Molecular Cancer Therapeutics

Characterization of EGFR T790M, L792F, and C797S Mutations as Mechanisms of Acquired Resistance to Afatinib in Lung Cancer

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Abstract

Lung cancers harboring common *EGFR* mutations respond to EGFR tyrosine kinase inhibitors (TKI). We previously reported that tumors with exon 18 mutations are particularly sensitive to irreversible second-generation (2G) afatinib compared with first-generation TKIs (1G-TKI). However, data on the mechanisms of acquired resistance to afatinib are limited. We established afatinib-resistant cells by transfecting Ba/F3 cells with common or exon 18 (G719A and Del18) mutations and subjecting them to chronic exposure to increasing concentrations of afatinib. Afatinib-resistant clones were separately established through N-ethyl-N-nitrosourea (ENU) mutagenesis and exposure to fixed concentrations of afatinib. Rebiopsy samples from patients whose tumors acquired resistance to afatinib were analyzed. Afatinib-resistant cells with Del19, L858R, or G719A developed T790M, whereas those with Del18 acquired novel L792F mutation. ENU mutagenesis screening established 84 afatinib-resistant clones. All Del19 clones and most of the other clones acquired only T790M. However, C797S occurred in subsets of L858R, G719A, and Del18 clones. In addition, subsets of Del18 clones acquired L792F. C797S-acquired cells were sensitive to 1G erlotinib. L792F demonstrated intermediate resistance between T790M and C797S to both 1G- and 3G-TKIs, whereas L792F was the least resistant to 2G-TKIs, particularly dacomitinib. Chronic exposure of Del18 + L792F cells to dacomitinib induced additional T790M. T790M was detected in one of four clinical samples. In conclusion, L792F and C797S, in addition to the major T790M, can develop in afatinib-resistant cells particularly using a low dose of afatinib, and these minor mutations appear to exhibit sensitivity to dacomitinib and erlotinib, respectively. These secondary mutations should be tested in clinical practice. *Mol Cancer Ther; 16(2); 357–64.* ©*2016 AACR.*

See related article by Talbert et al., p. 344.

Introduction

Gefitinib and erlotinib, first-generation (1G) EGFR tyrosine kinase inhibitors (EGFR-TKI), reversibly bind to the ATP-binding pocket of EGFR. These drugs prolonged progression-free survival (PFS) in patients with non–small cell lung cancer (NSCLC) harboring common *EGFR* mutations, that is, exon 19 deletions (Del19) and L858R, compared with chemotherapy (1–4). However, these cancers inevitably acquire resistance after an initial dramatic response. The secondary mutation T790M accounts for approximately 50% to 60% of the mechanisms of acquired resistance to 1G-TKIs (5, 6).

Afatinib is one of the irreversible ErbB family blockers, so-called second-generation (2G) TKIs, which were developed to overcome the T790M mutation. Despite promising preclinical data, the clinically available concentrations of the drug did not reach the treatment range for T790M tumors (7). However, afatinib prolonged overall survival compared with chemotherapy, particularly in patients with Del19 (8). Furthermore, in patients with NSCLC harboring common mutations, the LUX-lung 7 trial demonstrated the superiority of afatinib to gefitinib in terms of PFS, with HR of 0.73 and 95% CI of 0.57–0.95 (9). In addition, we previously demonstrated that tumors with exon 18 mutations, which account for 3.7% of all *EGFR* mutations (10), were particularly sensitive to afatinib compared with 1G-TKIs (11), and a combined analysis of the LUX-lung 2, 3, and 6 trials also indicated the efficacy of afatinib in these tumors (12). However, only limited data on the mechanisms of acquired resistance to afatinib are available (13–15).

This study aimed to clarify the mechanisms of acquired resistance to afatinib and establish a therapeutic strategy for overcoming resistance. We comprehensively investigated the secondary *EGFR* mutations of afatinib-resistant Ba/F3 cells with or without the aid of N-ethyl-N-nitrosourea (ENU) mutagenesis. Rebiopsy samples from patients with adenocarcinoma that acquired resistance to afatinib were also analyzed.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Cell culture and reagents

