Table 6. Response rates

	n	CR	PR	SD	PD	NE	Response rate (%) (95% CI)
All	44	4	35	3	0	2	88.6 (75.4–96.2)
Treated at RD	41	4	32	3	0	2	87.8 (73.8–95.9)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluated; 95% CI, 95% confidence interval; RD, recommended dose.

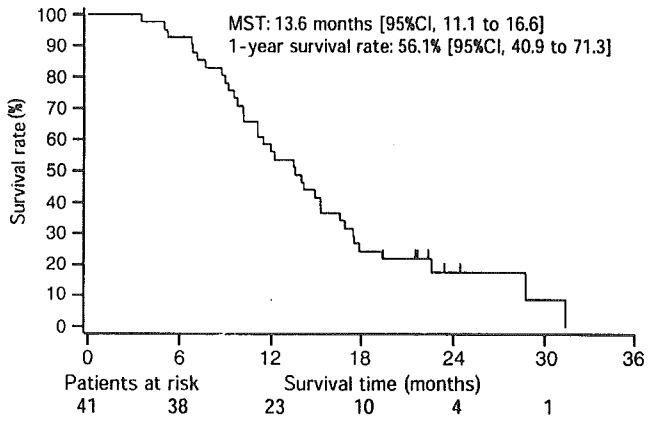

Figure 1. Overall survival of patients with extensive-stage small-cell lung cancer who were treated with amrubicin and cisplatin at the recommended dose. MST, median survival time; 95% CI, 95% confidence interval.

Toxicity in patients treated at the RD

The worst grades of hematological and non-hematological toxicities experienced by each patient are listed in Table 7. Hematological toxicity, especially leukopenia and neutropenia, was common and relatively severe. Grade 3 or worse leukopenia and neutropenia occurred in 65.9% and 95.1% of patients, respectively. Febrile neutropenia was observed in two patients at level 2. Grade 3 or worse anemia and thrombocytopenia occurred in 53.7% and 24.4% of patients, respectively. Four patients received platelet transfusions. Common non-hematological toxicities were gastrointestinal toxicity, such as anorexia, nausea, vomiting, constipation, diarrhea and stomatitis. Gastric ulcers developed in three patients. Hepatic and renal toxicity were not common in this study. Grade 3 or worse hyponatremia and hypokalemia occurred in 22% and 9.8% of patients, respectively. One patient developed myocardial infarction; however, cardiac toxicity was not common. No treatment-related deaths were observed.

Discussion

Doxorubicin and epirubicin are classified as active agents for SCLC, for which single-agent activity is a >20% response rate [19]. Doxorubicin has been used as a constituent of combination therapy for SCLC in the CAV (cyclophospamide, doxorubicin and vincristine) and CAP (cyclophosphamide, doxorubicin and cisplatin) regimens. Epirubicin has shown

Table 7. Toxicity in patients treated at the recommended dose ($n=41$)

	Grade (NCI CTC)					Grade 3/4 (%)
	0	1	2	3	4	
Leukopenia	1	0	13	20	7	65.9
Neutropenia	0	1	1	7	32	95.1
Febrile neutropenia	41	–	–	0	0	0.0
Hemoglobin	1	8	10	17	5	53.7
Thrombocytopenia	9	14	8	10	0	24.4
Stomatitis	22	13	5	1	0	2.4
Anorexia	1	14	13	13	0	31.7
Nausea	3	15	14	9	0	22.0
Vomiting	20	8	11	2	0	4.9
Constipation	24	1	13	3	0	7.3
Diarrhea	26	12	1	2	0	4.9
Gastric ulcer	38	0	1	2	0	4.9
Bilirubin	24	12	4	1	0	2.4
Hyponatremia	18	14	–	7	2	22.0
Hypokalemia	31	6	–	4	0	9.8
Hyperkalemia	33	3	4	1	0	2.4
Hypocalcemia	31	5	4	0	1	2.4

NCI CTC, National Cancer Institute Common Toxicity Criteria.

50% and 48% response rates in two clinical studies in 41 and 80 previously untreated patients, respectively, with ED-SCLC [20, 21]. However, currently, combination modalities containing doxorubicin or epirubicin are not being used in the therapy of SCLC, in preference to combination therapy with cisplatin and etoposide. Since amrubicin has shown excellent single-agent activity [15], it can be expected to be superior to other anthracyclines in the treatment of SCLC. Additionally, the present results of combination therapy with cisplatin support the view that amrubicin may be a promising agent that overcomes the therapeutic plateau of SCLC.

Amrubicin is one of the most promising new agents for the treatment of SCLC. In a previous phase II study of amrubicin 45 mg/m^2 on days 1–3 every 3 weeks as a monotherapy for chemonaive ED-SCLC, a 76% overall response rate and 11.7 month MST were observed [15]. The overall response rate and MST were comparable to those achieved with standard combination chemotherapy, such as etoposide plus cisplatin [5, 6]. Moreover, only a few patients treated in the phase II study received salvage chemotherapy consisting of cisplatin and etoposide [15]. The major toxicity of amrubicin as a monotherapy was hematological toxicity: grade 4 leukopenia and neutropenia were seen in 12.1% and 39.4% of patients, respectively, and thrombocytopenia and anemia of grade 3 or worse in 21.2%. Hepatic, renal and cardiac toxicities with amrubicin were not common. Cisplatin is a key drug for the treatment of SCLC and its hematological toxicity, such as leukopenia and neutropenia, is not severe. Thus, we conducted a phase I–II study of amrubicin and cisplatin treatment for chemonaive ED-SCLC to determine the MTD of this combination therapy, to

assess the efficacy and safety of the drugs delivered at their RD in chemonaive ED-SCLC, and to examine pharmacokinetics.

The topoisomerase I inhibitor, irinotecan, is also very effective for SCLC [6]. Combinations of topoisomerase I and topoisomerase II inhibitors, such as irinotecan plus etoposide, have been reported as active combination chemotherapy for SCLC [22]. Thus, combination of irinotecan and amrubicin is another candidate for new combination chemotherapy for SCLC. A phase I study of irinotecan and amrubicin for chemonaive non-SCLC was performed in National Cancer Center Hospital (unpublished data). However, the MTD was less than irinotecan 60 mg/m^2 on days 1 and 8 and amrubicin 35 mg/m^2 on days 2–4, due to relatively severe myelotoxicity. We considered that amrubicin <35 mg/m^2 on days 2–4 with irinotecan 60 mg/m^2 on days 1 and 8 was insufficient to treat SCLC.

In this study, we determined the RD to be amrubicin 40 mg/m^2 on days 1–3 and cisplatin 60 mg/m^2 on day 1 every 3 weeks, and 41 patients were treated at the RD. Main toxicities of this combination chemotherapy were myelosuppression, especially leukopenia and neutropenia, and gastrointestinal toxicities including anorexia, nausea, vomiting, constipation, diarrhea, stomatitis and gastric ulcer. Of 41 patients, 32 (78%) patients received four or more courses of chemotherapy, and 22 (54%) patients completed four courses of chemotherapy without dose modification. One patient developed myocardial infarction; however, other cardiac toxicity, including decrease in left ventricle ejection fraction, was not observed in up to six courses of chemotherapy. The total dose of amrubicin was 720 mg/m^2. Grade 3 or 4 hyponatremia occurred in nine (22%) patients; however, most of the patients were asymptomatic. No unexpected toxicities and no treatment-related deaths were observed in this study. Toxicities observed in this study were manageable.

