Table 4. Characteristics of patients with modified premedication protocol ($n = 22$)

Total no. of patients	22
Sex	
Male	20
Female	2
Median age, years (range)	65 (38–74)
Performance status	
0–1	19
2	3
Stage	
IIIA	8
IIIB	6
IV	8
Histological type	
Adenocarcinoma	17
Squamous cell carcinoma	2
Large cell carcinoma	3
Chemotherapy	
First-line	15
Second-line	6
Third-line	1

Table 5. Nonhematological toxicity observed in patients with modified premedication protocol ($n = 22$)

	Grade (no. of patients)							
	1	(%)	2	(%)	3	(%)	4	(%)
Nausea	11	(50)	1	(4)	1	(4)	–	
Vomiting	1	(4)	0	(0)	1	(4)	0	(0)
Appetite loss	12	(54)	1	(4)	2	(9)	0	(0)
Neuropathy	8	(36)	3	(13)	0	(0)	0	(0)
Myalgia	5	(22)	6	(27)	0	(0)	0	(0)
Arthralgia	6	(27)	8	(36)	0	(0)	0	(0)
Diarrhea	1	(4)	0	(0)	1	(4)	0	(0)
Alopecia	2	(9)	9	(40)	–	–		
Fatigue	14	(63)	2	(9)	1	(4)	0	(0)
Somnolence	11	(50)	0	(0)	0	(0)	0	(0)
Hypersensitivity reactions								
Flushing	14	(63)	0	(0)	0	(0)	0	(0)
Tachycardia[a]	1	(4)	0	(0)	0	(0)	0	(0)
Skin rash[a]	1	(4)	1	(4)	0	(0)	0	(0)

Toxicity assessed according to NCI-CTC version 2
[a]Three patients also showed flushing

HSRs were seen. All adverse events resolved naturally without corticosteroid administration. Other nonhematological toxicities, some of which were grade 3, were mainly digestive toxicities, such as appetite loss, vomiting, constipation, and diarrhea. In addition, 11 (50%) of the 22 patients had mild somnolence, which symptom disappeared immediately after the end of treatment.

Drug delivery

Overall, the median cumulative paclitaxel exposure of the 22 patients was 665 mg/m^2 (range, 175–1050 mg/m^2). The average number of cycles delivered was 3.5 (range, 1–6). The dose was reduced in 18% of the patients because of hematological toxicity.

Discussion

Although paclitaxel-based chemotherapy is widely used for patients with NSCLC,[14] severe HSRs are reported more frequently with paclitaxel treatment than with other cytotoxic chemotherapeutic drugs. If severe HSRs occur, the paclitaxel treatment is discontinued. This is a disadvantage for patients, so prophylactic treatment has been used.[4–7] Dexamethasone is a long-acting glucocorticoid with a biologic half-time of approximately 48 h.[15] Currently, a short premedication regimen including single-dose intravenous dexamethasone has been recommended.[8–11] A comparative prospective study has reported that the incidence of paclitaxel-related HSRs was not significantly different between conventional and short premedication regimens.[14] However, Kwon et al.[12] retrospectively showed that a single-dose intravenous corticosteroid prophylactic regimen was associated with a significantly higher rate of HSRs than the two-dose oral corticosteroid regimen. Moreover, Kloover et al.[13] have reported that short premedication may not be a suitable prophylactic therapy for paclitaxel-related HSR because of a fatal outcome.

We retrospectively analyzed, in a historical cohort, the incidence of paclitaxel-related HSRs in patients who had received oral diphenhydramine, plus a single dose or two doses of intravenous dexamethasone. We found that six of the patients with a short premedication regimen had severe HSRs, which events occurred as soon as paclitaxel was initiated in the second course. These events included chest or back pain, hypoxia, dyspnea, and bronchospasm, and the incidence of severe HSRs was significantly higher than that in the conventional premedication group. Since obtaining the results of the historical analysis, we immediately stopped using the short premedication regimen. The incidence of severe HSRs in the patients in the short premedication group was quite high compared with that in past reports. Possibly, our definition of severe HSR may have differed from that used previously. However, grade 2 chest or back pain should be considered as severe, and paclitaxel infusion should be stopped for safety, because such symptoms have a possibility to lead to serious toxicity, such as cardiac arrest.[13]

In Japan, oral diphenhydramine had usually been used as a prophylactic H1 antagonist, because pure intravenous diphenhydramine was not available. With the oral product, the blood concentration is thought to be more influenced by the patient's general condition (for example, by the presence of gastrointestinal disease, or advanced age) than when the intravenous form is used:. Bearing in mind its pharmacological properties, oral diphenhydramine plus single-dose intravenous dexamethasone is unlikely to result in an adequate level of immunosuppression during the infusion of paclitaxel. This may explain the results of our historical analysis. As a result of these concerns, we employed a modified premedication protocol, using Rescalmin (Nissin) intravenously instead of oral diphenhydramine, with a dose of oral dexamethasone being administered the night before the paclitaxel infusion.

Paclitaxel treatment using the modified premedication protocol was performed smoothly and good compliance was obtained. There were no severe HSRs, and no treatment was discontinued because of toxic allergic reactions. This treatment regimen seems to be effective for the prophylaxis of paclitaxel-related HSRs, although the number of patients in our study was small. This treatment regimen has several advantages, as follows. First, it ensures that an intravenous H1 antagonist is administered prior to paclitaxel, in contrast to the administration of the oral product. Second, because the dose of oral dexamethasone given the night before the paclitaxel infusion is lower than the conventional dose, patients' compliance is better. Third, mild somnolence seems to be a favorable effect of receiving chemotherapy in anxious patients. Finally, this treatment regimen might be useful not only for paclitaxel but also for other chemotherapeutic drugs such as docetaxel, oxaliplatin, and cetuximab. In fact, in a patient with docetaxel-related HSR, re-administration of docetaxel succeeded with the modified premedication protocol. Our modified protocol might also be useful with oxaliplatin, a platinum salt which is particularly effective in treating colorectal cancer, but with which, as a result of its increasing clinical use, a rising incidence of HSRs has been observed.[16] HSRs have also been observed with cetuximab, a monoclonal antibody that is an inhibitor of epidermal growth factor receptor.[17]

In conclusion, in our historical cohort analysis, the incidence of HSRs in the short premedication group tended to be higher than that in the conventional premedication group. Our modified premedication protocol was found to be feasible for preventing paclitaxel-related HSRs, but case accumulation is needed.

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Review

Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan

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Sekine, I., Nokihara, H., Yamamoto, N., Kunitoh, H., Ohe, Y., Saijo, N. and Tamura, T. *Problems with Registration-Directed Clinical Trials for Lung Cancer in Japan.* Tohoku J. Exp. Med., 2007, **213** (1), 17-23 —— New anticancer agents against lung cancer are needed because efficacy of chemotherapy is limited. The long time required, low quality, and considerable costs of registration-directed clinical trials in Japan ("Chiken") have been pointed out. The quality of 24 phase I and 41 phase II trials of an anticancer drug for lung cancer were analyzed according to the approval year of the drug. The human resources and infrastructure to support oncology clinical practice and clinical trials were compared between Japan and the USA. A maximum tolerated dose was not defined in any of seven phase I trials before 1989, and was determined in two of six trials between 1989 and 1996 and in seven of 10 trials thereafter. Before 1989, 29 (20%) of 142 patients registered in two trials were ineligible, and the number of ineligible patients was not reported in the five trials. Sample size calculations were not performed in any of seven phase II trials before 1989 and were performed in only four of 10 trials between 1989 and 1996 and in all 23 trials conducted thereafter. The shortage of human resources, including medical oncologists, oncology nurse practitioners and clinical research coordinators, is serious and acute. The infrastructure to support clinical trials also remains insufficient in Japan. In conclusion, registration-directed clinical trials of anticancer agents have advanced significantly during last three decades but remain unsatisfactory. The development of infrastructure and human resources is an urgent task to ensure high-quality clinical trials without unnecessary delays. ———— clinical trials; medical oncologists; nurse practitioners; lung cancer; anticancer agents

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and global lung caner incidence is increasing at a rate of 0.5% per year (Schottenfeld and Searle 2005). About 80% of patients with lung cancer have already developed distant metastases or pleural effusion, either by the time of the initial

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diagnosis or by the time recurrence is detected after surgery for local disease. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely live long. Therefore, new chemotherapeutic agents continue to be developed against lung cancer (Sekine and Saijo 2000).