The murine pro-B cell line Ba/F3 was provided by the RIKEN Bio Resource Center (Tsukuba, Japan). The *EGFR*-mutant NSCLC cell lines HCC4006 (del E746_A750), PC9 (del E746_A750), and 11_18 (L858R) were obtained from Dr. A.F. Gazdar (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, TX) in 2009, Dr. Y. Hayata (Tokyo Medical University, Tokyo, Japan) in 1993, and the RIKEN Bio Resource Center (Tsukuba, Japan) in 2015, respectively. All cells were maintained in RPMI1640 (Wako) medium with 10% FBS (Sigma-Aldrich) and cultured at 37°C in a humidified atmosphere with 5% CO_2. All human cell lines were analyzed using a short tandem repeat method and were authenticated in August 2015 as reported previously (11). The 1G-TKI erlotinib, 2G-TKIs (afatinib, dacomitinib, and neratinib), and the T790M mutation-specific third-generation (3G) TKI osimertinib were purchased from Selleck Chemicals, and each compound was dissolved in DMSO (Sigma-Aldrich). Chemical structures of dacomitinib and neratinib were previously shown (16, 17).

Construction of retroviral vector–transduced cell lines

EGFR mutations were retrovirally introduced into Ba/F3 cells as described previously (11). Briefly, the full-length cDNA fragment encoding the human *EGFR* gene was subcloned in a pQCLIN retroviral vector (Clontech). The pQCLIN constructs encoding *EGFR* Del19 (del E746_A750), L858R, G719A, and Del18 (delE709_T710insD) were generated using the Prime STAR Mutagenesis Basal Kit (Takara), and a pQCLIN construct carrying wild-type (WT) *EGFR* was used as a template. The pQCLIN constructs were cotransfected with a pVSV-G vector (Clontech) in gpIRES-293 cells using the FuGENE6 transfection reagent (Roche Diagnostics) to produce viral particles. *EGFR* Del18 + L792F, Del19 + L792F, L858R + L792F, L858R + T790M, L858R + C797S, and L858R + C797S + T790M were also introduced into Ba/F3 cells using the same method.

Establishment of resistant cells by chronic exposure to afatinib

Parental Ba/F3 cells expressing each *EGFR* mutation were chronically exposed to increasing concentrations of afatinib. Because the trough concentration in the phase I study was 69 nmol/L when 40 mg/day afatinib was administered (7), the final target concentration in this study was 100 nmol/L, and the established resistant cells were maintained with 100 nmol/L afatinib. Similarly, HCC4006, PC9, and 11_18 cells were also exposed to afatinib until acquiring resistance to 100 nmol/L afatinib.

Establishment of afatinib-resistant clones through ENU mutagenesis

Ba/F3 cells expressing *EGFR* Del19, L858R, G719A, and Del18 were exposed to 100 μg/ml ENU (Sigma-Aldrich) for 24 hours. The cells were then washed with RPMI containing 10% FBS and cultured in RPMI with 10% FBS for 24 hours. Similar to previous studies (18–20), 1×10^4 to 1×10^5 cells were plated in 96-well plates in the presence of afatinib. The concentrations of afatinib were set to 10 or 100 nmol/L to mimic the wide range of plasma concentrations achieved due to dose modification in the clinic. As a result of the relatively severe side effects of afatinib compared with those of 1G-TKIs, dose reduction was necessary for approximately half of the patients in the LUX lung-3 trial (21). The medium containing afatinib was changed twice weekly. The cells were observed by visual inspection for growth until confluence, and the resistant cells were maintained in the presence of the corresponding concentrations of afatinib.

EGFR mutation analyses

The total RNA from resistant cells or clinical samples was isolated using mirVana miRNA Isolation Kit (Qiagen). cDNA was then transcribed from the total RNA using ReverTra Ace (TOYOBO). The tyrosine kinase domains of *EGFR* exons 18 to 21 were amplified with primers as reported previously (22). Sanger sequencing was performed using Genetic Analyzer 3130 or 3500XL (Applied Biosystems). When RNA was not obtained, DNA was extracted from the samples using DNeasy Blood & Tissue Kit (Qiagen), and each exon from 18 to 21 was separately sequenced.

MET gene copy number analyses

DNA was extracted from clinical samples. Gene copy numbers of *MET* relative to *LINE1* were calculated by real-time PCR using normal DNA as a control. Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and StepOnePlus system (Applied Biosystems).

Cell growth inhibition assay

A colorimetric assay was performed as described previously (11, 23). Briefly, 2×10^3 cells were plated in 96-well plates and grown for 24 hours. The cells were then treated with EGFR-TKIs for 72 hours. After the addition of 10 μL of Cell Counting Kit-8 Reagent (Dojindo Laboratories), the absorbance at 450 nm was read using a multiplate reader (Tecan). The data are expressed as growth percentages relative to that of the DMSO-treated controls.

