EGFR Exon 18 Mutations in Lung Cancer: Molecular Predictors of Augmented Sensitivity to Afatinib or Neratinib as Compared with First- or Third-Generation TKIs

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Abstract

Purpose: Lung cancers harboring common EGFR mutations respond to EGFR tyrosine kinase inhibitors (TKI), whereas exon 20 insertions (Ins20) are resistant to them. However, little is known about mutations in exon 18.

Experimental Design: Mutational status of lung cancers between 2001 and 2015 was reviewed. Three representative mutations in exon 18, G719A, E709K, and exon 18 deletion (Del18: delE709_T710insD), were retrovirally introduced into Ba/F3 and NIH/3T3 cells. The 90% inhibitory concentrations (IC90s) of first-generation (1G; gefitinib and erlotinib), second-generation (2G; afatinib, dacomitinib, and neratinib), and third-generation TKIs (3G; AZD9291 and CO1686) were determined.

Results: Among 1,402 EGFR mutations, Del19, L858R, and Ins20 were detected in 40%, 47%, and 4%, respectively. Exon 18 mutations, including G719X, E709X, and Del18, were present in 3.2%. Transfected Ba/F3 cells grew in the absence of IL3, and NIH/3T3 cells formed foci with marked pile-up, indicating their oncogenic abilities. IC90s of 1G and 3G TKIs in G719A, E709K, and Del18 were much higher than those in Del19 (by >11–50-fold), whereas IC90s of afatinib were only 3- to 7-fold greater than those for Del19. Notably, cells transfected with G719A and E709K exhibited higher sensitivity to neratinib (by 5–25-fold) than those expressing Del19. Patients with lung cancers harboring G719X exhibited higher response rate to afatinib or neratinib (~80%) than to 1G TKIs (35%–56%) by compilation of data in the literature.

Conclusions: Lung cancers harboring exon 18 mutations should not be overlooked in clinical practice. These cases can be best treated with afatinib or neratinib, although the currently available in vitro diagnostic kits cannot detect all exon 18 mutations.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Introduction

Somatic mutations in the kinase domain of the EGFR gene are detected in approximately 40% and 17% of patients with non-small cell lung cancer in Asians (1) and in Caucasians (2), respectively. Common mutations, that is, exon 19 deletions (Del 19) and L858R mutation in exon 21, comprise approximately 90% of all the mutations and are associated with sensitivity to EGFR tyrosine kinase inhibitors (TKI; refs. 3–8). In contrast, lung cancers harboring exon 20 insertions (Ins 20; refs. 9, 10) or T790M in exon 20 (11–13) are known to be resistant to these drugs.

Mutations in exon 18 are detected in 3.6% (14) of all the mutations and G719X (where X indicates the substitution of the glycine residue for A, S, C, and D) mutations account for the majority of them. However, these mutations are not fully characterized because only limited data are available (15).

We recently treated a patient with lung adenocarcinoma harboring a rare exon 18 deletions (Del 18: deleE709_T710insD), and this tumor responded to afatinib, second-generation (2G) TKI. Inspired by this patient, this study aimed to establish a rational therapeutic strategy for lung cancers harboring exon 18 mutations. We investigated the in vitro sensitivities to three generations of EGFR-TKIs in retrovirally transfected cells and comprehensively clarified the frequency and clinical data on the treatment response of lung cancers harboring exon 18 mutations.

Materials and Methods

EGFR mutation analyses

To determine the frequency of exon 18 mutations in the EGFR gene, pathologic and genetic data were obtained from a database at the Department of Pathology and Molecular Diagnostics of
Translational Relevance

This study comprehensively focused on mutations in exon 18 of the EGFR gene and investigated the in vitro sensitivities to various EGFR tyrosine kinase inhibitors (TKI), including three generations of TKIs. Exon 18 mutations, including G719X, E709X, and exon 18 deletion, were present in 3% to 4% of all EGFR mutations. Lung cancers harboring these mutations appeared to have higher sensitivities to second-generation TKIs, especially afatinib and neratinib, than to first- and third-generation TKIs based on in vitro experiments as well as clinical data. Although the currently available in vitro diagnostic kits do not detect all exon 18 mutations, lung cancers harboring exon 18 mutations should not be overlooked in clinical practice, and patients with these tumors can be best treated with afatinib or neratinib.

