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EGFR exon 20 insertion mutation in Japanese lung cancer

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Summary Mutations of the epidermal growth factor receptor (*EGFR*) gene have been reported in non-small cell lung cancer (NSCLC), especially in female, never smoker patients with adenocarcinoma. Some common somatic mutations in *EGFR*, including deletion mutations in exon 19 and leucine to arginine substitution at amino acid position 858 (L858R) in exon 21, have been examined for their ability to predict sensitivity to gefitinib or erlotinib. On the other hand, previous report has shown that the insertion mutation at exon 20 is related to gefitinib resistance. We investigated the exon 20 *EGFR* mutation statuses in 322 surgically treated non-small cell lung cancer cases. Two hundred and five adenocarcinoma cases were included. The presence or absence of *EGFR* mutations of kinase domains was analyzed by direct sequences. *EGFR* insertion mutations at exon 20 were found from 7 of 322 (2.17%) lung cancer patients. We also detected the 18 deletion type mutations in exon 19, and 25 L858R type mutations in exon 21. There was a tendency towards higher exon 20 insertion ratio in never smoker (never smoker 4.4% versus smoker 1.3%, $p=0.0996$) and female (female 4.5% versus male 1.3%, $p=0.0917$). Two exon 20 insertion cases were treated with gefitinib and failed to response.

EGFR insertion mutation in exon 20 could not be ignored from Japanese lung cancers.

1. Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy [1]. There are much accumulated evidences that epidermal growth fac-

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tor receptor (*EGFR*) and its family members are strongly implicated in the development and progression of numerous human tumors, including lung cancer [2,3]. The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) since 2002. Original two reports showed that *EGFR* mutations statuses at ATP binding pockets in NSCLC patients were correlated with the clinico-pathological features related to good response to gefitinib [4,5]. These *EGFR* mutations are predominantly found in Japanese lung cancer patients (about 25–40%) [4,6–9] when compared to USA patients (about 8–10%) [4,5,7,10] or European patients [7,11]. Actually, *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy *in vivo* and *in vitro* [4,5,10]. Although many *EGFR* mutations have been reported, not all have been associated with responsiveness to gefitinib. The two most common *EGFR* mutations that have been identified, representing 85–90% of *EGFR* mutations, are the *EGFR* exon 19 deletion that eliminates a leucine–arginine–glutamate–alanine motif in the tyrosine kinase domain of *EGFR* and a thymine to guanine transversion that results in an arginine for leucine substitution at amino acid 858 (L858R). These two mutants responded significantly better for gefitinib therapy than other types of mutants [12,13]. However, Greulich et al. showed transformation by an exon 20 insertion, made cells resistant to gefitinib or erlotinib [14]. To determine the *EGFR* mutation status and correlation with clinico-pathological features in Japanese lung carcinoma, we investigated exon 20 insertion mutation status by direct sequences. The findings were compared to the clinico-pathological features of lung cancer.

2. Material and methods

2.1. Patients

The study group included 295 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1994 and 2005. We have also investigated *EGFR* mutation status for 27 lung cancer patients who had undergone surgery followed by treated with gefitinib at the National Hospital Organization, Kinki-chuo Chest Medical Center. Gefitinib was used after lung cancer recurrence, and clinical outcome was shown in reference [9]. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan [15]. All tumor samples were immediately frozen and stored at −80 °C until assayed. Written informed consent was obtained from the patients, and the institutional ethics committee of the Nagoya City University Medical School approved the study.

2.2. PCR assays for *EGFR* mutations

Genomic DNA was extracted using Wizard SV Genomic DNA Purification Systems (Promega) according to the manufacturers' instructions. The primers for exon 20 sequencing were designed with Primer Express 2.0 software (Applied Biosystems). The sequences of the primer sets used in the assay are: forward ACTTCACAGCCCTGCGTAAAC, and reverse: ATGGGACAGGCACTGATTTGT. The sequence results of exon 20 about 131 of 322 cases were already reported [4,16]. The cycling conditions were as follows: initial denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 30 s, 64 °C for 30 s, 72 °C for 60 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review form forward and reverse, both side.

2.3. Statistical analysis

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 means of 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. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA), and was considered significant when the p-value was less than 0.05.

3. Results

3.1. *EGFR* gene mutation status in Japanese lung cancer patients

The clinical and pathological characteristics of the 322 lung cancer patients are as follows: 234 (72.7%) were males and 88 were females. Two hundred and five (63.7%) were diagnosed as adenocarcinoma, and 117 were diagnosed as other types of carcinoma. Two hundred and thirty-one (71.7%) were smokers and 90 were non-smokers (one unknown). Of 295 lung cancer patients from Nagoya City University, 167 (56.6%) were stage I.

Most of the sequencing results about exon 18, 19 and 21 were already reported [4,16,17]. In exon 19, 18 patients had the deletion type mutation. In exon 18 or exon 21, 29 patients had the missense point mutations (2 G719S, 1 G719C, 25 L858R and 1 L861Q). Of these 47 patients, 17 were males and 30 were females. Thirty were non-smokers and 17 were smokers. Forty-three patients had adenocarcinoma, one had squamous cell carcinoma and three had adenosquamous cell carcinoma. Thus *EGFR* mutation status at exon 18, 19 or 21 was significantly correlated with gender ($p < 0.0001$), tobacco-smoking ($p < 0.0001$) and pathological subtypes (adenocarcinoma versus non-adenocarcinoma, $p < 0.0001$).

For exon 20, 7 patients had the insertion mutations (Table 1). These mutations were exclusively associated with other *EGFR* mutation. Three were males and four were females. Four were non-smokers and three were smokers. Six patients had adenocarcinoma and one had squamous

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Table 1 Clinico-pathological features of 322 lung cancer patients

Factors	*EGFR* exon 20 mutations		
	Mutation patients	Wild type patients	*p*-Value
Mean age (65.5±9.3; years)	7	315	
Age			
≦60	1 (1.1%)	94 (98.9%)	0.6783
>60	6 (2.6%)	221 (97.4%)	
Gender			
Male	3 (1.3%)	231 (98.7%)	0.0917
Female	4 (4.5%)	84 (95.5%)	
Pathological subtypes			
Adeno	6 (3.0%)	197 (97.0%)	0.2666
Non-adeno	1 (0.8%)	118 (99.2%)	
Differentiation			
Well	4 (3.5%)	111 (96.5%)	0.4236
Moderately or poorly	2 (1.5%)	128 (98.5%)	
Lymph node metastasis			
N0	4 (1.9%)	205 (98.1%)	>0.9999
N+	2 (2.3%)	84 (97.7%)	
Smoking status			
Smoker	3 (1.3%)	228 (98.7%)	0.0996
Non-smoker	4 (4.5%)	86 (95.5%)	
Pathological stages			
I	4 (2.4%)	164 (97.6%)	0.7025
II–IV	2 (1.6%)	125 (98.4%)	

Adeno, adenocarcinoma; N+, lymph node metastasis positive.

cell carcinoma. Two were moderately differentiated, and four were well differentiated (one unclassified). There was a tendency towards higher exon 20 insertion mutation ratio in never smoker (never smoker 4.4% versus smoker 1.3%, $p=0.0996$) and female (female 4.5% versus male 1.3%, $p=0.0917$). Two female patients had 774_776 insertion NPH (2320-2328 insertion AACCCCCAC) mutations reported as D7 mutation by Shigematsu et al. (Fig. 1) [7]. A female patient had 770_772 insertion ASV (2308-2316 insertion GCCAGCGTG) mutation reported as D1 mutation by Shigematsu et al. [7]. A male patient had 771_773 insertion SVD (2311-19 insertion GCGTGGACA) mutation reported by Sonobe et al. [18]. A male patient had 772_773 insertion V (2312-14 insertion GGT) reported by Thomas et al. [19]. Two patients had 772_773insertion N (2312-14 insertion AAC) mutations (Fig. 1).

3.2. Relationship between clinical course of patients with lung cancer and *EGFR* mutations

The overall survival of 322 lung cancer patients with follow-up through December 30, 2006, was studied in reference to the *EGFR* mutation status. The prognosis from patients with exon 20 insertion mutation ($n=7$, 2 were dead) and the patient without exon 20 insertion mutation *EGFR* ($n=315$, 102 were dead) was not significantly different (Log-rank test, $p=0.7186$, Breslow–Gehan–Wilcoxon test, $p=0.8593$) (Fig. 2). Eighteen patients received adjuvant chemotherapy (five were with cisplatin base, seven were with calboplatin base and six were with Uracil-Ftegafur). Even if the 18 patients were excluded for survival analysis, the prognosis from patients with exon 20 mutation and without mutation was not significantly different ($p=0.7215$).