The Japanese Pharmaceutical Affairs Law (PAL) was enacted in 1948, and was first amended in 1960 to provide for regulations to ensure the maintenance of the quality, efficacy, and safety of drugs and medical devices, and to promote research and development of these medical and pharmaceutical products. Good Clinical Practice (GCP) was enforced by the Bureau Notification of the Ministry of Health and Welfare of Japan ("Kyokuchou-Tsuuchi") in 1989 (the former GCP). In 1996, the PAL and its related laws were amended to strengthen GCP (the new GCP), Good Laboratory Practice, Good Post-Marketing Surveillance Practice, and standard compliance reviews, conforming to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use. In contrast to the laws prevailing in the US and EU, marketing approval for anticancer agents in Japan has been granted based on reports of the anti-tumor effects of the new agents in phase II trials (Fujiwara and Kobayashi 2002).

Under this Japanese drug approval system regulated by the PAL, 23 anticancer drugs have been approved for use against lung cancer during the last five decades (Fig. 1). Of these, 9 drugs are original to Japan, some of which are routinely used all over the world. Several problems, however, have been pointed out in registration-directed clinical trials in Japan ("Chiken"), including the long time required, low quality, and considerable cost (The Ministry of Health, Labour and Welfare of Japan 2002; The Ministry of Education, Science and Culture and the Ministry of Health, Labour and Welfare 2003). As a result, Japanese cancer patients must wait for a long time

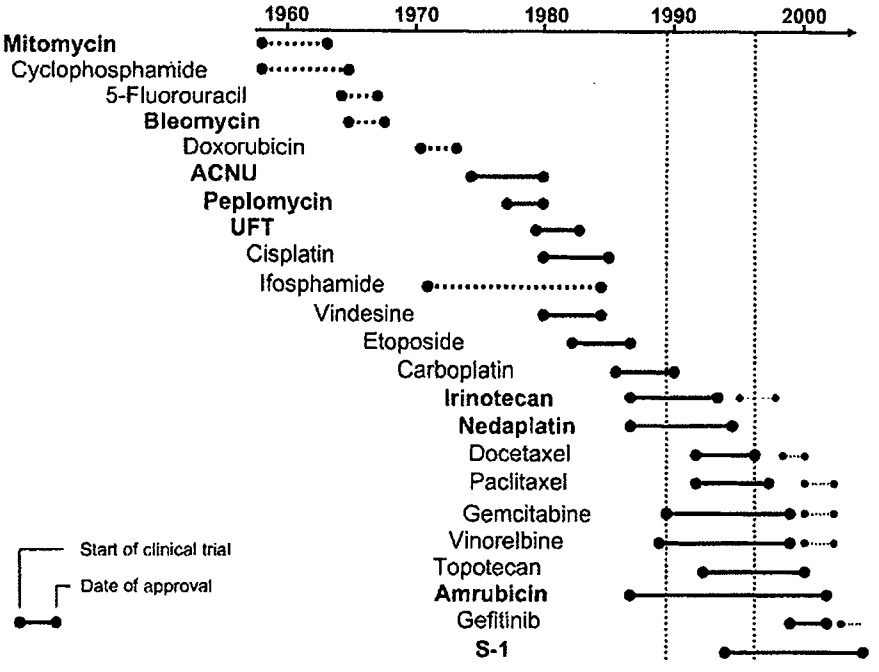

Fig. 1. Anticancer drugs approved for lung cancer in Japan.
Bold: original to Japan. Dotted line: case series studies, solid thick line: investigational new drug phase I-II trials for approval, and dotted thin line: post-marketing sponsored phase III trials. Vertical dotted lines indicate the year when the former and new GCP were issued.

until they receive new anticancer drugs which have been approved long before in other countries (The Ministry of Health, Labour and Welfare of Japan 2005). We discuss the aspects and issues of registration-directed trials in Japan by reviewing such trials for the 23 anticancer drugs.

Review of registration-directed clinical trials for the 23 anticancer drugs

A total of 65 phase I and II trials of an anticancer drug for approval were reviewed in terms of definition of eligibility criteria, maximum tolerated dose (MTD), sample size, response criteria, and extramural review for tumor responses. The MTD is the dose associated with seriouis but reversible toxicities in a sizeable proportion of patients and the one that offers the best chance for a favorable therapeutic ratio (Piantadosi 1997). The number of patients accrued in a trial, percentage of ineligible patients, number of participant hospitals in a trial, and the study period defined as the months between the first and last patient accrual were also analyzed. They were obtained from a published paper for 53 trials, from a meeting abstract and in-company resource for one trial, and from in-company resource alone for the remaining 11 trials. The clinical developmental period of an anticancer drug was defined as years between the start month of the first phase I trial and the month of the approval for lung cancer. These parameters are compared according to the approval year of the drug. We categorized three periods of approval: 1) before 1989, 2) between 1989 and 1996, and 3) between 1997 and 2004, because the former GCP was enforced in 1989, and the new GCP in 1997 (Fujiwara et al. 2002).

Of the 23 anticancer drugs, six drugs whose clinical development started before 1974 were approved on the basis of the clinical experience of the use of the drug without clinical trials (Fig. 1). A total of 24 phase I trials were identified (Table 1). The MTD was not defined in the protocol of any trials before 1989, but was defined in 33% of trials between 1989 and 1996, and in 70% of trials after 1996. Instead of the MTD, maximum acceptable dose, defined as the dose associated with grade 2 or severer toxicity in two thirds or more patients, was used in a trial after 1996. About twice more patients were registered in a trial before 1989 than thereafter, but 20% of the registered patients before 1989 were ineligible. The study period of a phase I trial got longer as the number of participant hospitals decreased, from 7 months and 11 hospitals before 1989 to 13 months and 4 hospitals after 1996, respectively.

In this review, 41 phase II trials for approval were analyzed (Table 2). Calculation of the sample size was not made in any trials before 1989, was seen in 40% of trials between 1989 and 1996, and in all trials thereafter. Response criteria were

TABLE 1. Investigational new drug phase I trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	6	11
Defined, number (%) of trials			
Eligibility criteria	4 (57)	6 (100)	11 (100)
Maximum tolerated dose*	0 (0)	2 (33)	7 (70) ‡
Results of trials, median (range)			
Number of patients**	61 (32-170)	24 (18-54)	29 (9-43)
% of ineligible patients	20 (20-21) †	8 (0-33)	6 (0-22)
Number of hospitals	11 (1-21)	9 (1-18)	4 (1-17)
Study period in months	7 (5-30)	10 (5-11)	13 (8-24)

*Statistically significant difference obtained ($p = 0.014$ by the chi-square test); **Statistically significant difference obtained ($p < 0.01$ by the Kruskal Wallis test); †Data were available in 2 trials only; ‡Data were available in 10 trials only.

TABLE 2. Investigational new drug phase II trials for approval.

	Before 1989	1989-1996	1997 or thereafter
Total number of trials	7	11	23
Defined, number (%) of trials			
Eligibility criteria	4 (57)	11 (100)	23 (100)
Sample size calculation*	0 (0)	4 (40) [‡]	23 (100)
Response criteria	6 (86)	11 (100)	23 (100)
Extramural review	3 (43)	9 (82)	23 (100)
Results of trials, median (range)			
Number of patients	71 (10-127)	68 (18-153)	61 (11-102)
% of ineligible patients	18 (0-29) [†]	3 (0-22)	3 (0-12)
Number of hospitals	27 (3-103)	17 (1-30)	20 (5-46)
Study period in months	18 (12-36)	12 (6-34)	26 (4-48) [§]

*Statistically significant difference obtained ($p < 0.01$ by the chi-square test); [†]Data were available in 5 trials only; [‡]Data were available in 10 trials only; [§]Data were available in 22 trials only.

defined in almost all studies, but an extramural review was conducted only after 1989. The median number of registered patients in a trial was constant through the three periods, but the percentage of ineligible patients was high in trials conducted before 1989. The number of patients in a trial, and the number of hospitals in a trial were similar regardless of the year. The median study period in recent trials was 26 months.

The clinical development period was evaluated in the 23 drugs. Cisplatin was approved for germ cell tumors in 1983 and additionally approved for non-small cell lung cancer (NSCLC) in 1986. S-1 was firstly approved for gastric cancer in 1999, and additionally approved for NSCLC in 2004. The other drugs were approved for lung cancer for the first time. The median (range) clinical development period was 5.2 (3.2-14.5) years before 1989, 6.0 (4.8-9.1) years between 1989 and 1996, and 9.0 (3.9-15.4) years in 1997 or thereafter.

