A New Mechanism for Primary Resistance to Gefitinib in Lung Adenocarcinoma: The Role of a Novel G796A Mutation in Exon 20 of EGFR

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Abstract. *Subsets of non-small cell lung cancer (NSCLC) patients who carry activating somatic mutations of the epidermal growth factor receptor (EGFR) have demonstrated an increased probability of obtaining objective responses to the receptor tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib. However, a substantial proportion of the cases with somatic mutations, which suggest sensitivity to gefitinib, are primary resistant to it. A primary resistant case of lung adenocarcinoma that was found to carry both delE746-A750 and a G796A mutation in the EGFR is reported. In vitro, a stable clone of cells bearing the G796A mutation was approximately 50,000-fold less sensitive to gefitinib in comparison to cells carrying the delE746-A750 mutant EGFR. This study suggests that screening tumour samples for a range of EGFR mutations may improve our ability to identify the patients most likely to benefit from EFGR TKIs.*

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related mortality in the world because of both the limited number of patients for whom surgery is cindicated and its resistance to currently available chemotherapeutic agents (1, 2). The response rates are 10 to 20 percent when tyrosine kinase inhibitors (TKI) such as gefitinib or erlotonib are used as second- or third-line treatments for advanced disease (3-5). Although such factors as female sex, adenocarcinoma, and no history of smoking are all considered to be probable markers for a favorable response to TKI (6), activating epidermal growth factor receptor (*EGFR*) mutations have also been reported to be highly significant predictors of the response (7-16). Although patients with activating *EGFR* mutations may be expected to respond to gefitinib, a proportion of them actually do not obtain an objective response. The molecular mechanism of such primary resistance remains unresolved. This is the first description of primary resistance to gefitinib associated with mutations of both G796A (a substitution of alanine for glycine at position 796) and delE746-A750 in *EGFR*.

Abbreviations: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; TK, tyrosine kinase; TKI, tyrosine kinase inhibitor; SD, stable disease; CT, computed tomography; T790M, substitution of methionine for threonine at position 790 in EGFR; G796A, substitution of alanine for glycine at position 796 in EGFR; IC_{50}, 50% inhibitory concentration.

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Key Words: EGFR, TKI, gefitinib, mutation, lung cancer, resistance.

Materials and Methods

Case and tissue procurement. A 68-year-old post-menopausal woman who had no history of smoking visited our hospital in June 2002 after the discovery of a single abnormal opacity on chest X-ray in her right middle lobe at another hospital. The patient also had a tumor in her left breast. Transbronchial lung washing cytology revealed a pulmonary adenocarcinoma. Cytological examination of fine-needle aspirates of the mammary tumor demonstrated breast cancer. The patient underwent a right middle lobectomy with mediastinal lymph node dissection for the lung cancer, followed by a modified radical mastectomy with lymph node dissection due to intraductal spread for the breast with an interval of 16 days between operations. The lung was found to have a well-differentiated adenocarcinoma with lymph node metastasis (pathological stage IIA; T1N1). The breast tumor was diagnosed as invasive ductal carcinoma (pathological stage IA T1N0). Adjuvant chemotherapy for lung cancer was not administered, however the patient received anastrozole as adjuvant therapy for her breast cancer because of the high histological grade and positive hormone receptor status. In April 2004, recurrence of the NSCLC with pleural dissemination and bone metastasis occurred.

