The Allelic Context of the C797S Mutation Acquired upon Treatment with Third-Generation EGFR Inhibitors Impacts Sensitivity to Subsequent Treatment Strategies

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Abstract

Purpose: A secondary *EGFR* mutation, T790M, is the most common resistance mechanism in *EGFR*-mutant adenocarcinomas that have progressed on erlotinib. Third-generation EGFR inhibitors capable of inhibiting mutant EGFR with T790M produce responses in nearly two thirds of patients. However, acquired resistance mechanisms in patients treated with these drugs are yet to be described.

Experimental Design: To study acquired resistance to third-generation EGFR inhibitors, T790M-positive cells derived from an erlotinib-resistant cancer were made resistant to a third-generation TKI and then characterized using cell and molecular analyses.

Results: Cells resistant to a third-generation TKI acquired an additional *EGFR* mutation, C797S, which prevented suppression of EGFR. Our results demonstrate that the allelic context in which C797S was acquired may predict responsiveness to alternative treatments. If the C797S and T790M mutations are in trans, cells will be resistant to third-generation EGFR TKIs, but will be sensitive to a combination of first- and third-generation TKIs. If the mutations are in cis, no EGFR TKIs alone or in combination can suppress activity. If C797S develops in cells wild-type for T790 (when third-generation TKIs are administered in the first-line setting), the cells are resistant to third-generation TKIs, but retain sensitivity to first-generation TKIs.

Conclusions: Mutation of C797S in *EGFR* is a novel mechanism of acquired resistance to third-generation TKIs. The context in which the C797S develops with respect to the other *EGFR* alleles affects the efficacy of subsequent treatments. *Clin Cancer Res; 21(17); 3924–33. ©2015 AACR.*

See related commentary by Ayeni et al., p. 3818

Introduction

The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) gefitinib, erlotinib, and afatinib are effective treatments for *EGFR*-mutant non–small cell lung cancers (NSCLCs; refs. 1–5). Although most patients with *EGFR*-mutant NSCLC respond to these therapies, the responses are not permanent, and patients typically develop resistance after an average of 1 year on treatment (6). There are several mechanisms of acquired resistance to erlotinib, including the development of a "gatekeeper" point mutation, T790M, which prevents the TKI from effectively inhibiting EGFR (7, 8), reactivation of downstream signaling pathways via bypass tracks (9–14), and phenotypic/histological changes such as epithelial-to-mesenchymal transition (EMT) or small cell lung cancer (SCLC) transformation (12, 14, 15). T790M is the most common resistance mechanism in these cancers and is observed in over 50% of resistant biopsies (12, 14).

Second-generation EGFR inhibitors, including afatinib (BIBW2992) and dacomitinib (PF00299804), are irreversible EGFR inhibitors that bind to Cys797 and have been shown in preclinical experiments to effectively inhibit EGFR with activating mutations (exon 19 deletion or L858R) as well as those with the T790M resistance mutation (16, 17). However, their activity in patients with erlotinib-resistant cancers harboring T790M has been minimal (18, 19). The discordance between laboratory and clinical results is likely due to a poor therapeutic window. These drugs are equally potent against wild-type EGFR and EGFR T790M, and thus the toxicity resulting from inhibiting wild-type EGFR (rash and diarrhea) precludes the use of doses that would be needed to effectively suppress T790M (20). More recently, third-generation EGFR TKIs, including WZ4002, CO-1686, AZD-9291, and EGF816, have been developed to target mutant EGFR harboring T790M (21–24). This class of inhibitor also binds covalently to Cys797, and largely spares WT EGFR, thereby decreasing toxicity and permitting the use of doses that fully suppress T790M. This large therapeutic window likely underlies the greater than 50% response rates observed in clinical trials with CO-1686 and AZD-9291 in erlotinib-resistant, T790M-positive NSCLCs (25, 26). Based on these promising results, both drugs have received FDA "breakthrough therapy designation," and this class of inhibitors is on the verge of becoming widely implemented for treatment of this patient population. Previous studies have generated acquired resistance to third-generation inhibitors in well-studied cell lines, and have identified mechanisms that have also been observed in cancers with acquired resistance to

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Translational Relevance

Acquired resistance to first-generation EGFR inhibitors is mediated by a secondary mutation to *EGFR*, T790M, in just over half of *EGFR*-mutant non–small cell lung cancer patients. Third-generation EGFR inhibitors that inhibit EGFR with T790M have recently entered clinical testing and have led to remissions in a majority of these cancers. Despite these promising initial results, acquired resistance to these drugs will likely develop. Little is known, however, about mechanisms of resistance to third-generation EGFR TKIs. Herein, we identify the C797S *EGFR* mutation in cells made resistant to a third-generation inhibitor and demonstrate that it is sufficient to promote resistance to third-generation TKIs. Moreover, whether the C797S mutation occurs in cis or trans with T790M determines which subsequent EGFR TKIs, alone and in combination, may be effective. These data suggest an important clinical value in sequencing for this mutation in patients with acquired resistance to third-generation TKI.

first-generation EGFR inhibitors. These include EMT (22), sustained activation of the MAPK kinase pathway (27), and IGF1R bypass signaling as resistance mechanisms (28). Herein, we utilize T790M-positive cells derived from a biopsy of an erlotinib-resistant tumor to cultivate resistance to a third-generation EGFR TKI. In doing so, we identify a widely anticipated resistance mechanism specific to third-generation EGFR inhibitors, a C797S resistance mutation, that prevents this class of drugs from effectively suppressing EGFR activity. We also determine that the presence of T790M, whether in cis or trans to C797S, markedly affects efficacy of subsequent therapeutic strategies.

