we examined the inhibitory effect of gefitinib on site-specific phosphorylation of EGFR. Phosphorylation of several different EGFR tyrosine residues (Tyr845, Tyr992 and Tyr1068) was dose-dependently inhibited by gefitinib in PC-9 cells, whereas no clear inhibitory effects of gefitinib on phosphorylation at Tyr 845 and Tyr1068 residues in PC-9/ZD cells was observed (Fig. 5*b*,*c*,*e*). The most marked difference of in inhibition between the cells was observed at Tyr1068 (Fig. 5*e*). Tyr1045 showed resistance to inhibition of autophosphorylation by gefitinib in both PC-9 and PC-9/ZD cells (Fig. 5*d*).

Complex formation of EGFR and its adaptor proteins

Tyr1068 of EGFR is the tyrosine that is most resistant to inhibition of autophosphorylation by gefitinib in PC-9/ZD cells. Because the Tyr 1068 is a direct binding site for the GRB2/SH2 domain, and its phosphorylation is related to the complex formation of EGFR-adaptor proteins and their signaling, we examined complex formation between EGFR and the adaptor proteins GRB2, SOS, Shc, and PI3K by immunoprecipitation. The level of expression of these proteins in PC-9 and PC-9/ZD cells were similar (Fig. 3*a*). A smaller amount of EGFR-GRB2 complex was observed in PC-9/ZD cells and no EGFR-SOS complex was detected at all (Fig. 6). The amount of HER2- or HER3-GRB2 complex in PC-9 and PC-9/ZD cells was similar, and no decreases in complex formation were observed after exposure to gefitinib. A decreased amount of HER2-SOS complex and inability to detect HER3-SOS complex were also observed in PC-9/ZD cells. HER2-PI3K complex increased in PC-9/ZD. There are no significant differences in complex formation between SHC and EGFR, HER2, or HER3 between PC-9 and PC-9/ZD cells. These results suggest that GRB2-SOS-mediated signaling may be inactivated in PC-9/ZD cells.

Heterodimerization of HER family member in PC-9/ZD cells

Dimerization of members of the HER family is essential for activation of their catalytic activity and their signaling. We examined the effect of gefitinib on the dimerization of HER family members by immunoblotting, immunoprecipitation and chemical cross-linking analysis (Figs. 3*a*, 5*a*, 7*a*). The expression levels of EGFR and HER2 were similar and the HER3 level was lower in PC-9/ZD cells by immunoblotting (Fig. 3*a*). A chemical cross-

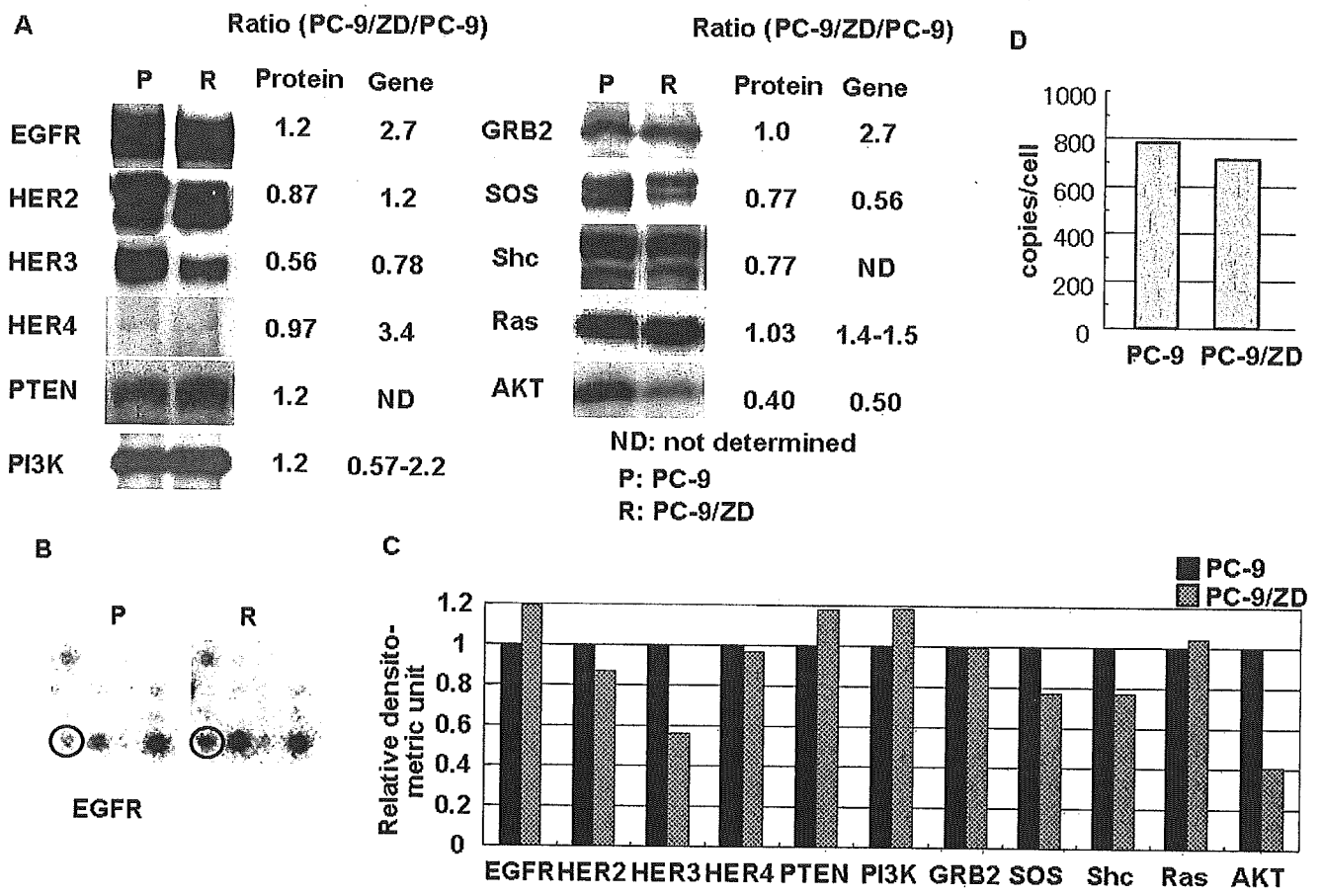


FIGURE 3 – Expresssion of HER family members and related molecules in PC-9 (P) and PC-9/ZD (R) cells. (*a*) Western blot analysis; a 20 μg sample of total cell lysates was separated by SDS-PAGE, transferred to a PVDF membrane, and incubated with a specific anti-human antibody as the first antibody and then with horseradish peroxidase-conjugated secondary antibody. The ratios of the levels of expression of proteins and genes in PC-9 cells to the levels in PC-9/ZD cells are shown. (*b*) cDNA expression array; Poly A RNA was converted into ^{32}P-labeled first-strand cDNA with MMLV reverse transcriptase. The ^{32}P-labeled cDNA fraction was hybridized to the membrane on which fragments of 777 genes were spotted. The close-up view shows *EGFR* mRNA expression. (*c*) Each band was quantified by a densitometry and with NIH image software. The levels of protein expression are shown in a graph. (*d*) Absolute amounts of *EGFR* transcripts of PC-9 cells and PC-9/ZD cells measured by real-time quantitative RT-PCR. The values were calculated back to the initial cell numbers for RNA extraction in Material and Methods.