Clinical specimens with acquired resistance to afatinib

Rebiopsy samples from patients with NSCLC that acquired resistance to afatinib were collected at Kurume University Hospital (Kurume, Japan) and Kyoto University Hospital (Kyoto, Japan). Acquired resistance was defined as progression after partial response or durable stable disease (≥6 months; ref. 24). Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was adapted to evaluate the treatment response (25). Appropriate approval was obtained from the institutional review committee, and written informed consent was obtained from the patients.

Results

Secondary *EGFR* mutations of afatinib-resistant Ba/F3 cells and NSCLC cell lines

The concentrations for 50% inhibition (IC_{50}) of parental Ba/F3 cells transfected with Del19, L858R, G719A, or Del18 were markedly lower than the clinically available trough concentration of afatinib (Fig. 1A). The cells with Del18 were the least sensitive, and those with G719A exhibited relatively low sensitivity, consistent with our previous study (11). After acquiring resistance to 100 nmol/L afatinib, IC_{50}s for these resistant cells increased >50-fold compared with those for the

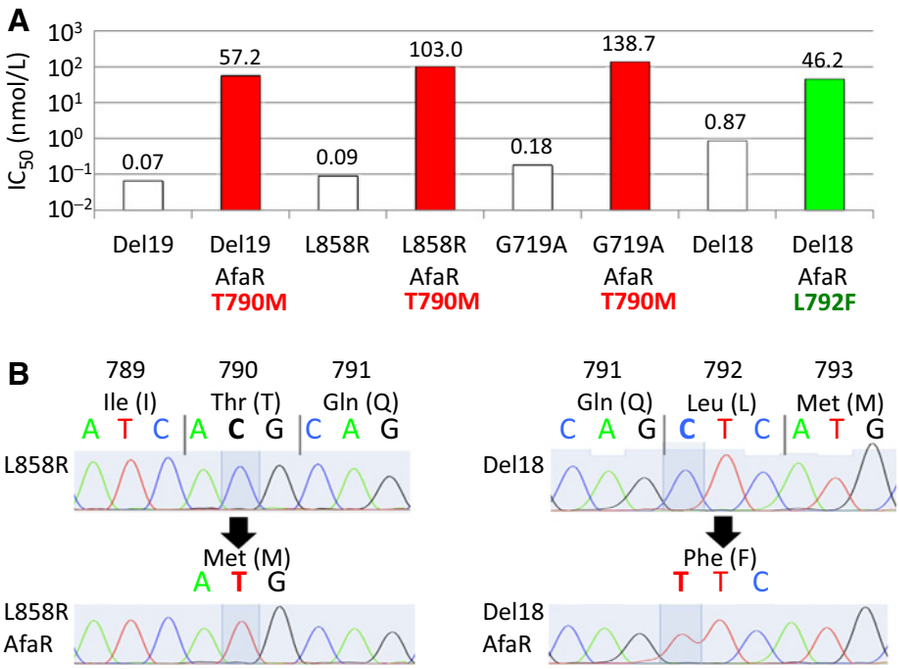


Figure 1.
Secondary *EGFR* mutations of Ba/F3 cells expressing Del19, L858R, G719A, or Del18 that acquired resistance to 100 nmol/L afatinib. **A,** Cell viability assay of parental Ba/F3 cells expressing each *EGFR* mutation and afatinib-resistant cells established by chronic exposure to increasing concentrations of afatinib. Cells were treated with afatinib for 72 hours, and the concentrations for 50% inhibition (IC_{50}) were determined. The secondary *EGFR* mutations for each afatinib-resistant cell line are labeled. **B,** Chromatograms of *EGFR* derived from parental Ba/F3 cells expressing L858R or Del18 and afatinib-resistant cells. Threonine (ACG) at codon 790 was substituted by methionine (ATG) in afatinib-resistant cells expressing L858R, whereas leucine (CTC) at codon 792 was substituted by phenylalanine (TTC) in afatinib-resistant cells expressing Del18.

parental cells. Afatinib-resistant cells expressing Del19, L858R, and G719A developed T790M, whereas those harboring Del18 acquired a novel L792F mutation (Fig. 1A and B). No secondary *EGFR* mutations were detected in the afatinib-resistant HCC4006, PC9, or 11_18 cells.