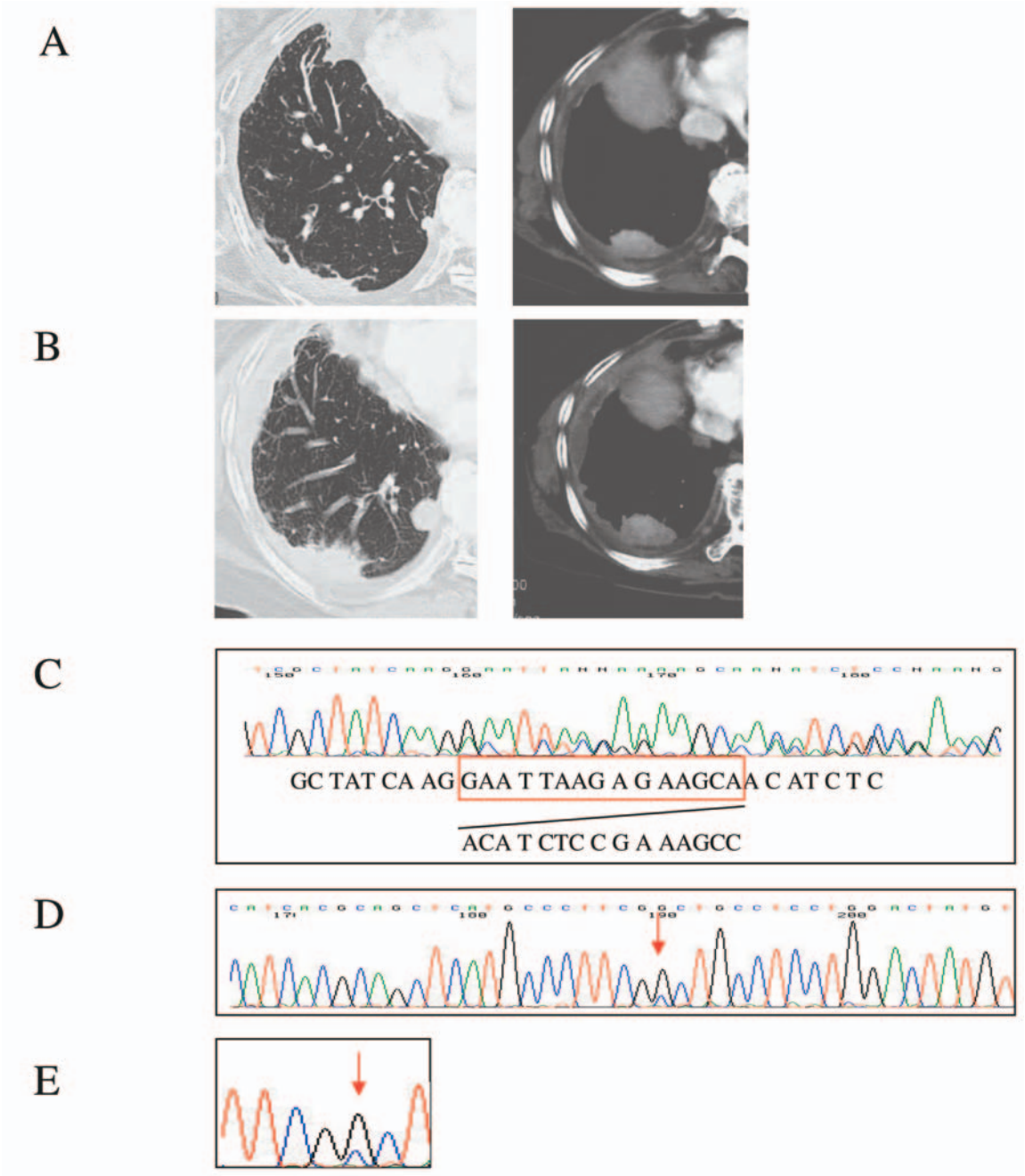

Figure 1. *Chest CT revealing pleural disseminations and pulmonary metastases in the right lung before (A) and after (B) treatment with gefitinib. C) The nucleotide sequence of the EGFR gene in the original specimen with heterozygous in-frame deletions in exon 19 within the TK domain (double peaks); the wild-type nucleotide sequence is shown in the red frame, and the mutant sequence is shown below it. D) The missense mutations (arrow) resulting in amino acid substitutions within the TK domain in a progressive lung lesion; the single peaks represent one nucleotide's mutation from G to C, thus resulting in a glycine for alanine amino acid change at position 796 of EGFR. E) The nucleotide sequences are shown by the extension of the square in Figure 1D.*

The disease was diagnosed as a recurrence of lung cancer based on a cytological examination from the pleural effusion. Systemic chemotherapy with paclitaxel and carboplatin was administered while her disease remained stable (SD) for nine months. When her disease progressed, she was treated with oral uracil-tegafur. However, one month later, her symptoms worsened and treatment with gefitinib 250 mg daily was started in March 2005. Comparison of computed tomography (CT) obtained prior to starting gefitinib (Figure 1A) and two months later (Figure 1B) showed progressive disease (PD) consistent with primary resistance to gefitinib. Gefitinib was stopped and the patient was treated with docetaxel and gemcitabine and obtained SD lasting for nine months.