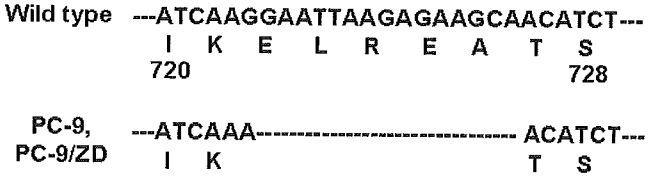


FIGURE 4 – Detection of a deleted position of EGFR. Direct sequencing of a PC-9 and PC-9/ZD-derived, amplified cDNA fragment containing the ATP-binding site of EGFR. *Top*, wild-type EGFR; *bottom*, PC-9 and PC-9/ZD.

linking assay showed that in the absence of gefitinib the amount of high molecular weight complexes (~400 kDa) that are recognized by anti-EGFR antibody (EGFR dimers), including formations of homodimers and heterodimers (EGFR-EGFR, EGFR-HER2 or EGFR-HER3), was almost the same in PC-9 and PC-9/ZD cells, whereas HER2 dimerization detected by anti-HER2 antibody was remarkably lower in PC-9/ZD cells (Fig. 7*a*). Increased EGFR/HER2 (and EGFR/HER3) heterodimer formation was detected in PC-9/ZD cells by immunoprecipitation analysis (Fig. 5*a*). The proportion of EGFR heterodimer to homodimer is increased significantly in PC-9/ZD (Fig. 7*b*). When exposed to gefitinib at a concentration of 0.2 μM for 6 hr the amount of dimer-formation increased similarly in PC-9 and PC-9/ZD cells (Fig. 7*a*), whereas marked induction of hetero-dimerization of EGFR-HER2 was observed only in PC-9 cells (Fig. 5*a*). These results suggest that a difference in hetero- or homo-dimerization is a possible determinant factor of gefitinib sensitivity.

AKT and MAPK pathways in PC-9/ZD cells

Because phosphorylation at Tyr 1068 of EGFR plays an important role for transduction of the signal to downstream of MAPK and AKT pathway,[28,29] we examined the difference between PC-9 and PC-9/ZD cells in downstream signaling. The basal level of phosphorylated AKT is higher in PC-9 cells than in PC-9/ZD cells, and although gefitinib inhibited AKT phosphorylation in a dose-dependent manner (Fig. 8*a*), the inhibitory effect of gefitinib on phosphorylation of AKT in PC-9/ZD cells was significantly less than in PC-9 cells (Fig. 8*a*). This difference in the inhibitory effect of gefitinib on AKT phosphorylation between PC-9 and PC-9/ZD cells is very similar to the difference in effect on EGFR autophosphorylation. No inhibition of phosphorylation of MAPK by gefitinib was observed in either cell line (Fig. 8*b*). These results suggest that downregulation of activated AKT is closely correlated with the cellular sensitivity to gefitinib, but that inhibition of the MAPK pathway does not contribute to drug sensitivity.