Secondary *EGFR* mutations of afatinib-resistant clones established through ENU mutagenesis

To clarify the frequency of secondary mutations, we developed a number of resistant clones through ENU mutagenesis. ENU mutagenesis screening in the presence of 10 or 100 nmol/L afatinib yielded 74 resistant clones (Fig. 2). T790M accounted for 86% of the mutations (64/74), and cells with Del19 tended to exclusively acquire T790M. However, C797S occurred in L858R-mutated cells (2/20), G719A-mutated cells (1/17), and Del18-mutated cells (4/20). In addition, three clones of Del18-mutated cells acquired the L792F mutation.

Notably, eight clones developed C797S or L792F in the presence of 10 nmol/L afatinib, whereas only the least sensitive Del18-mutated cells developed C797S with 100 nmol/L afatinib. Therefore, we attempted to assess whether lower concentrations of afatinib (0.1 or 1 nmol/L) could induce C797S or L792F even in the most sensitive Del19-mutated cells. However, no secondary mutations developed in the presence of a

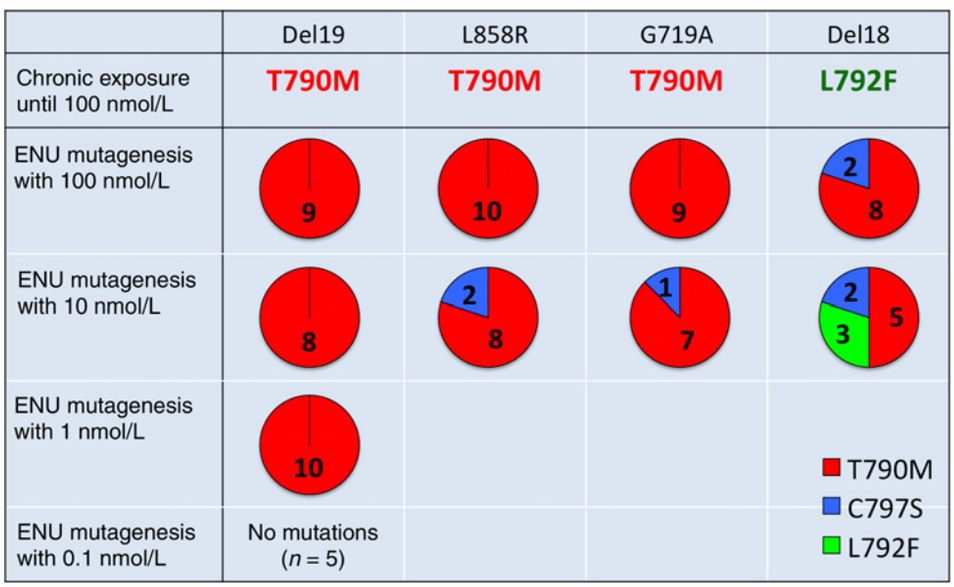


Figure 2.
Secondary *EGFR* mutations of afatinib-resistant clones established through ENU mutagenesis screening. Ba/F3 cells expressing *EGFR* Del19, L858R, G719A, and Del18 were exposed to 100 μg/mL ENU for 24 hours. After growing in medium for 24 hours, the cells were seeded in 96-well plates with the indicated concentrations of afatinib. The cells were observed by visual inspection for growth until reaching confluence. The secondary *EGFR* mutations of the resistant cells in each well were analyzed.

low concentration of 0.1 nmol/L (0/5), and the secondary mutations acquired with 1 nmol/L were exclusively T790M (10/10). In total, 88% (74/84) of the secondary mutations were T790M.

In vitro sensitivities of afatinib-resistant Ba/F3 cells to three generations of EGFR-TKIs

To characterize the novel L792F mutation, IC_{50}s of parental Ba/F3 cells expressing Del18 and the established afatinib-resistant cells (Del18 + T790M, Del18 + L792F, and Del18 + C797S) to various TKIs were determined (Fig. 3A; Supplementary Fig. S1; Supplementary Table S1). L792F cells exhibited intermediate resistance between T790M and C797S cells to both 1G- and 3G-TKIs. However, L792F presented the least resistance to 2G-TKIs. Compared with the trough concentrations of each drug in phase I studies (7, 26–29), cells with C797S, L792F, and T790M appeared to be particularly sensitive to erlotinib, dacomitinib, and osimertinib, respectively.