Development and recent problems of phase I and phase II trials in Japan

The concept of the "clinical trial" was not widely followed in Japan until 1974, when a phase I trial of nimustine hydrochloride (ACNU) was launched as one part of the United States-Japan Cooperation Cancer Research Program on the basis of the agreement between the National Cancer Institute and Japan Society for the Promotion of Science (Sugano 1982; Niitani 1999). Phase I trials before 1989 required the accrual of many patients, because 1) the maximum tolerated dose was not defined, 2) many patients were treated at unnecessary dose levels because the modified Fibonacci dose escalation schedule was not applied, and 3) the percentage of ineligible patients was high. Some of these issues were improved in 1997 or thereafter, but the maximum tolerated dose is still not defined in as many as 40% of trials. Recently, oncology phase I trials came to be conducted among fewer hospitals than before, as more participants were recruited in each hospital. This facilitated communication among phase I investigators, which is important to complete phase I trials safely.

Phase II trials play the central role in anticancer agent approval in Japan, because the approval can be granted based on the response rate in these trials. The quality of protocols for phase II trials suggested by eligibility criteria, sample size calculation, response criteria, and extramural review has been improved significantly. The study period of phase II trials, however, was and is still too long, as long as 4 years in recent trials. To increase participant hospitals, however, is not necessarily a desirable solution,

because a certain number of patients per hospital are needed to maintain the quality of trials by training doctors in the application of a new drug. Thus, enhancing patient recruitment in each hospital participating in the trial is the most important consideration.

A high standard of oncology clinical practice as the basis for clinical trials

Since a high standard of clinical practice is the basis for all clinical trials, the infrastructure for oncological clinical practice should be promptly advanced. The shortage of human resources including medical oncologists and oncology nurse practitioners in Japan is serious and acute. In the United States, medical oncology was established as a separate discipline by the American Board of Internal Medicine in 1971, and approximately 8,000 certified internists as of 2003 have been further certified by the Board in the subspecialty of medical oncology (Holland et al. 2003). In contrast, medical oncology has not been established as an academic unit or a regular university course in many medical schools in Japan. The Japanese Society of Medical Oncology was launched as an association in 1993, and framed the system of cancer medical specialists in 2003. A total of 1,479 doctors were certified as a tentative medical oncology supervisor between 2003 and 2005, and 47 doctors as a medical oncology specialist in 2005 (Table 3) (Japanese Society of Medical Oncology 2005).

To deal with complex cancer care, oncology nurse practitioners in the United States have become an integral part of the multidisciplinary team in the care of patients. As of 2002, more than 19,000 oncology nurse practitioners have been certified by the Oncology Nursing Society in the United States (Rieger 2003). In contrast, the number of oncology nurse practitioners registered in the Japanese Nursing Association was only 44 as of 2005 (Table 3) (Japanese Nursing Association 2005). Introduction of oncology nurse practitioners in clinical practice should lessen the burden on oncologists significantly and help them to have the incentive to take part in registration-directed clinical trials.

The infrastructure and human resources to support clinical trials

The infrastructure to support in-house clinical trials remains insufficient and even lacking in almost all institutes in Japan, while it has been advanced systematically in the United States. In the 1960s, General Clinical Research Centers were founded with the support of National Institutes of Health in 80 universities and academic institutions to provide the primary resources and optimal environment necessary for investigators to conduct clinical research. They include experienced nursing, laboratory, computer system, and biostatistical staff (Robertson and Tung 2001; General Clinical Research Centers 2005). To carry out a multicenter trial, a central data center

TABLE 3. Medical oncology professionals in Japan and the USA.

Professionals	*n* of medical oncology professionals	
	Japan	USA
Medical oncologists	47 [1]	8,000 [2]
Oncology nurse practitioners	44 [3]	19,000 < [4]
Clinical research coordinators	335 [5]	10,723 [6]

[1] Certified by the Japanese Society of Medical Oncology in 2005.
[2] Certified by the American Board of Internal Medicine as of 2003.
[3] Certified by the Japanese Nursing Association as of 2005.
[4] Certified by the Oncology Nursing Society as of 2002.
[5] Certified by the Japanese Society of Clinical Pharmacology and Therapeutics as of 2005.
[6] Certified by the Association of Clinical Research Professionals as of 2005.

is needed to deal with the increased administrative difficulties and quality assurance problems associated with this type of trial (Pollock 1994). The quality control and quality assurance system of the Japan Clinical Oncology Group has been significantly developed during the last two decades (Japan Clinical Oncology Group 2005). Using Internet resources may facilitate developing national and regional networks for clinical trials by reducing the burden associated with the extensive research time and considerable cost of all these processes (Paul et al. 2005).

The new GCP demands more of the clinical researchers in time, resources and money to enhance the science, credibility, and ethics of clinical trials for approval (Sweatman 2003). The clinical research coordinator (CRC) plays a key role in the clinical trial process by supporting investigators. The CRCs are involved in every aspect of registration-directed clinical trials, including protocol development, checking eligibility criteria, informed consent, organizing study schedules, checking clinical tests, filling in case report forms, and providing support for monitoring and auditing the trials (Rico-Villademoros et al. 2004; Sakamoto 2004). Association of Clinical Research Professionals in the USA has offered the CRC certification since 1992, and there are 10,723 CRCs to date (Association of Clinical Research Professionals 2006). The Japanese Society of Clinical Pharmacology and Therapeutics launched the certified CRC system in 2003, and there were 335 certified CRCs as of 2005 (Table 3) (The Japanese Society of Clinical Pharmacology and Therapeutics 2005).

In conclusion, clinical trials of anticancer agents for approval have been developing significantly, but still remain at an unsatisfactory level. Development of the infrastructure and human resources for clinical trials is an urgent task to complete good quality clinical trials for approval without delay.

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Prospective Validation for Prediction of Gefitinib Sensitivity by Epidermal Growth Factor Receptor Gene Mutation in Patients with Non-Small Cell Lung Cancer

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Introduction: We evaluated the efficacy of gefitinib monotherapy prospectively in patients with advanced or pretreated non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations.

Methods: Patients with NSCLC were examined for EGFR exon 19 deletion mutations by fragment analysis and for EGFR L858R point mutations by the Cycleave polymerase chain reaction technique. EGFR mutation-positive patients with locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable with surgery or thoracic radiotherapy were candidates for gefitinib treatment administered at 250 mg/day until disease progression.

Results: Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten had exon 19 deletion, and 17 had L858R. Twenty-one patients harboring EGFR mutations were treated with gefitinib and were considered assessable for responses and adverse events. Nineteen patients with EGFR mutations achieved objective responses (three complete responses and 16 partial responses), resulting in an overall response rate of 90.5% (95% confidence interval, 69.6%–98.8%). The median progression-free survival was 7.7 months (95% confidence interval, 6.0 mo to not reached). The median overall survival has not been reached. Common adverse events were skin toxicity, diarrhea, and elevated aminotransferases, but no pulmonary toxicity was observed.

Conclusions: Detection of common EGFR mutations seems to be useful for selecting patients with NSCLC who would likely benefit from gefitinib monotherapy.

Key Words: EGFR, Gefitinib, Lung cancer, Mutations, Drug sensitivity.

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Lung cancer remains the most common cause of cancer death in both men and women worldwide. Lung cancer frequently presents at an advanced and biologically aggressive stage, resulting in poor prognosis. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancers. Currently, platinum-based combination chemotherapy regimens, including several active new chemotherapeutic agents, comprise the standard option for patients with advanced NSCLC. However, various combinations of drugs have similar efficacy, producing objective response rates of 30 to 40%, median survival time of eight to 10 months, and 1-year survival rates of 30 to 40%.[1,2] These results remain unsatisfactory, and new modalities of treatment are urgently awaited. Recently, novel molecular targeted strategies that block cancer progression pathways have been suggested as the ideal treatment to control cancer and are considered an exciting therapeutic approach for treating NSCLC.[3]

The epidermal growth factor receptor (EGFR) is a 170 kDa receptor tyrosine kinase and a member of the erbB receptor family that plays a pivotal role in the signaling processes of tumor progression.[4–6] EGFR is overexpressed in several solid tumors, including NSCLC, and it is one of the leading therapeutic molecular targets.[7] Gefitinib is an orally bioavailable, selective EGFR tyrosine kinase inhibitor (TKI) and was the first targeted drug for NSCLC. Phase II and III monotherapy trials for patients pretreated for NSCLC demonstrated objective response rates of only 8 to 18%.[8–10] However, subset analyses of these trials and a retrospective study[11] showed a small group of clinical responders comprising women, patients with adenocarcinomas, nonsmokers, and Japanese or Asian patients. These results suggest that identifying predictive molecular or genetic biomarkers for gefitinib sensitivity may be useful for selecting patients who are most likely to benefit from treatment.

