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The T790M "gatekeeper" mutation in *EGFR* mediates resistance to low concentrations of an irreversible EGFR inhibitor

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Abstract

Patients with non–small cell lung cancer (NSCLC) harboring activating mutations in the epidermal growth factor receptor (*EGFR*) kinase domain tend to respond well to the tyrosine kinase inhibitors, gefitinib and erlotinib. However, following clinical response, these patients typically relapse within a year of treatment. In many cases, resistance is caused by an acquired secondary *EGFR* kinase domain mutation, *T790M*. *In vitro* studies have shown that a new class of EGFR-irreversible inhibitors could overcome the resistance conferred by *T790M*. Clinical trials are under way to examine the efficacy of one of these inhibitors, HKI-272, in patients with NSCLC who initially responded to gefitinib/erlotinib and subsequently relapsed. To anticipate the possibility that patients who respond to irreversible inhibitors will develop secondary resistance to such inhibitors, as has been seen in other similar settings, we modeled acquired resistance to the dual EGFR/HER2-irreversible tyrosine kinase inhibitor HKI-272 in a NSCLC cell culture model. We found that HKI-272–resistant clones fall into two biochemical groups based on the retention of EGFR phosphorylation in the presence of the drug. Cells that retain phosphorylated EGFR have acquired the secondary mutation *T790M*. Moreover, HKI-272 can overcome *T790M* resistance only at supra-pharmacologic concentrations. We further model mutations at *EGFR C797* as a mechanism of resistance to irreversible EGFR inhibitors and show that although these mutants are resistant to the irreversible inhibitor, they retain erlotinib sensitivity. Our findings suggest that HKI-272 treatment at maximally tolerated dosing may lead to the emergence of *T790M*-mediated resistance, whereas treatment with a more potent irreversible inhibitor could yield a resistance mutation at *EGFR C797*. [Mol Cancer Ther 2008;7(4):874–9]

Introduction

Tyrosine kinase inhibitors (TKI) have emerged as important agents in the treatment of several different human cancers. For example, non–small cell lung cancers (NSCLC) harboring mutant epidermal growth factor receptor (EGFR) often respond well to treatment with the EGFR kinase inhibitors gefitinib and erlotinib (1–3). These TKIs compete with ATP in a reversible manner to bind the kinase domain of their targets. Although the initial response to treatment with these agents can be dramatic, most patients eventually relapse due to the acquisition of drug resistance. In approximately half of NSCLC cases that showed an initial response to EGFR TKIs and subsequently progressed, resistance is associated with the emergence of a single recurrent missense mutation *T790M* within the EGFR kinase domain (4–6). The bulkier methionine residue at position 790 sterically hinders the interaction with inhibitor, effectively preventing binding to the EGFR kinase domain while preserving catalytic activity. An analogous "gatekeeper" mutation (T315I) in the BCR-ABL fusion kinase in chronic myelogenous leukemia cancer cells renders these leukemias resistant to the ABL kinase inhibitors gleevec and dasatinib, suggesting a conserved mechanism of resistance to TKIs (7–9).

Cell-based studies have suggested that an alternative class of irreversible EGFR inhibitors, which bind covalently to EGFR, may be capable of overcoming the effects of the EGFR *T790M* resistance mutation, thereby potentially providing a second-line treatment to manage such resistance (5, 10). Several of these inhibitors are currently undergoing clinical testing in patients that initially responded to gefitinib or erlotinib and subsequently relapsed (11). Other irreversible inhibitors that target both EGFR and the related protein ErbB2 are also being tested in clinical trials of NSCLC as well as breast cancer. Although the clinical efficacy of this class of inhibitors has yet to be

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shown, previous experience with TKIs indicates that tumor cells will eventually acquire resistance to even the most effective TKIs. To anticipate molecular mechanisms that may underlie acquired resistance to irreversible TKIs, we modeled acquired resistance to the dual EGFR/HER2-irreversible kinase inhibitor HKI-272 in a NSCLC cell culture model.