L858R and three types of resistant mutations, that is, T790M, C797S, and T790M + C797S, were retrovirally introduced into Ba/F3 cells to characterize the C797S mutation. IC_{50}s of these cells to various TKIs were also determined (Fig. 3A). Similar to the results obtained for afatinib-resistant Del18 cells, cells expressing L858R + C797S and L858R + T790M were sensitive to 1G- and 3G-TKIs, respectively. Interestingly, the degree of resistance to 2G-TKIs increased in order from C797S to T790M to T790M + C797S, and the cells with L858R + C797S exhibited moderate sensitivity to afatinib.

To confirm the role of the L792F mutation as a resistant mechanism, *EGFR* Del18 + L792F, Del19 + L792F, or L858R + L792F mutations were introduced into Ba/F3 cells using retroviral vector. IC_{50}s of these cells with additional L792F mutation to afatinib were 4- to 10-fold greater than those of the parental cells with Del18, Del19, or L858R mutations (Supplementary Fig. S2). In addition, they were lower than the clinically available trough concentration of afatinib. As compared with above studies,

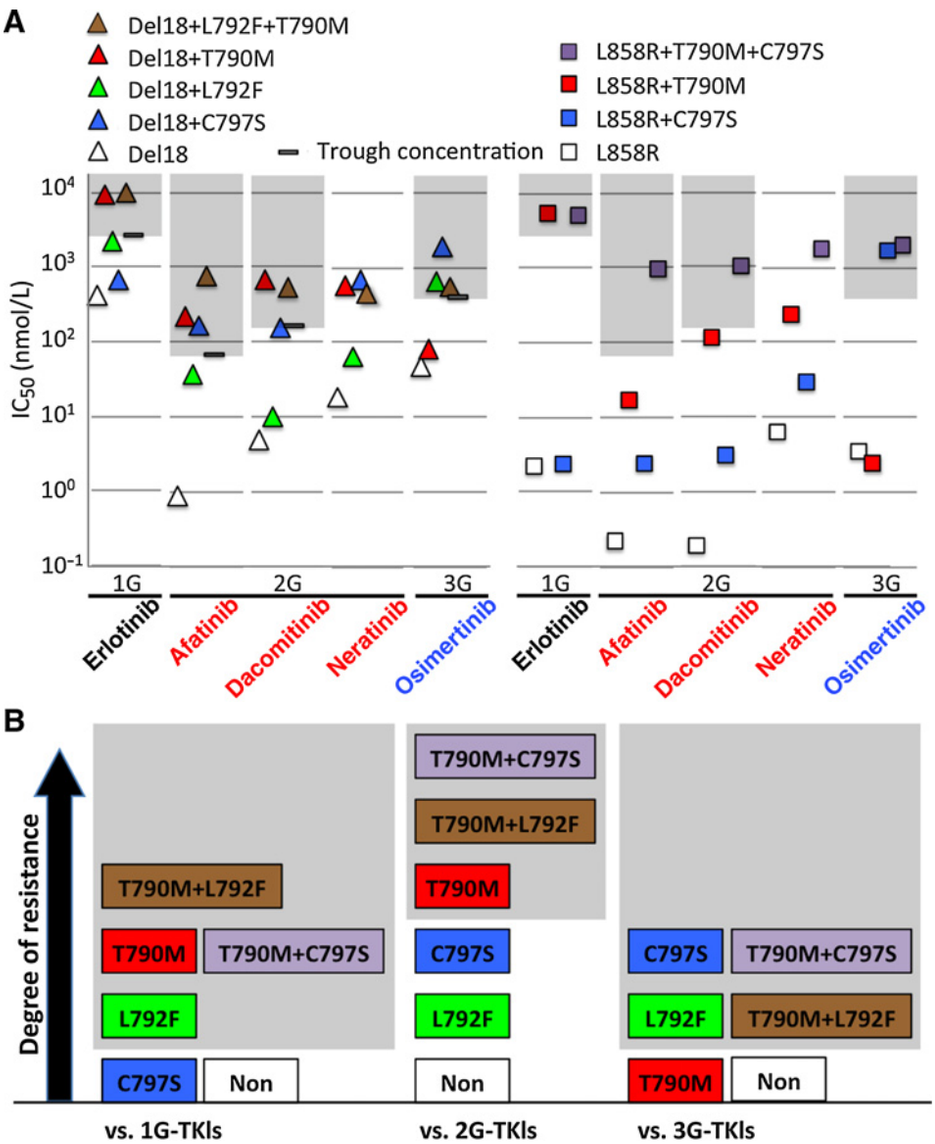


Figure 3.
Characterization of L792F, C797S, and T790M mutations against various EGFR TKIs. **A,** *In vitro* sensitivities of afatinib-resistant Ba/F3 cells expressing *EGFR* Del18 that acquired T790M, L792F, or C797S to various EGFR-TKIs. The sensitivities of the parental Ba/F3 cells expressing *EGFR* L858R, L858R + C797S, L858R + T790M, and L858R + T790M + C797S to EGFR-TKIs are also shown. Cells treated with each of the EGFR-TKIs for 72 hours were subjected to a cell viability assay, and the concentrations for 50% inhibition (IC_{50}) were determined. The trough concentrations for each drug at the recommended doses were obtained from the literature (7, 26–29), and concentrations higher than the trough concentration are shown in gray. **B,** Summary of the characteristics of various resistant mutations against 1G, 2G, and 3G-TKIs. Concentrations higher than the trough concentration are shown in gray.

IC_{50} of cells transfected with Del18 + L792F was lower than those of Del18 cells that acquired C797S or T790M. Similarly, IC_{50} of cells transfected with L858R + L792F was lower than those of cells transfected with L858R + C797S or L858R + T790M. In summary, L792F certainly presented the least resistance to afatinib compared with C797S or T790M.

On the basis of these *in vitro* data, the characteristics of the various resistant mutations against 1G-, 2G-, and 3G-TKIs were summarized in Fig. 3B.

Outcome after the treatment of L792F cells with dacomitinib

The chronic exposure of cells with the Del18 + L792F mutations to dacomitinib until these became resistant to 200 nmol/L, which was selected because the trough concentration in a phase I study was 166 nmol/L (28), induced the additional acquisition of T790M (Del18 + L792F + T790M). These cells were resistant to any single TKIs (Fig. 3A) and even combinations of 2G- and 3G-TKIs: IC_{50}s of osimertinib were 217 and 410 nmol/L in the presence of 200 nmol/L dacomitinib and 100 nmol/L afatinib, respectively.