In 2004, three independent groups reported that somatic EGFR mutations correlated with sensitivity of NSCLC to gefitinib or erlotinib, another EGFR TKI.[12–14] Subsequently, several groups confirmed this striking correlation between EGFR mutations and gefitinib sensitivity, yielding a response rate of about 60 to 94% in retrospective analyses.[15–22] EGFR mutations are likely to be significantly associated with survival benefit attributed to gefitinib treatment.[17,18,21] In con-

trast to these results, recent reports concerning molecular analyses of large-scale phase II and III trials showed lower response rates than previously reported and no survival benefit in patients with mutations treated with TKIs.[23–26] Around the same time, the EGFR gene amplification/copy number was demonstrated as another useful predictive molecular marker of TKI efficacy.[23,26–28] However, these contradictory results were obtained through the retrospective collection of tumor samples, and prospective validation studies that predict TKI efficacy by EGFR mutations are needed.

Data from previous reports show that in-frame deletions in exon 19 and specific missense mutation of codon 858 in exon 21 (L858R) account for about 90% of all EGFR mutations, and about 80% of responders to gefitinib or erlotinib harbor either of these two hotspot mutations. Therefore, we developed a rapid, sensitive screening assay of two hotspot mutations[29] and conducted a prospective cohort study to explore the prediction of gefitinib sensitivity in EGFR mutation-positive patients.

MATERIALS AND METHODS

Study Design

This prospective cohort study was conducted to identify patients with NSCLC who would most likely benefit from gefitinib treatment according to their EGFR mutation. Patients with EGFR mutation were treated with oral administration of gefitinib at a dose of 250 mg once a day until disease progression or intolerable toxicity occurred, or until the patient refused to continue treatment. The primary endpoint was objective tumor response rate. Secondary endpoints included adverse effects, disease control rate (response + stable disease), progression-free survival (PFS), and overall survival (OS). This study was approved by the institutional review board of Aichi Cancer Center Hospital.

Patient Eligibility

Eligibility criteria for gefitinib treatment were adult (age ≥20 yr) with cytologic or histologic confirmation; locally advanced, metastatic, or recurrent/refractory NSCLC that was not curable by surgery or radiotherapy; harboring EGFR mutation; and one or more measurable or assessable lesions. All patients were admitted to the study regardless of prior treatment, extent of performance status (PS), or main organ functions. The exclusion criteria were pulmonary fibrosis, interstitial pneumonia, or prior treatment with an EGFR TKI or antibody. All patients gave written informed consent in accordance with institutional regulations before entering the study.

Efficacy and Toxicity Evaluation

Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors[30] and were confirmed by repeated imaging studies after 4 to 8 weeks of gefitinib treatment. During the treatment and for 30 days after the last dose of gefitinib, patients were monitored for adverse events, which were graded using Common Terminology Criteria for Adverse Events, version 3.0. PFS was assessed from the date of gefitinib treatment until the date of objective disease progression, death from any cause, or the last follow-up. OS was assessed from the date of gefitinib treatment until the date of death from any cause, or the last follow-up.

Detection of EGFR Mutations

Genomic DNA was extracted from tumors embedded in paraffin blocks or from aspirated tumors obtained in pleural effusions, superficial lymph nodes, or subcutaneous metastasis. All specimens were reviewed by a single reference pathologist (Y.Y.) and marked grossly near the tumor-rich lesion on an unstained slide to enrich the tumor cell population as much as possible.

We performed mutational analyses of exon 19 deletion and the L858R point mutation of the EGFR gene, as previously described.[29] Briefly, exon 19 deletion was determined by common fragment analysis using polymerase chain reaction (PCR) with an FAM-labeled primer set, and the PCR products were electrophoresed on an ABI PRISM 310 (Applied Biosystems, Foster City, CA). The shorter segment of DNA amplified by PCR showed a deletion mutation in a new peak in an electropherogram. The L858R mutation was detected by the Cycleave real-time quantitative PCR technique using the Cycleave PCR core kit (Takara Co. Ltd., Ohtsu, Japan) with an L858R-specific cycling probe and a wild-type probe. Fluorescence intensity was measured with a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA).

Statistical Analysis

Data were analyzed using the chi-square test; $p < 0.05$ was regarded as significant. Confidence intervals (CI) were calculated using binomial CIs. PFS and OS were calculated using the Kaplan–Meier method and compared between two EGFR mutation groups using log-rank test. All the analyses were performed with Stata 8.2 for Macintosh (Stata Corp, College Station, TX).

RESULTS

Sampling Procedure for Detecting EGFR Mutations

Sixty-six consecutive patients with NSCLC were examined to detect the EGFR mutations from November 2004 through August 2005 at Aichi Cancer Center Hospital. Of these patients' samples, 23 specimens were obtained from bronchoscopic biopsy, 22 from computed tomography/ultrasound-guided needle biopsy, 13 from percutaneous aspiration (seven from pleural effusion, four from lymph nodes, and two from skin metastases), two from biopsy (one from tonsil metastasis and one from skin metastasis), and six from surgery with general anesthesia (three from thoracotomy, two from thoracoscopy, and one from mediastinoscopy (Table 1). Sixty samples (91%) were obtained from the biopsy or aspiration method. Tumor tissues or aspirates were procured at the time of initial diagnosis in 52 patients and at the time of tumor progression in 14 patients.

Patient Characteristics and EGFR Mutations

Mutations of the EGFR gene were detected in 27 (41%) of 66 patients. Ten of these had the deletion in exon 19, and

TABLE 1. Patient Characteristics and Sample Procurement According to EGFR Mutation Status

	EGFR Mutation Status			
	All	Mutation	Wild type	*p*
All cases	66	27 (21)	39	
Sex				0.175
Male	36	10 (8)	26	
Female	30	17 (13)	13	
Age (yr)				0.5084
≤64	31	14 (11)	17	
>64	35	13 (10)	22	
Histology				0.0199
Adenocarcinoma[a]	59	27 (21)	32	*p* ([a] vs. [b])
Squamous cell[b]	2	0	2	
Large cell[b]	2	0	2	
Pleomorphic[b]	1	0	1	
NSCLC NOS[b]	2	0	2	
Smoking status				0.0002
Never smoker[c]	24	17 (13)	7	*p* ([c] vs. [d])
Former smoker[d]	17	9 (7)	8	
Current smoker[d]	25	1 (1)	24	
Stage at initial diagnosis				0.6348
IA[e]	2	1	1	*p* ([e] vs. [f])
IIB[e]	4	2 (2)	2	
IIIA[f]	3	0	3	
IIIB[f]	16	3 (2)	13	
IV[f]	41	21 (17)	20	
Performance status				0.6059
0/1	51	20 (14)	31	*p* (0/1 vs. ≥2)
2	7	3 (3)	4	
3	3	1 (1)	2	
4	5	3 (3)	2	
Prior first treatment				ND
No	8	5 (5)	3	
Surgery	3	3 (1)	0	
Thoracic irradiation	4	2 (2)	2	
Chemoradiotherapy	10	2 (1)	8	
Bone irradiation	6	3 (3)	3	
Brain irradiation	6	3 (2)	3	
Sclerotherapy for effusion	1	1 (1)	0	
Chemotherapy	28	8 (6)	20	
Prior chemotherapy				0.4337
0	28	13 (12)	15	*p* (0 vs. ≥1)
One regimen	28	10 (6)	18	
Two regimens	8	4 (3)	4	
Three regimens	2	0	2	
Method for sample procurement				ND
Bronchoscopic biopsy	23	11	12	
CT/US-guided needle biopsy	22	6	16	
Pleural effusion aspiration	7	4	3	
LN/skin aspiration	6	2	4	
Tonsil/skin biopsy	2	0	2	
Thoracotomy	3	2	1	
VATS	2	1	1	
Mediastinoscopy	1	1	0	

EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer; NOS, not otherwise specified; ND, not done; CT/US, computed tomography/ultrasound; LN, lymph node; VATS, video-assisted thoracoscopy. Superscript letters indicate groups compared in the statistical analysis. Numbers in parentheses represent the numbers of patients receiving gefitinib treatment.