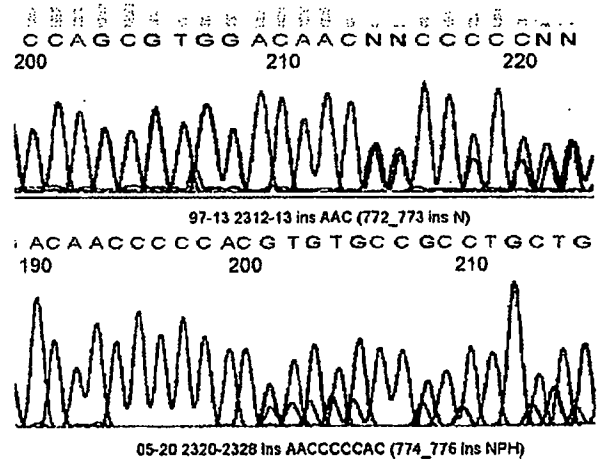

Fig. 1 Two patients had 774_776 insertion NPH (2320-2328 insertion AACCCCCAC) mutations reported as D7 mutation (upper). Two patients had 772_773insertion N (2312-14 insertion AAC) mutations (below).

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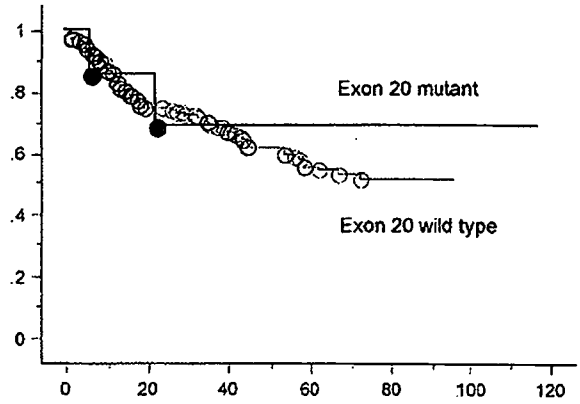

Fig. 2 The prognosis from patients with exon 20 insertion mutation ($n=7$, 2 were dead) and the patient without exon 20 insertion mutation *EGFR* ($n=315$, 92 were dead) was not significantly different (Log-rank test, $p=0.7186$, Breslow–Gehan–Wilcoxon test, $p=0.8593$).

3.3. Clinical course of two recurrent lung cancer patients treated with gefitinib

Case 1: 58-year-old adenocarcinoma woman with no history of smoking underwent surgery at Kinki-chuo Chest Medical Center. A molecular analysis revealed 772_773 insertion N (2312-14 insertion AAC) mutations at *EGFR* exon 20. Three years later, the recurrent lung cancer was treated with gemcitabine, vinorelbine and Uracil-Ftegafur in addition to radiotherapy. Because the treatment failed, gefitinib treatment was started at 2004. The patient died from progressive disease about 6 months after gefitinib administration. Case 2: 72-year-old adenocarcinoma man with no history of smoking underwent surgery at Nagoya City University Hospital. A molecular analysis revealed 772_773 insertion V (2312-14 insertion GGT) at *EGFR* exon 20 (Fig. 3), and wild type at *Kras* codon 12/13. Multiple lung metastasis were treated with Uracil-Ftegafur, however, the treatment failed. Gefitinib treatment was started at 2005. But the tumor size was increased (Fig. 3) and the treatment was quitted at 3 months.

4. Discussion

We obtained findings that exon 20 insertion type *EGFR* mutations tend to be higher in female gender and never smoker, as like as other *EGFR* mutation subtypes [8–14]. From the original three papers published by Lynch et al., Paez et al. and Pao et al., there was no *EGFR* exon 20 insertion subtypes. Shigematsu et al. reported that 12 of 617 (1.9%) had exon 20 insertion mutation, however, 356 of 617 patients were either from Japan or Taiwan [11]. Sonobe et al. reported that the 2 of 154 cases (1.3%) had *EGFR* exon 20 insertion mutations. These data suggested that *EGFR* mutations at exon 20 might be also higher in East Asian. More interestingly, patients with exon 20 mutation did not respond to gefitinib therapy.

Although many reports have identified more than 30 different mutations in the tyrosine kinase domains of *EGFR*, the vast majority of which can be grouped into three major types, including in-frame deletion at exon 19, single-nucleotide substitution at exon 18 or 21 and in-frame duplication at exon 20 [8–14]. To date, only the L858R missense mutation in exon 21 and deletions in exon 19 have been proven to be activating mutations [4,5,10,14]. On the

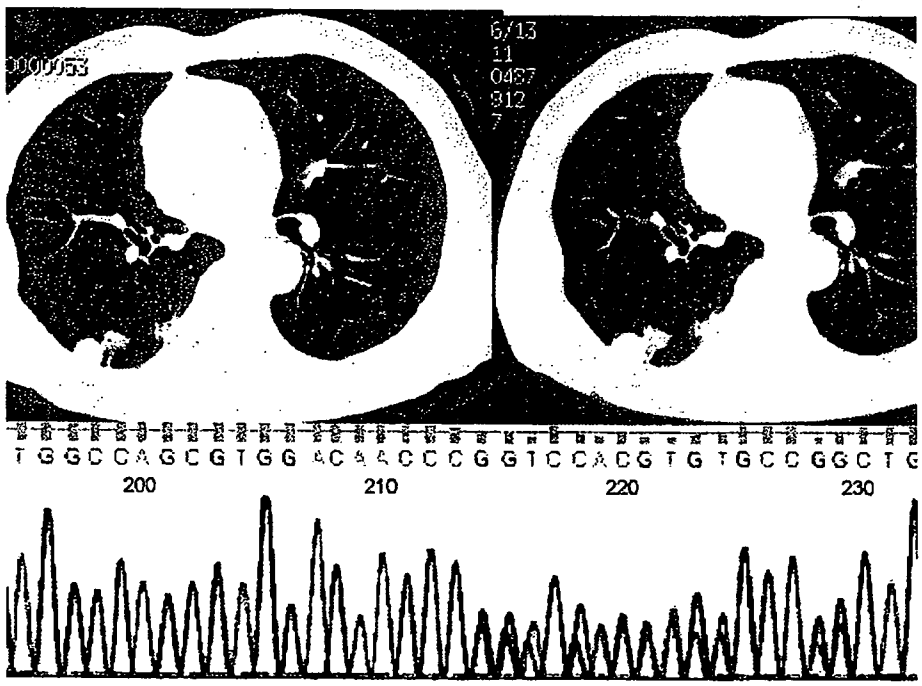

Fig. 3 CT examination before (left) and after (right) gefitinib therapy revealed increased tumor size. A molecular analysis revealed 772_773 insertion V (2312-14 insertion GGT) at *EGFR* exon 20 (below).

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other hands, Greulich et al. reported that transformation by the D770_N771 ins NPG (exon 20) *EGFR* insertion mutant was remarkably insensitive to gefitinib and erlotinib, as inhibition of colony growth in soft agar required exposure to 100-fold higher concentrations (>1 mM) of these agents than was required to inhibit colony formation by cells expressing the *EGFR* missense mutants or deletion mutant [14]. No significant inhibition of anchorage-independent growth of cells expressing D770_N771ins NPG *EGFR* was observed at 3 mM gefitinib or erlotinib [14]. Greulich et al. also reported that all three lung adenocarcinoma patients with known exon 20 insertion mutants of *EGFR* have failed to show a clinical response to treatment and have instead achieved only stable disease with erlotinib [14]. *In vitro* analysis, cells expressing the *EGFR* deletion and insertion mutants formed colonies in soft agar with a higher efficiency than that of cells expressing the missense mutants, comparable to the colony formation efficiency of cells expressing polyoma middle T antigen, suggested these mutants were oncogenic [14]. Interestingly, the irreversible *EGFR* inhibitor CL-387, 785 [20] is more effective than gefitinib or erlotinib for inhibition of colony formation by cells expressing the exon 20 insertion mutant [14]. CL-387, 785 had an even greater effect on colony formation by cells expressing L858R [14], and this compound was previously found to be active against *EGFR* containing the exon 20 point mutation T790M, associated with resistance to gefitinib and erlotinib [21]. Thus the distinct inhibitor sensitivity of various *EGFR* mutants argues that therapies may need to be targeted against specific mutant forms of a protein, whereas generalized inhibition of a particular oncogenic target may not be sufficient.

Conflict of interest

None declared.