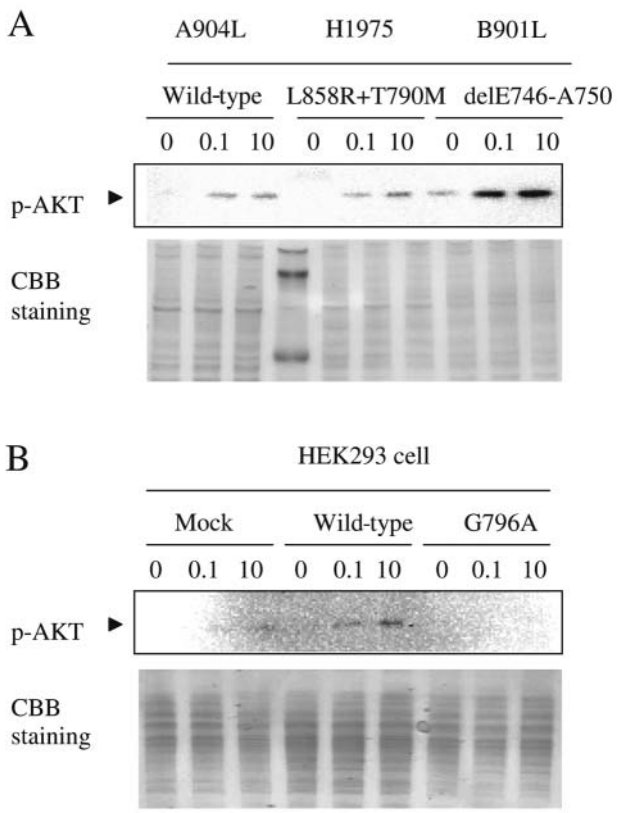

Figure 2. *Induction of Akt phosphorylation by gefitinib. A) A904L, H1975 and B901L cells treated with gefitinib for 1 h. Coomassie Brilliant Blue (CBB) staining of the gel, demonstrating equal sample loading is shown in the bottom panel. B) HEK 293T cells stably transfected with plasmids encoding mock vector, wild-type EGFR or EGFR mutants (G796A) treated with gefitinib for 1 h.*

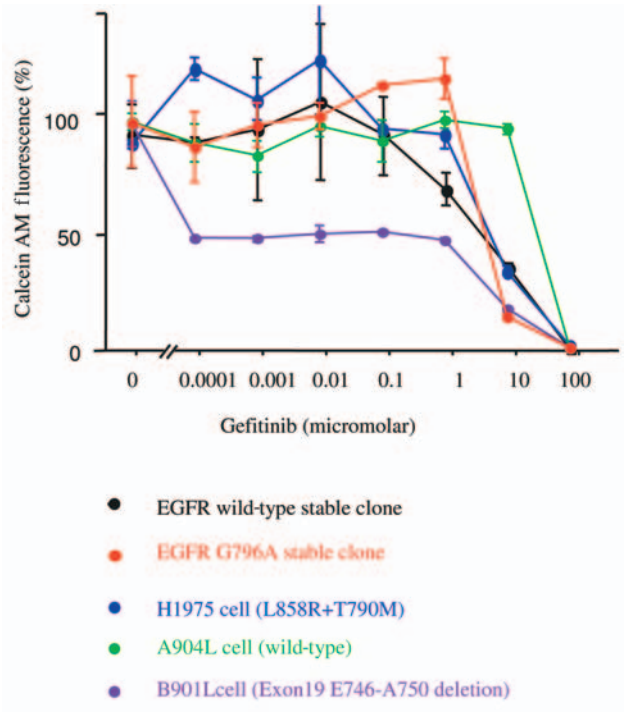

Figure 3. *The three indicated NSCLC cell lines, B901L (delE746-A750), H1975 (both T790M and L858R mutations), and A904L (wild-type EGFR) and stable clone transfected wild-type EGFR or EGFR G796A mutant were grown in increasing concentrations of gefitinib, and the density of live cells after 48 h of treatment was measured using a Calcein AM fluorescence assay. Fluorescence in vehicle-treated cells is expressed as 100%. The data are the mean and standard errors of two independent experiments with duplicate samples.*

Prior to starting gefitinib, tumor samples were collected from surgically resected specimens. Genomic DNA was extracted, as previously described (16). Informed consent for the use of such samples to perform these investigational studies was obtained from the patient according to the protocol approved by the institutional review board of the University of Occupational and Environmental Health.

Mutational analyses of EGFR. For a mutational analysis of the *EGFR* coding sequence, exons 18-23 (the area of the *EGFR* gene coding for the tyrosine kinase (TK) domain) of *EGFR* were amplified by polymerase chain reaction (PCR) using Taq polymerase (Takara, Tokyo, Japan). The sequences of the primers were as published (16). All sequencing reactions were performed on an ABI PRISM 310 automated sequencer (PE Biosystems, Tokyo, Japan).