17 were the point mutation at codon 858. As previously reported,[12–14,17] the EGFR mutations were significantly associated with adenocarcinoma histology and never-smoking status (Table 1). However, the EGFR mutation status was not significantly correlated with sex, age, PS, stage at initial diagnosis, or prior chemotherapy. Twelve patients received gefitinib treatment as the first-line chemotherapy; five patients desired first-line gefitinib therapy, and the other seven were unfit for conventional chemotherapy because of age (one patient, age 84 yr), cardiac disease (one patient), widespread bone metastases (two patients), and poor PS (3–4 in three patients).

Clinical Response and Survival

Of 27 patients harboring EGFR mutation, 21 were treated with gefitinib and were assessable for objective responses (Table 2) and adverse events (Table 3). The median interval of gefitinib treatment was 5.9 months (range, 0.67 to 11.4 mo). Of the assessable 21 patients, 19 patients achieved objective responses (three complete response and 16 partial response), for an overall response rate of 90.5% (95% CI, 69.6–98.8%). One patient had stable disease, giving an overall disease control rate of 95.2% (95% CI, 76.2–99.9%). According to EGFR mutation classes and PS, the objective responses were seven of eight for the exon 19 deletion, 12 of 13 for the L858R point mutation, 13 of 14 in PS 0 to PS 1 patients, and 6 of seven in PS 2 to PS 4 patients. The response to gefitinib did not differ significantly according to the mutation class or PS.

The median PFS was 7.7 months (95% CI, 6.0 mo to not reached) (Figure 1A). The median OS has not been reached at present (Figure 1B). Subset analyses showed that PFS was greater in patients with the exon 19 deletion than in those with the L858R point mutation (log rank test, $p = 0.04$; Fig 2*A*). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo). OS did not differ significantly between the two types of mutations (Figure 2B). No difference was observed in PFS and OS between never-smokers and current/former smokers (data not shown).

TABLE 2. Response of EGFR Mutation-Positive Patients to Gefitinib Treatment

	EGFR Mutation Status		
	Exon 19 Deletion ($n = 8$)	L858R Mutation ($n = 13$)	Total ($n = 21$)
CR	1 (12.5%)	2 (15.4%)	3 (14.3%)
PR	6 (75%)	10 (76.9%)	16 (76.2%)
Overall response rate (CR + PR)	7 (87.5%)	12 (92.3%)	19 (90.5%)
SD	1 (12.5%)	0	1 (4.8%)
Disease control (CR + PR + SD)	8 (100%)	12 (92.3%)	20 (95.2%)
Progressive disease	0	1 (7.7%)	1 (4.8%)

EGFR, epidermal growth factor receptor; CR, complete response; PR, partial response; SD, stable disease.

TABLE 3. Number (%) of Patients with Treatment-Related Adverse Events ($n = 21$)

	Grade				
	0	1	2	3	4
Skin toxicity	15 (71)	4 (19)	2 (10)	0	0
Diarrhea	13 (62)	3 (14)	3 (14)	2 (10)	0
Elevated aspartate aminotransferase/ alanine aminotransferase	15 (71)	1 (5)	2 (10)	3 (14)	0
Nail changes	17 (81)	3 (14)	1 (5)	0	0
Mucositis	20 (95)	1 (5)	0	0	0
Joint pain	20 (95)	1 (5)	0	0	0

Adverse Events

All 21 patients were evaluated for drug-related adverse events. The most common adverse events were skin toxicity, diarrhea, and elevated asparatate aminotransferase/alanine aminotransferase (AST/ALT) (Table 3). The grade 3 adverse events of diarrhea and elevated AST/ALT occurred in two (10%) and three (14%) patients, respectively. These events occurred slightly more frequently than in previous studies.[8,9] No grade 4 adverse events or pulmonary toxicity were observed. Seven patients required an interruption of treatment, lasting 2 to 4 weeks, because of grade 2/3 diarrhea or grade 3 elevated transaminases. Two patients withdrew: one after 3 weeks of gefitinib treatment because of grade 3 diarrhea, and the other after 9 weeks of gefitinib treatment because of grade 2 nail changes.

DISCUSSION

In the present study, we have observed that the objective response rate in our patients was similar to that in previous reports. We also found that PFS and OS seem promising in identifying gefitinib-sensitive patients regardless of whether the study includes patients unsuited for conventional cytotoxic chemotherapy because of age, cardiac disease, widespread bone metastases, or poor PS (3 to 4). Our favorable data might have resulted because we selected patients harboring one of two hotspot mutations (exon 19 deletion and exon 21 L858R mutation). Greulich et al.[31] examined NIH-3T3 cells transformed with various EGFR mutants and showed that a distinct EGFR mutation confers differential sensitivity to TKIs. They demonstrated greater sensitivity to TKIs in cell lines with the two hotspot mutations than with the G719S mutation, and insensitivity to TKIs in cell lines with exon 20 insertion (D770-N771 ins) mutation. These in vitro data may explain, at least partially, our promising results for detecting these two sensitive mutations.

We previously reported that patients with the EGFR exon 19 deletion respond significantly better to gefitinib than those with the L858R mutation ($p = 0.0108$).[17] Our current data show no difference in gefitinib sensitivity and OS after

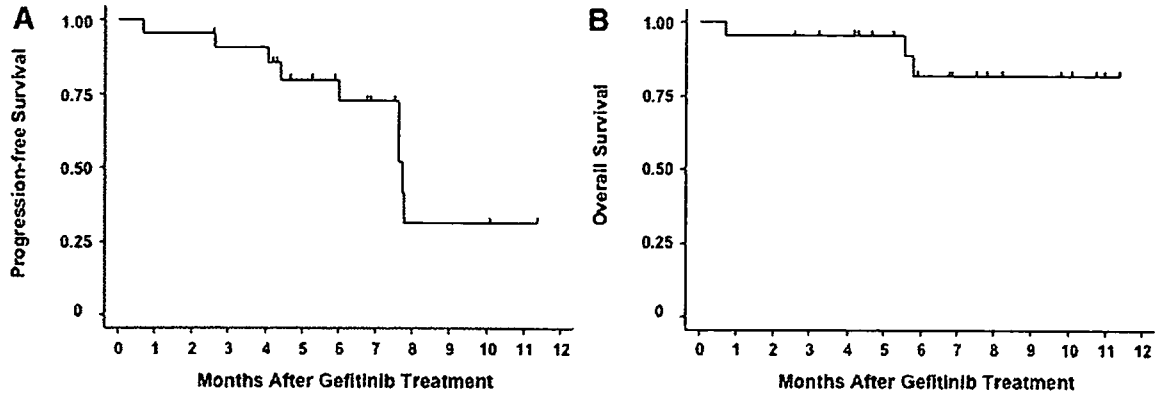

FIGURE 1. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations (n = 21). The median progression-free survival was 7.7 months (95% CI, 6.0 mo to not reached). The median survival was not reached.