Four CRs and 32 PRs occurred, for an objective response rate of 87.8% (95% CI 73.8% to 95.9%) in 41 patients treated at the RD. In most patients, ProGRP levels changed in parallel with tumor responses. The MST of the 41 patients was 13.6 months, and the 1-year survival rate was 56.1%. These results were better than recently reported results for irinotecan and cisplatin in chemonaive ED-SCLC: an objective response rate of 84% and MST of 12.8 months [6]. The combination of amrubicin and cisplatin has demonstrated an impressive response rate and MST in patients with previously untreated ED-SCLC. A possible reason for the better results is overselection of patients, because we used unusual exclusion criteria such as non-steroidal anti-inflammatory drug or adrenal cortical steroid use for >50 days, and gastric and/or duodenal ulcer. However, in a phase II study, this kind of bias is not uncommon.

Combination chemotherapy with etoposide plus cisplatin or etoposide plus cisplatin, alternating with cyclophosphamide, doxorubicin and vincristine, had been considered as standard chemotherapy for SCLC in North America and Japan. A Japanese phase III trial (JCOG 9511) demonstrated that treatment with four cycles of irinotecan plus cisplatin every 4 weeks yielded a highly significant improvement in survival in ED-SCLC patients over standard etoposide plus cisplatin, with less myelosuppression [6]. Based on the results of the JCOG 9511 trial, irinotecan plus cisplatin is considered to be the reference chemotherapy arm for ED-SCLC in future trials in Japan [23]. The JCOG are preparing a phase III clinical trial of amrubicin and cisplatin for previously untreated ED-SCLC to compare combination therapy of irinotecan with cisplatin.

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Genomewide cDNA Microarray Screening of Genes Related to Benefits and Toxicities of Platinum-Based Chemotherapy in Patients With Advanced Lung Cancer

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Abstract: The authors conducted a study using cDNA microarray analysis to determine whether expression levels of genes in tumors were correlated with the outcome of chemotherapy. Forty-seven patients were studied, and all except 3 received platinum-based chemotherapy. The expression levels of 1176 genes in transbronchial biopsy specimens of tumors that were obtained before chemotherapy were analyzed using the Atlas Human Cancer 1.2 Array. Multivariate regression analysis revealed that 3 genes were each independent factors related to tumor resistance to chemotherapy and patient survival ($P < 0.01$). Among various chemotherapy-related toxicities, 1, 3, 3, 1, and 1 genes were also revealed to be independent factors that were correlated with neutropenia, anemia, diarrhea, infection, and increased serum creatinine respectively ($P < 0.01$). It is concluded that not only the benefits but also the toxicities of chemotherapy can be predicted by cDNA microarray using tumor specimens obtained before chemotherapy.

Key Words: microarray, gene, lung, cancer

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Lung cancer is a disseminated disease, and most affected patients are candidates for chemotherapy. Although responders to chemotherapy may have a better prognosis than nonresponders,[1] even the most effective chemotherapy cannot always reduce the tumor volume of lung cancer. The properties of cancer cells are determined by complicated interactions among all the gene products they express, and it is certain that many proteins—including enzymes involved in apoptosis, DNA repair, and the metabolism and detoxification of drugs—have individual responses. The cDNA microarray method is now widely used to analyze the expression of thousands of genes simultaneously in cancer tissues, and its development has facilitated the analysis of genomewide expression profiles. Using the cDNA microarray technique on tumor tissues obtained before chemotherapy, we previously identified 3 independent genes, each of which is correlated with chemoresistance and patient survival.[2,3] However, another important aspect of chemotherapy apart from tumor susceptibility and patient survival is the extent of adverse effects. Some cancer patients suffer severe adverse effects of chemotherapy regardless of whether their tumors are chemosensitive. Such patients are unable to receive repeat courses of chemotherapy, even if they have shown a tumor response. Accordingly, it is important to be able to predict not only patients who are likely to respond to chemotherapy, but also those who will probably experience severe treatment-related toxicities.

The current study analyzed the correlation between the expressions of various genes in tumor specimens and chemotherapy-related toxicities, and compared the genes related with the beneficial and toxic effects of chemotherapy.

PATIENTS AND METHODS

Patients

This study was approved by the institutional review board of Kanagawa Cancer Center. Patients with histologically proved lung cancer treated with chemotherapy were entered into the study. All were eligible for treatment. They had an expected survival of at least 6 weeks, measurable lesions, Eastern Cooperative Oncology Group performance status score ≤3 points, a white blood count of ≥4000 cells/μL, hemoglobin ≥10 g/dL, platelet count ≥100,000 platelets/μL, total serum bilirubin less than 2 mg/dL, aspar-

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tate aminotransferase and alanine aminotransferase less than twice the upper limit of the normal range, serum creatinine ≤1.5 mg/dL, and creatinine clearance more than 50 mL/minute. None of the patients had received prior chemotherapy for the primary lesion. Written informed consent for chemotherapy and a genetic analysis of tumor tissue was obtained in every case.

Chemotherapy

All patients with nonprogressive cancer were treated with 2 or more courses of chemotherapy. Response criteria were evaluated according to the World Health Organization criteria.[4] Toxicities were evaluated according to the NCI-CTC version 2 criteria.[5]

Tumor Samples

Transbronchial biopsy specimens of tumors were obtained before chemotherapy. Half the specimens were fixed in formalin for pathologic diagnosis and the other half were immediately frozen for storage at −80°C until genetic analysis.

Extraction and Purification of RNA and Preparation of Probes

The total RNA of each sample was isolated and treated with DNase I to avoid contamination of genomic DNA by silica membrane affinity chromatography using Macherey–Nagel's total RNA isolation kit (MACHEREY–NAGEL GmbH & Co. KG, Germany). One hundred nanograms of the total RNA for each sample was reverse transcribed into cDNA and amplified by SMART polymerase chain reaction (PCR) technology[6] using the Super SMART PCR cDNA Synthesis kit (BD Biosciences Clontech, CA) according to the manufacturer's instructions. Each cDNA sample was subjected to microarray expression profiling using the BD Atlas Human Cancer 1.2 Array (Clontech) based on the manufacturer's protocol described previously.[2,3]

cDNA Microarray

Each labeled probe was then hybridized into a separate Atlas Array. The signal intensity for each spot, which corresponds to each gene examined, was determined using a STORM image analyzer (Amersham Bioscience, Picataway, NJ). The hybridization pattern and signal intensity were analyzed to determine changes in gene expression levels using AtlasImage 2.01 software (CLONTECH, Laboratory, Inc., Japan).

