EGFR mutations and resistance to Irreversible pyrimidine based EGFR inhibitors

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Statement of Translational Relevance

The most common mechanism of acquired drug resistance following treatment with an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor is the *EGFR* T790M mutation. Current treatment strategies include covalent pyrimidine based mutant selective EGFR inhibitors and to date these agents have led to tumor regressions in the majority of lung cancer patients with *EGFR* T790M mediated drug resistance. By modeling drug resistance *in vitro*, we identify *EGFR* mutations that mediate resistance to the tool compound WZ4002 and clinical compounds AZD9291 and CO-1686. These findings may help predict mechanism of acquired resistance that will develop in the clinic and can be used to develop potential treatment strategies that can be implemented clinically.

Abstract

Purpose: Mutant selective irreversible pyrimidine based EGFR kinase inhibitors, including WZ4002, CO-1686 and AZD9291, are effective in preclinical models and in lung cancer patients harboring the *EGFR* T790M gefitinib/erlotinib resistance mutation. However, little is known about how cancers develop acquired resistance to this class of EGFR inhibitors. We sought to identify and study *EGFR* mutations that confer resistance to this class of agents.

Experimental Design: We performed an N-ethyl-N-nitrosourea (ENU) mutagenesis screen in *EGFR* mutant (sensitizing alone or with concurrent *EGFR* T790M) Ba/F3 cells and selected drug resistant clones. We evaluated the sensitivity of EGFR inhibitors in models harboring drug resistant *EGFR* mutations.

Results: We identified 3 major drug resistance mutations. *EGFR* L718Q, L844V and C797S cause resistance to both WZ4002 and CO-1686 while, in contrast, only *EGFR* C797S leads to AZD9291 resistance. Cells containing an *EGFR* sensitizing mutation, Del 19 or L858R, in conjunction with L718Q, L844V or C797S retain sensitivity to quinazoline based EGFR inhibitors gefitinib and afatinib. The C797S mutation, in the presence of Del 19 or L858R and T790M,causes resistance to all current EGFR inhibitors, but L858R/T790M/C797S remains partially sensitive to cetuximab which leads to disruption of EGFR dimerization.

Conclusions: Our findings provide insights into resistance mechanisms to irreversible pyrimidine based EGFR inhibitors and identify specific genomic contexts in which sensitivity is retained to existing clinical EGFR inhibitors. These findings will guide the development of new strategies to inhibit EGFR.

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INTRODUCTION

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) are effective clinical therapies for *EGFR* mutant advanced non small cell lung cancer (NSCLC) patients (1-4). Several randomized clinical trials have demonstrated that EGFR TKIs are more effective, as measured by response rate (RR) and progression free survival (PFS), than chemotherapy when used as initial systemic treatment for advanced *EGFR* mutant NSCLC (1, 4-9). However, the vast majority of patients will develop disease progression following successful treatment with an EGFR TKI. The most common mechanism of acquired resistance, detected in 60% of patients, is a secondary mutation in *EGFR* at position T790 (T790M)(10). This mutation, leads to an increase in ATP affinity, thus making it more difficult for reversible EGFR TKIs gefitinib and erlotinib to bind the EGFR TKI domain (11).

Covalent EGFR inhibitors have emerged as strategies to inhibit *EGFR* T790M containing cancers. In preclinical models, afatinib, a covalent quinazoline based EGFR inhibitor, is effective both in models harboring only an *EGFR* activating mutation and in those with a concomitant T790M resistance mutation (12). However, in lung cancer patients, afatinib is only effective in EGFR TKI naive *EGFR* mutant cancers and has a RR of < 10% in patients with NSCLC that have developed resistance to gefitinib or erlotinib (13). Afatinib is a potent inhibitor of both mutant and wild type (WT) EGFR. Inhibition of WT EGFR leads to toxicities, including skin rash and diarrhea, which limits the ability to escalate afatinib doses in patients to those necessary to inhibit EGFR T790M. Irreversible pyrimidine EGFR inhibitors, including the tool compound WZ4002 and clinical compounds CO-1686 and AZD9291, overcome many of the limitations of afatinib (14-16). They are not only more potent on EGFR T790M, but also

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selectively inhibit mutant over WT EGFR and hence should lead to increased clinical efficacy and less toxicity compared with afatinib (14-16). In phase I studies to date, treatment with either CO-1686 or AZD9291, has resulted in a RR > 50% in *EGFR* mutant EGFR T790M NSCLC patients that have developed resistance to gefitinib or erlotinib (17, 18). In addition, both agents are associated with substantially less skin toxicity than typically observed for EGFR TKIs (17, 18).