Materials and Methods

Cell Culture Studies

To generate resistant subclones of PC-9 cells, these were treated with 400 μg/mL of ethyl methane sulfonate overnight. They were allowed to recover for several passages, and then seeded at a density of 6×10^4 cells/10 cm^2 dish in 0.2 μmol/L of HKI-272. The relative resistance of these cells to HKI-272, compared with the reversible inhibitors gefitinib and erlotinib, was achieved by seeding 3,000 cells/well in a 96-well plate in the presence of varying concentrations of drugs, followed after 72 h by fixing cells with 4% formaldehyde, staining with Syto 60, and quantifying cell mass by using the Odyssey Infrared Imaging System (LI-COR Biosciences).

NIH3T3 cells were cultured in DMEM supplemented with 10% bovine serum, glutamine and penicillin/streptomycin. Epidermal growth factor (Sigma) was used at a final concentration of 100 ng/mL. Gefitinib and erlotinib were obtained from the Massachusetts General Hospital pharmacy. HKI-272 was provided by Wyeth Pharmaceuticals. *EGFR* wild-type, *EGFR C797A, EGFR L858R,* and *EGFR C797A/L858R* were subcloned into pUSEamp for transient transfection of 293T cells (LipofectAMINE 2000, Invitrogen). *EGFR* wild-type and *EGFR T790M* pRV-HygR constructs were previously described (12). *EGFR L858R, EGFR Del746-750, EGFR C797A/L858R,* and *EGFR C797A/Del746-750* were subcloned into pBABE puro for infection of NIH3T3 cells. For retroviral infection studies, *EGFR* pRV-HygR or pBABE puro constructs were transfected into the 293T-derived BOSC-23 packaging cell lines. NIH3T3 cells were infected with the retroviral supernatant twice in the presence of polybrene. Infected cells were selected using hygromycin (700 μg/mL; Roche) or puromycin (2.5 μg/mL; Sigma).

Immunoblot Analysis

Cells were lysed with ice-cold buffer [150 mmol/L NaCl, 1% Triton X-100, 50 mmol/L Tris-HCl (pH 8.0), and 1 mmol/L EDTA] containing 1 mmol/L of sodium orthovanadate and 1× protease inhibitor cocktail (Roche). Debris was removed by centrifugation. Clarified lysates were boiled in gel-loading buffer and separated by 4% to 15% gradient SDS-PAGE. Proteins were electrotransferred to nitrocellulose and detected with specific antibodies. The phosphorylated EGFR Y992 antibody was obtained from Biosource, total EGFR antibody was from Santa Cruz Biotechnology, phosphorylated tyrosine antibody was from BD Transduction Laboratory, and β-tubulin antibody was from Abcam. This was followed by a horseradish peroxidase–conjugated secondary antibody. The bands were visualized with enhanced chemiluminescence (Perkin-Elmer) followed by autoradiography.

Analysis of EGFR Mutations

Multiple clones of exon 20 were sequenced to examine codon 790. Scorpion ARMS primers for the detection of *E746_A750del* and *T790M* was done with an EGFR Scorpion Kit (DxS Ltd.) as described by Kimura et al. (13). Briefly, all reactions were done in 25-μL volumes using 5 μL of template DNA, 16 μL of reaction buffer mix (including primers), 3.2 μL of water, and 0.8 μL of Taq polymerase. All reagents were included in this kit. Real-time PCR was carried out using Applied Biosystems 7500 under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, and 61°C for 60 s with fluorescence reading (set to FAM that allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was done with ABI 7500 Prism software (SDS version 1.3). The cycle threshold (Ct) was defined as the cycle at the highest peak of the second derivative curve, which represented the point of maximum curvature of the growth curve. Both Ct and Run versus Cycle were used for interpretation of the results.