Data collection from COSMIC database

Data of EGFR mutations in lung cancers were extracted from the Catalogue of Somatic Mutations in Cancer (COSMIC) database, release version 71 (20). To determine the distribution of mutations in exon 18 of the EGFR gene, total numbers of mutated tumors at each codon were counted.

Cell culture and reagents

The IL3-independent murine pro-B cell line Ba/F3 and myelomonocytic, macrophage-like, Balb/C mouse leukemia cells (WEHI-3) were provided by the RIKEN Bio Resource Center. The Ba/F3 cell was maintained in RPMI1640 (Wako) medium with 10% FBS (Sigma-Aldrich) and 5 ng/mL recombinant murine IL3 (Cell Signaling Technology). Conditioned media from WEHI-3 (10%) were also used as a source of IL3 for maintenance of Ba/F3. The human embryonic kidney cell line HEK293 and the murine embryo fibroblast cell line NIH/3T3 were obtained from the ATCC. The HEK 293 and NIH/3T3 cells were maintained in DMEM (Sigma-Aldrich) with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere with 5% CO2. The HEK293 cell line was analyzed using a short tandem repeat method, and was authenticated as previously reported (21). The reversible first-generation (1G) TKIs (gefitinib and erlotinib), irreversible 2G TKIs (afatinib, dacomitinib, and neratinib) and mutation-specific third-generation (3G) TKIs (AZD9291 and CO1686) were purchased from Selleck Chemicals and each compound was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Construction of retroviral-vector-transduced cell lines

We constructed retroviral vectors expressing G719A, E709K, and Del 18 (delE709_T710insD; Fig. 1A–D) as previously described (22). Briefly, a full-length cDNA fragment encoding the human EGFR gene was subcloned to a pQCLIN retroviral vector (Clontech) together with enhanced GFP (eGFP) to monitor the expression of the inserts. The pQCLIN constructs encoding EGFR Del18, E709K, G719A, and Del 19 were generated using the Prime STAR Mutagenesis Basal Kit (Takara); pQCLIN carrying wild-type (WT) EGFR was used as a template. All of the mutations were confirmed by sequencing. The pQCLIN constructs were cotransfected with a pVSV-G vector (Clontech) to generate the viral envelope in pglRES-293 cells using the FuGENE6 transfection reagent (Roche Diagnostics) to produce viral particles. After 48 hours of transfection, the culture medium was collected and the viral particles were concentrated by centrifugation at 15,000 × g for 3 hours at 4°C. The viral pellet was then resuspended in DMEM and was added to Ba/F3, HEK293, and NIH/3T3 cells. Infected Ba/F3, HEK293, and NIH/3T3 cells were purified by GFP-based fluorescence-activated cell sorting using BD FACS Aria Cell Sorter Special Order Research Product (BD Biosciences).

IL3-independent cell growth assay

A total of 3 × 10^4 transfected Ba/F3 cells were plated in 6-well plates and grown in RPMI with 10% FBS in the absence of IL3. Total numbers of cells in each well were manually counted every 24 hours using OneCell Counter (Bio Medical Science) in triplicate.

Focus formation assay

Transfected NIH/3T3 cells (2 × 10^5 cells/well) were seeded in each well of 6-well plates and grown in DMEM with 10% FBS. The medium was changed every 3 to 4 days, and the cells were photographed every 7 days.

Cell growth-inhibition assay

A total of 2 × 10^3 transfected Ba/F3 cells were plated in each well of 96-well plates and grown in RPMI with 10% FBS. IL3 was added to only the Ba/F3-WT cells. After 24 hours, the cells were treated with EGFR-TKIs at the indicated drug concentrations for 72 hours. A colorimetric assay was performed after the addition of 10 μL of Cell Counting Kit-8 reagent (Dojingo Laboratories) to each well, and the plates were incubated at 37°C for 2 to 4 hours. The absorbance at 450 nm was read using a multiplate reader (Tecan). Data are expressed as the percentages of growth relative to the DMSO-treated controls.