Despite the clinical efficacy of CO-1686 and AZD9291, it is fully anticipated that patients will ultimately develop acquired resistance to these agents. To date little is known about the mechanisms of acquired resistance and whether cross resistance will occur to all irreversible pyrimidine based and to existing EGFR inhibitors. Understanding the mechanism (s) of resistance to this class of agents may both help predict the mechanism(s) that will occur in patients and allow the development of subsequent treatment strategies. Prior studies, using chronic exposure models, have identified reactivation of mitogen activated protein kinase (MAPK) signaling and insulin like growth factor 1 (IGF1R) signaling as mechanisms of acquired resistance to WZ4002 (19, 20). Secondary mutations in *EGFR* itself have not been described as a mechanism of acquired resistance.

In the current study, we used a mutagenesis strategy to identify secondary *EGFR* mutations that impart resistance to WZ4002 and to CO-1686 and AZD9291. We further evaluate how the secondary mutations cause resistance and evaluate their impact on cross resistance to other EGFR targeted therapies.

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MATERIALS AND METHODS

Kinase inhibitors

WZ4002, AZD9291 and CO-1686 were synthesized using previously published methods (14-16). Gefitinib, Afatinib, Neratinib, CL-387,785 were obtained from Selleck chemicals. The synthesis of TX2-30 is described in supplementary methods. Stock solutions of all drugs were prepared in DMSO and stored at -80°C.

Cell Culture and Reagents

EGFR mutant NSCLC cell lines HCC827 (del E746_A750), H3255 (L858R), H3255GR (L858R/T790M), H3255DR (L858R/T790M Amplified), HCC827EPR (del E746_A750/T790M), H1975 (L858R/T790M) PC9 (del E746_A750), PC9 GR (del E746_A750/T790M), PC9 DR (del E746_A750/T790M amplified) and SNU2315 (del E746_A750/T790M), were obtained from Dr. Adi Gazdar (UT Southwestern, Dallas, TX), American Type Culture Collection, or from the Korean Cell Line Bank (Seoul National University, Seoul, Korea) and have been previously characterized (14, 21-23). All cell lines were authenticated in September 2014 using the Promega Geneprint 16 cell ID system and was performed at the Research Technology Support Facility at Michigan State University. All cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. H3255, H3255GR and H3255DR were maintained in ACL-4 media (Invitrogen, Carlsbad, CA) supplemented with 5% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mM glutamine. The *EGFR mutant* Ba/F3 cells and the NIH-3T3 cells have been previously characterized (14).

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N-ethyl-N-nitrosurea (ENU) Mutagenesis Drug Screen

N-ethyl-N-nitrosourea (ENU) was purchased from Sigma Aldrich and mutagenesis was carried

out as previously described (24). Briefly, L858R,L858R/T790M, DelE746_A750 and

DelE746_A750 Ba/F3 cells (1 X 10^6 cells/ml) were exposed to ENU (50 μg/ml) for 24 hours.

Cells were then washed 3 times with RPMI, and expanded in growth media for 5-7 days. Cells

were subsequently cultured in 96-well plates (1 X 10^4 cells/well; total 5 X 10^5 cells per inhibitor)

in the presence of 100 nM WZ4002, 1 μM WZ4002. Wells were observed for growth by visual

inspection and resistant wells were expanded in the presence of the corresponding inhibitor.

Total RNA was isolated from drug resistant cell lines using Trizol^{TM} (Invitrogen, Carlsbad, CA)

and purified using RNeasy^{TM} minielute cleanup kit (Qiagen,Valencia, CA). cDNA was

transcribed from 2μg of total RNA with Superscript II Reverse Transcriptase (Invitrogen Life

technologies, Carlsbad, CA). The cDNA was used as template for subsequent sequencing of the

EGFR tyrosine kinase domain (exons 18-21).