Materials and Methods

Reagents and cell culture

MGH121, MGH121 Res#1 and PC9 cells were cultured in RPMI with 10% serum. 293FT cells were cultured in DMEM with 10% serum. PC9 cells were a gift from Pasi Janne (Dana Farber Cancer Institute), 293FT cells are from Invitrogen. MGH121 cells were generated from a pleural effusion of an erlotinib-resistant NSCLC patient on July 19, 2011, and were originally developed in ACL4 supplemented with 10% serum. Once completed, the cell line was sequenced to confirm that it matched the patient effusion sample. Experiments involving the 293FT cells were completed within 6 months of purchasing from Invitrogen and did not undergo any further testing. PC9 cells were verified by STR analysis within 6 months to 1 year of experimentation. Gefitinib, afatinib, WZ4002, CO-1686, and AZD-9291 were purchased from Selleck and resuspended in DMSO. pEGFR antibody (pY1068) was from Abcam, and total EGFR was from Santa Cruz Biotechnology. pERK (T202/Y204), total ERK, pS6 (S240/244), total S6, Actin, pAKT (T308), and total AKT were purchased from Cell Signaling Technology. All antibodies were used at a concentration of 1:1,000.

Generating *in vitro*–resistant cell line

MGH121 was derived from a pleural effusion of an erlotinib-resistant patient. The procedure for establishing this line has been described previously (15, 29, 30). To generate resistance in this model, MGH121 parental cells were grown in increasing doses of

WZ4002 starting at 10 nmol/L and increasing to 30, 100, 300, and 1,000 nmol/L incrementally once the cells began to grow through the given dose.

Generating lentiviral constructs, lentivirus, and stable expression cell lines

Lentiviral constructs and lentivirus were generated using the Vira-Power Lentiviral Directional TOPO Expression Kit (Life Technologies) per the manufacturer's protocol. Briefly, *EGFR* exon 19 del and exon 19 del/T790M plasmids were purchased from Addgene (32062, 32072). Quikchange site-directed mutagenesis kit (Stratagene) was used to introduce the C797S mutation into both constructs using the following primers: C797S F – CATGCCCTTCGGCTCCCTCCTG-GACTA and C797S R – TAGTCCAGGAGGGAGCCGAAGGGCATG. The resulting *EGFR* exon 19 del/C797S and *EGFR* exon 19 del/C797S/T790M constructs were used as a template to amplify EGFR for ligation into the pLENTI6/V5-D-TOPO vector. Virus was made by transfecting the pLENTI6 constructs along with helper plasmids in 293FT cells. Virus production, collection, and infection were completed following the manufacturer's protocol. The PC9 and MGH121-infected cells were selected beginning 24 hours after infection using Blasticidin at a concentration of 5 μg/mL.

Overexpressing EGFR mutants in 293FT cells

EGFR-mutant constructs (see section on generating lentivirus) were transfected into 200k/well 293FT cells in a 6-well dish using TransIT-LT1 transfection reagent (Mirus) per the manufacturer's protocol. Forty-eight hours after infection, cells were treated with TKI inhibitor for 6 hours prior to lysis.

Cell viability assays

Three-day viability assays were carried out by plating 4000 cells/well into black-bottom 96-well plates. The following day, cells were drugged with TKI across a 10-dose range from 1 nmol/L to 30 μmol/L. Seventy-two hours after drug treatment, cell viability was measured using CellTiter-Glo (Promega).

Crystal violet viability assays were completed by plating 100k cells/well into 6-well plates. The following day, cells were drugged with TKI across a 5-dose range from 10 nmol/L to 1,000 nmol/L and redrugged after 4 days. The crystal violet staining was carried out 7 days following the initial drug treatment.

DNA sequencing and allele quantification assay

gDNA was prepared from the MGH121 parental and MGH121 Res#1 cells using the DNeasy kit from Qiagen. *EGFR* exons 19 and 20 were PCR amplified using the following primers (31): Exon 19 F–GGTAACATCCACCCAGATCAC, exon 19 R–TGAGCAGGGTC-TAGAGCAGAG; exon 20 F–GAAGCCACACTGACGTGC and exon 20 R–CTCCTTATCTCCCCTCCCCG. Following amplification, Sanger sequencing was completed by the Sequencing group of the CCIB DNA Core Facility at Massachusetts General Hospital. To quantify the allele frequencies, the PCR products were ligated into the TOPO TA for sequencing vector (Life Technologies). The ligations were transformed into competent bacteria and plated. Each bacterial colony should carry a plasmid that contains DNA that started as a single PCR product. For each sequencing reaction, plasmid DNA from approximately 100 bacterial colonies was isolated and sequenced (CCIB DNA Core Facility at Massachusetts General Hospital). Sequencing data were analyzed, and the relative frequency of the exon 19 del, T790M, and C797S mutations was quantified.

Quantification of EGFR gDNA levels

EGFR gDNA levels were quantified by a qPCR-based method. Ten nanograms of MGH121 parental and MGH121 Res#1 DNA were used as a template for a SBYR green–based qPCR assay using primers 20F and 20R to amplify genomic DNA of EGFR. LINE-1 was used as a loading control: LINE1 F–AAAGCCGCTCAACTA-CATGG, LINE 1 R–TGCTTTGAATGCGTCCCAGAG. Normal female DNA served as a diploid control.