Statistical Methods

t-tests were used to identify differences in mean expression levels among benefits and toxicities of chemotherapy. We compared the differences of gene expression between grade 3 or grade 4 (worst grade) and others for hematologic toxicities, and between grade 0 and others for nonhematologic toxicities. To determine whether gene expression profiles were associated with variety in cases of survival, Kaplan–Meier survival plots and log-rank tests were used. The influence of each gene expression on each outcome of chemotherapy was examined in stepwise multivariate regression analysis. $P < 0.01$ was considered significant.

RESULTS

Between September 2000 and December 2001, 47 patients were registered in the study (Table 1). Thirty-six patients were men and 11 were women, with a median age of 66 years (range, 35–81 years). Eighteen patients had small cell lung cancer (SCLC), and the rest had nonsmall cell lung cancer (NSCLC). Of the patients with SCLC, 2 had limited disease and the other 16 had extensive disease. Of the patients with NSCLC, 12 had locally advanced disease and 17 had metastatic disease. No patients had received prior chemotherapy. All the patients, except for 3 who had been prescribed paclitaxel and irinotecan, were given full-dose platinum-based chemotherapy. Sixteen of the 18 patients with SCLC (89%) and 12 of the 29 patients with NSCLC (41%) responded to chemotherapy.

The expression levels of 1176 genes in the tumor specimens were analyzed using cDNA microarray screening. Four housekeeping genes that were expressed in all 47 tumor

TABLE 1. Patient Characteristics

	No. of Patients
Total	47
Gender	
Male	36
Female	11
Smoker	38
PS(ECOG)	
0	5
1	30
2	9
3	3
Pathology	
SCLC	
Stage	
LD	2
ED	16
NSCLC	
Stage	
IIB/IIIA	4
IIIB	8
IV	17

PS, performance status; ECOG, Eastern Cooperative Oncology Group; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; LD, limited disease; ED, extensive disease.

samples were used as controls for gene expression: ubiquitin, liver glyceraldehyde 3-phosphate dehydrogenase, 23-kDa highly basic protein, 60S ribosomal protein L13A, and 40S ribosomal protein S9.

When we analyzed the relationship between gene expression and chemotherapy-related hematologic toxicity, 2 and 22 genes were identified as showing significantly higher expression in patients with grade 4 neutropenia and grade 3 anemia in comparison with grade 0 to grade 3 neutropenia and grade 0 to grade 2 anemia respectively. We also identified 17, 19, 4, and 1 genes that showed significantly higher expression in patients who experienced diarrhea, infection, increased serum creatinine, and pneumonitis respectively than in patients who did not (grade 0). Stepwise multivariate regression analysis revealed that 1, 3, 3, 1, and 1 genes were independent factors, each of which was correlated with toxicities such as neutropenia, anemia, diarrhea, infection, and increased serum creatinine respectively (Table 2, $P < 0.01$). We were unable to identify any genes that were correlated with thrombocytopenia, emesis, increased total bilirubin, and increased GPT.

As previously presented, stepwise multivariate regression analysis revealed that 3 genes—allograft inflammatory factor 1, HLA-DR antigen-associated invariant subunit, and MHC class HLA-DR-β precursor—were factors independently associated with chemoresistance ($P < 0.0001$, Table 3). When we analyzed the relationship between gene expression level and survival, G1/S-specific cyclin, type II cGMP-dependent protein kinase, and hepatocyte growth factor-like protein were significantly correlated (log-rank test, $P < 0.01$, Table 3). Thus, not only chemotherapeutic benefits but also some toxicities were predicted by cDNA microarray using tumor specimens obtained before chemotherapy.

DISCUSSION

We examined the expression of cancer-related genes in samples of lung cancer obtained before chemotherapy using cDNA microarray screening, and analyzed the relationship between gene expression levels and clinical outcome after chemotherapy. We previously reported 3 genes with expression levels that were each correlated with the tumor response to chemotherapy[2] or patient survival.[3] One surprising finding was that chemoresistant genes related to host immunity were different from survival-related genes. This is because patient survival is influenced by not only the effect of chemotherapy on the tumor but also by tumor growth and metastasis.

The current study revealed some specific genes with expression levels that were correlated with chemotherapy-related toxicity. Cytohesin-1 was identified as a genetic factor that predicted neutropenia resulting from chemotherapy. This is a guanine nucleotide exchange factor that regulates members of the ADP–ribosylation factor family of small GTPases. An analysis of granulocytic maturation of HL-60 cells has revealed a marked increase in the level of cytohesin-1 expression during dibutyryl–cyclic AMP-induced granulocyte differentiation.[7] These data suggest that cytohesin-1 may be useful as a potential marker of granulocytic differentiation.

Three genes—MAD3, DNAX activation protein 12, and interleukin-1β precursor—were identified as predictors of anemia induced by chemotherapy. MAD3 is one of the

TABLE 2. Genes Closely Associated With Chemotherapeutic Toxicities

Factor	Description	Gene Expression (mean ± SD)		Coefficient	SE	*P*
Neutrophil		grade 0–3 (n = 35)	grade 4 (n = 12)			0.0056
	Cytohesin-1	1.8 ± 3.5	6.6 ± 7.9	0.033	0.011	
Hemoglobin		grade 0–2 (n = 43)	grade 3 (n = 4)			<0.0001
	Major histocompatibility complex enhancer-binding protein MAD3	7.0 ± 9.4	43.5 ± 83.7	−0.009	0.004	
	DNAX activation protein 12	10.2 ± 13.1	53.8 ± 63.7	0.005	0.002	
	Interleukin-1 beta precursor	13.1 ± 13.5	529.3 ± 1034.6	0.001	0.0003	
Infection		grade 0 (n = 43)	grade 1–3 (n = 4)			0.0003
	Hemoglobin alpha subunit	7.7 ± 8.8	44.3 ± 61.0	0.007	0.002	
Creatinine		grade 0 (n = 41)	grade 1–2 (n = 6)			0.0021
	Matrix metalloproteinase 10	12.3 ± 19.1	62.2 ± 64.3	0.005	0.001	
Diarrhea		grade 0 (n = 35)	grade 1–3 (n = 12)			0.0002
	ICH-2 protease	16.1 ± 17.5	42.4 ± 36.8	0.008	0.073	
	Interferon-inducible RNA-dependent protein kinase	4.3 ± 6.8	12.9 ± 14.0	−0.028	0.013	
	Collagen 16 alpha 1 subunit precursor	2.8 ± 4.5	15.9 ± 20.0	0.031	0.01	

TABLE 3. Genes Closely Associated With Chemotherapeutic Benefits

Factor	Description	Coefficient	SE	P
Survival	G1/S-specific cyclin D2			0.0055
	Type II cGMP-dependent protein kinase			0.0016
	Hepatocyte growth factor-like protein			0.0075
Tumor effect on chemotherapy	Allograft inflammatory factor 1			<0.0001
	HLA-DR antigen-associated invariant subunit	−0.014	0.002	
	MHC class II HLA-DR-beta precursor	−0.001	0.0003	
		−0.01	0.002	

metaphase checkpoint proteins involved in cell division, and interleukin-1 is one of the monokines that can elicit many of the defective host responses to infection. DNAX activation protein 12 is a membrane adaptor molecule that contains an immunoreceptor tyrosine-based activation motif, which activates calcium signaling in immune cells. However, the mechanisms by which these 3 genes influence the incidence of chemotherapy-related anemia remain unclear.