Generation of Drug-Resistant Cells Lines

The *EGFR* L718Q, L844V and C797S mutations were introduced via site directed mutagenesis

using the Quick Change Site-Directed Mutagenesis kit (Stratagene; La Jolla, CA) according to

the manufacturer's instructions. The L718Q mutation was generated using forward primer 5'-

aaaaagatcaaagtgcagggctccggtgcgttc-3' and reverse primer 5' gaacgcaccggagccctgcactttgatctttt-

3'. The L844V mutation was generated using forward primer 5'-

cctggcagccaggaacgtagtggtgaaaaca-3' and reverse primer 5'-tgttttcaccactacgttcctggctgccagg-3'

The C797S mutation was generated using forward primer 5'-catgcccttcggctccctcctggacta-3' and

reverse primer 5'-tagtccaggagggagccgaagggcatg-3'.All constructs were confirmed by DNA

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sequencing. The constructs were shuttled into the retroviral vector JP1540 or lentiviral vector

JP1698 using the BD Creator^TM System (BD Biosciences). Ba/F3, NIH-3T3, cells were infected

with retrovirus and, PC9, PC9GR4 and HCC827 EPR cells infected with lentivirus according to

standard protocols, as described previously (25). Stable clones were obtained by selection in

puromycin (2 µg/ml).

Cell proliferation assays and Growth Assays

Growth and inhibition of growth was assessed by MTS assay and was performed according to

previously established methods (14, 19). NSCLC or Ba/F3 cells were exposed to treatment for 72

hours and the number of cells used per experiment determined empirically and has been

previously established (14, 19). All experimental points were set up in six wells and all

experiments were repeated at least three times. The data was graphically displayed using

GraphPad Prism version 5.0 for Windows, (GraphPad Software; www.graphpad.com). The

curves were fitted using a non-linear regression model with a sigmoidal dose response.

For clonogenic assays, 1000 cells were seeded in a 6 well plate and allowed to adhere overnight

then treated with 400nM of indicated drug. After 7 days, wells were fixed with 0.5% Crystal

Violet solution. Number of colonies was quantified using Adobe Photoshop version CS4

Extended analysis tool. All experimental points were set up in three wells and all experiments

were repeated at least twice. Data were graphically displayed using GraphPad Prism version 5.0

for Windows.

Colony formation was measured using the CytoSelect 96-well Cell Transformation Assay (Cell

BIOLABS, San Diego,CA), according to manufacturer's protocol and as previously described

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(26). Briefly, $1x10^3$ cells per well (6 wells per condition) were treated with 1.0 µM Gefitinib, 400nM AZD9291, 200nM WZ4002, 200 nM Afatinib alone or in combination with 10µg/ml, Cetuximab for seven days and growth assayed as described in (26) .The experiment was repeated three independent times.

Antibodies and Western Blotting

Cells grown under the previously specified conditions were lysed in NP-40 buffer (Cell Signaling Technology). Western blot analyses were conducted after separation by SDS/PAGE electrophoresis and transfer to polyvinylidene difluoride-P membrane (Millipore). Immunoblotting was performed according to the antibody manufacturers' recommendations. Antibody binding was detected using an enhanced chemiluminescence system (Perkin Elmer Inc.). Anti-phospho-Akt (Ser-473), anti-total Akt antibodies were obtained from Cell Signaling Technology. The phospho-specific EGFR (pY1068), total ERK1/2, phospho-ERK1/2 (pT185/pY187) antibodies were purchased from Invitrogen. Total EGFR antibody was purchased from Bethyl Laboratories. Tubulin antibody was purchased from Sigma.