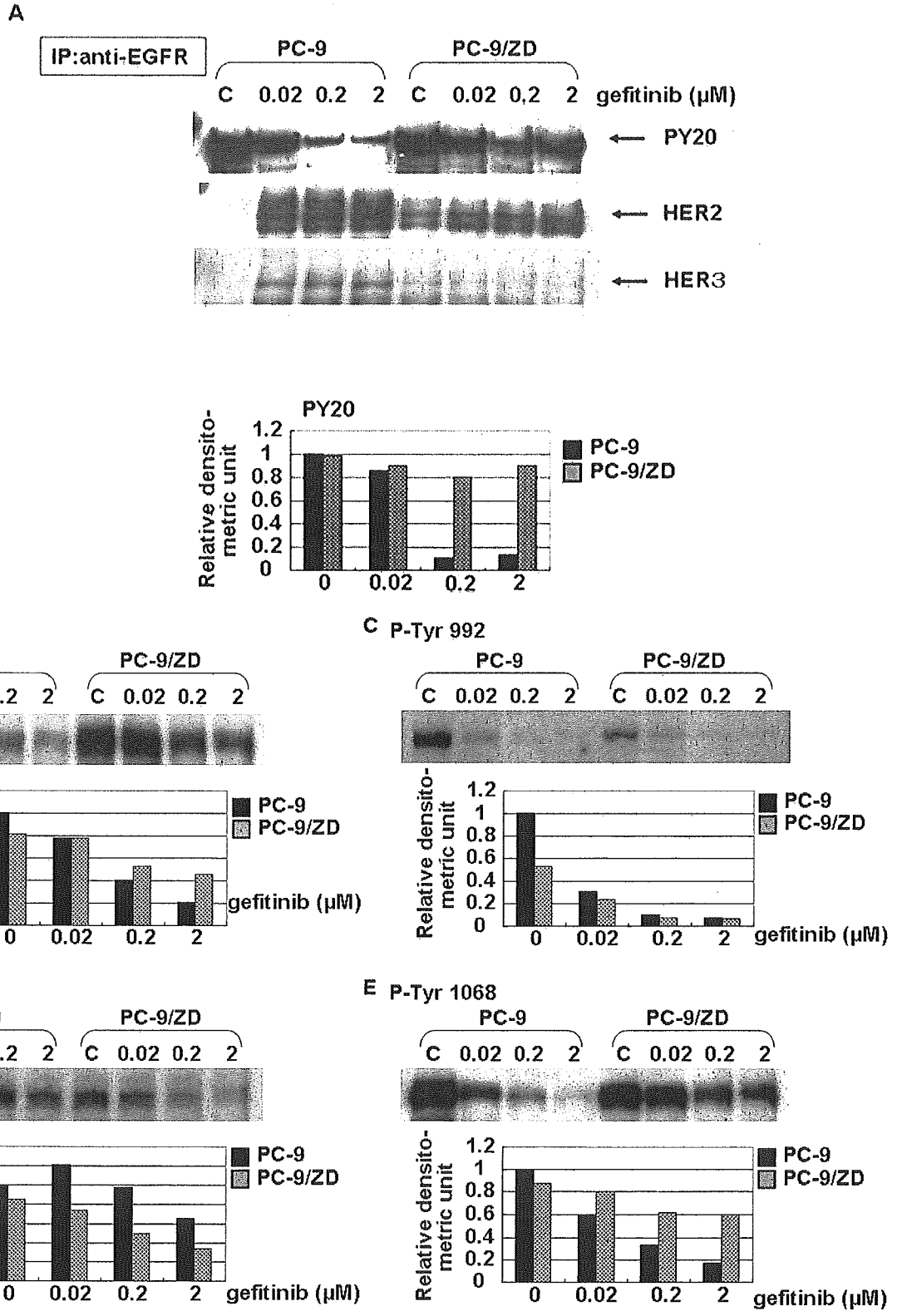


FIGURE 5 – Effect of gefitinib on autophosphorylation of EGFR. (*a*) PC-9 and PC-9/ZD cells (5×10^6) were exposed to 0.02, 0.2 or 2 μM gefitinib for 6 hr. The 1,500 μg of total cell lysate was immunoprecipitated with an anti-EGFR antibody. The immunoprecipitates were subjected to gel electrophoresis and Western blotting with anti-phosphotyrosine, anti-HER2 and anti-HER3 antibodies. Tyrosine-phosphorylated EGFR was determined with an anti-phosphotyrosine antibody. Heterodimer formation of EGFR was analyzed with anti-HER2 and anti-HER3 antibodies. The expression levels have been plotted in a graph. (*b–e*) PC-9 and PC-9/ZD cells were exposed to 0.02, 0.2 and 2 μM gefitinib for 6 hr. A 20 μg of protein of each sample was analyzed by Western blotting by using anti phospho-EGFR (Tyr845, Tyr992, Tyr 1045, Tyr 1068) antibodies.

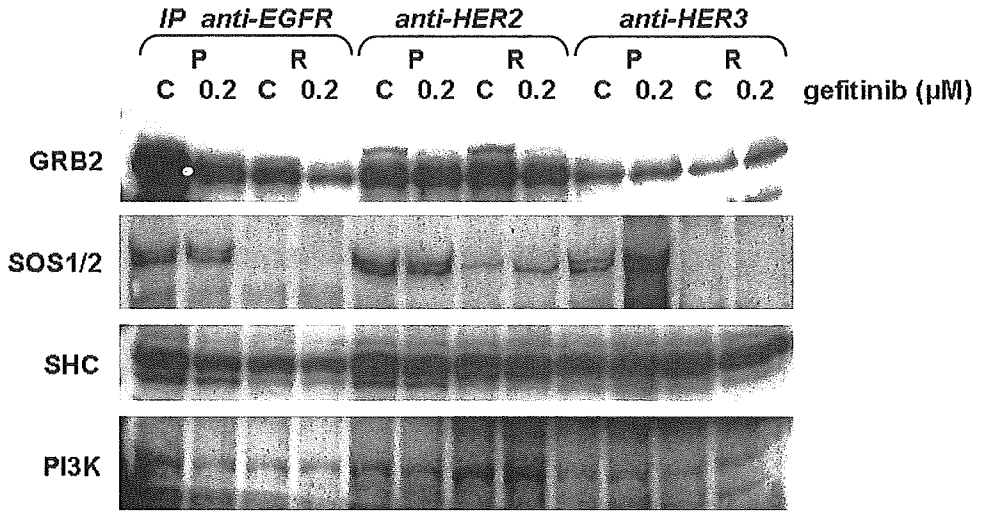


FIGURE 6 – Protein interaction between EGFR and its adaptor proteins. Cells (P: PC-9, R: PC-9/ZD) were exposed to 0 and 0.2 μM of gefitinib for 6 hr. The cells were lysed and immunoprecipitated with ant-EGFR, anti-HER2, and anti-HER3 antibodies, and the amounts of the Grb2, SOS1/2, SHC and PI3K precipitated were monitored by immunoblotting with their specific Abs.

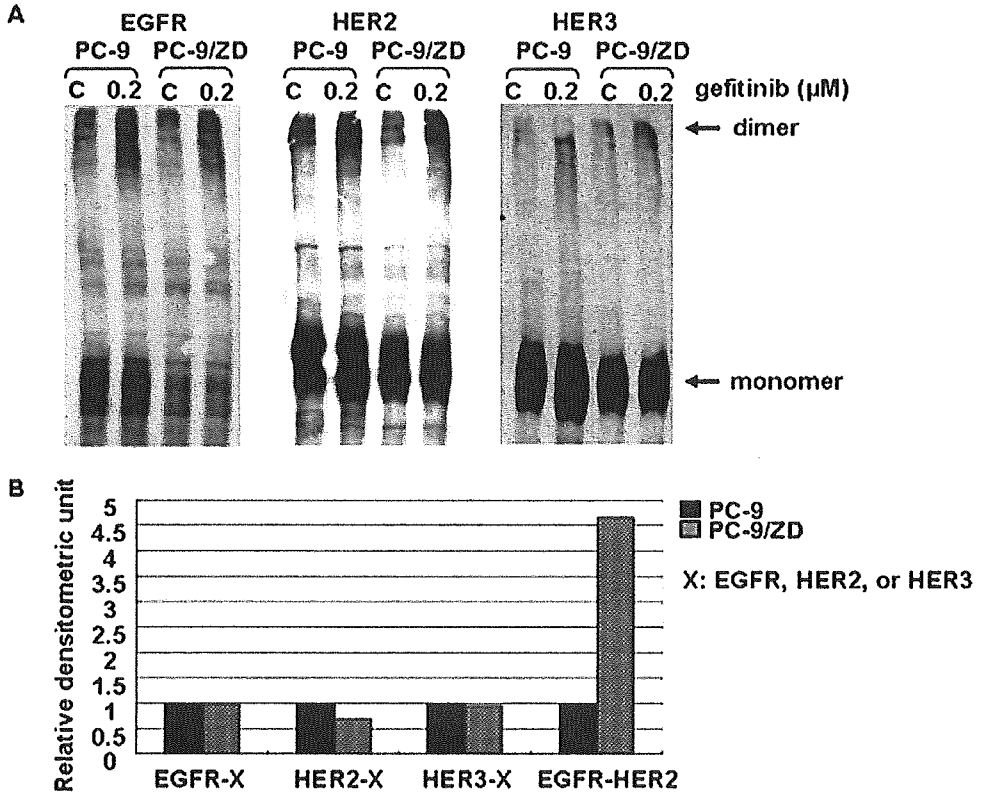


FIGURE 7 – Chemical cross-linking of PC-9 and PC-9/ZD cells. (*a*) After 6 hr exposure to 1.5 mM bis (sulfosuccinimidyl) substrate dissolved in PBS as indicated in Material and Methods. The cross-linking reaction was quenched and the cell lysates were prepared and subjected to immunoblot analysis of EGFR, HER2 and HER3. (*b*) Ratio of dimmers formed by PC-9 cells to those by PC-9/ZD cells in the absence of gefitinib. The density of the bands in (*a*) for EGFR-X, HER2-X and HER3-X were quantified densitometrically. The ratio of EGFR-HER2 was calculated by the band density obtained in Figure 5*a*. X = EGFR, HER2 or HER3.