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Full Paper

Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status

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The expression and activity of the epidermal growth factor receptor (EGFR) are determinants of radiosensitivity in several tumour types, including non-small cell lung cancer (NSCLC). However, little is known of whether genetic alterations of *EGFR* in NSCLC cells affect the therapeutic response to monoclonal antibodies (mAbs) to EGFR in combination with radiation. We examined the effects of nimotuzumab, a humanised mAb to EGFR, in combination with ionising radiation on human NSCLC cell lines of differing *EGFR* status. Flow cytometry revealed that H292 and Ma-1 cells expressed high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression. Immunoblot analysis revealed that EGFR phosphorylation was inhibited by nimotuzumab in H292 and Ma-1 cells but not in H460, H1299, or H1975 cells. Nimotuzumab augmented the cytotoxic effect of radiation in H292 and Ma-1 cells in a clonogenic assay *in vitro*, with a dose enhancement factor of 1.5 and 1.3, respectively. It also enhanced the antitumor effect of radiation on H292 and Ma-1 cell xenografts in nude mice, with an enhancement factor of 1.3 and 4.0, respectively. Nimotuzumab did not affect the radioresponse of H460 cells *in vitro* or *in vivo*. Nimotuzumab enhanced the antitumor efficacy of radiation in certain human NSCLC cell lines *in vitro* and *in vivo*. This effect may be related to the level of EGFR expression on the cell surface rather than to *EGFR* mutation.
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Keywords: epidermal growth factor receptor; non-small cell lung cancer; nimotuzumab; monoclonal antibody; genetic alteration; radiosensitisation

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally upregulated and activated in a variety of tumours (Baselga, 2002). Deregulation of receptor tyrosine kinases as a result of overexpression or activating mutations is frequently associated with human cancers and leads to the promotion of cell proliferation or migration, inhibition of cell death, or the induction of angiogenesis (Gschwind *et al*, 2004). The epidermal growth factor receptor has thus been identified as an important target in cancer therapy (Baselga and Arteaga, 2005). Several agents, including small-molecule inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKIs) and monoclonal antibodies (mAbs) specific for EGFR, have been designed to block EGFR signalling selectively (Ettinger, 2006; Harari and Huang, 2006; Imai and Takaoka, 2006). Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in non-small cell lung cancer (NSCLC), and sensitivity to these drugs has been associated with the presence of somatic mutations in the EGFR kinase domain or with *EGFR* amplification (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Various mAbs to EGFR are also undergoing preclinical and clinical trials of their efficacy as anticancer agents. However, biological markers able to predict the response to such antibodies have remained elusive.

The possibility of combining chemotherapy or radiation therapy with anti-EGFR mAb treatment has generated much interest, because the cellular targets for these agents and their mechanisms of action are different (Baumann and Krause, 2004). Studies have thus been undertaken to determine whether inhibition of EGFR signalling improves the response to chemotherapy or radiation therapy. Preclinical studies have shown that the anti-EGFR mAb cetuximab markedly increases the cytotoxic effect of chemotherapy or radiation therapy in various EGFR-expressing tumour cell lines (Huang *et al*, 1999; Milas *et al*, 2000; Buchsbaum *et al*, 2002; Prewett *et al*, 2002; Raben *et al*, 2005; Ettinger, 2006). A phase III clinical trial also showed that the combination of cetuximab with

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radiation therapy resulted in a significant improvement in local control and survival compared with radiation therapy alone, without an increase in radiation-induced side effects, in patients with locally advanced head and neck cancer (Bonner *et al*, 2006).

Nimotuzumab (also known as h-R3) is a humanised anti-EGFR mAb, which is currently undergoing clinical evaluation. In a preclinical study, nimotuzumab showed marked antiproliferative, proapoptotic, and antiangiogenic effects in tumours that overexpress EGFR (Crombet-Ramos *et al*, 2002). In early clinical trials, nimotuzumab has shown a longer half-life and a greater area under the curve (AUC) in comparison with other anti-EGFR antibodies (Crombet *et al*, 2003). A phase I/II trial showed that nimotuzumab was well tolerated and enhanced the curative potential of radiation in patients with advanced head and neck cancer (Crombet *et al*, 2004). Given that little is known of the antitumor action of nimotuzumab in NSCLC, we investigated the growth-inhibitory effects of this mAb alone and in combination with radiation in NSCLC cell lines with differing patterns of EGFR expression. We also examined whether genetic alterations of *EGFR* affect the antitumor action of combined treatment with nimotuzumab and radiation.

MATERIALS AND METHODS

Cell lines and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460), Ma-1, NCI-H1299 (H1299), and NCI-H1975 (H1975) were obtained as previously described (Okabe *et al*, 2007) and were maintained under a humidified atmosphere of 5% CO_2 in air at 37.0°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Nimotuzumab was provided by Daiichi Sankyo Co Ltd (Tokyo, Japan), and gefitinib was obtained from AstraZeneca (Macclesfield, UK).

Flow cytometric analysis of surface EGFR expression

Cells (1.0×10^6) were stained for 2 h at 4°C with an R-phycoerythrin-conjugated mAb to EGFR (BD Biosciences, San Jose, CA, USA) or an isotype-matched control mAb (BD Biosciences). The cells were washed three times before measurement of fluorescence with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously (Okabe *et al*, 2007). Primary antibodies to phosphorylated EGFR (pY1068) were obtained from Cell Signaling Technology (Beverly, MA, USA), and those to EGFR were from Zymed (South San Francisco, CA, USA). Horseradish peroxidase-conjugated goat secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK).

Clonogenic assay

Exponentially growing cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm^2 flasks containing 10 ml of medium supplemented with 1% fetal bovine serum in the absence or presence of 700 nM nimotuzumab. After incubation for 24 h, the cells were exposed to various doses of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.82 Gy min^{-1} and at room temperature. The cells were then washed with phosphate-buffered saline, cultured in drug-free medium for 10–14 days, fixed with methanol : acetic acid (10 : 1, v/v), and stained with crystal violet. Colonies containing > 50 cells were counted. The surviving fraction was calculated as (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for control cultures not exposed to nimotuzumab or radiation. The surviving fraction for combined treatment was corrected by that for nimotuzumab treatment alone. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.5 for vehicle-treated cells divided by that for nimotuzumab-treated cells (after correction for drug toxicity).

Antitumor activity of nimotuzumab with or without radiation *in vivo*

Animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University, and they met the requirements of the UKCCCR guidelines (Workman *et al*, 1998). Tumour cells (2×10^6) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. tumour volume was determined from caliper measurement of tumour length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Treatment groups consisted of control, nimotuzumab alone, radiation alone, and the combination of nimotuzumab and radiation, with each group containing seven or eight mice. Nimotuzumab was administered intraperitoneally in a single dose of 1.0 mg per mouse; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the right hind leg of mice were exposed to 10 Gy of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.32 Gy min^{-1} beginning 6 h after drug treatment. Growth delay (GD) was calculated as the time required for treated tumours to achieve a fivefold increase in volume minus the corresponding time required for control tumours. The enhancement factor was then determined as $(GD_{combination} - GD_{nimotuzumab})/(GD_{radiation})$.

RESULTS

Surface EGFR expression in NSCLC cell lines of differing *EGFR* status

We first examined the surface expression of EGFR in five NSCLC cell lines by flow cytometry. The *EGFR* status for the cell lines was determined in our previous study (Okabe *et al*, 2007). Three cell lines (H460, H292, and H1299) possess wild-type *EGFR* alleles, whereas the other two cell lines (Ma-1 and H1975) harbour *EGFR* mutations (Table 1). Ma-1 cells have an in-frame deletion in

Table 1 Characteristics of NSCLC cell lines

Cell line	EGFR surface expression	EGFR status
H460	Low	Wild type
H292	High	Wild type
H1299	Low	Wild type
Ma-1	Moderate	del(E746–A750)
H1975	Low	L858R/T790M

EGFR = epidermal growth factor receptor; NSCLC = non-small cell lung cancer

exon 19 (E746–A750). H1975 cells harbour the I.858R mutation in exon 21 and a secondary mutation in exon 20 (T790M). Activating mutations in exons 19 and 21 are associated with sensitivity to EGFR-TKIs (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005), whereas the T790M mutation contributes to the development of resistance to these drugs (Kobayashi *et al*, 2005; Pao *et al*, 2005). Our flow cytometric analysis demonstrated that H292 and Ma-1 cells express high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression (Figure 1).

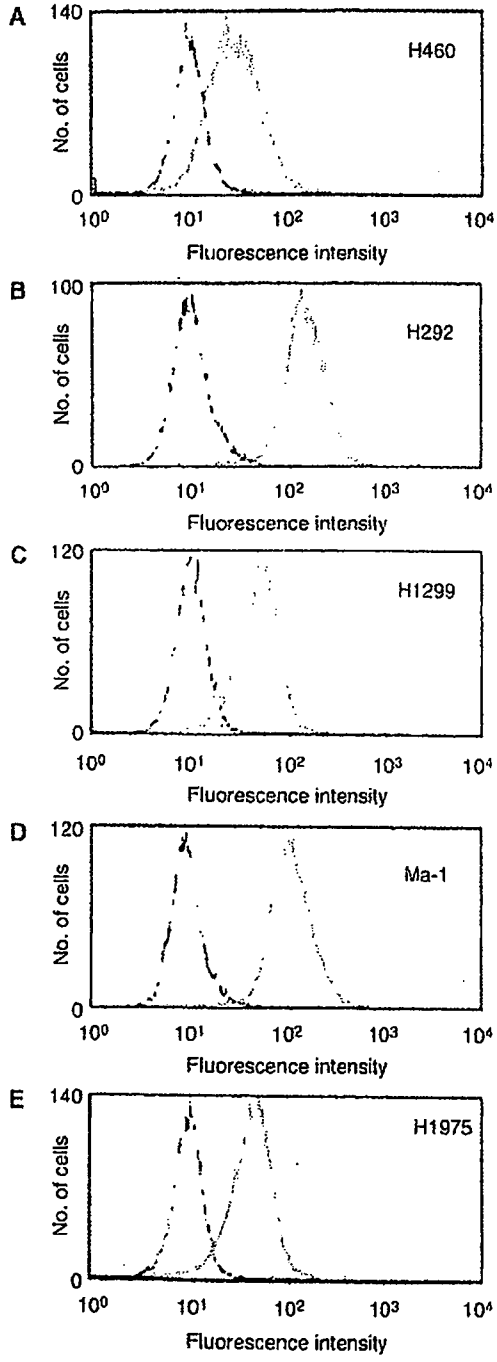

Figure 1 Expression of EGFR on the surface of NSCLC cells. Surface expression of EGFR on H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells was determined by flow cytometry. Representative histograms of cells stained with an anti-EGFR mAb (red peak) or with an isotype-matched control mAb (black peak) are shown.