Results and Discussion

Generation of NSCLC Cells Resistant to an Irreversible EGFR Inhibitor

To model the acquisition of resistance to an irreversible EGFR kinase inhibitor in the context of NSCLC treatment, we used the PC-9 NCSLC-derived cell line. These cells harbor an amplified *EGFR* allele containing an in-frame deletion in the kinase domain (delE746-A750), and they exhibit exquisite sensitivity to EGFR inhibitors (IC_{50} ~20 nmol/L). To establish drug-resistant PC-9 clones through mutational mechanisms, we exposed PC-9 cells to the mutagen ethyl methane sulfonate, and then cultured them continuously in HKI-272, an irreversible dual EGFR/HER2 kinase inhibitor that is currently being evaluated for clinical activity. The cells were maintained in 0.2 μmol/L of HKI-272, which approximates the pharmacologic concentration. Although the vast majority of PC-9 cells were rapidly killed by this treatment, approximately 1 in 2,000 cells gave rise to colonies after ~4 to 6 weeks in the presence of the drug. Several individual colonies were expanded and they were all found to exhibit an average relative increase in HKI-272 resistance of ~10-fold compared with the parental PC-9 cells, as measured by their IC_{50} in a cell survival assay (Fig. 1A). When grown in the absence of drug for up to 6 weeks, all clones maintained their HKI-272–resistant phenotype, suggesting a stable mechanism of resistance (data not shown; Fig. 1B).

Although all of the resistant clones exhibit similar degrees of HKI-272 resistance, further biochemical analyses revealed two distinct classes of clones: those in which phosphorylated EGFR is efficiently suppressed by drug (clones 8, 19, and 23), and those that retain phosphorylated EGFR in the presence of drug (clones 37, 47, and 49; Fig. 2A and B). For simplicity, we refer to clones with

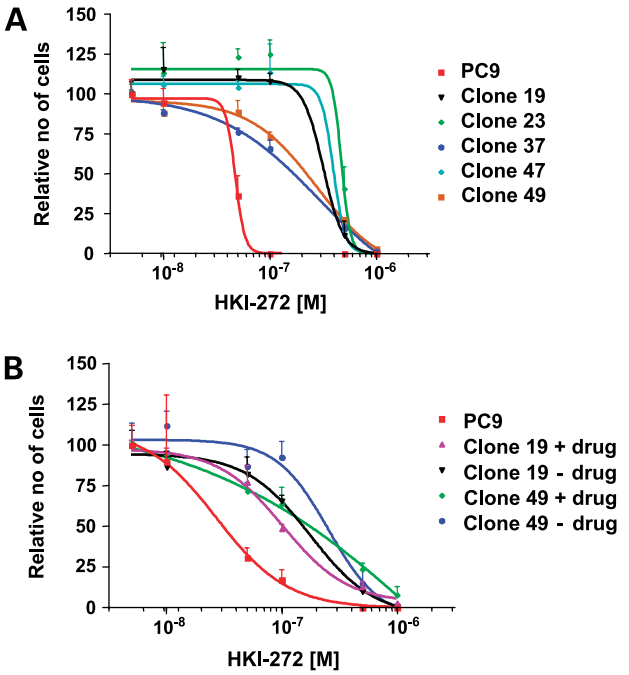


Figure 1. Acquired HKI-272 resistance in PC-9 NSCLC cells. **A,** dose-dependent growth inhibition of PC-9 cells and representative HKI-272–resistant cell lines treated with HKI-272. PC-9 remains 10-fold more sensitive to HKI-272 than its resistance derivatives. *Points,* mean of four samples. **B,** resistant clones 19 and 49 were grown in the presence or absence of 0.2 μmol/L of HKI-272 for up to 6 weeks, after which they were treated with HKI-272 at the indicated dose for 72 h. Parental PC-9 cells are shown as control. *Points,* mean of four samples. The results are representative of all HKI-272–resistant clones established.

drug-suppressible phosphorylated EGFR as class A, and those with nonsuppressible phosphorylated EGFR as class B. Notably, the suppression of phosphorylated EGFR by drugs in class A clones is not a permanent phenotype as EGFR is reactivated when cells are grown in the absence of drug (Fig. 2B). The absence of EGFR activity in class A clones suggests that these cells may have become dependent on an alternative survival pathway. It has been reported that *MET* gene amplification or activation of the IGF-IR pathway are possible mechanisms of acquired resistance to gefitinib *in vitro* (14, 15). We therefore tested whether either of these pathways was activated in the class A–resistant clones, but found that neither MET nor IGF-IR proteins exhibited increased phosphorylation levels in the HKI-272–resistant clones relative to the parental PC-9 cells (data not shown).