Cell lines and drugs. The NSCLC cell line H1975 was purchased from American Type Culture Collection (Manassas, Virginia, United States). The cells were grown in RPMI growth medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum at 37˚C and 5% CO_2. The HEK293 human embryonic kidney fibroblast cell line was grown in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum (17). A904L and B901L human lung cancer cell lines, which had been established and reported previously (18), were grown in RPMI medium supplemented with 10% fetal bovine serum. Gefitinib was provided by AstraZeneca (London, UK).

EGFR expression plasmid constructs. The entire length of *EGFR* was amplified by PCR from a human cDNA library (19). The forward primer was 5'-GATCAAGCTTATGCGACCCTCCGGGACGGCC-3' (EGFR5) and the reverse primer was 5'-CATGCTCGAGTCATG CTCCAATAAATTCACTGC-3' (EGFR3). To construct pCMV plasmids suitable for expression in mammalian cells, the PCR amplified products were digested with HindIII and XhoI and the fragments were ligated into the pcDNA3 vector (Invitrogen, CA, USA). Site-directed mutagenesis of the *EGFR* (G796A) was performed using a PCR-based method. To obtain *EGFR* G796A mutant, the first and second half lengths of the *EGFR* (G796A) sequences were amplified first, by using the forward primer EGFR5 and reverse primer 5'-CATGCCCTTCGCCTGCCTCCTGG-3' (EGFR5m), and the forward primer 5'-CCAGGAGGCAGGC GAAGGGCATG-3' (EGFR3m) and reverse primer EGFR3. A second PCR was then performed with the first PCR products as templates by using the EGFR5 and EGFR3 primers. The PCR

products were cloned into the HindIII-XhoI-digested pcDNA3 vector in order to generate the pcDNA-*EGFR* (G796A) plasmids. For construction of the enhanced GFP expression vectors, NruI-PvuII fragments of pcDNA-*EGFR* wild-type or *EGFR* G796A mutant were inserted into the VspI-site of pEGFP-N3 (Clontech, CA, USA). All constructs were confirmed by sequencing using a DNA-sequencing system (PE Biosystems).

Transfection. For transient-transfection experiments, HEK293 cells were plated at a concentration of $5x10^4$ cells per well in six-well plates. The following day, these cells were transfected with 1 μg of the expression constructs with the use of Fugene 6 (Roche, Mannheim, Germany), as previously described (20). Cells stably overexpressing the wild-type *EGFR* or the *EGFR* G796A mutant were first selected with G418, as described by us previously (19). A single clone was then isolated by checking GFP protein expression by fluorescence microscopy (Carl Zeiss Inc, Tokyo, Japan). To assess more precisely the contribution of the biological effect of the wild-type *EGFR* or the *EGFR* G796A mutant, the HEK293 cells were subjected to limiting dilution cloning in drug-free medium using 96-well culture plates, as previously described (21).

Western blot analysis. The cells were lysed in 50 mM Tris-HCl, pH 8.0 150 mM sodium chloride 5 mM magnesium chloride 1% Triton X-100 0.5% sodium deoxycholate 0.1% SDS 40 mM sodium fluoride and 1 mM sodium orthovanadate. The cytoplasmic proteins, after quantitation by Bio-Rad protein assay, were electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, MA, USA) and HRP-conjugated anti-rabbit Ig 1:5,000 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Detection was performed using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Viability assay functional analyses of EGFR mutant. For viability studies, the cells were seeded in complete growth medium in white 96-well clear microwell plates (MUNC, Roskilde, Denmark) at a density of 10,000 cells per well. Following overnight incubation, the cells were grown for 24 h in the supplemented RPMI-1640 or Dulbecco's modified Eagle's medium with 0.1% serum. The cells (in supplemented medium containing 0.1% serum) were then incubated for 48 h in the continued presence of gefitinib. The cell viability was assayed using a fluorometric assay based on cell lysis and staining methods (Wako, Tokyo, Japan) (22). Following incubation with gefitinib, monolayers were washed twice with PBS and incubated with 7.5 μmol Calcein AM in medium (no serum) for 30 min. The labeling medium was removed and the cells were washed three times with phosphate-buffered saline (PBS). Calcein fluorescence was detected immediately using a Fluoroscan fluorometer (Labsystems, Helsinki, Finland). All the drugs were added directly to the culture medium at the indicated times with the indicated concentrations.