Effect of nimotuzumab on EGFR phosphorylation

Next, we determined whether nimotuzumab inhibits ligand-induced EGFR phosphorylation in the five NSCLC cell lines. The cells were deprived of serum overnight, exposed to various concentrations of nimotuzumab, or to gefitinib, for 15 min, and then stimulated with EGF for 15 min. In the NSCLC cells that harbour wild-type *EGFR* (H460, H292, and H1299), phosphorylation of EGFR was undetectable in the absence of EGF, but was markedly induced on exposure of the cells to this growth factor. The EGF-induced phosphorylation of EGFR in these cells was completely inhibited by the EGFR-TKI gefitinib. Nimotuzumab also inhibited the EGF-induced EGFR phosphorylation in a concentration-dependent manner in H292 cells (which have a high level of surface EGFR expression), whereas it did not substantially affect such phosphorylation in H460 or H1299 cells (both of which have a low level of surface EGFR expression) (Figure 2A–C). We previously showed that the basal level of EGFR phosphorylation was increased in the *EGFR* mutant NSCLC cell lines Ma-1 and H1975, indicative of constitutive activation of the EGFR tyrosine kinase (Okabe *et al*, 2007). The phosphorylation of EGFR in EGF-treated Ma-1 cells (which have a moderate level of surface EGFR expression) was inhibited by gefitinib as well as by nimotuzumab in a concentration-dependent manner (Figure 2D). In contrast, the constitutive activation of EGFR in H1975 cells (which have a low level of surface EGFR expression) was inhibited partially by gefitinib but was unaffected by nimotuzumab (Figure 2E). These results suggested that the inhibition of EGFR phosphorylation by nimotuzumab may be related to the surface expression level of EGFR rather than to the mutational status of *EGFR*.

Augmentation of the cytotoxic effect of radiation in NSCLC cells by nimotuzumab *in vitro*

We examined whether nimotuzumab might enhance the anticancer effect of γ-radiation in the five NSCLC cell lines with the use of a clonogenic assay. Tumour cells were incubated with or without nimotuzumab for 24 h, exposed to various doses of γ-radiation, and then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that, whereas nimotuzumab had no effect on the radiation sensitivity of H460, H1299, or H1975 cells, it enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, with a dose enhancement factor of 1.5 and 1.3, respectively (Figure 3). These results showed that nimotuzumab increased the radiosensitivity of the NSCLC cell lines with high or moderate levels of surface EGFR expression, consistent with the inhibitory effects of this antibody on EGFR signalling.

Augmentation of the antitumor effect of radiation in NSCLC cells by nimotuzumab *in vivo*

To determine whether the nimotuzumab-induced potentiation of the response of NSCLC cells to radiation observed *in vitro* might also be apparent *in vivo*, we injected three of the cell lines into nude mice to elicit the formation of solid tumours. The mice were then treated with nimotuzumab, radiation, or both modalities. In the H460 xenograft model, tumour growth was inhibited by radiation alone but not by nimotuzumab alone, and the effect of radiation was not promoted by nimotuzumab (Figure 4A). In contrast, radiation and nimotuzumab each inhibited the growth of tumours formed by H292 (Figure 4B) or Ma-1 (Figure 4C) cells during the first few weeks after treatment. Thereafter, the rate of

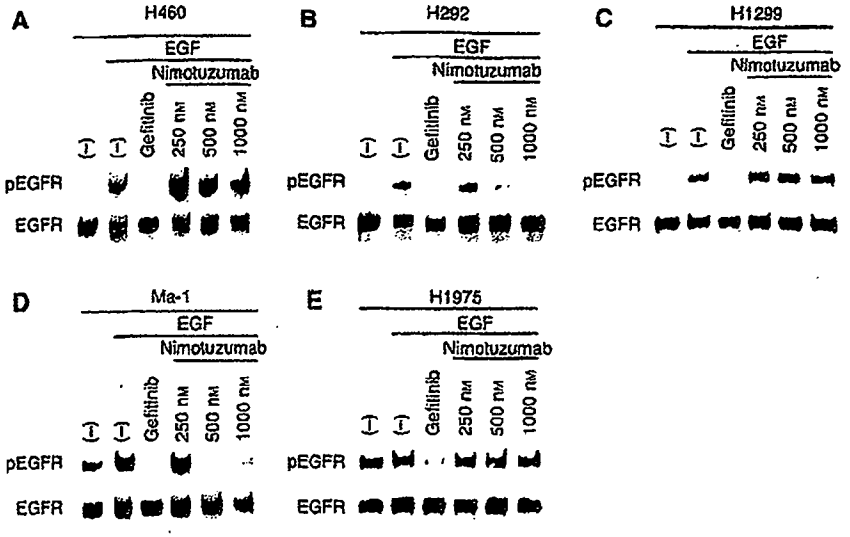

Figure 2 Effect of nimotuzumab on EGFR phosphorylation in NSCLC cells. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of the indicated concentrations of nimotuzumab or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng ml^{-1}). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr1068-phosphorylated form of EGFR (pEGFR) as well as with those to total EGFR.

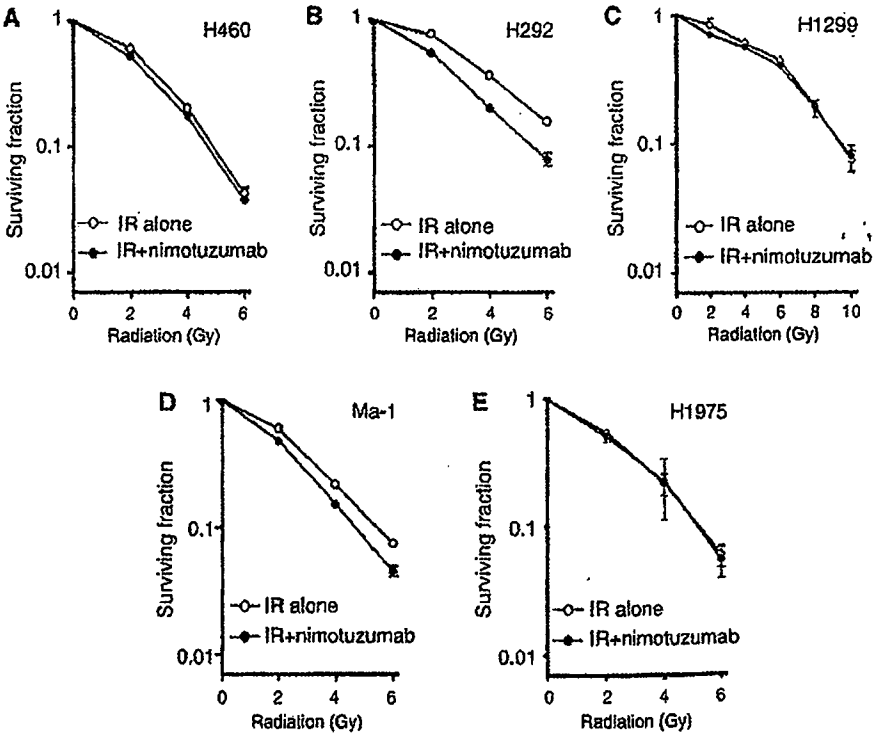

Figure 3 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vitro*. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were incubated with or without 700 nM nimotuzumab in medium supplemented with 1% fetal bovine serum for 24 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium supplemented with 10% serum for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and nimotuzumab by that apparent for treatment with nimotuzumab alone. Data are means ± s.d. of triplicates from a representative experiment.

tumour growth increased to a value similar to that seen in control animals. Combined treatment with radiation and nimotuzumab resulted in a substantial delay in tumour growth and subsequent inhibition of the growth rate of H292 and Ma-1 xenografts. The growth delay after treatment with nimotuzumab alone, radiation alone, or both nimotuzumab and radiation was thus 27.2, 19.6, and 53.6 days, respectively, for H292 cells and 26.7, 13.0, and 78.3 days, respectively, for Ma-1 cells (Table 2). The enhancement factor for the effect of nimotuzumab on the efficacy of radiation was 1.3 for H292 cells and 4.0 for Ma-1 cells, revealing the effect to be more