ICH-2, found to be a predictor of diarrhea, is a novel human gene encoding a member of the interleukin-1β converting enzyme cysteine protease family. ICH-2 mRNA is widely expressed in human tissue and appears to play a primary role in apoptosis.[8] Another predictor of diarrhea, protein kinase regulated by RNA, plays an important role in many cellular processes, including virus multiplication and cell growth, differentiation, and apoptosis.[9] It is also still unclear how these genes, including collagen 16, participate in susceptibility to chemotherapy-related diarrhea.

Although this study revealed a number of genes related to the beneficial and toxic effects of chemotherapy, their mechanisms of action remain to be explained. This may be because we used mononuclear cells from peripheral blood of healthy volunteers as a control for gene expression. A major objective of this study was to clarify predictors of not only beneficial but also toxic effects of cancer chemotherapy. The genetic characteristics of various tissues are believed to differ from one another. Therefore cancer cells need to be examined to clarify the factors related to tumor susceptibility to chemotherapy, and blood cells need to be examined for susceptibility to hematologic toxicities. Malignant tumor tissues are heterogeneous and contain a number of cell types, and specimens of lung cancer obtained by transbronchial biopsy are not considered to reflect the general characteristics of tumor tissue. The fact that genetic information on tumor cells can predict not only tumor susceptibility to chemotherapy but also toxicity suggests that certain genetic characteristics may be common to all somatic cells, irrespective of whether they are malignant or normal. If this hypothesis is correct, then nonmalignant normal cells may also be used for analysis of informative genetic factors that can predict the antitumor effects and toxicities of chemotherapy.

We need to undertake prospective evaluations to determine whether the genes revealed in this study are truly important and potentially useful for predicting the beneficial or toxic effects of chemotherapy. Accumulation of such data could eventually allow chemotherapy to become "personalized" using anticancer drugs that would be effective and nontoxic in individual patients.

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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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Is radiotherapy optimally combined with chemotherapy in elderly patients with limited-stage small-cell lung cancer?

GLOSSARY

ECOG PERFORMANCE STATUS (ECOG PS)
A scoring system to assess the wellbeing of cancer patients and their ability to perform ordinary tasks (0 = fully active to 5 = dead)

Original article Schild SE *et al.* (2005) Results of combined-modality therapy for limited-stage small cell lung carcinoma in the elderly. *Cancer* **103:** 2349–2354

SYNOPSIS

KEYWORDS cisplatin, combined-modality therapy, etoposide, radiotherapy, small-cell lung cancer

BACKGROUND

It is important to understand the effects of modern combined-modality therapy in elderly patients with lung carcinoma. Half of the patients who are diagnosed with lung carcinoma are ≥70 years of age.

OBJECTIVES

To determine the relationship between age and outcome in patients with limited-stage small-cell lung cancer (SCLC) treated with etoposide and cisplatin in addition to once-daily or twice-daily radiotherapy (QDRT or BIDRT respectively).

DESIGN AND INTERVENTION

From September 1990 to November 1996, this North Central Cancer Treatment Group phase III trial enrolled patients with limited-stage disease confirmed by pathology as SCLC, with ECOG PERFORMANCE STATUS (ECOG PS) ≤2 and sufficient organ function. Six 3-day cycles of etoposide and cisplatin were given, with a 28-day interval between cycles. Cisplatin (30 mg/m^2 given intravenously over 30–60 minutes), and etoposide (130 mg/m^2 given intravenously over 45 minutes) were administered on each chemotherapy day. After the first three cycles, the dose of etoposide was reduced to 100 mg/m^2 per cycle. Patients were randomized to receive thoracic radiotherapy (in parallel to chemotherapy cycles 4–5), either QDRT (50.4 Gy in 28 fractions) or BIDRT (48 Gy in 32 fractions).

OUTCOME MEASURES

Toxicity, disease control and survival.

RESULTS

Of 263 evaluable patients (median age 63 years, range 37–81 years), followed for a median of 8.1 years (range 4.6–11.9 years), 209 were younger than 70 years old and 54 were 70 years old or older. Baseline ECOG PS and weight loss were worse in the older group. Tumor progression rates, survival, local control, and overall, hematologic and nonhematologic toxicities did not differ according to patient age. The 2-year and 5-year survival rates were 48% and 22% respectively, in patients aged <70 years, versus 33% and 17% in older patients (P=0.14). Hematologic toxicities ≥grade 3 or ≥grade 4 did not occur more frequently in elderly patients. Grade 3 toxicity or worse occurred in 91% of patients aged <70 years compared with 94% of elderly patients (P=0.58). Toxicities of grade 4 or more occurred in 46% of patients aged <70 years compared with 50% of older patients (P=0.65). Grade ≥3 nonhematologic toxicity occurred in 46% of those aged <70 years compared with 52% of older patients (P=0.45). Grade ≥4 nonhematologic toxicity occurred in 12% of patients aged <70 years compared with 11% of elderly patients (P=1.0). Of the nonhematologic toxicities, only grade ≥4 pneumonitis occurred more frequently in elderly patients. Grade ≥3 esophagitis occurred in similar numbers of patients in the two age groups. Treatment-related toxicity caused death in 4 of 263 patients (2%)—3 in the elderly group (pneumonitis) and 1 in the younger group (infection).

CONCLUSION

Elderly patients should be encouraged to receive combined-modality therapy, especially within clinical trials.

COMMENTARY

Nagahiro Saijo

Cisplatin plus etoposide with concurrent thoracic radiotherapy is the standard treatment for limited-disease small-cell lung carcinoma (LD-SCLC) in the elderly.[1,2] In Intergroup study 0096, Turrisi *et al.* found that, when combined with etoposide plus cisplatin chemotherapy, a total radiation dose of 45 Gy administered as a twice-daily therapy (1.5 Gy twice daily) produced superior survival to the same total dose administered as a once-daily therapy (1.8 Gy once daily).[1] The Japan Clinical Oncology Group also obtained excellent survival data (median survival time 27 months) using concurrent chemotherapy and twice-daily irradiation (Japan Clinical Oncology Group 9104).[2] In 2004, Schild *et al.* reported that equivalent survival benefit was achieved with twice-daily and once-daily irradiation with etoposide plus cisplatin chemotherapy.[3] Once-daily radiotherapy was administered continuously, and twice-daily radiotherapy was administered with a 2.5-week intermission after 24 Gy. The treatment schedule of the Intergroup study differed from that of the present study in that concurrent radiotherapy was given from the start of chemotherapy, and radiotherapy was given without a break. The dose intensity of the combination of chemotherapy and radiotherapy in the Intergroup study was higher in the twice-daily group. Efficacy improved with increased intensity of combined-modality therapy, as did adverse events. Elderly patients usually experience more toxicity than younger patients, and cannot tolerate intensive treatment. Few studies have specifically targeted elderly populations.