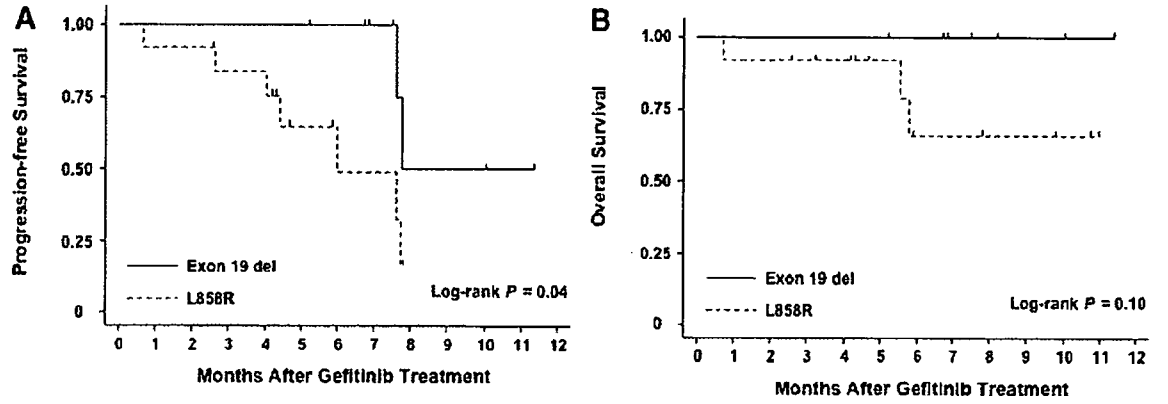

FIGURE 2. Kaplan–Meier estimates of (*A*) progression-free survival and (*B*) overall survival for patients with EGFR mutations according to the exon 19 deletion (n = 8) and L858R mutation (n = 13). The median PFS for the exon 19 deletion group was 7.8 months (95% CI, 7.6 mo to not reached); for the L858R mutation group, median PFS was 6.0 months (95% CI, 2.6 to 7.7 mo).

gefitinib treatment between these two groups of patients, although we observed a greater PFS in the EGFR exon 19 deletion group than in the L858R group. It is possible that the number of patients (eight with exon 19 deletion and 13 with L858R) was too small to detect a statistically significant difference in OS. Riely et al.[32] reported recently that patients with exon 19 deletion have a significantly longer survival after TKI treatment than those with the L858R mutation (p = 0.01). These findings suggest that the EGFR exon 19 deletion might be a better predictor of the efficacy of TKIs than the L858R mutation.

EGFR mutations are significantly associated with patients with adenocarcinomas, patients of Asian origin, females, and patients who had never smoked—clinical factors also associated with patients who respond to gefitinib.[13,14,24,33] A phase II trial using gefitinib monotherapy as the first-line therapy for patients with adenocarcinoma histology and never-smoking status was recently completed in South Korea and reported promising data (e.g., an objective response rate of 69% and estimated 1-year survival rate of 73%).[34] However, this trial did not select patients using biomarkers, and we believe the benefit of gefitinib therapy could be enhanced by selecting individual patients according to appropriate biomarkers. Very recently, two prospective phase II studies that had selected patients based on molecular biomarkers demonstrated that EGFR mutations[35] and gene copy number assessed by fluorescence in situ hybridization (FISH)[36] can predict clinical outcomes in TKI-treated NSCLC patients.

The grade 3 adverse events of diarrhea and elevated AST/ALT were observed in five patients (24%); this is a higher rate than that reported in two previous phase II studies that reported rates of adverse events of 1.5%[8] and 7%[9] at a gefitinib dose of 250 mg per day. The reasons for our higher rate of adverse events are unknown. Although adverse events related to gefitinib treatment are generally thought to be mild and tolerable, they should not be discounted.

Most studies have detected EGFR mutations using direct sequencing or single-strand conformation polymorphism analysis for exons 18 to 21.[37] These techniques are less sensitive when applied to a small amount of tumor cells from the biopsy or aspiration samples.[38] We were able to detect

two hotspot mutations with our sensitive rapid screening assay in most biopsy or aspiration samples in the routine clinical setting. Although this assay needs precise assessment of tumor samples by a pathologist to enrich the tumor cells, it is very sensitive and accurate for detection, and it can be completed within 4 hours without need for microdissection or nested PCR process.[29]

The key genetic event for TKI sensitivity has not been perfectly identified and is the subject of a growing debate about the role of EGFR mutations versus EGFR gene amplification/copy number in NSCLC. EGFR mutant NSCLC cell lines are strongly associated with increased EGFR gene copy number.[39,40] Cappuzzo et al.[27] and Takano et al.[22] found that EGFR mutations in NSCLC patients correlate significantly with gene copy number assessed by FISH and quantitative real-time PCR, respectively. However, Cappuzzo et al.[27] demonstrated that in patients treated with gefitinib, a high EGFR gene copy number is a better predictor of survival than EGFR mutations.[27] In contrast, Takano et al.[22] reported that the status of the EGFR mutations, rather than gene copy number, is the major determinant of gefitinib efficacy. Recent reports of the molecular analyses from the largest phase III TKI monotherapy trials failed to show that the EGFR mutation is superior to gene copy number in predicting the efficacy of TKIs.[23,26] These conflicting results on EGFR mutations and gene amplification/copy number could be explained by (i) differences in the detection methodologies and assessment of mutation and gene amplification/copy number (e.g., direct sequence versus PCR-based DNA testing for detecting EGFR mutations, or FISH versus PCR-based amplification for detecting EGFR gene amplification/copy number), (ii) failure to reconfirm these results in other institutions, and (iii) other unknown factors underlying drug sensitivity, especially those related to ethnicity. Further prospective studies are needed to investigate the crucial molecular markers involved in the EGFR network, using adequate tissue samples and assays to more precisely detect molecular events.

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Proteomic Signature Corresponding to the Response to Gefitinib (Iressa, ZD1839), an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor in Lung Adenocarcinoma

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Abstract **Purpose:** We aimed to identify candidate proteins for tumor markers to predict the response to gefitinib treatment.

Experimental Design: We did two-dimensional difference gel electrophoresis to create the protein expression profile of lung adenocarcinoma tissues from patients who showed a different response to gefitinib treatment. We used a support vector machine algorithm to select the proteins that best distinguished 31 responders from 16 nonresponders. The prediction performance of the selected spots was validated by an external sample set, including six responders and eight nonresponders. The results were validated using specific antibodies.

Results: We selected nine proteins that distinguish responders from nonresponders. The predictive performance of the nine proteins was validated examining an additional six responders and eight nonresponders, resulting in positive and negative predictive values of 100% (six of six) and 87.5% (seven of eight), respectively. The differential expression of one of the nine proteins, heart-type fatty acid–binding protein, was successfully validated by ELISA. We also identified 12 proteins as a signature to distinguish tumors based on their *epidermal growth factor receptor* gene mutation status.

Conclusions: Study of these proteins may contribute to the development of personalized therapy for lung cancer patients.

Non-small cell lung carcinoma (NSCLC) accounts for ~85% of lung cancer cases (1). Biomarker(s) that predict the response to gefitinib (Iressa; AstraZeneca, Macclesfield, United Kingdom), an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, may help to improve the choice of therapeutic strategy in patients with NSCLC. Gefitinib improves NSCLC-related symptoms and quality of life in some patients with advanced NSCLC who do not respond to platinum-based chemotherapy. However, the response rate for gefitinib remains <20% in patients with NSCLC (2–4), and treatment with gefitinib is associated with serious adverse effects, such as severe acute interstitial pneumonia in 5.4% of the patients who received the treatment (5, 6). Thus, it is imperative to select appropriate patients for treatment with gefitinib and exclude patients in whom gefitinib is unlikely to exhibit any clinical benefit. Women, patients who have never smoked, patients with adenocarcinoma, and East Asians are major subgroups of responders (3, 4, 6–8). Recently, gain-of-function somatic mutation in the tyrosine kinase domain of the EGFR has been correlated with the response to gefitinib (9, 10). However, other studies have revealed that correction of the phenotype arising from EGFR mutation may not account for all of the clinical benefits of gefitinib (11, 12), and both preclinical and clinical studies have reported that the efficacy of gefitinib is independent of EGFR expression level (11, 13–15). Although molecular features of the *EGFR* gene, including mutation and high copy number, (16, 17) are associated with response to gefitinib, other molecular markers in the tumor, such as HER2 overexpression (18), Akt phosphorylation (19), and other EGFR downstream molecules (20), also correlate with response. These observations suggest a role for unknown, but important, factors in gefitinib sensitivity. Identification and elucidation of such factors will improve existing therapeutic protocols and contribute to further understanding of the mechanisms of gefitinib sensitivity.

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To identify the gene products correlated with the efficacy of gefitinib, genome-wide screening was done recently for NSCLC. A global mRNA expression study using DNA microarrays and biopsy samples identified 51 genes associated with the sensitivity to gefitinib and established a numerical scoring system to predict the response (21). This expression study also led to the establishment of ELISA assays for the identified gene products in serum. Preclinical studies involving mRNA profiling of NSCLC xenografts resulted in the identification of a set of genes that were differentially expressed between tumors that were sensitive and insensitive to gefitinib treatment (22, 23). These studies will lead to the identification of novel biomarkers to predict the response to gefitinib treatment. However, mRNA expression does not necessarily correlate with protein level, and posttranslational modifications, such as phosphorylation, cannot be predicted from the amount of RNA or from the DNA sequence (24). With this background, comprehensive expression studies at the protein level, an approach called proteomics, have been conducted in patients with lung cancer to develop biomarkers that predict clinical outcomes (25). However, no global protein expression study has yet been done on the mechanism of response to gefitinib.