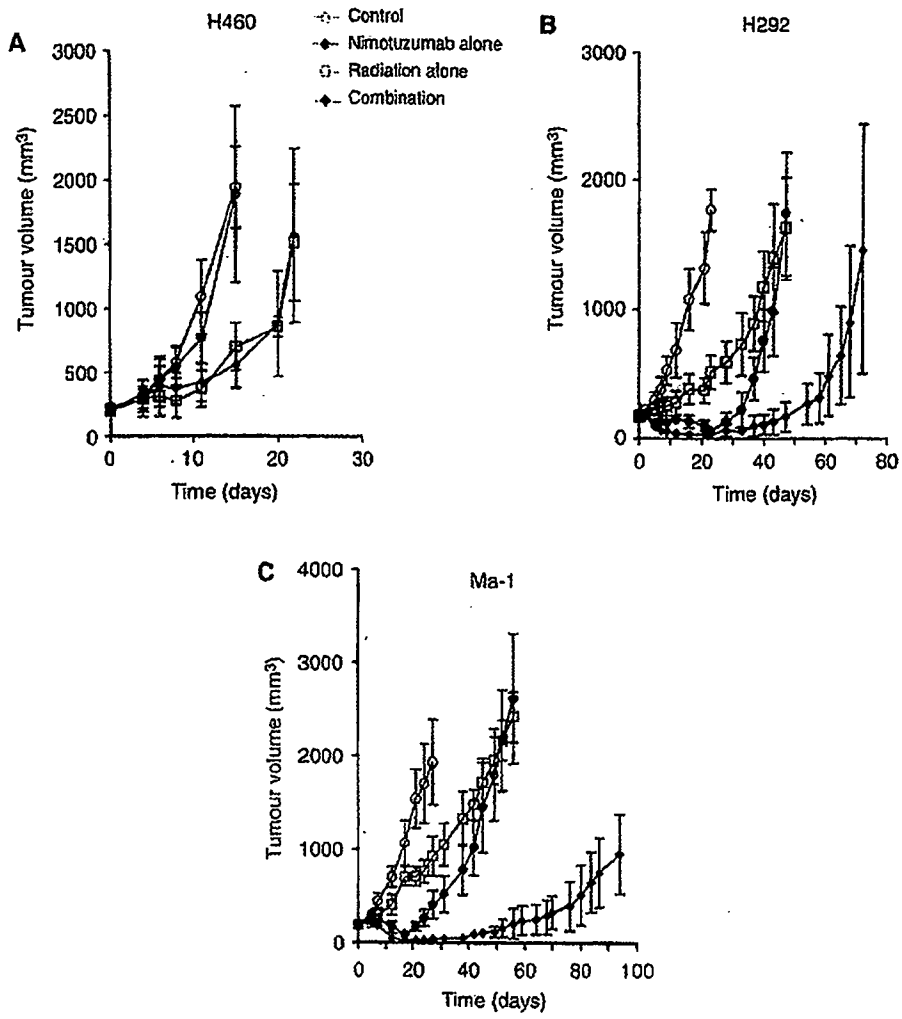

Figure 4 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (**A**), H292 (**B**), or Ma-1 (**C**) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of γ-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means ± s.d. for seven to eight mice per group.

Table 2 Tumour growth delay in nude mice treated with nimotuzumab, radiation, or both modalities

	H460		H292		Ma-1	
Treatment	**Days[a]**	**GD[b]**	**Days**	**GD**	**Days**	**GD**
Control	10.4		13.2		15.1	
Nimotuzumab alone	11.8	1.4	40.4	27.2	41.8	26.7
Radiation alone	20.4	10.0	32.8	19.6	28.1	13.0
Nimotuzumab+radiation	20.5	10.1	66.8	53.6	93.4	78.3
Enhancement factor	0.86		1.3		4.0	

GD = growth delay. [a]Time required for xenografts in each group to achieve a fivefold increase in volume. [b]The additional time (days) required for xenografts in each treatment group to achieve a fivefold increase in volume relative to the corresponding time for xenografts in the control group.

than additive. No pronounced tissue damage or toxicities such as diarrhoea or a decrease in body weight of > 10% were observed in mice in any of the four treatment groups. These results thus suggested that nimotuzumab potentiated the antitumor activity of radiation in H292 and Ma-1 cells *in vivo* as well as *in vitro*.

DISCUSSION

Somatic mutations in the EGFR kinase domain and *EGFR* amplification have been associated with a better response to EGFR-TKIs, such as gefitinib and erlotinib, in patients with NSCLC (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Given that little is known of the relation between such *EGFR* alterations and the response to treatment with anti-EGFR mAbs, we investigated the antitumor effect of combined treatment with the anti-EGFR mAb nimotuzumab and radiation in NSCLC cell lines of differing *EGFR* status.

The antitumor effect of EGFR-specific mAbs has been thought to result from inhibition of ligand binding to EGFR and consequent inhibition of EGFR activation (Li *et al*, 2005; Marshall, 2006). We, therefore, examined the effect of nimotuzumab on EGF-dependent EGFR signalling. Nimotuzumab inhibited the EGF-induced or constitutive phosphorylation of EGFR in H292 and Ma-1 cells (with high and moderate levels of surface EGFR expression, respectively), consistent with the mode of action of this antibody. However, nimotuzumab did not block EGF-induced or constitutive EGFR phosphorylation in H460, H1299, or H1975 cells (all with a

low level of surface EGFR expression). These observations suggest that the inhibitory effect of nimotuzumab on EGFR signalling depends on the expression level of EGFR on the cell surface. A clonogenic cell survival assay revealed that nimotuzumab enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, but not that in H460, H1299, or H1975 cells. These findings support the notion that the inhibition of EGFR signalling by nimotuzumab is responsible, at least in part, for the enhancement of the cytotoxic effect of radiation by this antibody. Irradiation of tumour cells has been shown to activate EGFR via ligand-independent and ligand-dependent mechanisms, possibly accounting for radiation-induced acceleration of tumour cell repopulation and the development of radioresistance (Schmidt-Ullrich *et al*, 1997, 2003; Dent *et al*, 2003). Such radiation-induced activation of EGFR-dependent processes may represent a rationale for combined treatment with radiation and EGFR inhibitors. It remains to be determined whether nimotuzumab is able to block radiation-induced activation of EGFR.

Consistent with our *in vitro* results, we found that nimotuzumab enhanced the antitumor effect of radiation on H292 or Ma-1 cells in nude mice. Such enhancement was not apparent for tumours formed by H460 cells. Nimotuzumab alone also manifested a substantial antitumor effect for xenografts formed by H292 or Ma-1 cells but not for those formed by H460 cells. Together these results suggest that the efficacy of nimotuzumab monotherapy is a prerequisite for augmentation of radioresponse by this mAb. Nimotuzumab was previously shown to induce the regression of A431 tumour xenografts *in vivo* as a result of inhibition of both tumour cell proliferation and tumour angiogenesis (Crombet-Ramos *et al*, 2002). Immunohistochemical analysis of tumour specimens from head and neck cancer patients treated with the combination of nimotuzumab and radiation also showed evidence of antiproliferative and antiangiogenic effects (Crombet *et al*, 2004). These observations suggest that effects of nimotuzumab on both NSCLC cell proliferation and tumour angiogenesis might contribute to the enhancement of the antitumor efficacy of radiation by this antibody observed in the present study. Enhancement of the anticancer effect of radiation by the anti-EGFR mAb cetuximab was previously shown to be increased by transfection of cells to upregulate the level of EGFR expression, suggesting that potentiation of the antitumor efficacy of radiation by anti-EGFR mAbs is related to the absolute level of EGFR expression (Liang *et al*, 2003; Bonner *et al*, 2004). This finding is consistent with our present results showing that potentiation of the antitumor activity of radiation by nimotuzumab was related to the level of surface EGFR expression. The nimotuzumab-resistant cell line H460 harbours a mutant form of *KRAS* (Balko *et al*, 2006) that has been associated with resistance to cetuximab (Lievre *et al*, 2006). However, we found that nimotuzumab also failed to inhibit EGF-induced EGFR phosphorylation and to enhance the cytotoxic effect of radiation in H1299 cells, which harbour wild-type *KRAS* (Coldren *et al*, 2006). These observations thus support the notion that a low level of EGFR expression at the cell surface is related to resistance to combined treatment with nimotuzumab and radiation, irrespective of *KRAS* status.

We demonstrated that nimotuzumab inhibited EGFR phosphorylation and enhanced the antitumor effect of radiation in *EGFR* mutant Ma-1 cells (with a moderate level of surface EGFR expression) but not in *EGFR*-mutant H1975 cells (with a low level of surface EGFR expression). Nimotuzumab also potentiated the cytotoxic effect of radiation in H292 cells, which harbour wild-type *EGFR* alleles and have a high level of surface EGFR expression. These findings support the notion that *EGFR* mutation is not the major determining factor for enhancement of the antitumor effect of radiation by nimotuzumab, consistent with previous observations with cetuximab (Barber *et al*, 2004; Tsuchihashi *et al*, 2005). However, the mechanisms underlying such enhancement of the antitumor effect of radiation may differ between NSCLC cells harbouring wild-type or mutant *EGFR* alleles. We and others have previously shown that mutations in the tyrosine kinase domain of EGFR are associated with increased ligand-independent tyrosine kinase activity of EGFR (Lynch *et al*, 2004) and aberrant EGFR signalling (Amann *et al*, 2005; Okabe *et al*, 2007). Given that cell-cycle checkpoints activated by ionising radiation are defective in *EGFR*-mutant NSCLC cell lines (Das *et al*, 2006), the constitutive activity of EGFR in such cells may result in unchecked DNA synthesis and in apoptosis on exposure to ionising radiation. It is possible that these defects in *EGFR*-mutant cells affect the enhancement of the antitumor efficacy of radiation by nimotuzumab.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro* and *in vivo*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy. Our data suggest that potentiation of the antitumor activity of radiation by nimotuzumab may be related to the level of EGFR expression at the cell surface rather than to *EGFR* mutation. The preselection of patients on the basis of genetic factors that predict treatment sensitivity or resistance may thus be required for the combination therapy with nimotuzumab and radiation.