Results

Two tumor specimens from the patient, one that was obtained from the lung tumor and the other from the breast tumor, were analyzed. The lung tumor samples showed an in-frame deletion (delE746-A750) in exon 19 of *EGFR* (Figure 1C). Notably, a careful analysis of the exon 20 sequence of the lung tumor demonstrated an additional small peak at nucleotide 2386, thus revealing a G→C mutation. This nucleotide change leads to a substitution of alanine for glycine at position 796 (G796A) (Figure 1D). However, an examination of the sequences of exon 18-23 of the breast tumor confirmed the absence of these mutations (wild-type *EGFR*, data not shown). The substitution of methionine for threonine at position 790 in the *EGFR* (T790M) and the K-*ras* mutation (23) were not found in any of the surgical specimens. The sequence analysis of *EGFR* in genomic DNA from the NSCLC cell lines showed the delE746-A750 mutations and wild-type in the B901L and the A904L cell lines, respectively. To determine how the G796A mutation would affect the kinase activity of Akt, which is one of the main pathways (10), the expression level of phosphorylated Akt was examined. The induction of Akt phosphorylation by gefitinib in the B901L cell was seen in a concentration-dependent manner, in contrast to the A904L and the H1975 cells (Figure 2A). This finding is consistent with those of a previous report (22). Two types of HEK293 transfectants were constructed with the wild-type *EGFR* and the *EGFR* G796A mutant, respectively. Interestingly, the *EGFR* G796A mutant completely lacked induction of Akt phosphorylation by gefitinib (Figure 2B). These results suggest that the G796A mutation may impair the ability of gefitinib to inhibit Akt serine kinase activity. The lack of protein expression such as phospho-Akt and EGFR for the primary lung cancer specimen was confirmed by immunohistochemical staining using phospho-Akt (Ser473) antibody and total-EGFR (data not shown). We hypothesized that the primary resistance of the lung tumor to gefitinib in spite of the presence of delE746-A750 may have been due to the *EGFR* G796A mutation. Therefore, we investigated the biological significance of this mutation on *in vitro* sensitivity to gefitinib. The B901L cell harboring the E746-A750 deletion mutation of *EGFR* (delE746-A750) was used as the gefitinib-sensitive control. The A904L cell with wild-type *EGFR* and the H1975 cells having both L858R and T790M mutations of *EGFR* (22) served as the gefitinib-resistant control. The B901L cell line was sensitive to treatment with gefitinib at a 50% inhibitory concentration (IC_{50}) of 0.0001 mmol. In contrast, the IC_{50} of the stable transfected G796A clone was 5.0 μmol, the same as the wild-type *EGFR* stable clone, the H1975 cells and the A904L cells. The G796A stable clone was approximately 50,000-fold less sensitive to gefinitib in comparison to B901L cells carrying the *EGFR* delE746-A750 mutant (Figure 3).

Discussion

Drug therapy is an important treatment modality for patients with lung cancer. However, drug resistance remains a major and poorly understood problem (24). Recently, responsiveness to gefitinib treatment has been reported to be associated with

activating somatic mutations in the *EGFR* gene (7-16). However, the somatic mutation of *EGFR* alone cannot completely account for the responsiveness to gefitinib. There are a few problems regarding the responsiveness to gefitinib.

Despite an initial response to gefitinib in drug-sensitive tumors, most patients eventually demonstrate a recurrence of the disease (22). Recently, the discovery of T790M was reported partially to explain the phenomenon of gefitinib-resistance (22, 25, 26).

A proportion of patients have been found to respond to gefitinib in spite of possessing wild-type *EGFR*. It has also been reported that *EGFR* mutations are virtually absent in patients with stable disease to gefitinib therapy (27). BR-21 study, a randomized trial to determine survival benefit, demonstrated that disease stabilization contributed to overall survival advantage (28). The responsiveness of patients with wild-type *EGFR* may in part be related to amplification of *EGFR* (29), the expression of E-cadherin (30), the modulation of STAT3 serine phosphorylation (31).