Results

Cell lines derived from biopsies of cancers that have become resistant to targeted therapies have been a valuable tool for studying acquired resistance to targeted therapies (32, 29). In addition, cultivating resistance to targeted therapies *in vitro* has yielded clinically validated resistance mechanisms (10, 14, 33–36). To study acquired resistance to third-generation EGFR TKIs, we took advantage of a cell line model that was derived from a biopsy of a resistant *EGFR*-mutant (exon 19 del) tumor that had developed T790M in the clinic after 7 months on erlotinib. This cell line, MGH121, was resistant to the first-generation EGFR TKI gefitinib, consistent with the presence of the T790M resistance mutation, but was highly sensitive to the third-generation TKI WZ4002 (Fig. 1A). To model acquired resistance to third-generation inhibitors in these cells, we cultured them in increasing doses of WZ4002, starting at 10 nmol/L and raising the concentration incrementally until the cells were growing in 1 μmol/L. This common approach for generating resistance *in vitro* has proven to consistently produce clinically relevant resistance mechanisms (10, 32). The resulting resistant model, MGH121
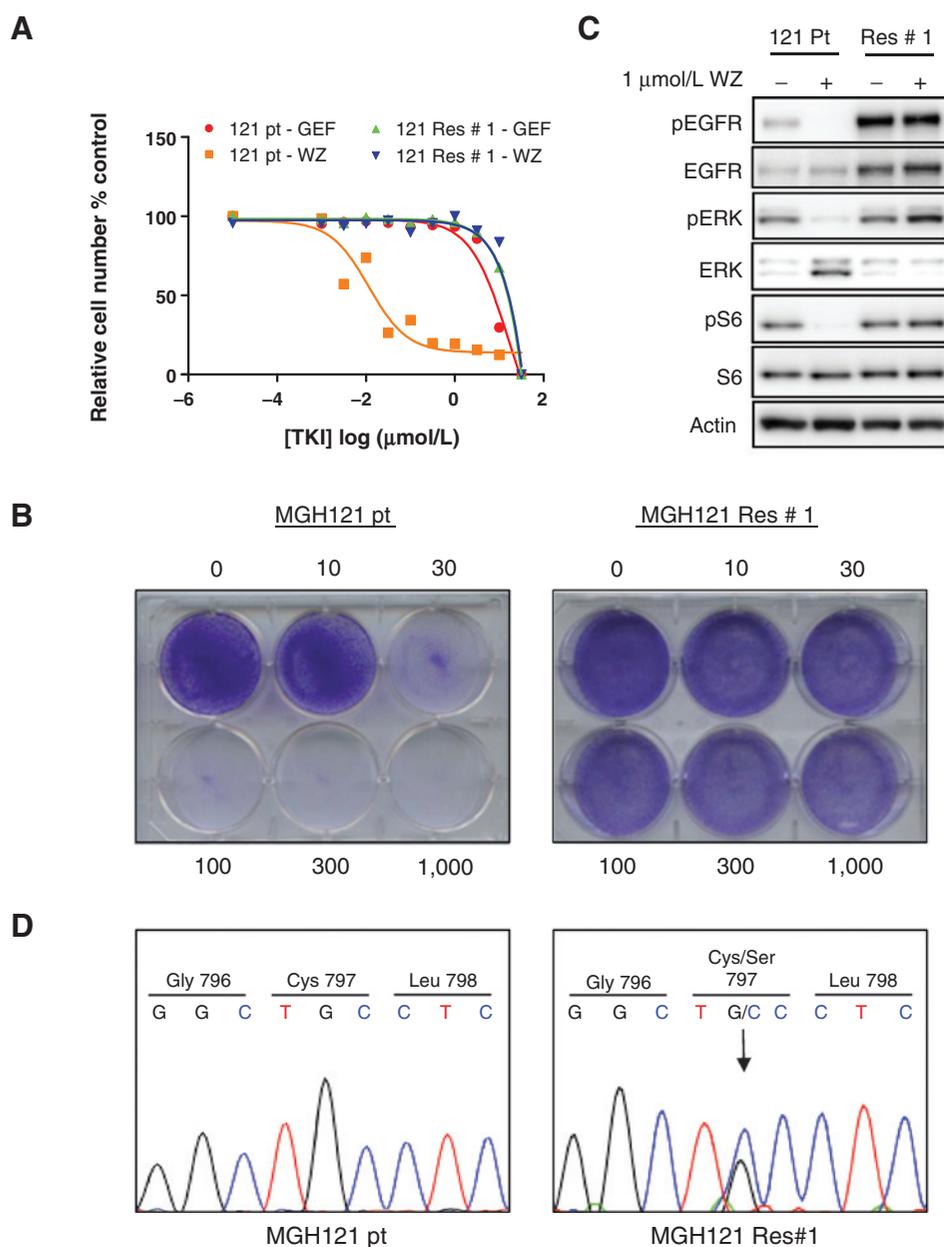

Figure 1.
A WZ4002-resistant cell line acquires a C797S mutation and maintains EGFR activity in the presence of TKI. A, MGH121 parental (pt) and MGH121 WZ4002 Resistant#1 (Res#1) cells were treated with the indicated concentrations of the first-generation EGFR TKI gefitinib (GEF) or the third-generation EGFR TKI WZ4002 (WZ) for 72 hours. Cell viability was measured by CellTiter-Glo. Experiments were performed in quadruplicate, and error bars depict SEM. B, MGH121 parental and Res#1 cells were treated with the indicated concentrations of WZ4002 (nmol/L) for 1 week and then stained with crystal violet. C, lysates from MGH121 parental and MGH121 Resistant#1 cells treated with DMSO or 1 μmol/L WZ4002 for 6 hours were probed with the indicated antibodies. D, chromatograms depicting the acquired C797S mutation present in MGH121 Resistant#1, but not in MGH121 parental cells. The arrow is pointing to the mutated base (G is WT-Cys, C is mt–Ser).

Res#1, was highly resistant to WZ4002 (Fig. 1A and B), and, in addition, WZ4002 treatment failed to suppress EGFR phosphorylation and downstream signaling in these cells (Fig. 1C). We hypothesized that this failure of the third-generation TKI to inhibit the target may result from an additional *EGFR* mutation. Sequencing of the kinase domain of *EGFR* revealed a single point mutation resulting in a cysteine to serine change at position 797 (C797S). This mutation was present in the MGH121 Res#1 cells, but not in the MGH121 parental cells (Fig. 1D). C797 is located opposite T790 in the ATP-binding pocket and forms a covalent bond with all third-generation TKIs and is necessary for the inhibitor to suppress EGFR (21). Thus, C797S is an excellent candidate for a resistance mutation.