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Original Article

Epidermal Growth Factor Receptor Gene Mutations in Early Pulmonary Adenocarcinomas

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Background: Epidermal growth factor receptor (*EGFR*) gene mutations are frequently found in pulmonary adenocarcinomas.

Materials and Methods: Various lung cancers (n=30) including 8 small adenocarcinomas were examined for *EGFR* gene mutations in three exons.

Results: Mutations were detected in 32% of adenocarcinomas. Exon 19 mutations were detected in 5 tumors, often advanced stages: 1 in Noguchi's pathologic type C, 2 in type D, and 2 in type F. Exon 21 mutations were detected in 3 tumors, all small adenocarcinomas in type C, at pathologic stage IA.

Conclusion: We suspect that exon 21 mutations are early events in small bronchioloalveolar carcinomas, while exon 19 mutations are later events occurring in adenocarcinomas of various types. (Ann Thorac Cardiovasc Surg 2007; 13: 87–92)

Key words: epidermal growth factor, lung cancer, adenocarcinoma, mutation, bronchioloalveolar carcinoma

Introduction

Lung cancer is the leading cause of cancer death worldwide[1]; despite much effort to conquer this disease, the overall survival rate is approximately 10%. Recently, molecular therapy targeting the epidermal growth factor receptor (EGFR) has become the second- or third-line treatment for selected patients with non-small-cell lung cancer (NSCLC).[2,3] Tyrosine kinase inhibitors such as gefitinib[4,5] and erlotinib,[6] were developed to inhibit signal transduction pathways mediated by the EGFR, thus selectively suppressing proliferation of lung cancer cells that carry activating mutations of the region encoding the cleft within the EGFR protein that binds adenosine triphosphate (ATP).[7,8] The mutations detected were single-base substitutions or small in-frame deletions occurring in the known "hot spot" for mutation, exons 18, 19 and 21. Importantly, these mutations were detected selectively in tumors responding to gefitinib or erlotinib,[7,8] which frequently were adenocarcinomas in women who never smoked. As these activating mutations are more frequent tumors in Asians than Westerners, the significantly higher response rates to gefitinib in Japanese reported in multi-institutional clinical trials[2] were attributed to high prevalence of *EGFR* gene mutations in this population.

While an association between gefitinib responsiveness and *EGFR* mutations has been demonstrated, when *EGFR* mutations occur during carcinogenesis in the lung still is unclear. Noguchi et al.[9] classified small peripheral adenocarcinomas into six types based on tumor growth patterns; types A and B represented in situ peripheral bronchioloalveolar carcinomas that did not involve lymph nodes[10] and required computed tomography (CT) for detection. Type C appears to be advanced slightly beyond types A and B, showing active fibroblastic proliferation. On the other hand, types D (poorly differentiated adenocarcinoma), E (tubular adenocarcinoma), and F (papillary adenocarcinoma with compressive and destructive

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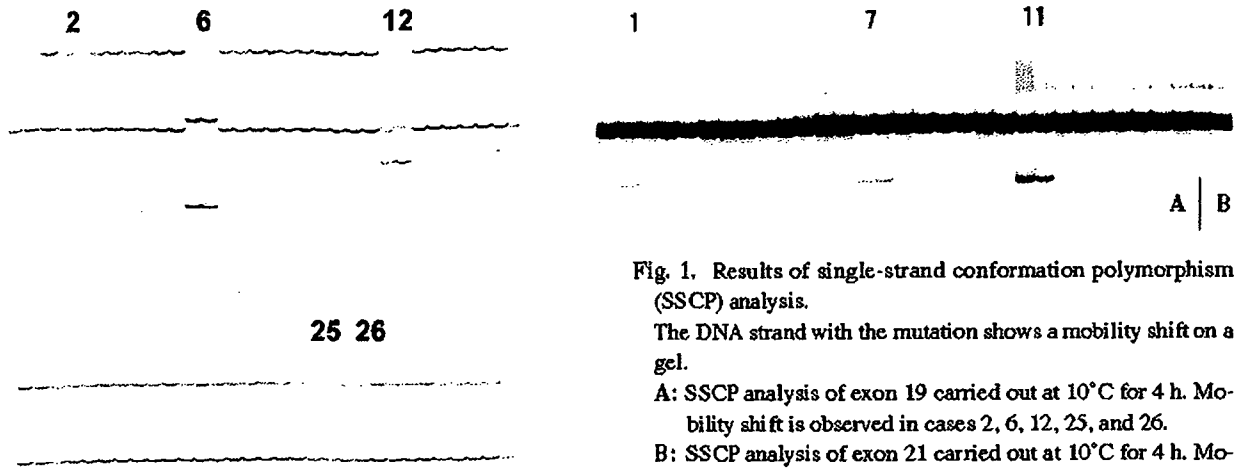

Fig. 1. Results of single-strand conformation polymorphism (SSCP) analysis.
The DNA strand with the mutation shows a mobility shift on a gel.
A: SSCP analysis of exon 19 carried out at 10°C for 4 h. Mobility shift is observed in cases 2, 6, 12, 25, and 26.
B: SSCP analysis of exon 21 carried out at 10°C for 4 h. Mobility shift is observed in cases 1, 7, and 11. All of these abnormal DNA strands were sequenced to identify the altered bases.

growth) are regarded as more aggressive cancers. This classification is widely considered to accurately depict the diverse array of pulmonary adenocarcinomas. We therefore sought to clarify when *EGFR* mutation occurred during development of small pulmonary adenocarcinomas, using the Noguchi's classification to estimate relative time points in tumor development.

Materials and Methods

Patient characteristics

Resected lung cancer tissues from 30 patients who underwent lobectomy and systematic lymph node dissection in Tokyo Medical University Hospital were studied with respect to *EGFR* gene mutations. Histologic types included 25 adenocarcinomas and 5 other carcinomas including 2 squamous cell carcinomas, 2 large cell carcinomas, and 1 small cell carcinoma. Noguchi's pathologic classification was applied to all adenocarcinomas, including some tumors larger than 20 mm. Adenocarcinomas included 2 in type A, 1 in type B, 7 in type C, 10 in type D, 2 in type E, and 3 in type F. Pathologic (p-) stages of the 30 carcinomas according to international staging criteria[11] were IA in 15, IB in 5, and IIA to IIIB in 10. The p-IA tumors included 8 small adenocarcinomas with a largest dimension below 20 mm. Written informed consent for genetic analysis of the resected tumor was obtained from all patients. In the operating room, immediately upon resection of a pulmonary lobe containing a primary lung cancer, about 500-mg sample was removed from the tumor, immersed in liquid nitrogen, and stored at −80°C until genetic study.

Detection of the *EGFR* gene mutation

Genomic DNA was extracted from the stored tumor using a REDExtract -N-Amp Tissue PCR kit (Sigma, St. Louis, MO). The three exons in the *EGFR* gene (exons 18, 19, and 21) reported to include frequent mutation sites were amplified by polymerase chain reaction (PCR). Primer sequences of 5′-AGGTGACCCTTGTCTCTGTGTTCT-3′ and 5′-CACCAGACCATGAGAGGCCCTGCG-3′ were used to amplify 216 base pairs in exon 18 by two-step PCR at an annealing temperature of 68°C; 5′-GATCACTGGGCAGCATGTGGCACC-3′ and 5′-TGGACCCCCACACAGCAAAGCAGA-3′ to amplify 199 base pairs in exon 19 by two-step PCR at annealing temperature of 68°C; and 5′-TTCCCATGATGATCTGTC-3′ and 5′-ATGCTGGCTGACCTAAA-3′ to amplify 232 base pairs in exon 21 by three-step PCR at an annealing temperature of 55°C. Amplified sequences within each exon initially were screened for mutations by single-strand conformation polymorphism (SSCP) analysis using 14% polyacrylamide gels. Samples were electrophoresed at 72 V/cm under two different conditions, 10°C for 4 h and 20°C for 2 h. Isolated DNA strands showing a mobility shift on gels were cut from gels, and these isolated DNA strands were sequenced using cycle sequencing kit (BigDye Terminator version 3.1, Applied Biosystems, Foster City, CA) in a DNA analyzer (Applied Biosystems 3730x).

Statistical analysis

Differences in distribution of EGFR mutations between two groups were tested by Fisher's exact probability test.