Immunoprecipitation using Biotinylated WZ4002 (TX2-30)

For the synthesis of TX2-30, the acrylamide modified pyrimidine of WZ4002 was synthesized as previously reported (Nature 2009). The biotin tail was introduced following the procedure from ZW, HW et al Chem. Biol 2010. Briefly, cell lysate was incubated with indicated concentrations of TX2-30 for 1 hour at 4C. Excess compound was removed using DG-10 column (Bio-Rad, Hercules, CA). The protein was further denatured with 8M Urea solution. The addition of

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Streptavidin beads (Sigma) was followed by 1 hour incubation at room temperature. Beads were washed 3 times and biotin labeled EGFR was released by heating beads to 95ºC in SDS buffer.

Chemical Cross-linking of EGFR Ba/F3 intact cells

Cells were treated with Cetuximab (10μg/ml) for 16 hours. Following treatment, cells were washed twice with cold PBS and incubated with 1.0 mM bis(sulfosuccinimidy) suberate (Thermo Scientific, Rockfrod, IL) for 30 minutes at room temperature. The reactions were quenched with the addition of 20mM Tris, (pH7.4). Cells were then washed twice with PBS and then lysed with NP40 buffer.

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RESULTS

Identification of novel drug resistant mutations

In order to identify *EGFR* mutations that may confer resistance to WZ4002, we performed an N-ethyl-N-nitrosourea (ENU) mutagenesis screen as previously described (14). Following ENU exposure of Ba/F3 cells harboring the common *EGFR* activating mutations (L858R and Del E746_A750) with or without the a concomitant T790M mutation, we selected resistant clones in the presence of either 100 nM or 1 µM WZ4002 for two weeks. Three mutations, L718Q (codon 2153, T>A), L844V (codon 2530, C>G) and C797S (codon 2389, T>A) were identified with different frequencies in the 4 different Ba/F3 cell lines (Figures 1A and S1 and Table S1). C797 is the site of covalent binding of WZ4002 and previously demonstrated to lead to drug resistance if mutated to serine (14). The L844V mutation has been previously observed in a NSCLC patient (27). Mutations at L718 resulting in L718P, L718V and L718M mutations have been described but not *EGFR* L718Q (28-31). The clinical significance of these mutations is presently unknown. We next tested whether L718Q and L844V were oncogenic and caused drug resistance or only the latter. The *EGFR* T790M mutation is both oncogenic and leads to drug resistance (32, 33). Neither EGFR L718Q nor L844V mutations were sufficient to lead to IL-3 independent cell growth in the background of wild type (WT) EGFR when introduced into Ba/F3 cells (Figure 1B). Intriguingly, while these mutations led to resistance to WZ4002, they remained sensitive to irreversible quinazoline EGFR inhibitors afatinib, neratinib and CL-387,785 as demonstrated by growth inhibition using Ba/F3 cells and by the ability to inhibit EGFR phosphorylation (Figure 1C and Table S2). Gefitinib, a reversible quinazoline EGFR inhibitor, was moderately effective, with greater efficacy in the L844V model (Figure 1C and Table S2). We were not able to identify any secondary mutations using the Del E746_A750 Ba/F3 cells

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using our ENU mutagenesis assay (Figure 1A and Table S1). Engineered *EGFR* Del

E746_A750/L718Q but not *EGFR* L858R/L718Q Ba/F3 cells demonstrated a growth

disadvantage providing one explanation as to why these were not recovered in our original assay

(Figure 1D). In support of this, in the presence of the L718Q mutation, basal EGFR

phosphorylation was reduced in *EGFR* Del E746_A750 cells (Figure S2).

EGFR L718Q and L844V impact WZ4002 binding and retain sensitivity to quinazoline based

EGFR inhibitors

We next aimed to determine how EGFR L718Q and L844V may cause resistance to WZ4002.

Examination of the crystal structure of WZ4002 in complex with EGFR T790M (14) reveals that

both L718 and L844 are in direct contact with the inhibitor; L718 primarily with the aniline ring

and L844 with the pyrimidine ring (Figure 2A). The L718Q substitution likely sterically

interferes with binding of the compound, while the L844V mutation can be expected to remove

favorable contacts with the inhibitor (Figure 2B). In order to experimentally determine whether

WZ4002 binding was affected in the presence of these secondary mutations, we synthesized a

biotinylated form of WZ4002, TX2-30 (Figure S3), and used it as an affinity reagent to label

EGFR in cell lysates. We exposed NIH-3T3 cells expressing WT or mutant EGFR to TX2-30,

performed an affinity purification of the biotin followed by western blotting for EGFR. TX2-30

labeled the mutant EGFR proteins more efficiently than WT, consistent with the 'mutant'

selectivity exhibited by the parental WZ4002 compound (Figure 2C). However, in the presence

of either the L718Q or the L844V mutation, we recovered less WT and mutant EGFR suggesting

that these mutations impact drug binding (Figure 2C).