The higher levels of EGFR protein in the MGH121 Res#1 cells compared with the parental cells (Fig. 1C) combined with an increased relative abundance of the mutated nucleotide that encodes for the serine on the chromatogram (Fig. 1D) raised the possibility that amplification of *EGFR* carrying C797S may have occurred in the MGH121 Res#1 cells. We carried out qPCR of the *EGFR* gene using genomic DNA as a template to measure the relative *EGFR* copy number. Compared with normal diploid control DNA, the parental MGH121 cells have over 3.5-fold more *EGFR* gDNA, equal to roughly 7 copies of *EGFR* per cell on average (Supplementary Fig. S1A). MGH121 Res#1 have over 9-fold more *EGFR* gDNA compared with normal control DNA, equal to roughly 18 copies of *EGFR* and consistent with amplification versus the MGH121 parental cells. Further, variant allele frequency analysis revealed that the C797S mutant allele was specifically amplified in the MGH121 Res#1, as the majority of *EGFR* alleles harbor this mutation (Supplementary Fig. S1B).

As a group, the second- and third-generation irreversible EGFR inhibitors are able to suppress the *in vitro* growth of cells with an *EGFR*-activating mutation and T790M. Indeed, MGH121 parental cells are also sensitive to the second-generation inhibitor afatinib and other third-generation inhibitors CO-1686 and AZD-9291 (Fig. 2A, left). These inhibitors similarly bind covalently to C797 of EGFR (22, 23, 37) and are ineffective at suppressing the growth of MGH121 Res#1 cells (Fig. 2A right; although, as discussed in the Introduction, the dose of the second-generation inhibitors required to inhibit T790M cannot be achieved in patients). The sensitivity of the MGH121 parental and Res#1 cells to different EGFR TKIs correlates with the ability of the inhibitors to block EGFR signaling. In the MGH121 parental cells, gefitinib failed to suppress EGFR phosphorylation and downstream pathway components ERK and S6, whereas afatinib, WZ4002, CO-1686, and AZD-9291 were all able to do so (Fig. 2B). In contrast, none of the EGFR TKIs suppressed EGFR signaling in MGH121 Res#1 cells. To confirm that the sustained EGFR phosphorylation in the presence of the third-generation TKI was due to the C797S mutation, we engineered different *EGFR* mutants and overexpressed them in 293T cells. These include *EGFR* exon 19 del/T790M and *EGFR* exon 19 del/T790M/C797S (Fig. 2C). In this system, EGFR exon 19 del/T790M phosphorylation was effectively inhibited by second- and third-generation TKIs, while phosphorylation of EGFR exon 19 del/T790M/C797S was not suppressed by any of the inhibitors tested (Fig. 2C). To determine if the C797S mutation alone was sufficient to promote resistance to second- and third-generation TKIs in an *EGFR*-mutant cell line, we stably expressed the *EGFR* exon 19 del/T790M/C797S in MGH121 parental cells. Neither afatinib nor WZ4002 was able to block EGFR phosphorylation or the downstream signaling in these cells (Fig. 2D).

Accordingly, the growth of these cells was resistant to all generations of EGFR TKIs (Fig. 2E). Together, these data demonstrate that the C797S mutation is sufficient to promote resistance to second- and third-generation EGFR inhibitors by preventing suppression of the target.

We next wanted to identify whether the C797S mutation occurred in cis (on the same allele) or trans (on a different allele) with T790M in MGH121 Res#1 cells. To address this question, we amplified exon 20 by PCR (exon 20 contains the nucleotides encoding for both T790 and C797) using a genomic DNA template and assessed 119 individual PCR products via a TOPO cloning protocol (see Materials and Methods). This analysis revealed that the T790M and C797S were in cis in MGH121 Res#1 cells, as the C797S mutation coexisted with T790M on 85% of alleles (n = 101). The other 15% of alleles had no mutations in exon 20. Thus, there were no exon 20 alleles harboring only T790M or C797S (n = 18; Fig. 3A).

Whether the C797S mutation is in cis or trans with T790M may have important biological implications. As demonstrated above, *EGFR* with an activating mutation, T790M and C797S all in cis, as observed in the MGH 121 Res#1 cells, leads to resistance to all three generations of EGFR TKI (Fig. 2). To determine what would happen if the C797S and T790M mutations were both present in the same cell but on different *EGFR* alleles (i.e., in trans), we overexpressed several potential *EGFR*-mutant allele configurations in 293T cells and examined their response to different EGFR TKIs. First, we tested the exon 19 del/T790M and exon 19 del/C797S double mutants independently. As expected for exon 19 del/T790M, the first-generation TKI gefitinib did not inhibit EGFR phosphorylation, while afatinib and WZ4002 suppressed it (Fig. 3B, lanes 1–4). Conversely, EGFR with the exon 19 del/C797S double mutant was inhibited by gefitinib and afatinib, but not by WZ4002 (Fig. 3B, lanes 5–8). Thus, a C797S mutant, even in the absence of T790M, would clearly lead to resistance to a third-generation TKI. This scenario might develop when *EGFR*-mutant cancers are treated with third-generation inhibitors in the first-line setting, and importantly, such cancers might be sensitive to single-agent first-generation EGFR inhibitors. In addition, these data predict that a combination of first- and third-generation inhibitors would be effective if the C797S and T790M mutations were in trans. When exon 19 del/T790M and exon 19 del/C797S were coexpressed in the same cells to model the trans scenario, treatment with either gefitinib or WZ4002 alone each partially suppressed EGFR phosphorylation, consistent with each drug suppressing a fraction of the EGFR protein expressed in the cells. The combination of gefitinib and WZ4002, however, completely inhibited EGFR phosphorylation (Fig. 3B, lanes 9–13). Finally, EGFR in the triple mutant (cis scenario) was resistant to all three generations of TKI as well as the combination of gefitinib and WZ4002 (Fig. 3B, lanes 14–18). Of note, the second-generation inhibitor, afatinib, suppressed EGFR when C797S and T790M were in trans. However, we know from clinical experience that afatinib is unable to inhibit T790M in the clinic due to a lack of a therapeutic window, and thus the combination of a first- and third-generation TKI holds more promise.