We next determined that all of the HKI-272–resistant clones were cross-resistant to both gefitinib and erlotinib, suggesting that the acquired resistance does not specifically reflect a mechanism unique to irreversible inhibitors (data not shown; Fig. 3A). Moreover, the clones were significantly more resistant to gefitinib and erlotinib than they were to HKI-272. Thus, these clones exhibit, on average, 10-fold increased resistance to HKI-272, but 100-fold more resistance to gefitinib and erlotinib. Notably, the parental PC-9 cells were slightly more sensitive to gefitinib and erlotinib than they were to HKI-272.

Acquired Resistance to the Irreversible EGFR Inhibitor is due to *T790M* Substitution

Acquired resistance to gefitinib and erlotinib is frequently associated with a *T790M* substitution mutation of the EGFR kinase domain, yielding a protein that exhibits reduced binding to drug, while preserving catalytic activity (4–6). Therefore, we determined whether the HKI-272–resistant clones harbor a *T790M* mutation. Because the endogenous mutant *EGFR* alleles are amplified in PC-9 cells, we used a sensitive assay that combines quantitative PCR and allele-specific amplification. In this assay, the primers are designed such that only the allele-specific mutation is amplified and the amount of relevant PCR product is then quantified. The number of PCR cycles required to detect the mutation provides a measure of the target molecule present in the reaction. The ΔCt defines the difference between the cycle threshold (Ct) of the mutant reaction and that of a positive control. Thus, the lower the ΔCt, the greater the representation of the mutation within the tested sample. Conversely, the closer the ΔCt is to the cutoff point, the fewer mutations these samples contained.

We first confirmed that all our HKI-272–resistant clones retained the *EGFR Del15* mutation found in the parental cell line, confirming that resistance did not arise within a

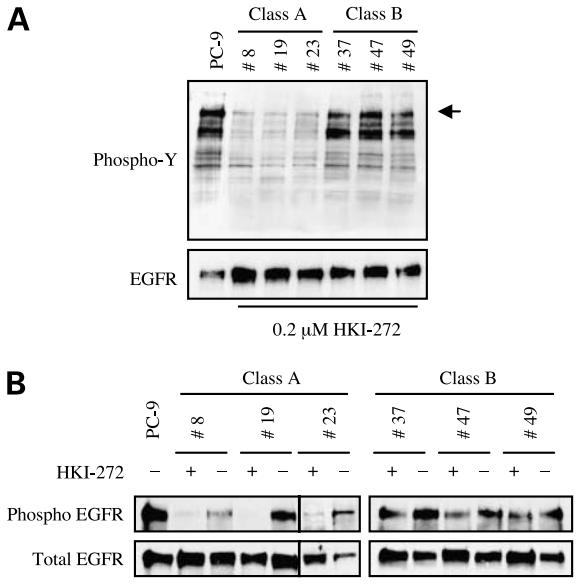
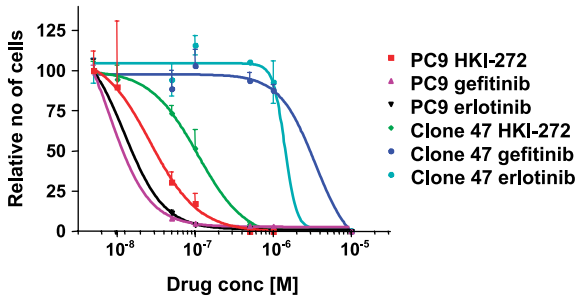


Figure 2. Two classes of HKI-272–resistant PC-9 cells were established. **A,** immunoblot of lysates from PC-9 parental cells and HKI-272–resistant cells (clones 8, 19, 23, 37, 47, and 49) probed with a phosphorylated tyrosine antibody, demonstrating two distinct groups of resistant cells (*top*). *Arrow,* phosphorylated EGFR. Total EGFR was used as a control (*bottom*). **B,** lysates of parental PC-9 cells and resistant clones 8, 19, 23, 37, 47, and 49 grown in the presence or absence of 0.2 μmol/L of HKI-272 for 8 days were probed with a phosphorylated EGFR antibody (*top*) and a total EGFR antibody (*bottom*). Note that EGFR phosphorylation levels reappear in the absence of HKI-272.