Outcome after the treatment of C797S cells with 1G-TKIs or afatinib

Tumors with the C797S mutation are sensitive to 1G-TKIs, but these tumors subsequently develop C797S + T790M (30). Cells with C797S + T790M in *trans* are sensitive to combinations of 1G- and 3G-TKIs, whereas those with C797S + T790M in *cis* are resistant to any TKI alone or combinations of TKIs (30). However, the allosteric inhibitor EAI045 is promising for the treatment of tumors with the L858R + C797S + T790M mutations (31).

Because cells with the L858R + C797S mutations exhibited moderate sensitivity to afatinib, we chronically exposed these cells to afatinib until they acquired resistance to 100 nmol/L afatinib, and the treated cells subsequently acquired T790M (L858R + C797S + T790M).

Secondary mutations in rebiopsy samples from patients whose tumors acquired resistance to afatinib

Rebiopsy samples from 5 patients with adenocarcinoma that acquired resistance to afatinib were analyzed, and sufficient amounts of cancer cells for analysis were obtained from four of the five samples (Table 1). T790M was detected in one sample, and the remaining samples did not harbor any secondary mutations, although the original mutations were detected. *MET* gene copy numbers of three evaluable rebiopsy samples (excluding one sample harboring T790M) were 1.8, 2.3, and 1.8 copies, which indicated that *MET* gene was not amplified.

Discussion

In this study, we detected the development of the novel secondary mutations L792F and C797S, in addition to the major T790M, in afatinib-resistant cells. Acquisition of the L792F mutation resulted in intermediate resistance between T790M and C797S to both 1G- and 3G-TKIs, whereas L792F was the least resistant to 2G-TKIs, particularly dacomitinib. In addition, cells with C797S were sensitive to erlotinib. Our study suggests that detecting these secondary mutations is important for overcoming acquired resistance to afatinib.

The novel L792F mutation is located near the gatekeeper T790 site in the ATP-binding pocket of EGFR. Therefore, L792F is anticipated to structurally influence binding between afatinib and EGFR (Fig. 4A). The homologies of protein structure among different receptor tyrosine kinases are well known: both *EGFR* T790M and anaplastic lymphoma kinase (*ALK*) L1196M are considered gatekeeper mutations, and *EGFR* L792 is homologous to *ALK* L1198 (Fig. 4B). Interestingly, it has been reported that *ALK* L1198F resensitizes NSCLC to crizotinib that had become resistant to crizotinib due to the acquisition of C1156Y. The initial response to crizotinib involved C1156Y mutation, and the subsequent administration of the 3G-ALK inhibitor lorlatinib shrunk the tumor and resulted in C1156Y + L1198F mutations. Rechallenge with crizotinib achieved partial response (32). Our data suggest that the initial response to afatinib causes L792F in a subset of tumors and that dacomitinib can be effective for resistant tumors. However, the tumor subsequently acquires L792F + T790M, which is resistant to all TKIs.