We also modeled both the cis and trans configurations in the parental MGH121 cells by stably expressing either the triple mutant to recapitulate the cis scenario or the exon 19 del/C797S for the trans situation (MGH121 parental cells carry an endogenous exon 19 del/T790M allele). We observed results consistent with those from the previous overexpression
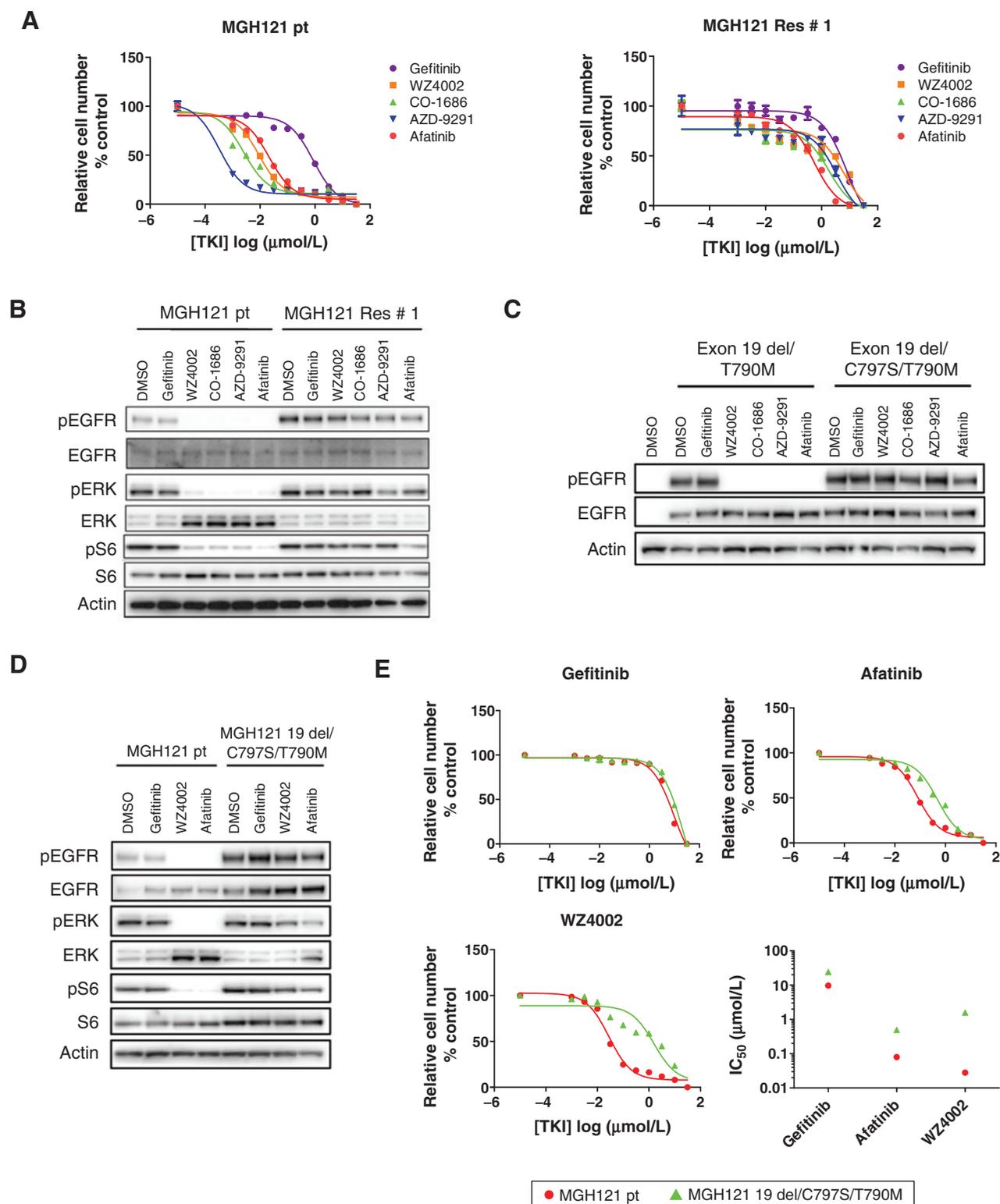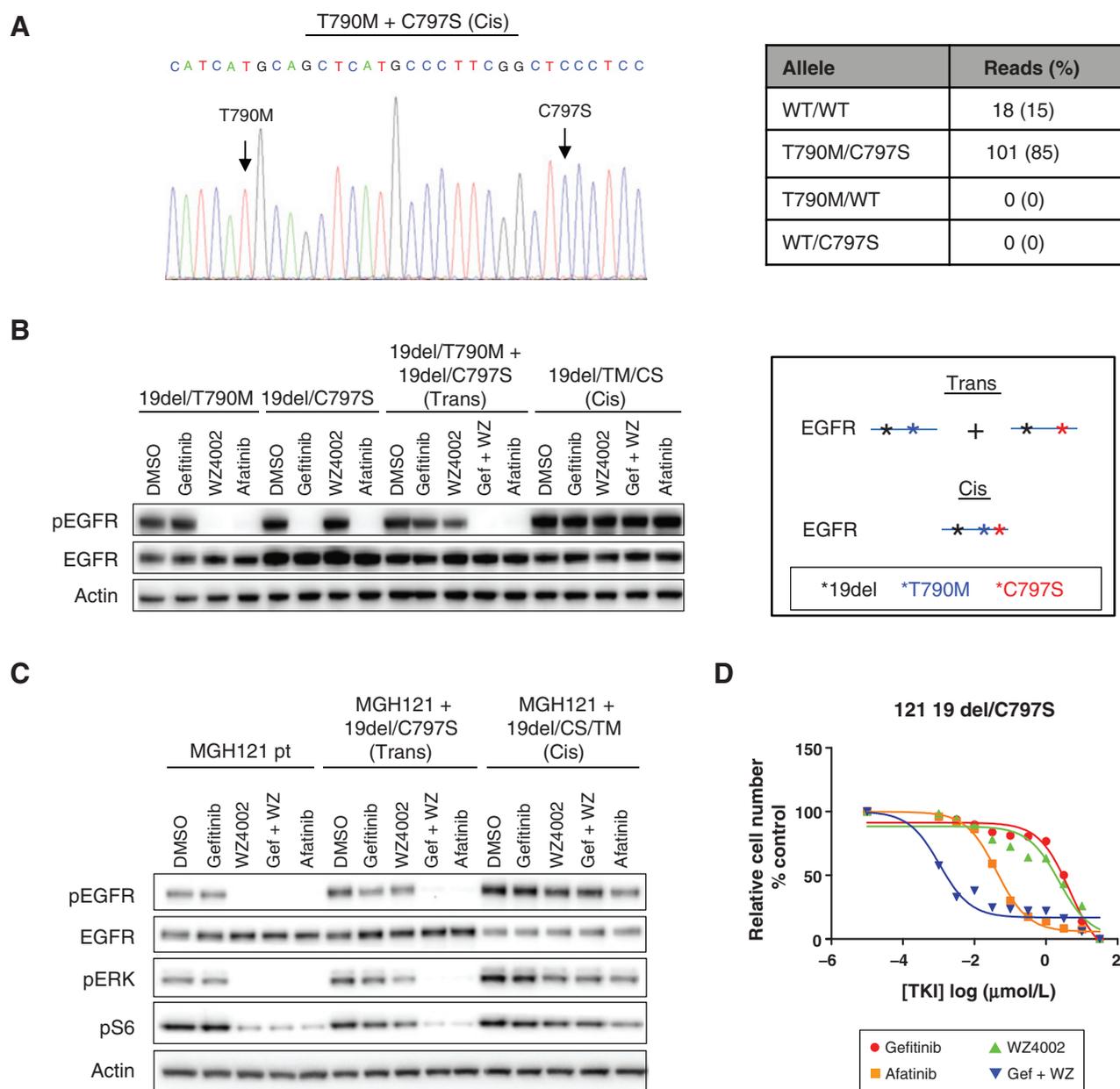
Figure 2.
EGFR C797S confers resistance to all second- and third-generation EGFR TKIs. A, MGH121 parental (left) and Resistant#1 (right) cells were treated with the indicated concentrations of EGFR TKIs for 72 hours. CellTiter-Glo was used to measure cell viability. Experiments were carried out in triplicate, and the error bars depict SEM. B and C, lysates from MGH121 parental and MGH121 Res#1 cells (B) and 293T cells overexpressing EGFR-mutant constructs (C) treated with 1 μmol/L of the indicated TKIs except for AZD9291 (160 nmol/L) for 6 hours were probed with the indicated antibodies. D, lysates from 121 parental and 121 parental stably overexpressing EGFR exon 19 del/T790M/C797S treated with 1 μmol/L of the indicated EGFR TKIs for 6 hours were probed with the indicated antibodies. E, the same cells were treated with the indicated doses of EGFR TKI for 72 hours. CellTiter-Glo was used to measure cell viability. Experiments were completed in triplicate, and error bars depict SEM. IC_{50} values for each TKI and cell line are shown (bottom right).