To identify the proteomic signature for sensitivity to gefitinib and to use that signature as a tumor marker to predict the response to gefitinib, we analyzed global protein expression levels in lung adenocarcinoma tissues for whom we have detailed information on *EGFR* gene status. The surgical specimens were obtained at the time of surgery from patients who subsequently had recurrence and received gefitinib monotherapy. We then used two-dimensional difference gel electrophoresis (2D-DIGE) covering ~2,000 proteins to identify a set of proteins of which expression was associated with sensitivity to gefitinib and with EGFR mutation. The predictive performance of the protein set was validated with an independent data set and compared with that of EGFR mutation.

Materials and Methods

Patients and tissue samples. We examined tumor tissues from patients who relapsed after surgery and received gefitinib monotherapy. Two hundred seventy-nine patients who received gefitinib at the National Cancer Center Hospital from July 2002 to December 2004 were evaluated for inclusion in this study. Ninety-two patients relapsed after surgical resection of primary NSCLC and started to receive monotherapy with gefitinib 250 mg/d for 14 days (n = 92). We used tumor tissues obtained at the time of surgery and stored in vapor nitrogen. Fifteen patients were excluded from our study for the following reasons: frozen tissues were not available (n = 10) and tumor histology showed squamous cell carcinoma (n = 4) or pleomorphic carcinoma (n = 1). The histologic features of the tissues were reviewed by two board-certified pathologists (Y.M and K.T.) and diagnosis was based on the latest WHO classification of lung adenocarcinoma (8, 26–28). The tumor responses were classified into complete response (CR), partial response (PR), and progressive disease (PD) using standard bidimensional measurements (29). In this study, patients without a marked reduction of tumor size were subdivided into minor response (MR) and stable disease (SD) groups. MR was defined as a 25% decrease in the sum of the products of perpendicular diameters of all measurable lesions at any point during gefitinib treatment. SD was defined as a <25% decrease in tumor size after treatment. The clinical information is summarized in Table 1, and further information, including EGFR mutation status, is summarized in Supplementary Table S1. Consent was obtained from all patients and the protocol was approved by the institutional review board of the National Cancer Center.

To identify the proteins associated with response to gefitinib, we compared the protein expression profiles of responders (CR and PR) and nonresponders (PD). Of 77 samples available, the effects of gefitinib treatment were not examined for six cases because the treatment was not completed. These six samples were excluded from this study. We constructed two sample sets in the following way (Table 2): a training sample set comprising 31 responders (2 CRs + 29 PRs) and 16 nonresponders (16 PDs) and a test set comprising six responders (6 PRs) and 8 nonresponders (8 PDs) from whom samples were obtained between June and December 2004 (Table 2). As no significant differences were observed between CRs and PRs (Supplementary Fig. S1A), we grouped CRs and PRs together in the responder group.

Protein extraction and protein expression profiling. The frozen tumor tissues were crushed to frozen powder with a Multi-Beads Shocker (Yasui-kikai, Osaka, Japan) under cooling with liquid nitrogen. The frozen powder was then treated with urea lysis buffer (7 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 1% Triton X-100) for 30 min on ice. After centrifugation at 15,000 rpm for 30 min, the supernatant was recovered as cellular protein for the protein expression study.

Protein samples were labeled with CyDye DIGE Fluor saturation dye (GE Healthcare Amersham Biosciences, Uppsala, Sweden) according to

Table 1. Patient characteristics

	No. patients	%
Gender		
Female	33	43
Male	44	57
Age (y)		
Median (range)	62.2 (32-80)	—
Histologic type		
Adenocarcinoma		100
Papillary/acinar/bronchioloalveolar/solid	30/16/9/6	49/26/15/10
Smoking history*		
Never smokers	37	48
Former smokers	12	16
Current smokers	28	36
ECOG performance status†		
0/1/2/3	24/39/9/5	31/51/12/6
Prior chemotherapy		
Yes	30	39
No	47	61
Response to gefinitib		
CR/PR/MR/SD/PD/NE	2/35/2/8/24/6	3/45/3/10/31/8
EGFR gene status		
Mutation L858R	18	23.4
DEL‡	18	23.4
G719§	2	2.6
Wild-type	35	45.4
Unknown	4	5.2

Abbbreviation: NE, not evaluated.
*Never-smokers: those who had never had a smoking habit; former smokers: those who had stopped smoking at least 1 yr before diagnosis; and current smokers: active smokers at diagnosis of NSCLC or those who had stopped smoking less than 1 yr before diagnosis.
†ECOG performance status was monitored according to the previous report (44).
‡Deletional mutations in exon 19.
§G719S and G719C.

Table 2. Training and test sets to develop the classifier for the response to gefitinib

	Training set			Test set		
	Responders, n = 31 (%)	Nonresponders, n = 16 (%)	P	Responders, n = 6 (%)	Nonresponders, n = 8 (%)	P
Age						
Mean ± SD	64.0 ± 8.9	60.5 ± 12.0	0.330	57.5 ± 12.8	62.8 ± 6.1	0.386
Gender						
Male	17 (55)	9 (56)	0.927	3 (50)	5 (62.5)	0.640
Female	14 (45)	7 (44)		3 (50)	3 (37.5)	
Smoking history						
Never smokers	17 (55)	9 (56)	0.286	4 (67)	4 (50)	0.054
Former smokers	7 (22.5)	1 (6)		2 (33)	0 (0)	
Current smokers	7 (22.5)	6 (38)		0 (0)	4 (50)	
EGFR gene status						
Mutation	27 (87)	1 (6)	<0.001	4 (66)	0 (0)	0.006
Wild type	3 (10)	13 (81)		1 (17)	8 (100)	
Unknown	1 (3)	2 (13)		1 (17)	0 (0)	
Prior chemotherapy						
(+)	12 (39)	5 (31)	0.614	6 (100)	0 (22)	<0.001
(−)	19 (61)	11 (69)		0 (0)	8 (100)	
Performance status						
0	11 (35.5)	6 (37.5)	0.945	2 (33)	1 (12.5)	0.347
1	11 (35.5)	10 (62.5)		4 (67)	7 (87.5)	
2	6 (19)	0 (0)		0 (0)	0 (0)	
3	3 (10)	0 (0)		0 (0)	0 (0)	

our previous report (30). We prepared an internal control consisting of a mixture of small portions of all protein samples obtained before May 2004 (31). The internal control sample and the individual experimental samples were labeled with Cy3 and Cy5 CyDye DIGE Fluor saturation dyes, respectively. Five micrograms of Cy3- or Cy5-labeled protein were mixed and coseparated by two-dimensional PAGE. The first-dimension separation was achieved on an Immobiline pH gradient gel (isoelectric point range, 4-7; 24 cm length) with a Multiphor II (GE Healthcare Amersham Biosciences). The second-dimension separation was done with an EttanDalt II (GE Healthcare Amersham Biosciences) with a 9% to 15% gradient polyacrylamide gel. After electrophoresis, the gels were scanned at appropriate wavelengths for Cy3 and Cy5 (Supplementary Fig. S2A). The ratio between Cy5 and Cy3 intensity was calculated for all protein spots in identical gels by the use of DeCyder software (GE Healthcare Amersham Biosciences; ref. 31). The standardized spot intensities were then logarithmically transformed and subjected to a data-mining package (Impressionist; GeneData, Basel, Switzerland). We ran triplicate gels for each sample and calculated the averaged standardized spot intensity.

To assess the reproducibility of the proteomic data with the internal control in our analyses, we generated triplicate protein profiles from identical samples (case 9; Supplementary Table S1) and compared the standardized intensity of the paired spots (Supplementary Fig. S2B). Scattergrams with 1,980, 1,646, and 1,873 spots showed that the intensities of 1,916 (93.7%), 1,599 (94.7%), and 1,770 (94.5%) spots, respectively, were scattered within a 2-fold difference, and the correlation values were also high (r values > 0.93; Supplementary Fig. S2B).

Data analysis. A bioinformatic approach based on a support vector machine (SVM) algorithm and a leave-one-out cross-validation was used to identify proteins of which expression was associated with tumor characteristics, including therapeutic response to gefitinib and the presence of EGFR mutation (32).