Table 1. Results of the EGFR mutation analysis

Case	Age	Gender	Smoking status (SI)	Pathologic type	Noguchi's classification	Largest dimension	Pathologic stage (mm)	v	ly	EGFR mutation analysis (exons 18, 19, and 21)	Changes in amino acids
1	71	F	Never smoked	W/D Ad	C	25	T1N0M0 IA	–	–	exon 21 T2573 → G	L858 → R
2	81	F	Never smoked	W/D Ad	F	25	T1N0M0 IA	–	–	exon 19 2235–2249 del (15 base)	E746–A750 del
3	77	M	Current smoker (1,000)	P/D Ad	D	31	T2M0N0 IB	2+	–	Normal	Normal
4	69	F	Current smoker (450)	W/D Ad	B	10	T1N0M0 IA	–	–	Normal	Normal
5	68	F	Never smoked	P/D Ad	D	25	T1N2M0 IIIA	1+	1+	Normal	Normal
6	71	M	Never smoked	P/D Ad	D	60	T2N1M0 IIB	2+	1+	exon 19 2239–2251 del (13 base), ins C	L747–T751 del, ins P
7	70	F	Never smoked	W/D Ad	C	15	T1N0M0 IA	–	1+	exon 21 T2573 → G	L858 → R
8	65	M	Never smoked	La	NA	70	T2M0N0 IB	2+	1+	Normal	Normal
9	65	M	Current smoker (1,140)	P/D Ad	D	15	T4N1M0 IIIB	2+	1+	Normal	Normal
10	55	M	Current smoker (2,960)	M/D Ad	F	25	T1N0M0 IA	2+	–	Normal	Normal
11	69	M	Current smoker (400)	W/D Ad	C	28	T1N0M0 IA	–	–	exon 21 T2573 → G	L858 → R
12	71	F	Never smoked	W/D Ad	F	20	T1N1M0 IIA	–	–	exon 19 2235–2249 del (15 base)	E746–A750 del
13	55	M	Current smoker (760)	P/D Ad	D	50	T2M0N0 IB	–	–	Normal	Normal
14	54	F	Never smoked	W/D Ad	A	10	T1N0M0 IA	–	–	Normal	Normal
15	72	F	Current smoker (1,060)	M/D Sq	NA	60	T2N1M0 IIB	–	–	Normal	Normal
16	76	M	Current smoker (340)	W/D Ad	E	60	T4N0M0 IIIB	–	–	Normal	Normal
17	74	M	Current smoker (1,650)	P/D Ad	D	20	T1N0M0 IA	1+	–	Normal	Normal
18	66	M	Current smoker (920)	M/D Sq	NA	37	T2M0N0 IB	–	–	Normal	Normal
19	71	F	Never smoked	La	NA	22	T4N0M0 IIIB	–	–	Normal	Normal
20	77	M	Current smoker (1,140)	Sm	NA	34	T2M0N0 IB	2+	–	Normal	Normal
21	64	M	Current smoker (150)	P/D Ad	D	15	T1N0M0 IA	–	–	Normal	Normal
22	43	M	Current smoker (500)	M/D Ad	E	35	T2N2M0 IIIA	1+	2+	Normal	Normal
23	57	M	Ex-smoker (150)	P/D Ad	D	45	T2N1M0 IIB	1+	1+	Normal	Normal
24	58	M	Current smoker (300)	P/D Ad	D	27	T1N0M0 IA	–	–	Normal	Normal
25	71	M	Current smoker (1,530)	P/D Ad	D	35	T2N2M0 IIIA	1+	2+	exon 19 G2203 → A	G735 → S
26	67	F	Never smoked	W/D Ad	C	28	T1N0M0 IA	–	–	exon 19 2239–2253 del (15 base)	L747–T751 del
27	78	M	Never smoked	W/D Ad	C	18	T1N0M0 IA	–	1+	Normal	Normal
28	76	M	Current smoker (560)	W/D Ad	C	15	T1N0M0 IA	–	1+	Normal	Normal
29	78	M	Ex-smoker (280)	W/D Ad	C	27	T1N0M0 IA	–	–	Normal	Normal
30	49	M	Current smoker (750)	W/D Ad	A	10	T1N0M0 IA	–	–	Normal	Normal

F, female; M, male; SI, smoking index (cigarettes/day × years); v, microscopic vascular invasion; ly, microscopic lymph vessel invasion; W/D, well-differentiated; M/D moderately differentiated; P/D poorly differentiated; Ad, adenocarcinoma; La, large cell carcinoma; Sm, small cell carcinoma; Sq, squamous cell carcinoma; NA, not applicable; G, guanine; C, cytosine; T, thymine; A, adenine; L, leucine; R, arginine; E, glutamic acid; A, alanine; T, threonine; P, proline; G, glycine; S, serine.

A p value less than 0.05 was considered to indicate significance.

Results

SSCP analysis detected shifts of amplified single-strand DNAs in electrophoretic gels in 8 samples (Fig. 1, A and B). DNA fragments showing abnormal mobility shifts on gels were cut and sequenced. Altered sequences were determined in all 8 samples. Patient characteristics and results of *EGFR* mutation screening are shown in Table 1. Mutations were detected only in adenocarcinomas.

Mutations in exon 19 were detected in 5 tumors including 1 in type C, 2 in type D, and 2 in type F according to Noguchi's classification. These include one point mutation resulting in replacement of G735 by S and four small deletions of 13 to 15 base pairs. The deletions caused omission of five amino acids (E746 to A750) in 2 tumors and omission of a slightly different sequence in 2 others (L747 to T751). One of the latter tumors also had insertion of cytosine at the deletion point, resulting in insertion of P where the others were omitted. P-stages included

Table 2. Association of EGFR mutations and clinicopathologic features

Factors	EGFR (exons 18, 19, 21)		p value*
	Mutation	No mutation	
Male	3	16	0.104
Female	5	6	
Smoker	2	17	0.028**
Non-smoker	6	5	
p-stage IA	5	10	0.682
p-stages IB–IIIB	3	12	

*, Fisher's exact probability test; **, significant difference.

stage IA in 2 tumors, stage IIA in 1, stage IIB in 1, and stage IIIA in 1.

Mutations in exon 21 were detected in three tumors, all in Noguchi type C and p-stage IA. All represented substitution of G for T at nucleotide 2573, resulting in an amino acid substitution (L858R). No mutations were detected in exon 18.

All *EGFR* mutations were detected only in adenocarcinomas, which showed a frequency of the *EGFR* mutations of 32% (8/25). Relationships between *EGFR* mutations and clinicopathologic features are shown in Table 2. Frequency of mutations did not differ between p-IA and the more advanced stages p-IB to IIIB (p=0.682). *EGFR* gene mutations were more frequent in patients who never smoked than in current or previous smokers (p=0.028). Although mutations were more frequent in women (50%) than in men (15%), this difference was not statistically significant (p=0.104).

Discussion

In this study we initially screened for mutations using PCR-SSCP, which enabled us to detect small amounts of abnormal tumor-derived DNA fragments among largest amounts of normal DNA derived from interstitial tissue. We successfully detected mutations within coding regions of the *EGFR* gene in 32% of unselected Japanese patients with adenocarcinoma. All gene mutations resulted in changes of amino acids. Lynch et al.[7] reported 10 tumors carrying five types of *EGFR* mutations causing amino acid alterations, 2 representing mutations that we also detected (E746–A750 del and L858R). Paez et al.[8] reported 22 tumors carrying four types of mutations, 3 being types that we detected. *EGFR* mutations detected in seven studies including our present one[7,8,12–15] are summarized in Table 3. In all studies exons 19 and 21 represented "hot spots" for mutations, which frequently were found in non smokers and in women.

Kosaka et al.[13] detected *EGFR* mutations more frequently in moderately and well differentiated adenocarcinomas than in poorly differentiated adenocarcinomas. This is of considerable interest as gene mutations occurring in less invasive cancers have been reported as relatively rare. Moreover, *EGFR* mutations are frequent in tumors affecting nonsmokers, while most altered genes in lung cancers such as *RAS*, p53, and *FHIT* were found more frequently in heavy smokers than in nonsmokers. According to the hypothesis of multistep carcinogenesis, gene mutations tend to accumulate in late-stage disease or highly malignant cancers, a generalization that seems not to apply to *EGFR* mutations.

Our present study disclosed *EGFR* mutations in early-stage adenocarcinomas. Noguchi's pathologic classification[9] represents an effort to depict the sequence of carcinogenesis for peripherally located adenocarcinomas. When chest CT is used to screen for lung cancer, most peripheral small shadows showing pure ground glass opacity prove to be atypical adenomatous hyperplasia or noninvasive bronchioloalveolar carcinoma, Noguchi types A and B. In our present study we found a point mutation in exon 21 in 3 Noguchi type C tumors, all representing p-IA disease. This suggests that exon 21 mutations in the *EGFR* gene may be relatively early occurences in the development of bronchioloalveolar carcinoma. In contrast, mutations in exon 19 were found in more advanced tumors such as Noguchi types D, E, and F. These results suggest the possibility that malignant grades of pulmonary adenocarcinoma may be related to mutation at different sites within the *EGFR* gene. Although a relationship between exons affected and disease stage or adenocarcinoma subtype was not mentioned in previous studies, Tokumo et al.[14] reported significantly higher prevalence of mutations in exon 19 in tumors from men than women. Minna et al.[16] also suggested different biologic activities of different affected exons, given that point mutations in exon 21 are heterozygous, including one normal allele, while the normal allele is severely underrepresented in tumors with small exon 19 deletions. These differences may be related to disease stages, histopathologic grade, and lineage of adenocarcinomas. We suspect that exon 21 is likely to be altered in the noninvasive Noguchi type A to C sequence (well differentiated bronchioloalveolar carcinoma), while exon 19 might be altered in more aggressive types such as D, E, and F.