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We further evaluated the impact of the L718Q and L844V mutations when present with both an

EGFR activating mutation and the T790M mutation or only in the presence of an *EGFR*

activating mutation on drug efficacy (Table S2). In the triple mutants (*EGFR* activating

mutation/T790M with L718Q or L844V), none of the quinazoline based EGFR inhibitors tested

showed any efficacy (Figure 2D, S4A and S4B). However, in the background of an EGFR

activating mutation alone, afatinib and CL-387,785, were particularly effective at inhibiting the

growth of either L718Q or L844V containing cells which was mirrored by inhibition of EGFR

phosphorylation (Figure 2E and S4C). There were some differences in the efficacy of gefitinib in

these models, the EGFR Del E746_A750/L844V and L858R/L844V being more sensitive than

the corresponding L718Q models (Figure 2E and S4C). Given the differential effects of gefitinib

and WZ4002 against Ba/F3 cells harboring *EGFR* L718Q/L844V and *EGFR* T790M mutations,

we evaluated gefitinib alone, WZ4002 alone or the combination using the ENU mutagenesis

assay in *EGFR* L858R Ba/F3 cells. With gefitinib we only recovered *EGFR* T790M containing

clones while with WZ4002 only L718Q containing clones were recovered (Figure 2F). However

no clones harboring *EGFR* secondary mutations were recovered with the combination.

Furthermore, the combination was very effective in Ba/F3 cells harboring L858R or Del

E746_A750 in conjunction with either L718Q or L844V (Figure 2G and S4D).

Differential efficacy of clinical mutant selective EGFR inhibitors in models harboring EGFR

mutations

Given the WZ4002 is a tool compound not in clinical development, while CO-1686 and

AZD9291 are in clinical studies, we compared the efficacy of all three covalent agents along

with afatinib in Ba/F3 cell models and endogenous NSCLC cell lines harboring *EGFR* activating

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mutations alone, or in conjunction with *EGFR* T790M. All 4 drugs effectively inhibited EGFR

Del E746_A750 and L858R containing cells while WZ4002, CO-1686 and AZD9291 were

significantly more effective in T790M containing models than afatinib (Table S3). None of the

drugs were effective in triple-mutant models harboring C797S or the *EGFR* exon 20 insertion

mutation A768_V769dupASV (Table S3). Afatinib was a substantially better inhibitor of WT

EGFR; AZD9291 inhibited WT EGFR at lower concentrations than WZ4002 or CO-1686

(Figure 3A). Similar trends were observed using endogenous lung cancer cell lines (Table S3). In

T790M containing endogenous lung cancer cell lines, including the PC9 GR4

(E746_A750/T790M) cells, AZD9291 was the most potent inhibitor (Table S3 and Figure 3B).

Analogously, EGFR phosphorylation, and downstream signaling, was inhibited at lower

concentrations of AZD9291 than WZ4002 or CO-1686 in these cells (Figure 3B).

Efficacy of clinical irreversible EGFR inhibitors on models harboring EGFR tertiary mutations
We further evaluated whether there were differences in the efficacy of the clinical irreversible

EGFR inhibitors in models harboring *EGFR* tertiary mutations. Afatinib was most effective in

models harboring an *EGFR* activating mutation (L858R or Del 19) along with either the L718Q

or L844V mutations (Figure 4A and B). Of the pyrimidine inhibitors, only AZD9291 inhibited

Del/L718Q, Del/L844V and L858R/L844V cells, but was less effective against the

L858R/L718Q cells (Figure 4A). In the triple mutant Ba/F3 cells, only AZD9291 was effective

in models harboring L844V while having a minimal effect against those with the L718Q

mutation (Figure 4A). The efficacy paralleled inhibition of EGFR phosphorylation (Figure 4C).