Protein identification. Proteins corresponding to the protein spots of interest were identified by mass spectrometry (30). The proteins were recovered in a gel plug by using an automated spot collector (SpotPicker; GE Healthcare Amersham Biosciences) and digested with sequence grade trypsin (Promega, Madison, WI; ref. 30). Trypsin digests were applied to liquid chromatography coupled with tandem mass spectrometry (LTQ, Thermo, Waltham, MA). A database search against Swiss-Prot was done with Mascot software. Patients with a Mascot score of 35 or more were used for protein identification. When multiple proteins were identified in a single spot, the proteins with the highest number of peptides were considered as those corresponding to the spot.

Mutations in the EGFR gene. EGFR mutations in the samples obtained between July 2002 and May 2004 were examined as described in our previous report (8). Analysis of samples obtained between June 2004 and December 2004 was done by high-resolution melting analysis with a LightCycler HR-1 system (Idaho Technology Inc., Salt Lake City, UT).

ELISA. The expression level of heart-type fatty acid–binding protein (H-FABP) in protein samples from 55 lung adenocarcinoma patients (2 CRs, 28 PRs, 6 SDs, 1 MR, and 18 PDs) was measured in a clinical laboratory (SRL, Tokyo, Japan) with a commercially available ELISA kit (MARKIT-M H-FABP, Dainippon Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions (Supplementary Table S1). All these 55 samples were included in a 2D-DIGE analysis set in this study.

Results

Proteomic signature for the response to gefitinib. We first selected 1,685 protein spots that appeared in at least 80% of the images of Cy3-labeled internal control. We further selected 87 protein spots that showed different intensities between responder and nonresponder groups ($P < 0.05$, Wilcoxon test). Although potentially resulting in a loss of information, this trimming process decreased the possibility that the classifier would be significantly influenced by irrelevant expression data. We selected protein sets for which expression was associated with response to gefitinib by using a SVM algorithm. Accuracy, plotted as a function of spot number, was constant until the number of spots decreased to less than nine, showing that accurate classification did not require all protein spots (Fig. 1A). The location on the two-dimensional map is shown for the selected nine spots (Fig. 1B; Supplementary Fig. S3).

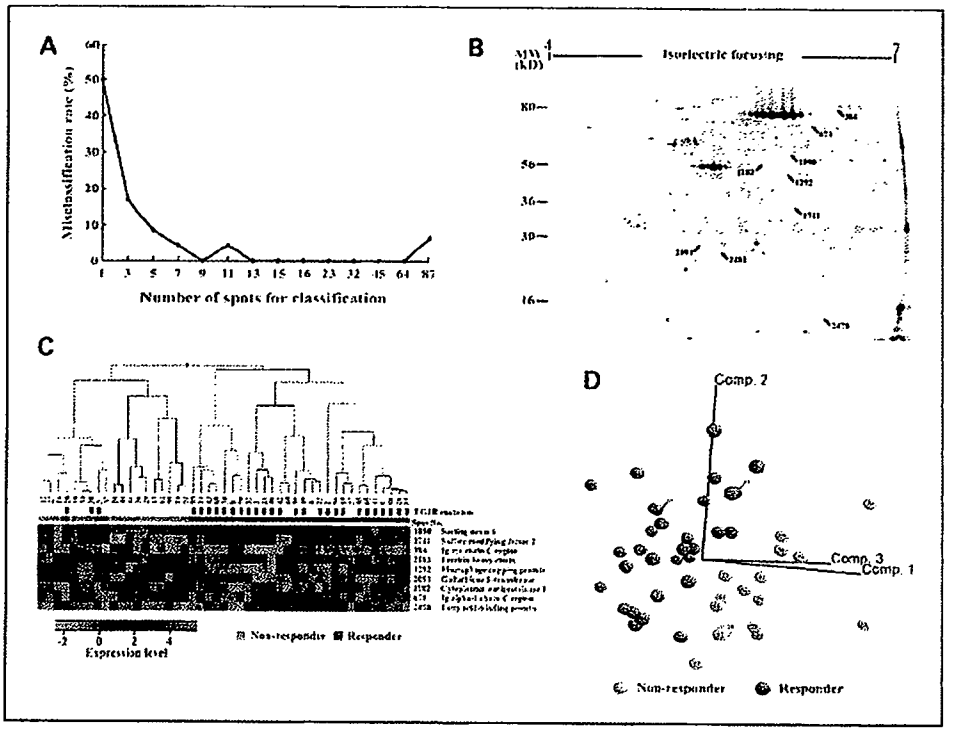

Fig. 1. Data-mining procedure to develop the prediction model for the response to gefitinib. *A*, a spot ranking method selected a few protein spots by which the cumulative error rate of a leave-one-out cross-validation became minimal. The spot ranking method indicated that the error rate was minimal when the prediction model was constructed by a particular nine protein spots. *B*, localization of the selected nine protein spots on the two-dimensional map. An enlarged two-dimensional image is shown in Supplementary Fig. S2. *C*, hierarchical clustering analysis of the samples in the learning set using the selected nine protein spots. Black bars, the presence of EGFR mutations within exons 18 to 21. *D*, principal component analysis of the samples in the learning set using the selected nine protein spots. Comp.1, 2, and 3, the first component 1, 2, and 3, respectively.

Mass spectrometry revealed that these nine spots corresponded to nine gene products (Table 3). Overall similarity of the selected spots is shown in Supplementary Fig. S1B and C. As the responder group in the training set consisted mainly of PRs, the obtained proteomic signature would presumably be more reflective of PR than CR.

The classification performance of the selected nine protein spots was validated by unsupervised classification. Hierarchical clustering showed that all tumor samples in the training set, except for cases 5, 20, and 37, were grouped according to their sensitivity to gefitinib based on the expression pattern of the nine proteins (Fig. 1C). In principal component analysis, all 47 samples seemed to be separated into two groups, although the border between these groups was not clear (Fig. 1D). Although hierarchical clustering and principal component analysis are crude methods of validation of classification, the results obtained using them were consistent.

To validate the predictive performance of the nine protein spots, we investigated a newly enrolled test sample set that was completely independent of the learning set. Based on the expression level of the nine protein spots, the distance of each sample from the hyperplane created by the SVM algorithm, defined as the SVM value, was calculated. The samples with a positive SVM value were grouped as responders and the samples with a negative SVM value were grouped as nonresponders. As a consequence, all training set samples were correctly classified in accordance with their clinical response to gefitinib (Fig. 2). All responders (six PRs) and seven of eight nonresponders (eight PDs) in the test set were also correctly classified. The expression pattern of the nine protein spots in the nonresponder patient (case 75) was more similar to that of the responder group, for unknown reasons. We also validated the results using the samples from patients who showed MR and SD. We found that the two patients showing MR were categorized as responders and that among the eight patients showing three SDs were classified into the responder group and five SDs into the nonresponder group. We did a leave-one-out cross validation for all 47 samples in the training set and the test set using nine protein spots with 1,000 times random permutation. All but two cases, cases 37 and 75, were correctly classified according to their status of response to the treatment. The overall misclassification error rate was 3.3%. Consequently, the model predicted the response to gefitinib in 13 of the 14 (92.8%) newly enrolled samples from the responders and nonresponders and may be useful for disease monitoring.

Proteomic signature for EGFR gene mutation. We studied the spots on the prediction for EGFR mutation. We set a training sample set, including 58 samples (34 mutation-positive samples and 24 mutation-negative samples; Supplementary Table S2). We found that the 12 protein spots showed the high correlation with the EGFR mutation (Supplementary Data; Supplementary Figs. S4-6). The classification and prediction performance of the selected 12 protein spots was successfully validated using the external validation sample set, including four mutation-positive samples and 11 mutation-negative samples (Supplementary Fig. S7). Only one protein, sulfate modifying factor 2, was shared between the signatures for the response and for the mutation (Table 3; Supplementary Table S3).

Expression of H-FABP measured by ELISA. We validated the differential expression of the identified proteins by the use of a widely available clinical assay. The expression level of H-FABP in the same tumor samples as those used in 2D-DIGE was measured with a commercially available ELISA kit intended for serum assays (Fig. 3). H-FABP expression measured by ELISA was highly correlated with that measured by 2D-DIGE (Pearson correlation, 0.76295; $P < 0.0001$). The ELISA study also showed