A



B

class	Cell line	ΔCt Del15	ΔCt T790M	T790M/ clone
	Cut-off point	12	11	
	PC9	2	undetectable	0/86
A	Clone 8	1	10	0/51
A	Clone 19	2	10	0/57
A	Clone 23	2	12	0/52
B	Clone 37	2	4	11/83
B	Clone 47	2	4	nd
B	Clone 49	2	4	nd

C

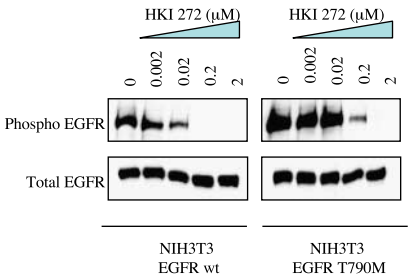


Figure 3. HKI-272–resistant cells are cross-resistant to gefitinib and erlotinib and a subset of these harbor a *EGFR T790M* mutation. **A,** inhibition of proliferation of resistant clone 47 and parental PC-9 cells. The effect of HKI-272 was compared with that of gefitinib and erlotinib. *Points,* mean of four samples. The results from clone 47 are representative of all HKI-272–resistant clones. **B,** determining the presence of *EGFR* mutations in PC-9 and resistant cells by Scorpion Amplified Refractory Mutation System technology. The low ΔCt values for deletion 15 in all cell lines shows that Del15 is present to a similar extent as the positive control *EGFR* exon 2. The resistant clones fall into two groups based on the presence of *T790M EGFR* allele. The low ΔCt values of clones 37, 47, and 49 show significant levels of *EGFR T790M*. Clones 8, 19, and 23 have high ΔCt around the cutoff point, suggesting that *EGFR T790M* is either absent in these cells or that the allele is present at such low levels that it is beyond the scope of sensitivity of this assay. Parental PC-9 cells contain no trace of EGFR T790M. Sequencing that the PCR products of exon 20 show the presence of a T790M missense mutation in 13% of EGFR alleles in clone 37. No mutations were detected in PC-9 cells, clones 8, 19, or 23. *nd,* not determined. **C,** autophosphorylation of EGFR (tyrosine 992) was detected by immunoblot of whole cell lysates isolated from NIH3T3 stably expressing *EGFR* wild-type or *EGFR T790M* (*top*). Cells were treated with drug for 2 h, then stimulated with 100 ng/mL of epidermal growth factor for 5 min prior to lysis. Phosphorylated EGFR (*top*) and total EGFR (*bottom*). *T790M* is ~10-fold more resistant to HKI-272 than wild-type EGFR.

contaminating subpopulation of distinct cells. Specifically, all of the HKI-272–resistant clones as well as the parental PC-9 cells exhibited a ΔCt value between 1 and 2, thereby confirming that the Del15 mutation is present at similarly high levels in all cells (Fig. 3B). When the presence of *EGFR T790M* was assayed, the resistant clones fell into two groups that corresponded precisely to the two groups defined biochemically. Resistant class B clones, which retain phosphorylated EGFR in the presence of HKI-272, exhibit high levels of T790M within their genomic DNA, as determined by the low ΔCt values. However, class A clones, in which EGFR phosphorylation is efficiently suppressed by drug, exhibit high ΔCt values. These values are near or beyond the cutoff point of 11, indicating either that these clones do not contain a *T790M* mutation or that this mutation is present at such low levels that they are below the sensitivity of the assay.

To confirm that *T790M* is present in only one of the two groups of resistant clones, we did a PCR reaction to specifically amplify exon 20, which contains codon 790, and we subsequently sequenced individual clones. Confirming the previous results, we found that *T790M* is present in a significant number of PCR subclones derived from clone 37 (13%; Fig. 3B). The parental PC-9 cells as well as the resistant clones of class A, however, only harbor wild-type sequences within exon 20. Thus, we conclude that the resistant clones which retain EGFR phosphorylation in the presence of the drug harbor the *EGFR T790M* mutation.