The elderly patients in the present analysis (aged ≥70 years) experienced significantly greater weight loss and poorer performance status than the younger patients (aged <70 years). The 2-year and 5-year survival rates were 48% and 22% for younger patients, compared with 33% and 17% for elderly patients. The incidence of grade 4 pneumonitis was higher in the elderly patients. Grade 5 toxicity occurred in 1 of 209 younger patients versus 3 of 54 older patients. Schild *et al.* concluded that LD-SCLC patients over 70 years of age are candidates for clinical trials of aggressive treatment if they do not have severe comorbidity. Yuen *et al.* reviewed the elderly subset results from the Intergroup 0096 study.[4] Quon *et al.* also studied the influence of age on the delivery, tolerance, and efficacy of thoracic irradiation in the combined-modality treatment of limited stage small-cell lung cancer.[5] In both analyses it was suggested that an elderly subset seems to be at risk of toxicity, but that those patients completing therapy do as well as their younger counterparts. It is extremely difficult, however, to distinguish those patients who are at risk of toxicity before toxicity occurs.

LD-SCLC is curable by chemotherapy and radiotherapy without surgery. Since the average age of LD-SCLC patients will increase year by year, fit elderly patients with LD-SCLC should be encouraged to undergo combined-modality therapy. An initial cycle of chemotherapy before concurrent treatment might unveil the vulnerable subset. The role of sequential chemotherapy should be evaluated in elderly patients considered marginal, to help us to distinguish those patients that are able to tolerate aggressive therapy from those that are too easily tipped over into a less-fit category. In conclusion, it is extremely important to establish a safe and effective standard treatment for the elderly patient population.

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Competing interests
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PRACTICE POINT
Further study of combined-modality therapy within clinical trials is needed to establish a safe and effective standard treatment for elderly patients with lung carcinoma

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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?

Supplementary information, in the form of a reference list, is available on the *Nature Clinical Practice Oncology* website.

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

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EGFR Mutation Status in Japanese Lung Cancer Patients: Genotyping Analysis Using LightCycler

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Abstract **Purpose:** Recently, somatic mutations of the epidermal growth factor receptor (*EGFR*) gene were found in ~25% of Japanese lung cancer patients. These *EGFR* mutations are reported to be correlated with clinical response to gefitinib therapy. However, DNA sequencing using the PCR methods described to date is time-consuming and requires significant quantities of DNA; thus, this existing approach is not suitable for a routine pretherapeutic screening program.
Experimental Design: We have genotyped *EGFR* mutation status in Japanese lung cancer patients, including 102 surgically treated lung cancer cases from Nagoya City University Hospital and 16 gefitinib-treated lung cancer cases from Kinki-chuo Chest Medical Center. The presence or absence of three common *EGFR* mutations were analyzed by real-time quantitative PCR with mutation-specific sensor and anchor probes.
Results: In exon 21, *EGFR* mutations (CTG → CGG; L858R) were found from 8 of 102 patients from Nagoya and 1 of 16 from Kinki. We also detected the deletion mutations in exon 19 from 7 of 102 patients from Nagoya (all were deletion type 1a) and 4 of 16 patients from Kinki (one was type 1a and three were type 1b). In exon 18, one example of G719S mutation was found from both Nagoya and Kinki. The L858R mutation was significantly correlated with gender (women versus men, $P < 0.0001$), Brinkman index (600 ≦ versus 600>, $P = 0.001$), pathologic subtypes (adenocarcinoma versus nonadenocarcinoma, $P = 0.007$), and differentiation status of the lung cancers (well versus moderately or poorly, $P = 0.0439$), whereas the deletion mutants were not. *EGFR* gene status, including the type of *EGFR* somatic mutation, was correlated with sensitivity to gefitinib therapy. For example, some of our gefitinib-responsive patients had L858R or deletion type 1a mutations. On the other hand, one of our gefitinib-resistant patients had a G719S mutation.
Conclusions: Using the LightCycler PCR assay, the *EGFR* L858R mutation status might correlate with gender, pathologic subtypes, and gefitinib sensitivity of lung cancers. However, further genotyping studies are needed to confirm the mechanisms of *EGFR* mutations for the sensitivity or resistance of gefitinib therapy for the lung cancer.

Lung cancer is a major cause of death from malignant diseases because of its high incidence, malignant behavior, and lack of major advancements in treatment strategy (1). Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan (2). More than 15,000 patients underwent surgical operation at Japanese institutions in 1998 (2). The clinical behavior of the lung cancer is largely associated with its stage. The cure of the disease by surgery is only achieved in cases representing an early stage of lung cancer (3).

The epidermal growth factor receptor (*EGFR*) tyrosine kinase inhibitor, gefitinib, has been approved in Japan for the treatment of non-small cell lung cancer from 2002. Although *EGFR* is more abundantly expressed in lung carcinoma (4, 5), *EGFR* expression, as detected by immunohistochemistry, did not reveal any obvious relationship with response to gefitinib (6). Clinical trial have revealed significant variability in the response to gefitinib, with higher response in Japanese patients than in predominantly European-derived population (27.5% versus 10.4%; ref. 7). The partial clinical responses to gefitinib have been observed most frequently in women, in nonsmokers, and in patients with adenocarcinoma (8–10). More recently, we have collaborated with Dana-Farber Cancer Institute and found that novel *EGFR* mutations status at ATP binding pockets in Japanese non-small cell lung cancer patients were correlated with the clinicopathologic features related to good response to gefitinib (11). Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro* (11–13).

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The standard for experimental detection of mutations is direct sequencing of DNA samples from the tissues. For known mutations, real-time polymerase chain detection followed by melting curve analysis, using hybridization probes, is highly sensitive, rapid, and an efficient alternative approach to mutation detection (14–16).

To determine the *EGFR* mutation status in Japanese lung carcinoma for screening and diagnostic purposes, we wanted to develop a faster and easy method to detect *EGFR* mutations. In this report, we investigated *EGFR* mutation status by the real-time reverse transcription-PCR assay using LightCycler (17) mutation-specific sensor and anchor probes. With this method, 32 samples were genotyped within 1 hour without the need of any post-PCR sample manipulation. The findings were compared with the clinicopathologic features of lung cancer.

Materials and Methods

Patients. The study group included 102 lung cancer patients who had undergone surgery (but did not receive gefitinib) at the Department of Surgery II, Nagoya City University Medical School, between 1997 and 2000. The study group also included 16 lung cancer patients who had undergone surgery at the Department of Surgery, National Hospital Organization, Kinki-chuo Chest Medical Center, and were subsequently treated with gefitinib. These 16 samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan, Ltd., Tokyo, Japan; data not shown) and analyzed by ABI prism Seq Scape version 2.1.1. The lung tumors were classified according to the general rule for clinical and pathologic record of lung cancer in Japan (18). All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathologic characteristics of the 102 lung cancer patients are as follows: 52 cases at stage I, 16 at stage II, and 34 at stage III to IV. The mean age was 65.5 years (range, 42-85). Among the 102 lung cancer patients, 49 (48%) were diagnosed as having adenocarcinoma, 32 (31.4%) squamous cell carcinoma, 9 (8.8%) adenosquamous cell carcinoma, and 7 (6.9%) small cell carcinoma.