We also repeated the ENU mutagenesis screen comparing all 3 irreversible pyrimidine EGFR

inhibitors and selecting using either 100 nM or 1 µM of each drug (Table S4 and Figure 4D).

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While multiple *EGFR* mutations were recovered at 100 nM for both WZ4002 and CO-1686, >

90% of the clones recovered using AZD9291 harbored a mutation in C797 mutation (Figure 4D

and Table S4). At 1 µM, only clones containing mutations in C797 (either C797S or C797G)

were recovered for both WZ4002 and AZD9291 while roughly equal numbers of L718Q and

C797S clones were recovered with CO-1686 (Figure 4D). Cells containing the Q791R (codon

2372, A>G) mutation were relatively resistant to W4002 and CO-1686 but remained sensitive to

AZD9291 and afatinib (Figure S5A-D).

EGFR tertiary mutations cause resistance in endogenous EGFR mutant lung cancer models

We expressed *EGFR* del E746_A750/T790M/L718Q or *EGFR* del E746_A750/T790M/L844V

in *trans* in NSCLC cell lines (PC9GR4 (Del E746_A750 or HCC827 EPR (Del

E746_A750/T790M)) to determine whether they were sufficient to cause resistance to WZ4002

in the context of an endogenous NSCLC cell line. While expression of green fluorescent protein

(GFP) was not associated with resistance, expression of either L718Q or L844V containing

constructs resulted in resistance to WZ4002, CO-1686 and afatinib (Figure 5A). The L844V

expressing cells remained sensitive to AZD9291. Similar results were obtained for HCC827

T790M containing cells (Figure S6A and S6B). In a 7 day clonogenic assays, PC9GR/GFP cells

remained sensitive to WZ4002, CO-1686, AZD9291 and afatinib (Figures S6C). However, the

L718Q and L844V expressing PC9 GR4 cells were only sensitive to AZD9291 (Figures S6 and

5B). Analogously, phosphorylation of EGFR and downstream signaling pathways were

inhibited by AZD9291 but not WZ4002 or CO-1686 in PC9 GR cells expressing either *EGFR*

del E746_A750/T790M/L718Q or *EGFR* del E746_A750/T790M/L844V (Figure 5B). PC9 GR

cells expressing *EGFR* del E746_A750/T790M/C797S were resistant to all tested agents (Figure

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5C and Figure S6D). Given the clinical efficacy of the combination of afatinib and cetuximab in gefitinib/erlotinib resistant cancers, we evaluated this combination as well as WZ4002/cetuximab in PC9 GR cells expressing the tertiary mutations using a previously established system evaluating cetuximab based combinations (26, 34). While afatinib/cetuximab had some effect on cell growth (Figure 5C) and EGFR signaling (Figure 5D) in cells expressing L718Q or L844V, in neither model was the effect as robust as observed with AZD9291 (Figure 5C).

Existing EGFR inhibitors are effective in some genomic contexts containing EGFR C797S
Given the potential resistance imparted by the *EGFR* C797S mutation to WZ4002, CO-1686 and AZD9291, we next evaluated whether C797S mediated resistance was dependent on the *EGFR* genomic context. Ba/F3 cells expressing an *EGFR* activating mutation and C797S in the absence of *EGFR* T790M remained sensitive to afatinib (Figure 4A and Table S2). Similarly, while PC9 cells (EGFR E746_A750) expressing EGFR DelE746_A750/C797S were resistant to WZ4002, CO-1686 and AZD9291, they remained sensitive to both afatinib and gefitinib (Figure 6A and 6B). Furthermore, in an ENU mutagenesis assay with *EGFR* L858R Ba/F3 cells followed by selection in gefitinib alone, in AZD9291 alone or in the combination, no resistant clones emerged following gefitinib & AZD9291 treatment (Figure 6C).