Uncovering *EGFR T790M* mutation as a mechanism of resistance to HKI-272 in the cultured cell model was unexpected, as it has previously been reported that HKI-272 could overcome *EGFR T790M* resistance to gefitinib and erlotinib in cell culture studies (5, 12). Additionally, a recently published report describing a mouse model of NSCLC associated with the expression of *EGFR L858R in cis* with *T790M* showed partial regression of adenocarcinomas after treatment with HKI-272 (16). The *in vitro* studies, however, were done using concentrations of 1 μmol/L or more, whereas dose-limiting toxicity, as established in a recently completed phase I clinical study of HKI-272 was ~0.2 μmol/L. Thus, it is possible that HKI-272 can overcome *T790M* only at relatively high doses that are not possible to safely achieve in patients. To test this directly, a dose-response curve was done with HKI-272 in NIH3T3 cells stably expressing *EGFR* wild-type or *EGFR T790M*. Indeed, 0.2 μmol/L of HKI-272 was sufficient to inhibit EGFR phosphorylation in cells expressing wild-type *EGFR,* but not in cells expressing *EGFR T790M* (Fig. 3C). Notably, EGFR *T790M* protein exhibits a higher basal level of phosphorylation relative to wild-type EGFR, confirming previous published results (12, 17).

Our findings suggest that the effectiveness seen with this drug at concentrations required *in vitro* may not be achievable in patients due to drug toxicity, and hence, HKI-272 might not effectively inhibit *EGFR T790M* at a clinically achievable concentration. Thus, there is clearly a need to develop a more potent irreversible EGFR inhibitor, with potentially reduced toxicity, to overcome the drug resistance associated with secondary *T790M* mutations. Furthermore, these findings suggest that the use of HKI-272 as first-line TKI therapy may yield EGFR T790M-associated disease progression similar to that seen with the reversible EGFR inhibitors.

Substitution of *EGFR* Cysteine 797 Confers Resistance to an Irreversible EGFR Inhibitor

Previous studies have shown efficient suppression of the *EGFR T790M* mutant signaling by relatively high concentrations of HKI-272, suggesting that a more potent irreversible EGFR inhibitor may be effective in the clinical setting of acquired TKI resistance. Notably, several such irreversible inhibitors are currently being tested in clinical studies. If any of these prove to be sufficiently potent to overcome the gefitinib/erlotinib resistance associated with *T790M* at pharmacologic concentrations, it will still be important to anticipate secondary resistance mechanisms that arise during treatment with those inhibitors. This phenomenon has been well exemplified by the experience with dasatanib as a second-line treatment for cases of chronic myelogenous leukemia with acquired imatinib resistance (18).

Therefore, to anticipate alternative mechanisms of acquired resistance to an irreversible EGFR kinase inhibitor, we considered the potential role of the cysteine residue within the EGFR kinase domain to which HKI-272 binds covalently. As with gefitinib and erlotinib, HKI-272 binds EGFR within its ATP-binding pocket. However, unlike gefitinib and erlotinib, HKI-272 forms a covalent bond with EGFR through a sulfhydryl group on cysteine 797 (19). We speculated that substituting this cysteine residue would result in the decreased ability of HKI-272 to inhibit EGFR, consequently leading to HKI-272 resistance. To test this possibility, we generated an *EGFR* variant in which cysteine 797 was changed to alanine (C797A) either in a wild-type *EGFR* background, a *L858R* background, or a *Del15* (*Del747–750*) background. Whereas EGFR wild-type, *L858R,* or *Del15* were effectively inhibited at HKI-272 concentrations as low as 0.02 μmol/L, EGFR variants *C797A, C797A/L858R,* and *C797A/Del* remained phosphorylated at concentrations of 1 μmol/L or higher (Fig. 4A and B). These findings, together with findings from a recent study, indicate that EGFR cysteine 797 is necessary for inhibition by irreversible inhibitors, and mutating this residue abolishes the activity of the irreversible inhibitors (20). Significantly, erlotinib can effectively inhibit EGFR C797A mutants, suggesting that this mutation is specifically resistant to irreversible inhibitors (Fig. 4C).