PCR assays for EGFR. The genomic DNA was extracted from lung cancer tissues and matched normal lymphocytes from the peripheral blood using the Wizard SV Genomic DNA purification system (Promega Corporation, Madison, WI). Initially, 58 DNA samples were also extracted from lung cancer tissues from Nagoya City University and sequenced as reported in our previous paper (11). These sets of DNA were used as a positive and negative control for genotyping. DNA concentration was determined by spectrophotometry and adjusted to a concentration of 50 ng/mL. We then used 1 μL of each DNA for LightCycler analyses. To ensure the fidelity of DNA extraction, all samples were subjected to PCR amplification with oligonucleotide primers specific for exon 18 of the *EGFR* gene and then digested by *Sac*I enzyme. The primer sequences for *EGFR* gene in exon 18 were as follows: the forward primer, 5-TCCAAATGAGCTGGCAAGTG-3, and the reverse primer, 5-TCCCAAACACTCAGTGAAACAAA-3 (397 bp). The cycling conditions were as follows: initial denaturation at 95°C for 15 minutes followed by 35 cycles at 95°C for 20 seconds, 57°C for 20 seconds, 72°C for 30 seconds, and one cycle of 72°C for 3 minutes. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA) and then digested with restriction enzyme at 37°C for 2 hours. The genotyping PCR reactions were done using LightCycler DNA Master Hybridization probes kit (Roche Molecular Biochemicals, Mannheim, Germany) in a 20 μL reaction volume. The primer sequences for *EGFR* gene in exon 18 were as follows: the forward primer, 5-TCCAATGAGCTGGCAAGTG-3, and the reverse primer, 5-TCCCAAACACTCAGTGAAACAAA-3 (397 bp). For the exon 18 genotyping, sensor (LC Red 640-GCACCGGAGCCCAGCA) and anchor (GCCAGGGACCTTATACACGTGCCGAA-Fluorescein) probes were used. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 16 seconds The primer sequences for *EGFR* gene in exon 19 were as follows: the forward primer, 5-CGTCTTCCTTCTCTCTCTGTC-3, and the reverse primer, 5-GACATGAGAAAAGGTGGGC-3 (175 bp). For the exon 19 genotyping, sensor (GCTATCAAAACATCTCC-Fluorescein) and anchor (LC Red 640-AAAGCCAACAAGGAAATCCTCGATGTGAGTTTCTGCTTTGCTGTGTGGGG) probes were used. The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes followed by 45 cycles at 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 7 seconds. The primer sequences for *EGFR* gene in exon 21 were as follows: the forward primer, 5-GCTCAGAGCCTGGCATGAA-3, and the reverse primer, 5-CATCCTCCCCTGCATGTGT-3 (349 bp). The cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, 57°C for 10 seconds, and 72°C for 14 seconds. For the exon 21 genotyping, sensor (Fluorescein-AGTTTGGCCCGCCCA) and anchor (LC Red 640-CCTCCTTCTGCATGGTATTCTTTCTCTTCCGCACCCAG) probes were used.

Statistical methods. Statistical analyses were done using the Mann-Whitney *U* test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan-Meier methods and differences were examined by the log-rank test, Breslow-Gehan-Wilcoxon test, and Cox proportional hazard regression model. All analyses were done using the StatView software package (Abacus Concepts, Inc., Berkeley, CA) and results were considered significant when $P < 0.05$.

Results

Fidelity of allele-specific PCR confirmed by conventional PCR assay in lung cancer tissues. Using the exon 18 primer sets, a PCR product of 397 bp was obtained. We have analyzed the product using PCR-RFLP method. The wild-type DNA does not have a *Sac*I site within the 397 bp. The PCR products digested with *Sac*I were loaded with 2% agarose gel and wild-type DNA should be visualized as one band. However, if the substitution mutation G719S were present, the PCR products digested by *Sac*I will be visualized as three bands. Using this method, PCR products were visualized from all lung cancer patients studied. In exon 18, a G719S mutation was found from one Nagoya specimen (stage Ia, well-differentiated adenocarcinoma with bronchioloalveolar carcinoma pattern at the edge of tumor, female, nonsmoker patient) and one Kinki specimen (Fig. 1A). These mutants were also analyzed by LightCycler. The anchor probe was matched for wild type. As shown in Fig. 1B for the G719S mutation in exon 18, the homozygous wild-type PCR product showed a single peak at 69°C, whereas the heterozygous products (mutant) showed an additional peak at 59°C. The LightCycler method using the mutation-specific probes confirmed the results with the restriction fragment analysis.

Genotyping of EGFR at exon 19 and exon 21 in lung cancer tissues. For exon 21 genotyping, the anchor probe was matched for L858R mutation. As shown in Fig. 2, for the L858R mutation in exon 21, the homozygous wild-type PCR product showed a single peak at 53°C, whereas the heterozygous products (mutant) showed an additional peak at 65°C. From the 102 lung cancer patients, 8 patients had the L858R mutation. One was male and seven were female. Seven were nonsmokers and one was a smoker (Brinkman index was 600). All eight patients had adenocarcinoma, one was moderately differentiated, and

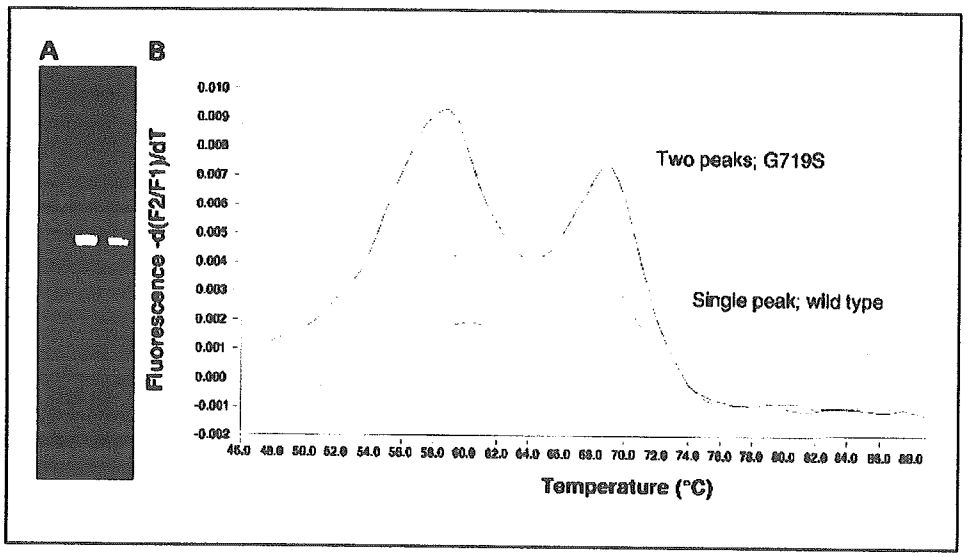

Fig. 1. *A*, analyzed data using PCR-RFLP. Left lane, the wild-type DNA within the 397 bp does not have *Sac*I site. The PCR products restricted with *Sac*I were loaded with 2% agarose gel and was visualized as one band. Right lane, the substitution mutation G719S caused *Sac*I site, and the PCR products restricted by *Sac*I was visualized as three bands. *B*, detection of a G719S mutation in the *EGFR* gene in genomic DNA extracted from lung cancer tissues. The negative derivative of the fluorescence ($-dF/dT$) versus temperature graph shows peaks with different T_m. The wild-type sample showed a single T_m at 69°C. The heterozygous mutant sample showed an additional peak at 59°C.

seven were well differentiated. Five of eight adenocarcinomas showed bronchioloalveolar carcinoma pattern at the edge of tumor. Thus, L858R mutation status was significantly correlated with gender, Brinkman index, pathologic subtypes, and differentiation of lung cancer (Table 1). Eight of eight PCR products from matched peripheral lymphocyte DNA showed a single peak, suggesting that the mutations were somatic. L858R mutation was also found in one nonsmoking female adenocarcinoma patient from Kinki-chuo Chest Medical Center.