Figure 3.
C797S located in cis or trans with T790M alters drug sensitivity. A, an exon 20 PCR reaction was ligated into a TOPO vector, and individual colonies representing a unique PCR product were sequenced. Left, representative chromatograph of the T790M and C797S mutations in cis. Right, quantification of allelic reads. B, left, Western blot of 293T cells overexpressing the indicated *EGFR*-mutant constructs and treated for 6 hours with the indicated drugs at a concentration of 1 μmol/L. Lysates were probed with the antibodies shown. Right, schematic representation of the T790M and C797S alleles in cis and in trans. C, Western blot of MGH121 parental cells and MGH121 cells stably overexpressing the indicated *EGFR*-mutant constructs were shown. Cells were treated as in B. Lysates were probed with the indicated antibodies. D, MGH121 cells stably expressing exon 19 del/C797S were treated with the indicated concentrations of EGFR TKIs for 72 hours. GEF + WZ is 1 μmol/L of gefitinib plus the indicated doses of WZ4002. CellTiter-Glo was used to measure cell viability. Experiments were carried out in triplicate, and the error bars depict SEM.

experiment: The trans setting induced resistance to third-generation TKI, but the combination of gefitinib and WZ4002 inhibited EGFR signaling (Fig. 3C) and cell growth (Fig. 3D). However, this combination was not effective when all the mutations were in cis. These experiments demonstrate an important difference in drug response if the T790M and C797S mutations occur on the same or on different alleles, a result that has clinical implications.

