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 EGFR 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 erlotinib 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.

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.
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 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.
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% CO₂. 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'-CATGCTCGAGTCATGCTCCAATAAATTCACTGC-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'-CCAGGAGGCAGGCAGGAGGACGGGC-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

![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.](image1)

![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.](image2)
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 5x10⁴ 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, 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) after separation on 10% SDS-PAGE. Immunoblot analysis was performed with a polyclonal phospho-Akt (Ser473) antibody (Cell Signaling Technology, Boston, MA, USA) after separation on 10% SDS-PAGE.

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 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 B901L cell line was sensitive to treatment with gefitinib at a 50% inhibitory concentration (IC₅₀) of 0.0001 nmol. In contrast, the IC₅₀ 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).

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 (IC₅₀) of 0.0001 nmol. In contrast, the IC₅₀ 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 IC50 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 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 IC50 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.

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