Of the patients with NSCLC tumors carrying activating *EGFR* mutations 12.3% still progress on TKI treatment (32, 33). Our data have demonstrated that the IC_{50} for gefitinib in stable tumor cell lines bearing the *EGFR* G796A mutant is 50,000 times greater than the B901L cells carrying the *EGFR* delE746-A750 mutant. It is unclear how common the G796A mutation is among the NSCLC patient population. In tumors from lung cancer patients, G796A might be extremely rare, as are T790M and E884K (22, 25, 34-36). Nagai Y *et al.* have speculated that the *EGFR* gene is unstable and specific mutations are only observed in a subpopulation of the cells with a background of genetic heterogeneity of *EGFR* (37). In fact, we could not find a single G796A clone in 30 clones randomly picked up from the transformed clones, which included three clones carrying the *EGFR* delE746-A750 mutant by cDNA of the sample, suggesting that the *EGFR* G796A mutant may lie in the other allele from the *EGFR* delE746-A750 mutant.

Recently, Choong *et al.* reported that E884K remains resistant in spite of the presence of a double or single mutation of *EGFR* (34). Residue G796 is conserved in other members of the EGFR family (data not shown). G796A might thus be one of the exclusion criteria, much as the K-*ras* mutation for primary resistance and T790M for acquired resistance, when selecting patients. Furthermore, this specific mutation does not account for all mechanisms of primary resistance to gefitinib, other possibilities include Amphiregulin (38) or Hsp90 (39).

Data concerning the difference of sensitivity between stable clones overexpressing a sensitive mutation of *EGFR* or the *EGFR* G796A mutant have not been shown. Arao *et al.* have reported that HEK293 stable transfectants expressing deletional *EGFR* exhibited a higher sensitivity to ZD6474 (agent which targets vascular endothelial growth factor receptor-2 TK and EGFR TK) which has a strong correlation with the IC_{50} values of gefitinib, in comparison to the transfectants expressing the wild-type *EGFR* (17).

To our knowledge, this is the first patient with a primary resistance to gefitinib associated with the newly identified G796A mutation. In the near future, the establishment of highly sensitive techniques, such as mutant allele-specific amplification (23, 37), to detect resistant mutations and clinical trials of newly developed drugs to overcome resistance in such cases (36) is expected. Eventually, the molecular profiling of lung carcinomas might thus be helpful for predicting the differential response to molecularly targeted drugs and this could also help in the selection of optimal treatments for individual therapy.

Acknowledgements

We thank Masahiko Hashimoto, Yukari Oshibunchi, Misako Fukumoto and Yuki Gotoh for their valuable technical assistance. This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 14370420, 14571292, 15591460, 15790756, 17390388, and 17591451) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

References

1 Parkin DM, Bray FI and Devesa SS: Cancer burden in the year 2000. The global picture. Eur J Cancer *8*: 4-66, 2001.

2 Rosell R, Taron M, Alberola V, Massuti B and Felip E: Genetic testing for chemotherapy in non-small cell lung cancer. Lung Cancer *1*: 97-102, 2003.

3 Fukuoka M, Yano S, Giaccone G, Tamura T, Nakagawa K, Douillard JY, Nishiwaki Y, Vansteenkiste J, Kudoh S, Rischin D, Eek R, Horai T, Noda K, Takata I, Smit E, Averbuch S, Macleod A, Feyereislova A, Dong RP and Baselga J: Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small cell lung cancer. J Clin Oncol *21*: 2237-2246, 2003.

4 Kris MG, Natale RB, Herbst RS, Lynch TJ Jr, Prager D, Belani CP, Schiller JH, Kelly K, Spiridonidis H, Sandler A, Albain KS, Cella D, Wolf MK, Averbuch SD, Ochs JJ and Kay AC: Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. JAMA *290*: 2149-2158, 2003.

5 Perez-Soler R, Chachoua A, Hammond LA, Rowinsky EK, Huberman M, Karp D, Rigas J, Clark GM, Santabarbara P and Bonomi P: Determinants of tumor response and survival with erlotinib in patients with non-small cell lung cancer. J Clin Oncol *22*: 3238-3247, 2004.

6 Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, Krug LM, Pao W, Rizvi N, Pizzo B, Tyson L, Venkatraman E, Ben-Porat L, Memoli N, Zakowski M, Rusch V and Heelan RT: Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small cell lung cancer. J Clin Oncol *22*: 1103-1109, 2004.

7 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, Louis DN, Christiani DC, Settleman J and Haber DA: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small cell lung cancer to gefitinib. N Engl J Med *350*: 2129-2139, 2004.

8 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, Naoki K, Sasaki H, Fujii Y, Eck MJ, Sellers WR, Johnson BE and Meyerson M: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*: 1497-1500, 2004.