To date, the majority of clinical trials of third-generation EGFR TKIs have been conducted in the setting of erlotinib-resistant disease, but clinical trials testing these therapies in TKI-naïve patients are currently ongoing (NCT02296125 and NCT02186301). The finding that the C797S mutation in the absence of T790M is sufficient to cause resistance to third-generation TKIs but retains sensitivity to gefitinib raises the
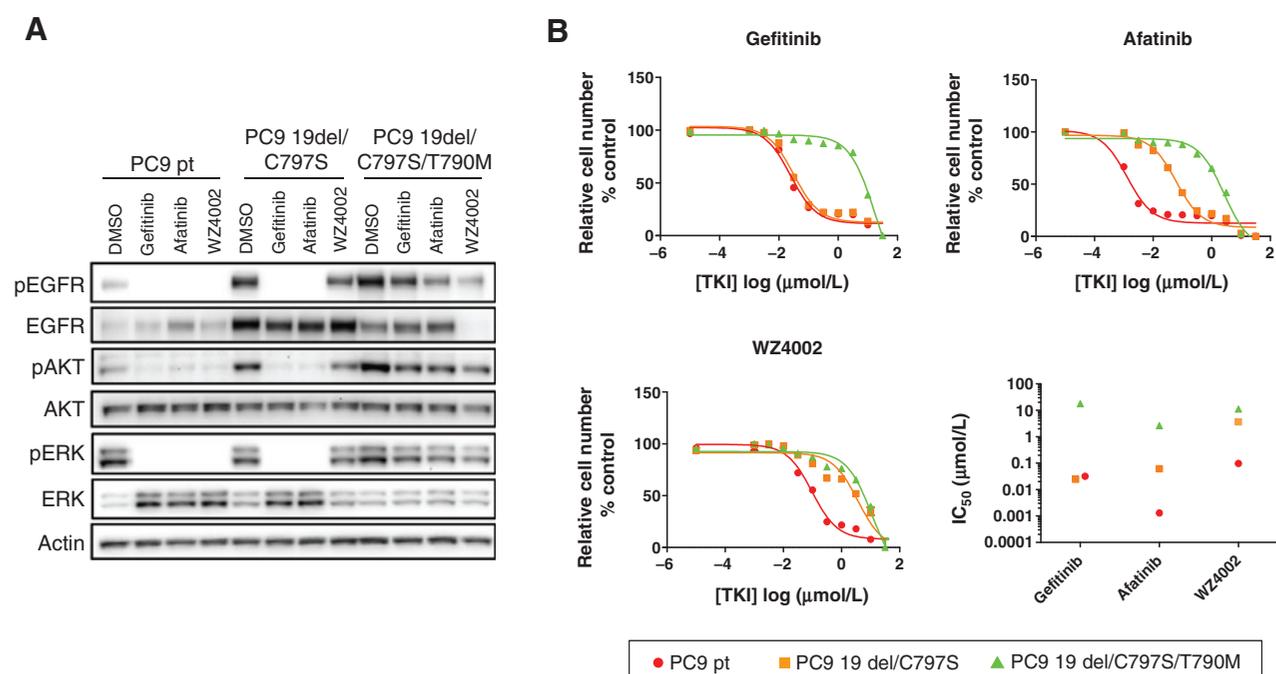
A

B

Figure 4.
Cells resistant to third-generation TKI in the first-line setting via C797S mutation retain sensitivity to first-generation TKI. A, Western blots of lysates from PC9 parental cells and PC9 cells stably overexpressing the indicated EGFR-mutant constructs treated with the indicated TKIs at 1 µmol/L. B, PC9 parental or PC9 cells stably expressing exon 19 del/C797S or exon 19 del/T790M/C797S were treated with the indicated concentrations of EGFR TKIs for 72 hours. CellTiter-Glo was used to measure cell viability. Experiments were carried out in triplicate, and the error bars depict SEM. IC$_{50}$ values for each TKI for both cell lines are shown (bottom right).

intriguing possibility that patients with acquired resistance to third-generation TKI in the first-line setting may subsequently respond to first- or second-generation TKIs in the second-line setting. To test this hypothesis, we stably expressed *EGFR* with exon 19 del/C797S in a TKI-naïve cell line, PC9, which is sensitive to all classes of EGFR inhibitors. PC9 exon 19 del/C797S cells were indeed resistant to WZ4002, but sensitive to gefitinib and afatinib (Fig. 4A and B). By comparison, PC9 triple-mutant cells were resistant to all EGFR inhibitors that were tested. This result supports the notion that patients that are treated with third-generation TKI as their first EGFR inhibitor and acquire resistance driven by C797S may subsequently respond to first-generation TKI. Of note, the concentration of afatinib required to suppress the growth of cells expressing the exon 19 del/C797S construct was markedly higher than the PC9 parental cells (Fig. 4B), suggesting that the C797S mutation leads to reduced potency of this drug. It remains to be determined whether the dose of afatinib necessary to inhibit mutant EGFR with a C797S mutation alone is achievable in patients given the aforementioned narrow therapeutic window.

Discussion

The efficacy of tyrosine kinase inhibitors has been limited by acquisition of resistance mutations that prevent inhibition of the target. For patients treated with the first-generation EGFR TKIs gefitinib and erlotinib, the T790M resistance mutation has been observed in over half of resistant biopsies (12, 14). In this paradigm and others, next-generation TKIs

have been developed to suppress oncogenic kinases that harbor resistance mutations. For T790M-positive *EGFR*-mutant NSCLCs, third-generation EGFR TKIs have been particularly effective and are poised to be approved by the FDA in this setting. In this study, we cultivated resistance to a third-generation EGFR TKI in a T790M-positive cell line established from an erlotinib-resistant biopsy. In these resistant cells, we report that another mutation to *EGFR*, C797S, prevents EGFR inhibition and promotes resistance to third-generation EGFR TKIs. The initial study characterizing WZ4002 revealed that the covalent interaction between EGFR and the inhibitor is mediated by this cysteine residue and is necessary for the activity of third-generation inhibitors in an *in vitro* kinase assay, and thus mutation of this amino acid is an excellent candidate for a resistance mutation (21). To our knowledge, our data serve as the first evidence that C797S can develop in an *EGFR*-mutant cancer model treated chronically with a third-generation EGFR TKI.