Discussion

Interest in resistance to target-based therapy (TBT) has been growing ever since clinical efficacy was first demonstrated.[11–13] Although CML patients respond to STI-571 well at first, most patients eventually relapse in the late stage of the disease.[25–27] It has been reported that some patients in whom treatment with gefitinib is effective at first, ultimately become refractory.[30] Resistance is likely to remain a hurdle that limits the long-term effectiveness of TBT. PC-9 had a deletion mutation within the kinase domain of *EGFR* and is highly sensitive. These characters are similar to those of NSCLC with clinical responsiveness to gefitinib. Analyzing the mechanism of resistance of PC-9/ZD subline might be clinically meaningful.

The mechanism of drug resistance is thought to be multifactorial. Because the growth-inhibitory assay in our present study showed no cross resistance to a variety of cytotoxic agents, the mechanism of the resistance differs from the mechanism of multidrug resistance patterns. Although expression of BCRP, one of the multidrug-resistance-related proteins has been reported to contribute to the resistance to gefitinib,[31] expression of *BCRP* mRNA is observed only in PC-9 cells (data not shown). Although mutations in the ATP-binding pocket of *BCR-ABL* gene have been identified recently in cells from CML patients who were refractory to STI-571 treatment or relapse,[25–27] there have been no reports of any such mutations for gefitinib resistance. PC-9/ZD also became refractory to gefitinib without secondary mutation in *EGFR* cDNA. These suggest the possibility of refractory tumor after treatment of gefitinib including this kind of phenotype.

There is no significant difference in expression level of EGFR between PC-9 and PC-9/ZD. Does the antitumor effect of gefitinib

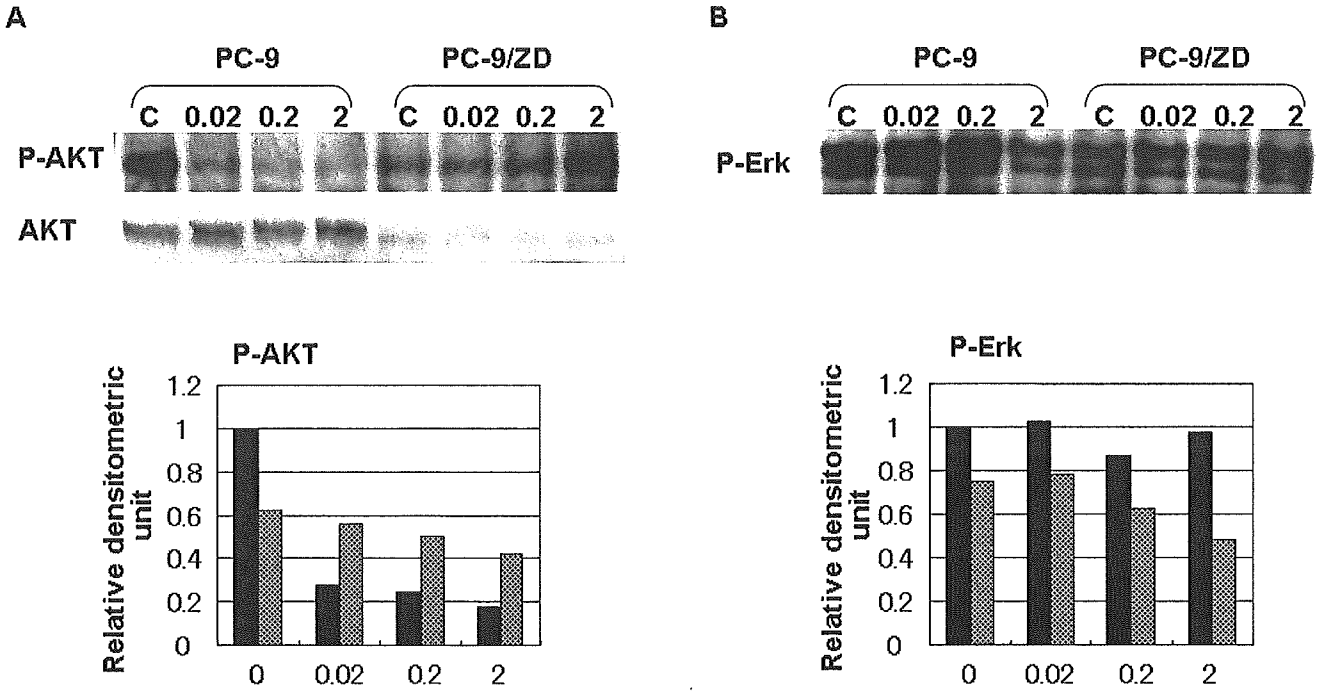


FIGURE 8 – Effect of gefitinib on the MAPK and AKT pathway. Cells were placed in medium containing 0, 0.02, 0.2 or 2 μM of gefitinib for 6 hr and harvested in EBC buffer. Total cellular lysates were separated on SDS-PAGE, transferred to a membrane and blotted with (*a*) anti-phospho-AKT (Ser473) and (*b*) anti-phospho-Erk (p44/42) antibodies. The expression levels are shown in a graph.

require EGFR expression? Naruse *et al.*[32] suggested that the high sensitivity of K562/TPA to gefitinib is due to acquired EGFR expression. In their study autophosphorylation of EGFR in K562/TPA cells was inhibited by 0.01 μM gefitinib, and the IC_{50}-value of gefitinib in parental K562 cells, which do not express EGFR, was approximately 400-fold higher than that in the K562/TPA subline. Furthermore, most patients who responded to gefitinib therapy have *EGFR* mutation in lung tumor.[18,19] These findings suggest strongly that gefitinib exerts its antitumor effect through an action on EGFR. Our present study showed similar EGFR expression and autophosphorylation levels in PC-9 and PC-9/ZD cells. The inhibitory effect of gefitinib on phosphorylation of EGFR is different. PC-9/ZD did not show cross-resistance to the specific EGFR TK inhibitors RG-14620 and Lavendustin A in an MTT assay, nor did inhibit the phosphorylation of EGFR at the cellular level (data not shown). Paez *et al.*[18] reported that phosphorylation of EGFR in gefitinib-resistant cell lines was inhibited only when gefitinib was present at high concentration. These findings suggest that the difference in the inhibitory-effect on EGFR phosphorylation may determine the efficacy of the drug.