For exon 19 genotyping, the anchor probe was matched for deletion type 1a (2,235-2,249 nucleotides deletion; deletion GGAATTAAGAGAAGC) mutation. As shown in Fig. 3, for the deletion 1a mutation in exon 19, the PCR product showed a single peak at 56°C, whereas the deletion 1b products (2,236-2,250 nucleotides deletion; deletion GAATTAAGAGAAGCA) showed a peak at 47°C. From the 102 lung cancer patients, seven patients had the deletion 1a mutation. Four were males and three were females. Three were nonsmokers and four were smokers. Four patients had adenocarcinoma, two had squamous cell carcinoma, and one had adenosquamous cell carcinoma. One of the tumors was moderately differentiated, two were poorly differentiated, and three were well differentiated. One of four adenocarcinomas showed bronchioloalveolar carcinoma pattern at the edge of tumor. Thus, deletion 1a mutation status was not significantly correlated with gender, Brinkman index, pathologic subtypes, and differentiation of lung cancer (Table 2). Five of seven PCR products from matched peripheral lymphocyte DNA were available and showed a single peak, suggesting that these mutations were somatic.

The mutations detected in lung cancer specimens from Kinki-chuo Chest Medical Center are summarized in Table 3. L858R mutation and deletion type 1a were found from partial response patients. On the other hand, G719S mutation was found from a patient with no response to gefitinib (progressive disease). A total of six mutations were found from 16 gefitinib-treated patients (37.5%). Taken together, 22 mutations were found from 117 examined samples in our analysis (18.8%).

The overall survival of 102 lung cancer patients from Nagoya City University, with follow-up through December 30, 2003, was studied in reference to the *EGFR* mutation status. There was no significant difference in the prognosis between the patients with wild-type *EGFR* (n = 86, 22 were dead) and the patients with

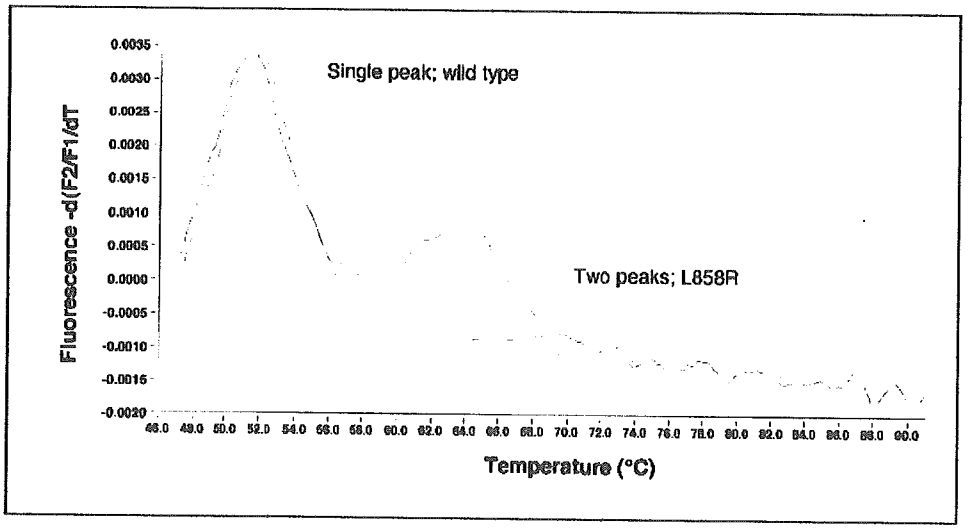

Fig. 2. The L858R mutation in exon 21 of the homozygous wild-type PCR product showed a single peak at 53°C, whereas the heterozygous products (mutant) showed an additional peak at 65°C.

Table 1. Clinicopathologic data of 102 lung cancer patients

Factors	L858R Mutation patients (%)	L858R Wild-type patients (%)	*P*
Mean age (y), 65.5 ± 9.3	8	94	
Stage			
I	7 (87.5)	45 (47.9)	0.0744
II-IV	1 (12.5)	49 (52.1)	
Lymph node metastasis			
N0	7 (87.5)	60 (63.8)	0.3341
N+	1 (12.5)	34 (36.2)	
BI			
≦600	8 (100)	32 (34.0)	0.001
>600	0 (0)	62 (66.0)	
Differentiation			
Well	7 (87.5)	31 (43.1)	0.0439
Moderately or poorly	1 (12.5)	41 (56.9)	
Pathologic subtypes			
Adenocarcinoma	8 (100)	41 (43.6)	0.007
Nonadenocarcinoma	0 (0)	53 (56.4)	
Age			
≦60	2 (25.0)	26 (27.7)	0.9999
>60	6 (75.0)	68 (72.3)	
Gender			
Male	1 (12.5)	80 (85.1)	<0.0001
Female	7 (87.5)	14 (14.9)	

Abbreviations: N+, lymph node metastasis positive; BI, Brinkman index.

mutation in the *EGFR* gene ($n = 16$, two were dead; log-rank test, $P = 0.3608$; Breslow-Gehan-Wilcoxon test, $P = 0.4761$), although the observation period was short.

Discussion

We obtained findings that L858R *EGFR* mutation status was significantly correlated with gender, smoking history, and pathologic subtypes of lung cancers. This was in agreement with the recent reports that *EGFR* gene mutations are common in lung cancers from never smokers (13) and females with adenocarcinoma (11). Our analysis also suggested that the type of *EGFR* mutation might be correlated with the sensitivity of gefitinib therapy for lung cancers.

When the PCR is used for the detection of mutations in very small amounts of DNA, although we would like to start from biopsy samples in the future, it is usually necessary to use "nested PCR." In this case, a DNA fragment is amplified with a first set of primers and part of the product is reamplified with a second set of primers complementary to sequences in the product. Recent developments in fast PCR and real-time detection of products make a more sensitive approach to detection of mutations possible (14–16, 19). We have optimized mutation detection, without nested PCR, using the LightCycler. This instrument measures fluorescence during PCR and can detect the SYBR Green dye when it is intercalated in double-stranded DNA, allowing the detection of double-stranded PCR product formation. The use of labeled probes homologous to the PCR product permits specific identification of PCR products (17). In the LightCycler, two adjacent probes were used, labeled with different fluorescent molecules. When the probes were bound to the single-stranded target, one to five bases apart, the 3′-end label of the 5′ probes came close to the 5′-end label of the 3′ probe, resulting in resonance and strong fluorescence at a specific wavelength. An advantage of this strategy is that hybridization of the probe is not restricted to the temperature range required for Taq polymerase to remove a base (19, 20). Further melting curves can be produced after PCR to assess the dissociation temperature of the probe. Mutations covered by the probe can be detected by a shift in melting temperature. The one-cycle analysis took ~1 hour and could examine 32 samples.