Antibodies and Western blot analysis

Transfected HEK293 cells were cultured to subconfluence. After 12 hours of serum starvation, the cells were treated with the indicated concentration of drug for 6 hours. Subsequently, 100 ng/mL EGF (Invitrogen) was added to only the HEK293-WT cells for 30 minutes. The cells were rinsed with PBS (Wako), lysed in SDS sample buffer and homogenized. Protein concentration was measured by colorimetric assay using Bio-Rad DC Protein Assay (Bio-Rad Laboratories). The total cell lysate (25 μg) was subjected to SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Bio-Rad Laboratories). After blocking with 2.5% nonfat dry milk and 2.5% bovine serum albumin in PBS, the membranes were incubated in primary and secondary antibodies, followed by visualization using an enhance.
chemiluminescence detection system (GE Healthcare) and an LAS-3000 camera (Fujifilm). Antibodies against total EGFR, phospho-EGFR (Tyr1068), total HER2, phospho-HER2 (Tyr1248), and β-actin were purchased from Cell Signaling Technology.

Clinical data on sensitivities to EGFR-TKIs in lung cancers harboring exon 18 mutations

Data on the treatment response to EGFR-TKI were extracted from COSMIC as well as the ACC database. Considering the mutational variations at the same codons, differences of sensitivities to TKIs between the top two variants were compared using the χ² test or Fisher exact test as appropriate. Statistical analyses were carried out with JMP version 11.1.1 (SAS Institute Inc.). Differences were considered statistically significant at a two-sided P value of <0.05.

Results

Frequency of exon 18 mutations in the EGFR gene

We identified 1,402 EGFR mutation-positive lung cancers at ACC (Fig. 1B). A majority of the tumors exhibited common mutations, i.e., Del 19 (40%) or L858R (47%). Ins 20 was detected in 4%. Of note, exon 18 mutations, including G719X (n=9), E709K (n=36) and G719A (n=163) were each identified in one tumor. E709K and G719A were each detected in two tumors, and G719X and L861Q were each identified in one tumor. E709K was the most common point mutation at codon 709, and G719A was the most frequent point mutation at codon 719 (Fig. 1D). We determined to focus on these three mutations.

EGFR Del 18, E709K, and G719A as driver mutations

We investigated the growth of Ba/F3 cells transfected with three common exon 18 mutations (Del18, E709K, and G719A) in the absence of IL3. Cells transfected with each mutant EGFR grew with a short doubling time of 8 hours, which was approximately equivalent to the doubling time of cells transfected with Del 19.
However, eGFP- and WT-transduced cells were unable to grow (Fig. 2A). NIH/3T3 cells transfected with exon 18 mutations formed foci with marked pile-up, whereas those transfected with eGFP or WT were inhibited to grow when they became confluent (Fig. 2B). These data showed that EGFR Del18, E709K, and G719A were actually oncogenic drivers.

Ba/F3 cells transfected with exon 18 mutations are more sensitive to 2G TKIs than to other TKIs at clinically relevant doses

To determine the rational TKI selection for lung cancers with exon 18 mutations, we evaluated the 90% inhibitory concentrations (IC90) of various EGFR TKIs in transfected Ba/F3 cells. We used the IC90 to assess whether most of the cells were nearly eliminated using clinically achievable concentrations of each drug. The IC90 for the exon 18 mutations were compared with the IC90 for Del 19 as well as to the trough concentrations (Ctrough) at the recommended doses for each drug, which were obtained from the literature for phase I studies (23–27). Ctrough for neratinib and CO1686 were not available.