Our results suggest that treatment history and genetic background may have important therapeutic implications for cancers that have acquired a C797S mutation (Fig. 5). For example, patients treated with a third-generation TKI as their first EGFR inhibitor may develop C797S in the absence of a T790M mutation. Our data indicate that first-generation TKIs do not depend on a cysteine at position 797 in order to block EGFR. As a result, a cancer that developed C797S as a resistance mechanism to first-line therapy with a third-generation EGFR TKI may subsequently respond to a first-generation inhibitor. In agreement with our findings, erlotinib has previously been shown to be capable of suppressing EGFR with a mutation at
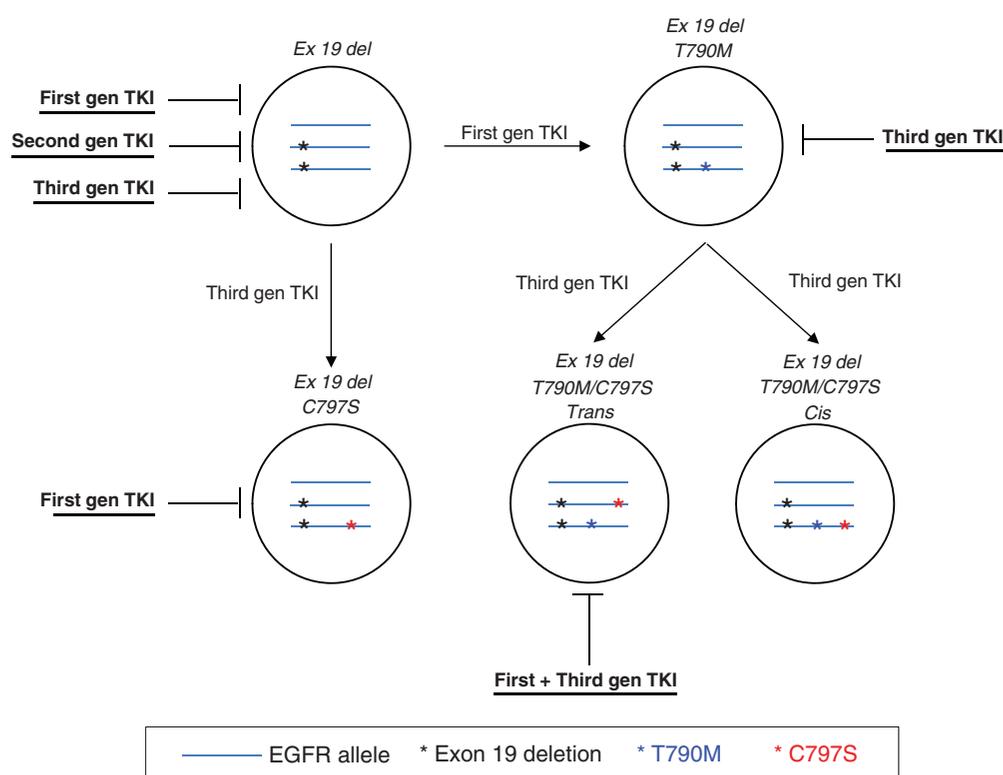
Figure 5.
Schematic representation of EGFR resistance mutations in response to TKI treatment and sensitivity to subsequent therapies. Arrows indicate the treatment that the cancers have developed resistance to. Inhibitors shown in bold and underlined indicate the therapies that would potentially be effective in that cancer. Italics represent the mutations present in each case.

C797 (38). This finding may also affect the current debate regarding whether it is better to give a third-generation inhibitor in the first-line or resistance setting. Importantly, these results suggest that combining first- and third-generation TKIs in the first-line setting may be particularly powerful because neither a T790M nor a C797S mutation alone would be sufficient to drive resistance to this combination.

For T790M-positive, erlotinib-resistant NSCLCs that develop a C797S mutation following treatment with a third-generation TKI, the configuration of the T790M and C797S mutations affects how the cells could respond to therapy. Indeed, if the two mutations are in trans (on separate alleles), we have shown that a combination of first- and third-generation TKIs can restore EGFR inhibition. Conversely, if the two mutations are in cis (on the same allele), the cells are refractory to any of the EGFR TKIs we tested as well as the combination of first- and third-generation inhibitors. Clinical assessment of the cis versus trans configurations can likely be determined by next-generation sequencing approaches, as the T790M and C797S mutations are in close enough proximity to coexist on a significant number of individual sequencing reads. PKC inhibitors such as midostaurin (PKC412) have recently been shown to noncovalently inhibit EGFR with T790M and thus may be able to suppress mutant EGFR with T790M and C797S in cis (39). However, these drugs are limited by their lack of specificity, and thus the potential for therapeutic window in this setting is uncertain.

The covalent interaction with C797 has also been demonstrated to be important to the activity of the pan-HER inhibitor CI-1033

(40, 41), and exogenous expression of a C797A mutant can confer resistance to another second-generation inhibitor, HKI-272 (38). Our data reveal that at a concentration of 1 μmol/L, afatinib can inhibit mutant EGFR with C797S in the absence of T790M. Importantly, however, expression of mutant EGFR with C797S in PC9 cells results in a significant increase in the IC_{50} of afatinib compared with parental PC9 cells. Of note, a similar reduction in activity of second-generation inhibitors toward EGFR is caused by T790M (38, 42). This decreased activity of second-generation TKIs toward mutant EGFR with T790M diminishes the therapeutic window in these cancers, resulting in the lack of clinical efficacy. It is likely that the same will be true for resistant *EGFR*-mutant cancers harboring C797S.

Third-generation EGFR inhibitors are proving to be effective treatments for erlotinib-resistant cancers with T790M mutations and will soon become clinically available. Our results indicate that mutation of the C797 residue may be a mechanism by which these cancers progress on this class of drugs. Sequencing biopsy samples from patients whose tumors have progressed on third-generation TKIs and determining if the C797S mutation is in cis or trans with T790M should be a priority going forward. Designing a treatment strategy that can suppress triple-mutant EGFR may soon be needed.

Disclosure of Potential Conflicts of Interest

Niederst et al.

Authors' Contributions

Grant Support

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