The inhibitory effect of gefitinib on EGFR phosphorylation is not significant in PC-9/ZD cells despite the absence of differences in the sequences of *EGFR*, *HER2*, and *HER3*. There are several possible explanations for the difference in inhibitory effect. First, the avidity of gefitinib for the ATP-binding site of EGFR may be decreased in PC-9/ZD cells due to a protein-protein interaction, *i.e.*, EGFR and a certain protein prevent gefitinib from binding to EGFR. Second, a change in the activity of specific protein-tyrosine kinase or phosphatase of EGFR in PC-9/ZD cells, especially after exposure to gefitinib, may result in resistance to inhibition of EGFR phosphorylation. The phosphorylation level is maintained in exquisite balance by the reciprocal activities of kinase and phosphatase,[33,34] and Wu reported that phosphatase plays a role in STI571-resistance.[35] Third, increased heterodimer formation by EGFR with other members of the HER family results in the limited inhibition. Heterodimer formation is increased in PC-9/ZD cells under basal conditions, and no increase in formation was observed after exposure to gefitinib, although marked heterodimer induction was observed in PC-9 cells. Calculations in *in vitro* studies have shown that the IC_{50}-value for inhibition of the tyrosine kinase activity of EGFR is 0.023–0.079 μM, whereas the IC_{50}-value for inhibition of HER2 is 100-fold higher.[36] We estimate that the inhibitory effect of gefitinib depends on the ratio of homodimer formation to heterodimer formation, and the heterodimer may be one of the routes of escape from the action of gefitinib.

Signal transduction by the HER family member is mediated by 2 major pathways, the MAPK signaling pathway and the AKT signaling pathway, which regulate cell proliferation and survival. Because phosphorylated AKT was inhibited completely by gefitinib in PC-9 cells, but inhibition of phosphorylated MAPK was not significant, inhibition of the AKT pathway may be more important to cell sensitivity than inhibition of MAPK. Moasser *et al.*[37] reported consistent results, showing that downregulation of AKT activity is predominantly seen in tumors that are sensitive to gefitinib. The phosphorylation of AKT and MAPK was not inhibited significantly by gefitinib in PC-9/ZD cells. This finding might be attributable to inactivation of Tyr 1068-GRB2-SOS-mediated signaling.

Based on the results of this comparative study, EGFR-GRB2-SOS complex formation, phosphorylation of Tyr1068, the ratio of the amount of homodimer formation to heterodimer formation, and the AKT signaling pathway are possible predictive biomarkers for gefitinib sensitivity. As a different approach, we are now looking for the genes associated with gefitinib resistance in PC-9/ZD cells compared to PC-9 cells by subtractive cloning.

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'Iressa' is a trademark of the AstraZeneca group of companies.

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ORIGINAL REPORT

First-Line Single Agent Treatment With Gefitinib in Patients With Advanced Non–Small-Cell Lung Cancer: A Phase II Study

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Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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ABSTRACT

Purpose
We conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced non–small-cell lung cancer (NSCLC) to assess its efficacy and toxicity.

Patients and Methods
Patients received 250 mg doses of gefitinib daily. Administration of gefitinib was terminated if partial response (PR) was not achieved within 8 weeks or if tumor reduction was not observed within 4 weeks. In these cases, platinum-based doublet chemotherapy was given as a salvage treatment. We evaluated mutation status of the epidermal growth factor receptor (EGFR) gene in cases with available tumor samples.

Results
Forty-two patients were enrolled between March and November 2003, with 40 of these patients being eligible. The response rate was 30% (95% CI, 17% to 47%). The most common toxicity included grade 1 or 2 acne-like rash (50%) and grade 1 diarrhea (18%). Grade 2 or 3 hepatic toxicity was observed in 8% of patients. Four patients developed grade 5 interstitial lung disease (ILD). Thirty patients received second-line chemotherapy. Median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%. Tumor samples were available in 13 patients, including four cases of PR, six cases of stable disease, and three cases of progressive disease. *EGFR* mutations (deletions in exon 19 or point mutations [L858R or E746V]) were detected in four tumor tissues. All four patients with *EGFR* mutation achieved PR with gefitinib treatment.

Conclusion
Single agent treatment with gefitinib is active in chemotherapy-naïve patients with advanced NSCLC, but produces unacceptably frequent ILD in the Japanese population.

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INTRODUCTION

Previous meta-analysis demonstrated that cisplatin-based chemotherapy yielded a modest but significant survival benefit over best supportive care in advanced non–small-cell lung cancer (NSCLC).[1-4] In the 1990s, new agents, including vinorelbine, gemcitabine, paclitaxel, docetaxel, and irinotecan became available for the treatment of NSCLC. Several phase III trials comparing doublet platinum-based chemotherapies demonstrated no significant difference with respect to response rate, survival, or quality of life.[5,6] Nonplatinum or triplet platinum-based combination chemotherapies have been investigated, but none of these produced longer survival than standard doublet platinum-based chemotherapy.[7-9]

Recently, molecular-targeted agents have been introduced for the treatment of NSCLC. Gefitinib is an orally active epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which displays activity against recurrent NSCLC after platinum-based chemotherapy. Two international, randomized phase II trials in patients with advanced or metastatic NSCLC after platinum-based chemotherapy demonstrated response rates of 12% to 18% (28% in the Japanese population).[10,11] Two international, randomized, double-blinded, placebo-controlled phase III trials investigated the role of gefitinib combined with platinum-based chemotherapy regimens, including carboplatin and paclitaxel, or cisplatin and gemcitabine in chemotherapy-naïve patients with advanced NSCLC.[12,13] Surprisingly, there were no improvements in overall survival,

time to progression, or response rate. There are no data available regarding first-line treatment with single agent gefitinib against NSCLC in the Japanese population. Here, we conducted a phase II study of single agent treatment with gefitinib in chemotherapy-naïve patients with advanced NSCLC. If a failure with gefitinib treatment was perceived, standard platinum-based doublet chemotherapy was performed as salvage. The primary end point of this phase II trial was response rate, and the secondary end points were toxicity, survival, and response rate of salvage chemotherapy.

PATIENTS AND METHODS

Patient Population

Patients were required to have histologically or cytologically confirmed stage IIIB (malignant pleural or pericardial effusion and/or metastasis in the same lobe) or stage IV NSCLC. Recurrences after surgical resection were permitted. Other criteria included: (1) age 20 years or older, but younger than 75 years; (2) Eastern Cooperative Oncology Group performance status (PS) 0 or 1; (3) measurable disease; (4) PaO2 $\geq$ 60 mmHg; (5) adequate organ function (ie, total bilirubin $\leq$ 2.0, AST and ALT $\leq$ 100 U/L, serum creatinine $\leq$ 1.5 mg/dL, leukocyte count 4,000 to 12,000/mm^3, neutrophil count $\geq$ 2,000/mm^3, hemoglobin $\geq$ 9.5 g/dL, and platelets $\geq$ 100,000/mm^3); (6) no prior chemotherapy or thoracic radiotherapy; (7) no interstitial pneumonia or pulmonary fibrosis, as determined by chest x-ray; (8) no paralytic ileus or vomiting, (9) no symptomatic brain metastases, (10) no active infection; (11) no active concomitant malignancy; (12) no pregnancy or breast-feeding; (13) no severe allergy to drugs. Patients with PaO2 less than 60 mmHg were excluded, because those patients might have pulmonary fibrosis, which is a risk factor of interstitial lung disease (ILD).[14] All patients were required to provide written informed consent and the institutional review board at the National Cancer Center approved the protocol.