Del 18 was the least sensitive mutation to all seven examined TKIs compared with G719A or E709K. However, the IC90 of erlotinib, afatinib, and dacomitinib were lower than the Ctrough of each drug (Fig. 3A). IC90 of the 1G and 3G TKIs in cells transfected with Del 18, E709K, and G719A were much greater than those in cells transfected with Del19 (by >50-, >25-, and >11-fold, respectively; Fig. 3B). In contrast, there were no such differences in IC90s between exon 18 mutations and Del 19 for afatinib or neratinib. IC90 of afatinib in these three mutant cell types ranged from only 3- to 7-fold greater than that in cells harboring Del 19 and was <1/40 of its Ctrough. Notably, cells harboring exon 18 mutations exhibited higher sensitivity to neratinib (by 25-fold for G719A, by 5-fold for E709K, and by a comparable extent for Del 18) than those harboring Del 19.

Western blot analyses of transfected HEK293 cells confirm the sensitivities of transfected Ba/F3 cells to EGFR-TKIs

To confirm the above data obtained from Ba/F3 cells, the phosphorylation levels of EGFR were evaluated after TKI exposure to HEK293 cells. The phosphorylation of EGFR was almost inhibited by 100 nmol/L afatinib in Del18, E709K, G719A, and Del19 cells (Fig. 4). Additionally, 10 nmol/L neratinib more effectively inhibited the phosphorylation of EGFR in G719A cells than in Del 19 cells. Cells transfected with Del 18 or E709K were also sensitive to neratinib. In contrast, 1G and 3G TKIs were particularly effective only in cells harboring Del 19. Overall, similar trends were observed between Western blot analysis and the growth inhibition assay. In addition, the phosphorylation of HER2 was inhibited to an extent comparable to that of phospho-EGFR in each transfected cell (Supplementary Fig. S1).

Case report

A 63-year-old man was diagnosed with stage IV adenocarcinoma of the lung with pleural disseminations on thoracoscopic lung biopsy. He was referred to ACC, and no mutation was detected on the basis of genetic analyses of EGFR, KRAS, ALK, HER2, and BRAF in DNA from FFPE samples. He was initially treated with two lines of platinum doublet (cisplatin + pemetrexed and carboplatin + gemcitabine). After that, direct sequencing of the RNA extracted from cells in pleural effusion revealed
and E709A. Furthermore, afatinib (28) and neratinib (29) displayed a high response rate of nearly 80% in G719X tumors according to the data from a small subset of prospective phase II/III studies.

Discussion

In this study, we found that lung cancers harboring exon 18 mutations accounted for 3.2% of all EGFR mutations. In addition, exon 18 mutations appeared to have the role as driver mutations and higher sensitivities to 2G TKIs than to 1G and 3G TKIs at clinically relevant doses. To the best of our knowledge, this is the first study to comprehensively focus on mutations in exon 18 and to investigate the in vitro sensitivities to various EGFR-TKIs, including three generations of TKIs.

Three patients with tumors harboring delE709_T710insD who received gefitinib or erlotinib have been reported, and their response rate was 33% (30, 31). The present case might be the first report on the administration of afatinib to a patient with adenocarcinoma harboring Del 18, and fortunately, this
treatment successfully shrank the tumor. Regarding tumors harboring G719X, afatinib achieved high response rate, that was comparable to that for tumors harboring Del 19 or L858R (28). Spectrum of efficacy in neratinib was unique in the phase II study; a partial response was observed only in patients with tumors harboring G719X, but not in those harboring Del 19 (29). Unfortunately, the development of neratinib for patients with lung cancer is abandoned because it is not active for tumors harboring major EGFR mutations. Currently, considering these preliminary clinical reports and our results of in vitro sensitivities, afatinib should be the appropriate TKI selection for patients with lung cancer harboring exon 18 mutations.

One of the primary mechanisms underlying the different sensitivities of lung cancers harboring each EGFR mutation to TKIs has been regarded as the different affinities between kinase and TKIs (32). Davis and colleagues reported comprehensive data on the dissociation constants between several types of EGFR kinases and TKIs (33). The affinity with geitinib was 2.0- to 3.7-fold higher in Del 19 than in G719C/S, whereas that with afatinib was only 0.9- to 1.7-fold. In addition, the affinity with neratinib was 2.5-6.2-fold lower in Del 19 than in G719C/S. These data concur with our data on the in vitro sensitivities to these TKIs. HER2 inhibition activity is one of the main differences between 2G and other TKIs. However, inhibition of HER2 does not seem to affect the different sensitivities we observed On the basis of the results of Western blot analyses.