9 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L, Mardis E, Kupfer D, Wilson R, Kris M and Varmus H: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA *101*: 13306-13311, 2004.

10 Sordella R, Bell DW, Haber DA and Settleman J: Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science *305*: 1163-1167, 2004.

11 Huang SF, Liu HP, Li LH, Ku YC, Fu YN, Tsai HY, Chen YT, Lin YF, Chang WC, Kuo HP, Wu YC, Chen YR and Tsai SF: High frequency of epidermal growth factor receptor mutations with complex patterns in non-small cell lung cancers related to gefitinib responsiveness in Taiwan. Clin Cancer Res *10*: 8195-8203, 2004.

12 Han SW, Kim TY, Hwang PG, Jeong S, Kim J, Choi IS, Oh DY, Kim JH, Kim DW, Chung DH, Im SA, Kim YT, Lee JS, Heo DS, Bang YJ and Kim NK: Predictive and prognostic impact of epidermal growth factor receptor mutation in non-small cell lung cancer patients treated with gefitinib. J Clin Oncol *23*: 2493-2501, 2005.

13 Tokumo M, Toyooka S, Kiura K, Shigematsu H, Tomii K, Aoe M, Ichimura K, Tsuda T, Yano M, Tsukuda K, Tabata M, Ueoka H, Tanimoto M, Date H, Gazdar AF and Shimizu N: The relationship between epidermal growth factor receptor mutations and clinicopathologic features in non-small cell lung cancers. Clin Cancer Res *11*: 1167-1173, 2005.

14 Mitsudomi T, Kosaka T, Endoh H, Horio Y, Hida T, Mori S, Hatooka S, Shinoda M, Takahashi T and Yatabe Y: Mutations of the epidermal growth factor receptor gene predict prolonged survival after gefitinib treatment in patients with non-small cell lung cancer with postoperative recurrence. J Clin Oncol *23*: 2513-2520, 2005.

15 Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, Fong KM, Lee H, Toyooka S, Shimizu N, Fujisawa T, Feng Z, Roth JA, Herz J, Minna JD and Gazdar AF: Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst *97*: 339-346, 2005.

16 Uramoto H, Sugio K, Oyama T, Ono K, Sugaya M, Yoshimatsu T, Hanagiri T, Morita M and Yasumoto K: Epidermal growth factor receptor mutations are associated with gefitinib sensitivity in non-small cell lung cancer in Japanese. Lung Cancer *51*: 71-77, 2006.

17 Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N and Nishio K: Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res *64*: 9101-9104, 2004.

18 Sugaya M, Takenoyama M, Osaki T, Yasuda M, Nagashima A, Sugio K and Yasumoto K: Establishment of 15 cancer cell lines from patients with lung cancer and the potential tools for immunotherapy. Chest *122*: 282-288, 2002.

19 Ohga T, Uchiumi T, Makino Y, Koike K, Wada M, Kuwano M and Kohno K: Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. J Biol Chem *273*: 5997-6000, 1998.

20 Uramoto H, Wetterskog D, Hackzell A, Matsumoto Y and Funa K: p73 Competes with co-activators and recruits histone deacetylase to NF-Y in the repression of PDGF {beta}-receptor. J Cell Sci *117*: 5323-5331, 2004.

21 Takenoyama M, Baurain JF, Yasuda M, So T, Sugaya M, Hanagiri T, Sugio K, Yasumoto K, Boon T and Coulie PG: A point mutation in the NFYC gene generates an antigenic peptide recognized by autologous cytolytic T lymphocytes on a human squamous cell lung carcinoma. Int J Cancer *118*: 1992-1997, 2006.

22 Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG and Varmus H: Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med *2*: 1-11, 2005.

23 Sugio K, Uramoto H, Ono K, Oyama T, Hanagiri T, Sugaya M, Ichiki Y, So T, Nakata S, Morita M and Yasumoto K: Mutations within the tyrosine kinase domain of EGFR gene specifically occur in lung adenocarcinoma patients with a low exposure of tobacco smoking. Br J Cancer *94*: 896-903, 2006.

24 Kohno K, Uchiumi T, Niina I, Wakasugi T, Igarashi T, Momii Y, Yoshida T, Matsuo K, Miyamoto N and Izumi H: Transcription factors and drug resistance. Eur J Cancer *41*: 2577-2586, 2005.