Treatment Plan

Treatment was started within a week after enrollment in the study. Patients received 250 mg of gefitinib orally daily. In the event of grade 3 or more and/or unacceptable toxicities, gefitinib was postponed until these toxicities were improved to grade 2 or less. Dose reduction was not performed. If treatment was postponed four times or more, the treatment was terminated. Therapy was continued unless the patient experienced unacceptable toxicity or progressive disease, partial response (PR) was not achieved within 8 weeks, or the sum of the longest diameters of the target lesions decreased less than 10% within 4 weeks. If the gefitinib treatment failed according to these criteria, platinum-based doublet chemotherapy was performed as a salvage regimen.

Previous trials of gefitinib for pretreated patients with NSCLC reported that most responding patients showed rapid tumor regression within 4 or 8 weeks.[11] Furthermore, most responses by gefitinib were extreme shrinkage of the tumor. Minor response, as frequently seen by the treatment with cytotoxic agents, was seldom experienced. Stable disease with gefitinib corresponded to no tumor reduction or slight progression. If patients with stable disease continued the treatment with gefitinib until progressive disease became obvious, those patients might not be able to receive platinum-based salvage chemotherapy because of poor PS due to progressive disease. Platinum-based combination chemotherapy is the standard care for patients with advanced NSCLC and good PS. Platinum-based chemotherapy was thought to be essential for patients with no response from the first-line single agent treatment with gefitinib. Therefore, we implemented these early stopping criteria for treatment with gefitinib.

Study Evaluations

Pretreatment evaluations consisted of a complete medical history, determination of performance status, physical examination, hematologic and biochemical profiles, arterial blood gas examination, ECG, chest x-ray, bone scan, and computed tomography (CT) scan of the chest, ultrasound or CT scan of the abdomen, and magnetic resonance imaging or CT scan of the whole brain. Evaluations performed included a weekly chest x-ray for 4 weeks, and once every 2 weeks for biochemistry, complete blood cell, platelet, leukocyte differential counts, physical examination, determination of performance status, and toxicity assessment. Imaging studies were scheduled to assess objective response every month.

Response and Toxicity Criteria

Response evaluation criteria in solid tumors (RECIST) guidelines were used for evaluation of antitumor activity.[15] The target lesions were defined as $\geq$ 2 cm in the longest diameter on CT scans. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A PR was defined as an at least 30% decrease in the sum of the longest diameters of the target lesions for more than 4 weeks with no new area of malignant disease. Progressive disease (PD) indicated at least a 20% increase in the sum of the longest diameter of the target lesions or a new malignant lesion. Stable disease was defined as insufficient shrinkage to qualify for PR and insufficient increase to qualify for PD. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 2.0.

Mutation Analysis of the EGFR Gene

Tumor specimens were obtained during diagnostic or surgical procedures. Biopsied or surgically resected specimens were fixed with formalin or 100% methanol, respectively. Tumor genomic DNA was prepared from paraffin-embedded sections using laser capture microdissection in biopsied specimens or macrodissection in surgically resected specimens at Mitsubishi Chemical Safety Institute LTD. Exons 18, 19, and 21 of the *EGFR* gene were amplified and sequenced as previously described.[16]

Statistical Analysis

In accordance with the minimax two-stage phase II study design by Simon,[17] the treatment program was designed to refuse response rates of 10% (P_0) and to provide a significance level of .05 with a statistical power of 80% in assessing the activity of the regimen as a 25% response rate (P_1). The upper limit for first-stage drug rejection was two responses in the 22 assessable patients; the upper limit of second-stage rejection was seven responses within the cohort of 40 assessable patients. Overall survival was defined as the interval between enrollment in this study and death or the final follow-up visit. Median overall survival was estimated by the Kaplan-Meier analysis method.[18] Fisher's exact test was used in a contingency table.

RESULTS

Patient Population

A total of 42 patients were enrolled in this study between March and November, 2003, with 40 of these patients being eligible. One patient was found ineligible due to anemia, the other because spinal magnetic resonance imaging could not confirm a positive bone scan. Patient characteristics are listed in Table 1. Sixty percent of patients were male; median age was 61 years. The most common histologic subtype was adenocarcinoma (75%). Most patients (93%) had stage IV disease or recurrence after surgical resection. Eighty percent of patients were current or former smokers.

Efficacy

One patient (3%) has been receiving gefitinib after 22 months. Four patients suspended gefitinib for 11, 14, 27, or 29 days, because of liver dysfunction (n = 3) and fever due to urinary tract infection (n = 1). Thirty-nine patients terminated gefitinib because of progressive disease (n = 20), no tumor reduction within 4 weeks (n = 12), not achieving PR within 8 weeks (n = 1), toxicities including pulmonary (n = 3), nausea and vomiting (n = 1), rash (n = 1), or hepatic dysfunction (n = 1).

There were 12 PRs in 40 eligible patients, and the objective response rate was 30% (95% CI, 17% to 47%; Table 2). All but one

Table 1. Patient Characteristics

Characteristic	No. of Patients
Patients enrolled	42
Patients eligible	40
Sex	
Male	24
Female	16
Age, years	
Median	61
Range	44-74
Performance status	
0	14
1	26
Stage	
IIIB	3
IV	34
Recurrence after surgery	3
Histologic type	
Adenocarcinoma	30
Squamous cell carcinoma	3
Large cell carcinoma	7
Smoking history	
Current	27
Former	5
Never	8

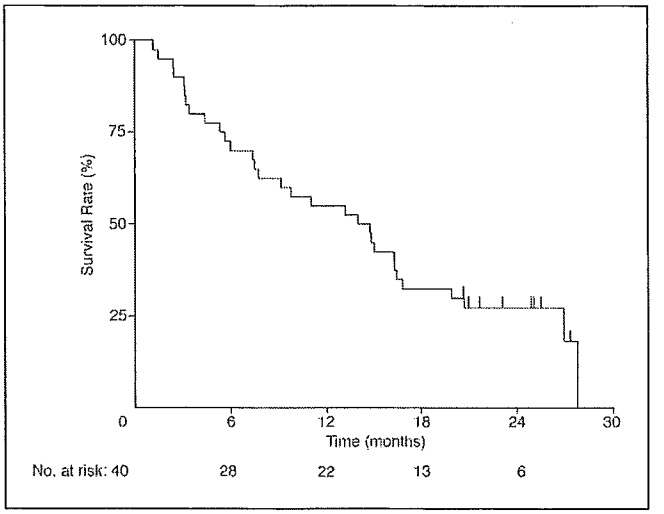

Fig 1. Overall survival of all eligible patients (n = 40) was calculated according to the Kaplan-Meier method. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55%.

patient from this subgroup achieved PR within 4 weeks, with the remaining patient achieving PR within 8 weeks. The background of the 12 responding patients was as follows: nine females, three males; 11 adenocarcinomas, one large-cell carcinoma; six individuals who never smoked, five current smokers, and one former smoker. Response rates based on patient characteristics were as follows: three of 24 (13%) males, nine of 16 (56%) females ($P = .0050$); 11 of 30 (37%) individuals with adenocarcinoma, one of 10 (10%) individuals with squamous or large-cell carcinoma ($P = .0048$); six of 32 (19%) current or former smokers, and six of eight (75%) individuals who never smoked ($P = .0048$).