Currently available in vitro diagnostic kits, therascreen (Qiagen) and cobas (Roche), which are approved by health authorities, cannot detect Del 18 (delE709_T710insX) or E709X. In the gene analyses at the ACC, Del 18 and E709X can be detected by RT-PCR direct sequencing only in cases frozen tissues were available. Therefore, these mutations should have been missed when RNA was not available and these mutations potentially would account for more than 3.2% of all mutations. Our data strongly suggest the improvement of these assays to enable the detection of alterations at E709 for the patients with these minor mutations. Comprehensive mutation searches of exon 18 to 21 by direct sequencing of RNA or by next-generation sequencing of DNA can also be useful for detecting these minor mutations. Detecting minor EGFR mutations has another significant benefit. Our data showed that these minor mutations certainly played roles as oncogenic drivers. Detecting minor driver mutations can allow us to skip searching for other driver mutations because driver mutations are generally mutually exclusive. Interestingly, next-generation sequencing identified Del 18 in two out of 31 driver-negative lung adenocarcinomas that had previously tested 'negative' for alterations in 11 genes (EGFR, ERBB2, KRAS, NRAS, BRAF, MAP2K1, PIK3CA, and AKT1, as well as fusions involving ALK,

Figure 4. Western blot analyses of transfected HEK293 cells. The cells were treated with the indicated concentration of drug for 6 hours. Subsequently, EGF was added to only the HEK293-WT cells for 30 minutes. Cell extracts were immunoblotted to detect the indicated proteins.
ROS1, and RET; ref. 34). This result indicates that additional patients with tumors harboring EGFR minor mutations would potentially benefit from treatment with 2G TKIs.

The LUX-lung 3 and 6 studies, which compared afatinib to platinum doublet chemotherapy, showed a survival benefit of afatinib for patients with tumors harboring Del 19 but not for patients with L858R-mutant tumors, which suggested that even these common mutations have different chemosensitivities (35). In addition, mutation-specific 3G TKIs showed promising response in tumors harboring T790M in clinical trials (27, 36). Our data suggest that conventionally interpreted sensitivity to EGFR-TKIs in lung cancers harboring each mutation should be reevaluated because the majority of them were obtained from only sensitivity to 1G TKIs.

In conclusion, we have shown that 2G TKIs have unique sensitivities in the context of EGFR exon 18 mutations compared with 1G and 3G TKIs. In particular, patients with lung cancers harboring EGFR Del 18, E709K, or G719A could be good candidates for treatment with afatinib. We should reconsider the methods of gene analyses to detect alterations at codon 709 to avoid missing the opportunity for patients with lung cancers harboring these minor mutations to benefit from 2G TKIs. Our

### Figure 5.
Clinical response to afatinib in a patient with adenocarcinoma harboring EGFR exon 18 deletion (delE709_T710insD). A, chromatogram of EGFR derived from pleural effusion of the patient. Three base pairs were deleted at nucleotides 2027 to 2029 (square), which led to a shift of subsequent nucleotides (underlined). Glutamic acid (GAA) at codon 709 and threonine (ACT) at codon 710 were substituted for aspartic acid (GAT) at codon 709. B, chest computed tomography images of the patient before and after 1 month’s treatment of afatinib.
data also suggest the significance of mutation-specific EGFR-TKI selection.

Disclosure of Potential Conflicts of Interest
T. Hida reports receiving speakers bureau honoraria from Boehringer Ingelheim. T. Mitsudomi reports receiving research grants and speakers bureau honoraria from and is a consultant/ advisory board member for AstraZeneca, Boehringer Ingelheim, and Chugai. No potential conflicts of interest were disclosed by the other authors.

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