25 Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, Johnson BE, Eck MJ, Tenen DG and Halmos B: EGFR mutation and resistance of non-small cell lung cancer to gefitinib. N Engl J Med *352*: 786-792, 2005.

26 Uramoto H, Sugio K, Oyama T, Sugaya M, Hanagiri T and Yasumoto K: A resistance to gefitinib. Int J Clin Oncol *11*: 487-491, 2006.

27 Cappuzzo F, Hirsch FR, Rossi E, Bartolini S, Ceresoli GL, Bemis L, Haney J, Witta S, Danenberg K, Domenichini I, Ludovini V, Magrini E, Gregorc V, Doglioni C, Sidoni A, Tonato M, Franklin WA, Crino L, Bunn PA Jr and Varella-Garcia M: Epidermal growth factor receptor gene and protein and gefitinib sensitivity in non-small cell lung cancer. J Natl Cancer Inst *97*: 643-655, 2005.

28 Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P and Seymour L: Erlotinib in previously treated non-small cell lung cancer. N Engl J Med *353*: 123-132, 2005.

29 Hirsch FR, Varella-Garcia M, McCoy J, West H, Xavier AC, Gumerlock P, Bunn PA Jr, Franklin WA, Crowley J and Gandara DR: Increased epidermal growth factor receptor gene copy number detected by fluorescence *in situ* hybridization associates with increased sensitivity to gefitinib in patients with bronchioloalveolar carcinoma subtypes. J Clin Oncol *23*: 6838-6845, 2005.

30 Witta SE, Gemmill RM, Hirsch FR, Coldren CD, Hedman K, Ravdel L, Helfrich B, Dziadziuszko R, Chan DC, Sugita M, Chan Z, Baron A, Franklin W, Drabkin HA, Girard L, Gazdar AF, Minna JD and Bunn PA Jr: Restoring E-cadherin expression increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. Cancer Res *66*: 944-950, 2006.
31 Alvarez JV, Greulich H, Sellers WR, Meyerson M and Frank DA: Signal transducer and activator of transcription 3 is required for the oncogenic effects of non-small cell lung cancer-associated mutations of the epidermal growth factor receptor. Cancer Res *66*: 3162-3168, 2006.
32 Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, Zakowski MF, Heelan RT, Kris MG and Varmus HE: KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. PLoS Med *2*: e17, 2005.
33 Chan SK, Gullick WJ and Hill ME: Mutations of the epidermal growth factor receptor in non-small cell lung cancer – search and destroy. Eur J Cancer *42*: 17-23, 2006.
34 Choong NW, Dietrich S, Seiwert TY, Tretiakova MS, Nallasura V, Davies GC, Lipkowitz S, Husain AN, Salgia R and Ma PC: Gefitinib response of erlotinib-refractory lung cancer involving meninges – role of EGFR mutation. Nat Clin Pract Oncol *3*: 50-57, 2006.
35 Toyooka S, Kiura K and Mitsudomi T: EGFR mutation and response of lung cancer to gefitinib. N Engl J Med *352*: 2136, 2005.
36 Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, Harris PL, Driscoll DR, Fidias P, Lynch TJ, Rabindran SK, McGinnis JP, Wissner A, Sharma SV, Isselbacher KJ, Settleman J and Haber DA: Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci USA *102*: 7665-7670, 2005.
37 Nagai Y, Miyazawa H, Huqun, Tanaka T, Udagawa K, Kato M, Fukuyama S, Yokote A, Kobayashi K, Kanaawa M and Hagiwara K: Genetic heterogeneity of the epidermal growth factor receptor in non-small cell lung cancer cell lines revealed by a rapid and sensitive detection system, the peptide nucleic acid-locked nucleic acid PCR clamp. Cancer Res *65*: 7276-7282, 2005.
38 Kakiuchi S, Daigo Y, Ishikawa N, Furukawa C, Tsunoda T, Yano S, Nakagawa K, Tsuruo T, Kohno N, Fukuoka M, Sone S and Nakamura Y: Prediction of sensitivity of advanced non-small cell lung cancers to gefitinib (Iressa, ZD1839). Hum Mol Genet *13*: 3029-3043, 2004.
39 Shimamura T, Lowell AM, Engelman J and Shapiro GI: Epidermal growth factor receptors harboring kinase domain mutations associate with the heat shock protein 90 chaperone and are destabilized following exposure to geldanamycins. Cancer Res *65*: 6401-6408, 2005.

Received January 19, 2007
Revised March 22, 2007
Accepted March 27, 2007