The median follow-up time was 23 months, and nine patients were still alive at the most recent follow-up. The median survival time was 13.9 months (95% CI, 9.1 to 18.7 months), and the 1-year survival rate was 55% (Fig 1).

Safety and Toxicity

Toxicity was evaluated in all eligible patients. The most common toxicity was rash (Table 3). Thirty-eight percent and 13% of patients experienced grade 1 or 2 rash, respectively. One patient experienced grade 3 nausea and vomiting, leading to gefitinib treatment being terminated. Grade 3 hepatic toxicity was observed in one patient, also causing termination of gefitinib treatment.

The most problematic toxicity was ILD. We reviewed the medical records, chest x-rays, and CT films of all the cases, which were suspected as ILD by the physician in charge. ILD was diagnosed on the basis of standard or high-resolution CT findings of the chest (diffuse ground-glass opacity, consolidation, or infiltrate) and no response to antibiotics. We diagnosed that four patients experienced grade 5 ILD during or after first-line treatment with gefitinib. The first patient was a 61-year-old man. He developed dyspnea and fever elevation (38.1°C) on day 23 of the treatment with gefitinib and administration of gefitinib was terminated. Chest CT demonstrated bilateral diffuse ground-glass opacity, and PaO2 was 43.7mmHg in the room air. KL-6 antigen, a serum marker of interstitial pneumonia, was not elevated

Table 2. Efficacy of Single Agent Treatment With Gefitinib in Patients With Stage IIIb or IV Non–Small-Cell Lung Cancer

Type of Response	No. of Patients	% of Patients
Complete	0	0
Partial	12	30
CR + PR	12	30
95% CI	17 to 47	
Stable disease	16	40
Progression	12	30

Abbreviations: CR, complete response; PR, partial response.

Table 3. Maximum Toxicity Grades Associated With Single Agent Treatment With Gefitinib in 40 Patients With Non–Small-Cell Lung Cancer

	Toxicity Grade									
	1		2		3		4		5	
Toxicity	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%	No. of Patients	%
Rash	15	38	5	13	0	0	0	0	0	0
Dry skin	4	10	0	0	0	0	0	0	0	0
Diarrhea	7	18	0	0	0	0	0	0	0	0
Nausea	3	8	0	0	1	3	0	0	0	0
Mucositis	6	15	0	0	0	0	0	0	0	0
Alopecia	4	10	0	0	0	0	0	0	0	0
Hyponatremia	24	60	0	0	3	8	0	0	0	0
Hypokalemia	12	30	0	0	0	0	0	0	0	0
Hepatic	11	28	2	5	1	3	0	0	0	0
Renal	4	10	1	3	0	0	0	0	0	0
ILD	0	0	0	0	0	0	0	0	4	10

Abbreviation: ILD; interstitial lung disease.

(351 U/mL) on day 24, but elevated on day 31 (1,400 U/mL). Beta-D-glucan, a serum marker of fungal infection and *Pneumocystis carinii* pneumonia, was also negative. Methylprednisolone and antibiotics were administered, with temporal improvement of ILD. However, subsequently, pulmonary function gradually deteriorated, leading to death. Autopsy revealed alveolar damage with organization around the bronchus and vessels in both neoplastic and non-neoplastic lesions, compatible with drug-induced ILD. The second patient was a 64-year-old man. Chest CT on day 27 showed stable disease, but administration of gefitinib was continued (protocol violation). Periodic chest x-ray film on day 45 showed abnormal shadow in the left lung field. High-resolution CT of the chest on the same day revealed reticular shadow on bilateral upper lobe. The treatment with gefitinib was terminated on day 45. KL-6 antigen was not elevated on day 49 (276 U/mL). Methylprednisolone and antibiotics were administered, but were not effective, leading to death. The third patient was a 67-year-old man. Chest CT on day 30 demonstrated enlargement of primary lesion and bilateral reticular shadow in subpleural lesions. Gefitinib was terminated on day 30. The patient developed dyspnea without fever elevation on day 37. Pao2 in the room air fell to 61.0 mmHg from 82.4 mmHg at pretreatment. Chest x-ray showed that the bilateral diffuse reticular shadow deteriorated. Methylprednisolone and antibiotics were administered, but were not effective, leading to death. Autopsy revealed severe fibrotic thickness of alveolar septum, compatible with severe interstitial pneumonia. There was no pathological evidence of carcinomatous lymphangiosis. The fourth patient was a 59-year-old woman. Chest x-ray showed consolidation in the left lung on day 21. Slight fever (37.9°C) developed on day 22. Blood culture was negative. Antibiotics were administered, but consolidation deteriorated and spread to both lungs on day 25. Gefitinib was terminated on day 25. KL-6 antigen was elevated to 3,590 U/mL. Methylprednisolone was administered, but was not effective, leading to death (Table 4). Four other patients experienced ILD after second-line or third-line chemotherapy. Two patients received second-line treatment with cisplatin plus vinorelbine (one and four courses), one patient received treatment with cisplatin plus gemcitabine (one course), and one patient received third-line treatment with docetaxel (four courses). Three of four patients received steroids, with temporal improvement of ILD being observed in two patients. However, ILD deteriorated during tapering of steroid treatment, with three patients subsequently dying. One patient stopped the third-line treatment with docetaxel, with the associated ILD showing improvement in this case without steroid treatment (Table 4).

We retrospectively reviewed the pretreatment chest x-rays and CT films of all patients. Interstitial shadow was not detected on pretreatment chest x-ray films in any patients. However, six patients showed evidence of interstitial shadow on pretreatment chest CT films. Three of the six patients with interstitial shadow, as determined by pretreatment chest CT, experienced ILD either during or following administration of gefitinib or second-line chemotherapy. None of the six patients responded to gefitinib treatment. On the other hand, four of 34 patients who showed no interstitial shadow on pretreatment chest CT films experienced ILD. Interstitial shadow as determined by pretreatment chest CT was not a statistically significant risk factor of ILD ($P = .0819$; Table 5).