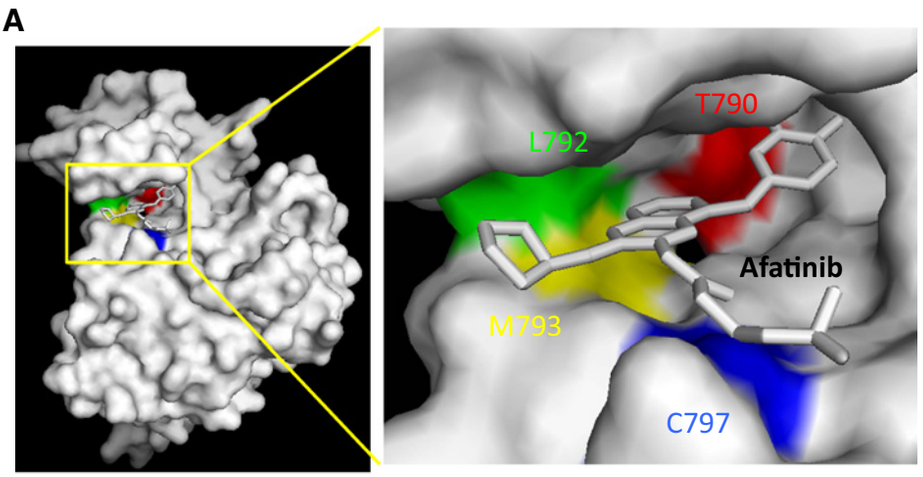
Our discovery of the C797S mutation in afatinib-resistant cells is reasonable because afatinib forms a covalent bond with cysteine 797 of the EGFR (Fig. 4A). The associations between the alteration at the C797 site and 2G-TKI resistance have been hypothesized and described using transfected cells: C797S for CL-387,785 (33), and C797A for neratinib (34). Consistent with previous studies, we observed that the 1G-TKI erlotinib is particularly effective for overcoming the resistant C797S mutation (20, 30, 34). We also found that afatinib moderately inhibited the growth of C797S cells, which concurs with recent other studies (20, 30). Afatinib shares an aniline–quinazoline core structure with erlotinib that is expected to inhibit even in the absence of a covalent bond with C797S. A hydrogen bond is formed between methionine 793 and quinazoline ring of afatinib (35). In contrast, 3G-TKIs do not have an aniline–quinazoline core structure.

The extent of resistance appears to be determined by combinations of the original mutations, secondary mutations, and drugs. As we previously reported, cells expressing Del18 and

Table 1. Summary of clinical data for patients with adenocarcinoma that acquired resistance to afatinib

	Rebiopsy sample	EGFR mutation prior to treatment	Prior EGFR-TKI	Line of afatinib	Best response to afatinib	Duration of afatinib treatment (months)	Secondary mutation after acquisition of resistance
Case 1	Pleural effusion	L858R	Non	2nd	PR	9	T790M
Case 2	Ascites fluid	L858R	Non	1st	PR	4.5	No
Case 3	Pleural effusion	Del19	Erlotinib[a]	1st[a]	PR	8	No
Case 4	Pleural effusion	Del19	Non	2nd	PR	8.5	No
Case 5	Pleural effusion	Del19	Non	2nd	PR	3	Not evaluable

Abbreviation: PR, partial response.
[a]Erlotinib therapy was discontinued because of serious rashes.



B

	Gate keeper							
EGFR	**T790M**	Q791	L792F	M793	P794	F795	G796	**C797S**
ALK	**L1196M**	E1197	**L1198F**	M1199	A1200	G1201	**G1202R**	D1203

Figure 4.
A, Each amino acid residue of the identified *EGFR* mutations was mapped on the crystal structure of EGFR with afatinib. Afatinib forms a covalent bond with C797 and a hydrogen bond with M793. T790, L792, and C797 are located in the ATP-binding pocket. Figures were drawn using the PyMOL Molecular Graphics System (Version 1.7.4 Schrödinger, LLC) based on the crystal structure information from PDB ID 4G5J. **B,** Homology between the EGFR and anaplastic lymphoma kinase (ALK) protein structures. Both *EGFR* T790M and *ALK* L1196M are referred to as gatekeeper mutations. The homologous residues are colored. The secondary mutations that have been reported to serve as mechanisms of acquired resistance to TKIs in patients with lung cancer are indicated in boldface.