Because so many *EGFR* mutation phenotypes were discovered, it would be of interest to determine whether resistance to *EGFR* inhibition emerges through secondary mutation as is the case in imatinib-treated chronic myelogenous leukemia (21). Our data showed that L858R mutation and deletion type 1a were found in gefitinib-sensitive patients; on the other hand, a G719S mutation was found in a gefitinib-resistant patient. Interestingly, recent data reported that L858R mutant (transfected cell) was inhibited at 10-fold lower concentrations of tyrosine kinase inhibitor; however, the deletion mutant seemed to have similar sensitivities as wild-type *EGFR*

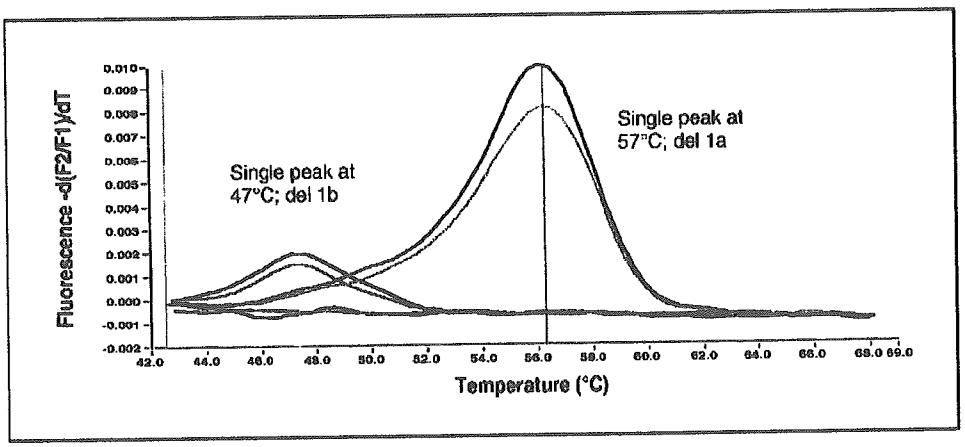

Fig. 3. Detection of the deletion mutations in the *EGFR* gene in genomic DNA extracted from lung cancer. The deletion 1a – type sample showed a single T_m at 56°C. The deletion type 1b sample showed a single peak at 47°C.

Table 2. Clinicopathologic data of 102 lung cancer patients

Factors	Exon 19 deletion: Mutation patients (%)	Exon 19 deletion: Wild-type patients (%)	*P*
Mean age (y), 65.5 ± 9.3	7	95	
Stage			
I	3 (42.9)	49 (51.6)	0.9571
II-IV	4 (57.1)	46 (48.4)	
Lymph node metastasis			
N0	3 (42.9)	64 (67.4)	0.3650
N+	4 (57.1)	31 (32.6)	
BI			
≦600	5 (71.4)	35 (36.8)	0.1592
>600	2 (28.6)	60 (63.2)	
Differentiation			
Well	3 (50.0)	35 (47.3)	0.9999
Moderately or poorly	3 (50.0)	39 (52.7)	
Pathologic subtypes			
Adenocarcinoma	4 (57.1)	45 (47.4)	0.9143
Nonadenocarcinoma	3 (42.9)	50 (52.6)	
Age			
≦60	2 (28.6)	26 (27.4)	0.9999
>60	5 (71.4)	69 (72.6)	
Gender			
Male	4 (57.1)	77 (81.1)	0.3051
Female	3 (42.9)	18 (18.9)	

to drug (13). Thus, mutation phenotypes might be correlated with sensitivity for gefitinib therapy. Substitution mutation L858R is located adjacent to the highly conserved DFG motif in the activation motif. The activation loop was known to be important for autoregulation in many kinases (22). For example, the mutation in the activation loop of insulin receptor tyrosine kinase substantially increases the ability of the unphosphorylated kinase to bind ATP (23). From our data, this mutation pattern (L858R) might be more correlated with the populations, such as women, smoking, and adenocarcinoma.

DNA sequencing using the PCR methods described to date is time-consuming and, therefore, may not be suitable for a regular pretherapeutic screening program. Genechip technology is promising but still in its infancy, and adapting this technology to new polymorphisms is time-consuming and expensive. Real-time PCR, on the other hand, allows for easy adoption of new polymorphisms and possibly provides the best means for pretherapeutic genotyping in a clinical setting at present. We, therefore, developed three different PCRs to detect *EGFR* gene mutations and deletions. The advantages of real-time PCR are extensive. The faster PCR method and elimination of additional steps to analyze PCR products save time and minimize the risks of DNA contamination. Handling is facilitated and potentially toxic reagents, such as ethidium bromide stain, are avoided. We have only found 16 of 101 surgically removed samples from Nagoya City University and 6 of 16 gefitinib-treated samples from Kinki-chuo Chest Medical Center. Other mutations might have existed for these patients, although we have only checked the three most frequent mutations. The difference in the ratio of *EGFR* mutation between Nagoya and Kinki patients might have been caused by selection bias because gefitinib was known to be sensitive for female, nonsmoker, and adenocarcinoma patients. Actually, we have checked seven small cell carcinoma and three large cell carcinoma patients from Nagoya and no mutations were found from these patients.

Using the LightCycler reverse transcription-PCR assay described here, the determination of *EGFR* mutation status may be of clinical importance in predicting the sensitivity or resistance to gefitinib therapy for lung cancer. With this method, 32 samples were genotyped within 1 hour without the need of any post-PCR sample manipulation. Mutation detection using real-time PCR with hybridization probes and

Table 3. Genotyping analyses data for the non – small cell lung cancer patients from Kinki-chuo Chest Medical Center

Age	Gender	Mutation	Exon	Mutation type	Pathology	Smoking history
59	F	+	19	del 1a	Adenocarcinoma	N
69	F	+	18	G719S	Adenocarcinoma	N
76	M	+	19	del 1b	Adenocarcinoma	N
56	M	+	19	del 1b	Adenocarcinoma	F/C
33	M	+	19	del 1b	Adenocarcinoma	F/C
59	F	+	21	L858R	Adenocarcinoma	N
47	M	–			Adenocarcinoma	F/C
65	F	–			Adenocarcinoma	N
51	F	–			Adenocarcinoma	N
66	M	–			Adenocarcinoma	F/C
82	M	–			Adenocarcinoma	F/C
71	F	–			BAC	N
66	F	–			BAC	N
71	F	–			Adenocarcinoma	N

Abbreviations: F, female; M, male; del, deletion; BAC, bronchioloalveolar carcinoma; N, never smoker; F/C, former or current smoker.

melting curve analysis can be used for the sensitive detection of DNA mutations. The fast detection of single base substitutions in small amounts of DNA has great potential in pretreated diagnosis and in oncology.

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