Second-Line Chemotherapy

A total of 30 patients received second-line chemotherapy. Twenty-seven patients received platinum-based chemotherapy (cisplatin plus vinorelbine; n = 17), carboplatin plus paclitaxel (n = 5), cisplatin plus gemcitabine (n = 3), cisplatin plus docetaxel (n = 1), and cisplatin plus irinotecan (n = 1). The remaining three patients received vinorelbine plus gemcitabine or vinorelbine alone. Nine of 30 patients achieved PR with these second-line chemotherapies. The objective response rate of second-line chemotherapy was 30% (95% CI, 15% to 50%).

Mutation Status of the EGFR Gene

Out of 42 enrolled patients, 16 patients were diagnosed pathologically, 22 were diagnosed cytologically, and four patients recurred after surgical resection. Biopsied specimens were available in nine patients. Therefore, tissue samples were available in a total of 13 patients. These 13 patients included four PRs, six with stable disease, and three PDs. *EGFR* mutations were detected in four tumor tissues, including the in-frame nucleotide deletions in exon 19 (n = 3) and an L858R mutation in exon 21 (n = 1). One tumor had an in-frame deletion and

Table 4. Four Patients Developed Interstitial Lung Disease During First-Line Chemotherapy With Gefitinib, With Another Four Patients Showing ILD During Either Second- or Third-Line Chemotherapy

Age (years)	Sex	Smoking Index	Pathology	Onset of ILD	Response to Gefitinib	Death From Chemotherapy
61	M	1,520	AD	Day 23*	PD	Day 74
64	M	880	AD	Day 45*	SD	Day 51
67	M	1,880	SQ	Day 37†	PD	Day 45
59	F	0	AD	Day 21*	PD	Day 35
61	M	820	AD	Day 131‡	SD	Day 154
68	M	2,000	LA	Day 37‡	PD	Day 106
68	M	705	AD	Day 22§	PR	Day 87
59	M	1,170	AD	Day 108‖	SD	Alive

Abbreviations: ILD, interstitial lung disease; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; LA, large-cell carcinoma; PD, progressive disease; SD, stable disease; PR, partial response.
*During gefitinib administration.
†One week after discontinuation of gefitinib.
‡ After 2nd-line chemotherapy of cisplatin and vinorelbine.
§ After 2nd-line chemotherapy of cisplatin and gemcitabine.
‖ After 3rd-line chemotherapy of docetaxel.

Table 5. Interstitial Shadow on Pretreatment Chest Computed Tomography Films and ILD

Interstitial Shadow on Pretreatment Chest Computed Tomography Scans	No ILD	ILD
No existence	29	5
Existence	3	3

NOTE. $P = .0819$.
Abbreviation: ILD interstitial lung disease.

an E746V mutation in exon 19. All four PR patients had *EGFR* mutations (Table 6).

DISCUSSION

This phase II study was designed to evaluate the efficacy and safety of first-line single agent treatment with gefitinib in patients with advanced NSCLC. There is no other paper that evaluates single agent treatment with gefitinib prospectively in patients with advanced NSCLC. The observed response rate of 30% (95% CI, 17% to 47%), median survival of 13.9 months and 1-year survival of 55% are promising. However, grade 5 ILD occurred in 10% (95% CI, 3% to 24%) of patients. This high rate of ILD was not acceptable. The incidence of ILD was seen to be less than 1% in two randomized controlled studies comparing gefitinib with placebo in combination with gemcitabine and cisplatin or paclitaxel and carboplatin.[12,13] The reason for the high incidence of ILD observed in our study is unknown. The West Japan Thoracic Oncology Group analyzed 1,976 patients receiving gefitinib retrospectively. In this case, the incidence of ILD was 3.2% (95% CI, 2.5% to 4.6%) and the death rate due to ILD was 1.3% (95% CI, 0.8% to1.9%). Multivariate analyses found that risk factors included being male, individuals who smoked, and complication of interstitial pneumonia.[14] Our retrospective analyses revealed that three of six patients with interstitial shadow on pretreatment chest CT films, but not detected on chest x-ray films developed ILD; on the other hand, five of 34 patients without interstitial shadow developed ILD. Interstitial shadow on pretreatment chest CT was a marginally significant risk factor of ILD ($P = .0819$). It might be suggested that patients with interstitial shadow on pretreatment chest CT films be excluded from administration of gefitinib; however, our analyses were biased because we analyzed retrospectively and did not blind patient clinical information. Prospective analysis is needed to evaluate interstitial shadow by chest CT before treatment with gefitinib.

The Southwest Oncology Group conducted a phase II trial to evaluate gefitinib in patients with advanced bronchioloalveolar carcinoma (SWOG 0126). Previously untreated (n = 102) and treated (n = 36) patients were entered and eligible in SWOG 0126. The response rate was 19% and the median survival time was 12 months in the untreated population.[19] These subset analyses were comparable to our results.

Recently, mutations in the tyrosine kinase domain of EGFR were found to be associated with gefitinib sensitivity in patients with NSCLC.[16,20,21] Our retrospective analyses demonstrated that *EGFR* mutations were detected in four of 13 patients, and those four patients achieved PR in the single agent treatment of gefitinib. These results were compatible with previous reports.[16,20,21]

Thirty patients received second-line chemotherapy, including platinum-based (n = 27) and nonplatinum-based (n = 3) regimens; the response rate was 30%. Pretreatment with gefitinib does not seem to adversely affect the response of second-line chemotherapy. However, our small-scale study does not suggest the best second-line regimen. Platinum combined with any third-generation agents including paclitaxel, docetaxel, vinorelbine,

Table 6. Mutation Status of the *EGFR* Gene

Sex	Age (years)	Pathologic Type	Smoking Status	Overall Survival (months)	*EGFR* Gene	Effect of Mutation	Response to Gefitinib	Response to Second Line Chemotherapy
M	68	AD	Current	14.9	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	67	AD	Current	16.2	Deletion of 15 nucleotides (2236-2250)	In-frame deletion (E746-A750)	PR	PD
F	54	AD	Current	5.6	Deletion of 18 nucleotides (2238-2255) and substitution of T for A at nucleotides 2237	In-frame deletion (L747-S752) and amino acid substitution (F746V)	PR	NR
F	57	AD	Never	25.4	Substitution of G for T at nucleotide 2573	Amino acid substitution (L858R)	PR	SD
M	61	AD	Current	7.5	Wild	—	SD	SD
M	54	AD	Current	9.7	Wild	—	SD	SD
M	45	AD	Current	16.2	Wild	—	SD	PR
M	59	AD	Current	14.7	Wild	—	SD	PR
M	67	SQ	Current	2.4	Wild	—	SD	NR
M	59	AD	Current	24.9	Wild	—	SD	PR
M	61	AD	Current	2.4	Wild	—	PD	NR
F	61	SQ	Current	3.4	Wild	—	PD	PD
F	61	AD	Current	16.3	Wild	—	PD	PR

Abbreviations: EGFR, epidermal growth factor receptor; M, male; F, female; AD, adenocarcinoma; SQ, squamous cell carcinoma; PR, partial response; SD, stable disease; PD, progressive disease; NR, not received.