G719A are sensitive to afatinib, but their IC_{90} values are increased several fold compared with those of cells with Del19 (11). In addition, acquisition of the L792F, C797S, and T790M mutations was associated with increasing resistance to afatinib in this study. Taken together, it may be reasonable to suggest that Del18 + L792F or C797S, G719A + C797S, and L858R + C797S cells exhibit resistance to the clinically available concentrations of afatinib. Therefore, these secondary mutations might be detected in patients whose tumors harbor moderately sensitive *EGFR* mutations or who require reductions of the afatinib dose.

Furthermore, tumors with different original mutations might have different tendencies to develop specific resistant mutations because even lower concentrations of afatinib were not able to induce L792F or C797S in cells with Del19. This hypothesis is supported by the following two lines of clinical evidence. First, approximately twice as many patients with Del19 + T790M tumors enrolled in the phase I/II trials for osimertinib (29) and rociletinib (36) compared with the number of patients with L858R + T790M tumors. Because the frequencies of the original Del19 and L858R mutations are nearly identical (37, 38), these data indicate that Del19 is more likely to develop T790M against 1G-TKIs compared with L858R. Second, only tumors with Del19 acquired C797S mutation after acquiring resistance to osimertinib (39, 40).

ENU mutagenesis screening using Ba/F3 cells is an efficient tool for the rapid development of resistant cells. This artificial method tends to cause specific base substitutions, such as GC to AT transitions (e.g., T790M and L792F) and AT to TA transversions (e.g., C797S; refs. 41, 42). However, novel mutations obtained through ENU mutagenesis screening have certainly been detected in clinical samples: *EGFR* C797S and L718Q mutations for osimertinib resistance (20, 39, 40, 43) and *ROS1* G2032R mutation for crizotinib resistance (19, 44). In addition, the novel L798I mutation was recently detected in a tumor that acquired resistance to rociletinib (45). Regarding afatinib, Wu and colleagues reported that T790M was detected in 50% (7/14) of rebiopsy samples from 1G-TKI–naïve patients whose tumor acquired resistance to afatinib (15). Our *in vitro* data for L792F and C797S mutations should be confirmed in clinical specimens to suggest that additional subsets other than T790M tumors can overcome acquired resistance to afatinib.

In conclusion, we demonstrate that L792F and C797S in addition to T790M can develop after the acquisition of resistance to afatinib. L792F and C797S should be tested in patients, particularly those whose tumors harbor moderately sensitive original mutations and those who require reductions of the afatinib dose. Patients with T790M tumors can be treated with osimertinib. Additional subsets with L792F or C797S mutations may be good candidates for the treatment with dacomitinib or erlotinib, respectively.

Disclosure of Potential Conflicts of Interest

Y. Kobayashi has received speakers bureau honoraria from Boehringer Ingelheim. Y. Togashi has received speakers bureau honoraria from Boehringer Ingelheim. T. Mitsudomi reports receiving a commercial research grant from Boehringer Ingelheim, has received speakers bureau honoraria from AstraZeneca, Boehringer Ingelheim, and Chugai Pharmaceuticals, and is a consultant/advisory board member for AstraZeneca and Boehringer Ingelheim. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: Y. Kobayashi, Y. Togashi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kobayashi, K. Azuma, H. Nagai, Y.H. Kim, Y. Togashi, Y. Sesumi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kobayashi, H. Nagai, Y.H. Kim, Y. Togashi, Y. Sesumi, M. Chiba, M. Shimoji, K. Sato, K. Tomizawa, T. Takemoto, T. Mitsudomi
Writing, review, and/or revision of the manuscript: Y. Kobayashi, H. Nagai, Y.H. Kim, Y. Togashi, Y. Sesumi, M. Chiba, M. Shimoji, K. Sato, K. Tomizawa, T. Takemoto, K. Nishio, T. Mitsudomi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kobayashi, Y. Togashi, K. Nishio, T. Mitsudomi
Study supervision: T. Mitsudomi

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