Table 3. Reported mutations in the EGFR gene in seven studies

Exon	Type of mutation	Number	Amino acid changes	Number
18	Point mutations	10 (4.0%)	G719S	5[a] (2.0%)
			G719C	2[a] (0.8%)
			Others	3 (1.2%)
19	Small deletions	118 (47.2%)	del E746–A750	65 (26.0%)
			Other deletions and/or insertions	53 (21.2%)
	Insertions or duplications	5 (2.0%)		
	Point mutations	1 (0.4%)		
20	Point mutations	2 (0.8%)	S768I	
	Insertions or duplications	2 (0.8%)		
21	Point mutations	112 (44.8%)	L858R	110[b] (44.0%)
			Other point mutations	2 (0.8%)

Studies summarized include our present results and those in references.[7,8,12-15]
G, glycine; S, serine; C, cysteine; E, glutamic acid; A, alanine; I, isoleucine; L, leucine; R, arginine; [a], A point mutation in another exon was also present in 1 tumor. [b], A point mutation in another exon was also present in 8 tumors.

Our previous study[17] revealed that lung cancer cells can be effectively detected in cytologic specimens using fluorescence in situ hybridization (FISH) techniques. If EGFR mutations might be closely associated with chromosomal aberrations around the *EGFR* gene locus, tumors carrying *EGFR* mutations could be detected by FISH more easily. This point should be further examined.

In conclusion, *EGFR* mutations were detected in early pulmonary adenocarcinomas. We believe that *EGFR* mutations in exon 21 are relatively early events during development of pulmonary adenocarcinomas, especially small bronchioloalveolar carcinomas (Noguchi type A to C). In contrast, mutations in exon 19 occur in various types of adenocarcinoma, often at later stages. These results of our small series should be examined further in larger numbers of patients.

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Epidermal Growth Factor Receptor Mutation Detection Using High-Resolution Melting Analysis Predicts Outcomes in Patients with Advanced Non–Small Cell Lung Cancer Treated with Gefitinib

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Abstract **Purpose:** Epidermal growth factor receptor (*EGFR*) mutations, especially deletional mutations in exon 19 (DEL) and L858R, predict gefitinib sensitivity in patients with non–small cell lung cancer (NSCLC). In this study, we validated *EGFR* mutation detection using high-resolution melting analysis (HRMA) and evaluated the associations between *EGFR* mutations and clinical outcomes in advanced NSCLC patients treated with gefitinib on a larger scale.

Experimental Design: The presence of DEL or L858R was evaluated using HRMA and paraffin-embedded tissues and/or cytologic slides from 212 patients. In 66 patients, the results were compared with direct sequencing data.

Results: HRMA using formalin-fixed tissues had a 92% sensitivity and a 100% specificity. The analysis was successfully completed in 207 patients, and DEL or L858R mutations were detected in 85 (41%) patients. The response rate (78% versus 8%), time-to-progression (median, 9.2 versus 1.6 months), and overall survival (median, 21.7 versus 8.7 months) were significantly better in patients with *EGFR* mutations ($P < 0.001$). Even among the 34 patients with stable diseases, the time-to-progression was significantly longer in patients with *EGFR* mutations. Patients with DEL ($n = 49$) tended to have better outcomes than those with L858R ($n = 36$); the response rates were 86% and 67%, respectively ($P = 0.037$), and the median time-to-progression was 10.5 and 7.4 months, respectively ($P = 0.11$).

Conclusions: HRMA is a precise method for detecting DEL and L858R mutations and is useful for predicting clinical outcomes in patients with advanced NSCLC treated with gefitinib.

Gefitinib (Iressa; AstraZeneca) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. Phase II studies have shown gefitinib antitumor activity in patients with advanced non–small cell lung cancer (NSCLC; refs. 1, 2). Several studies have shown that the response rate to gefitinib is higher in women, patients with adenocarcinoma, never smokers, and Japanese or East Asians (1–3); subsequently, somatic mutations in the kinase domain of *EGFR* were suggested to be a determinant of gefitinib sensitivity (4, 5). Since then, many retrospective studies have consistently revealed that *EGFR* mutations, mainly in-frame deletions including amino acids at codons 747 to 749 in exon 19 (DEL) and a missense mutation at codon 858 (L858R) in exon 21, are associated with tumor response, time-to-progtession, and overall survival in NSCLC patients treated with gefitinib (6–8).

In our previous study, which clearly showed a correlation between *EGFR* mutations and gefitinib sensitivity in patients with recurrent NSCLC after surgical resection of the primary tumor (6), we used methanol-fixed, paraffin-embedded surgical specimens and did laser capture microdissection and direct sequencing, which we considered to be the most precise methods available for identifying mutations at that time. However, these methods are not useful in clinical practice for the treatment of advanced NSCLC for two reasons. First, the diagnostic samples of advanced NSCLC tumors, unlike surgical specimens, contain a small amount of tumor cells and are highly contaminated with normal cells. Second, laser capture microdissection and direct sequencing require special

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Table 1. Patient characteristics (N = 212)

	n (%)
Age (y)	
Median (range)	62 (29-84)
Sex	
Women	92 (43)
Men	120 (57)
Smoking history*	
Never smokers	96 (45)
Former smokers	38 (18)
Current smokers	78 (37)
Histology	
Adenocarcinoma	193 (91)
Others	19 (9)
Performance status†	
0	59 (28)
1	123 (58)
2	22 (10)
3	8 (4)
Stage	
III	42 (20)
IV	75 (35)
Recurrence after surgery	95 (45)
Gefitinib therapy	
First line	89 (42)
Second line	66 (31)
Third or more line	57 (27)

*Never smokers were defined as patients who have never had a smoking habit and former smokers were defined as patients who had stopped smoking at least 1 y before diagnosis.
† At the beginning of gefitinib therapy.

instruments and cost time and money. Recently, high-resolution melting analysis (HRMA) using the dye LCGreen I (Idaho Technology) was introduced as an easy, quick, and precise method for mutation screening (9), and we established a method for detecting DEL and L858R mutations using HRMA. Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (10).

In this study, we validated *EGFR* mutation detection by HRMA using DNA extracted from archived paraffin-embedded tissues. We also did the HRMA in advanced NSCLC patients treated with gefitinib on a larger scale using archived tissues and/or cytologic slides.

Patients and Methods

Patients. Among 364 consecutive patients with NSCLC who began receiving gefitinib monotherapy (250 mg/d) at the National Cancer Center Hospital between July 2002 and December 2004, 212 patients were retrospectively analyzed using HRMA. One hundred fifty-two patients were excluded from the analysis because tumor samples were not available (n = 126) or their informed consent to the genetic analysis was not obtained (n = 26).

High-resolution melting analysis. On a protocol approved by the Institutional Review Board of the National Cancer Center Hospital, we did the following genetic analyses. Formalin-fixed, paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides containing sufficient tumor cells (at least 1% of nucleated cells) were selected after microscopic examination by a pathologist (K.T.). The detailed analysis method has been described previously (10). Briefly, DNA was extracted from the tissues and/or cytologic slides using a QIAamp DNA Micro kit (Qiagen). PCR was done using dye LCGreen I and primers designed to amplify a region containing E746-I759 of *EGFR* [DEL-specific primer, AAAATTCCCGTCGCTATC (forward) and AAGCAGAAACTCACATCG (reverse)] or L858 of *EGFR* [L858R-specific primer, AGATCACA-GATTTTGGGC (forward) and ATTCTTTCTCTTCCGCAC (reverse)] on a LightCycler (Roche Diagnostics). The PCR products were denatured at 95°C for 5 min and cooled to 40°C to form heteroduplexes. The LightCycler capillary was then transferred to an HR-1 (Idaho Technology), a HRMA instrument, and heated at a transition rate of 0.3°C per second. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as a control sample with wild-type (WT) *EGFR*. Samples revealing skewed or left-shifted curves from those of control samples were judged to have mutations. All analyses were done in a blinded fashion.

Table 2. Clinical validation of HRMA and direct sequencing without laser capture microdissection

	HRMA without LCM			Direct sequencing without LCM (6)
	Formalin-fixed tissues	Methanol-fixed tissues	Cytologic slides (10)	
n	66	66	29	66
Successfully analyzed, *n* (%)	63 (95)	66 (100)	28 (97)	66 (100)
True positive	34	36	14	28
True negative	26	29	12	29
False positive	0	0	0	0
False negative	3	1	2	9
Sensitivity (%)	92	97	88	76
Specificity (%)	100	100	100	100
Positive predictive value (%)	100	100	100	100
Negative predictive value (%)	90	97	86	76

NOTE: The results of these analyses were compared with those of direct sequencing with LCM (used as the "gold standard" method). True positive is defined as the correct detection of deletional mutations in exon 19 or L858R.
Abbreviation: LCM, laser capture microdissection.