Our findings also indicate that substituting cysteine 797 with alanine does not detectably affect the EGFR kinase activity. Thus, *EGFR C797A* mutants are autophosphorylated to a similar extent as the *EGFR C797* proteins under basal growth conditions. These findings suggest that treatment with an irreversible EGFR kinase inhibitor with sufficient potency to overcome the effect of the *T790M*

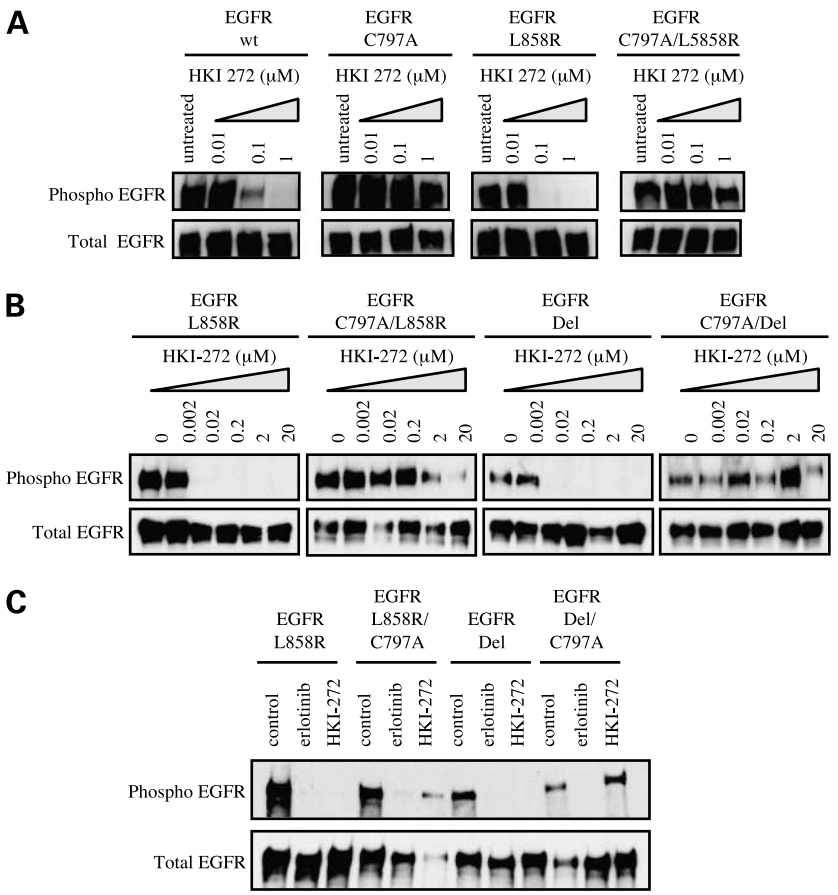


Figure 4. *EGFR C797A* confers resistance to HKI-272. **A,** 24 h after transfection with *EGFR* wild-type, *EGFR C797A, EGFR L858R,* or *EGFR C797A/L858R,* 293T cells were treated with HKI-272 at the indicated dose for 2 h, then stimulated with 100 ng/mL of epidermal growth factor for 5 min. Immunoblots of the lysates were probed with phosphorylated EGFR (*top*) or total EGFR antibody (*bottom*). **B,** NIH3T3 cells stably expressing *EGFR L858R, C797A/L858R, EGFR Del,* or *EGFR C797A/Del* were treated with HKI-272 at the indicated doses for 2 h, then stimulated with 100 ng/mL of epidermal growth factor for 5 min. Immunoblots of the lysates were probed with phosphorylated EGFR (*top*) or total EGFR antibody (*bottom*). **C,** same as in **B** with the exception that the cells were treated with 1 μmol/L of either erlotinib, HKI-272, or DMSO as a control for 2 h before stimulation with 100 ng/mL of epidermal growth factor. All cells expressing *EGFR* mutation *C797A* showed significant resistance to HKI-272, but remained sensitive to erlotinib.

mutation may still give rise to resistance conferred by the acquisition of a C797 substitution of EGFR. Notably, EGFR C797 is conserved in ErbB2/Her2 (C805). Because there are several irreversible TKIs currently undergoing clinical testing in patients with breast cancer harboring amplified *ErbB2,* it is possible that a mutation at *ErbB2* cysteine 805 could contribute to acquired resistance to these drugs. If the *EGFR C797* substitution does indeed emerge as a clinical mechanism of acquired resistance to irreversible inhibitors, a combination of gefitinib/erlotinib plus an irreversible inhibitor may prove beneficial, as that combination would overcome both the *T790M* and *C797* resistance mutations.

Acknowledgments

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