Prior studies have demonstrated that some EGFR mutant proteins, including L858R/T790M, do not require dimerization for oncogenic transformation (35). In contrast, other mutants, including EGFR L858R, require dimerization and are sensitive to the anti-EGFR antibody cetuximab which disrupts EGFR dimerization (35). Given these findings, we evaluated whether the triple EGFR mutant proteins existed as monomers or dimers in transformed Ba/F3 cells. While

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L858R/T790M/L718Q and L858R/T790M/L844V existed predominately as monomers, the

L858R/T790M/C797S was present in both monomers and dimers (Figure 6D). Treatment with

cetuximab disrupted dimerization, which resulted in inhibition of EGFR phosphorylation and

growth inhibition of the cells (Figure 6D and 6E). Unlike the L858R counterpart, Del

19/T790M/C797S exists predominately as a monomer with no effect on EGFR phosphorylation

of cell growth following cetuximab treatment (Figure S7A and S7B).

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DISCUSSION

Acquired drug resistance limits the long term clinical success of targeted therapies including

EGFR inhibitors in patients with *EGFR* mutant NSCLC. The mechanistic understanding of drug

resistance has led to the development of new and novel strategies that are currently being tested

in clinical trials. These include the development of mutant selective EGFR kinase inhibitors,

including WZ4002, CO-1686 and AZD9291, which to date have demonstrated encouraging

efficacy in both preclinical models and in *EGFR* mutant NSCLC patients with *EGFR* T790M

mediated drug resistance (14-18). However, it is fully anticipated that acquired resistance will

also develop to this class of EGFR inhibitors. Given that these EGFR inhibitors are structurally

distinct from currently approved EGFR inhibitors, including gefitinib, erlotinib and afatinib, it is

important to understand whether mechanisms of acquired resistance would also lead to cross

resistance to existing EGFR inhibitors. Furthermore, as both CO-1686 and AZD9291 are being

tested in patients with *EGFR* T790M mediated drug resistance, and in those that have an *EGFR*

mutation but are EGFR TKI naive, the mechanisms of acquired resistance may differ based on

the presence or absence of *EGFR* T790M. These differences may have clinical implications for

subsequent treatment with anti-EGFR based strategies.

Our findings identify C797 (C797S and C797G) as the most common site of secondary

mutations mediating resistance to WZ4002, C0-1686 and AZD9291 (Figure 4D). Cysteine 797

is the site of covalent binding for all three of these agents (14-16). Covalent binding is required

to overcome the increased ATP affinity mediated by T790M (11). In prior studies, introduction

of C797S into the background of *EGFR* L858R/T790M or *EGFR* Del 19/T790M resulted in

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significant loss of cellular potency for WZ4002 (14). Analogously, a non-covalent analogue of

WZ4002, WZ4003, was ineffective in *in vitro* models of EGFR T790M (14). Emerging clinical

data reveal that the C797S mutation is detected in ~ 40% of *EGFR* mutant NSCLC patients with

T790M who develop acquired resistance to AZD9291(36) . Furthermore, recent studies have also

identified mutations in C481 (C481S), the analogous cysteine residue to C797 in EGFR, in

patients with chronic lymphocytic leukemia treated with the Bruton's tyrosine kinase (BTK)

inhibitor ibrutinib suggesting that mutations in this conserved residue may be a common

mechanism of acquired resistance to covalent kinase inhibitors (37).

We also identify secondary mutations in *EGFR*, L718Q and L844V, both of which occur more

frequently in WZ4002 and CO-1686 resistant models (Figure 4D). These mutations are not

oncogenic but mediate drug resistance likely through steric hindrance and impact drug binding

(Figure 2A-C). AZD9291 retains activity against L844V and moderate activity in models

harboring *EGFR* L718Q, although only in the background on an *EGFR* exon 19 deletion, while

both mutations mediate resistance to CO-1686 (Figures 4A, 4C and 5A). Our findings suggest

that CO-1686 and AZD9291 are distinct from one another and that AZD9291 may still be

clinically effective in some CO-1686 treated patients if they were to develop resistance mediated

by L844V or L718Q. CO-1686 is structurally more closely related to WZ4002 than to AZD9291

and in all of the models tested CO-1686 is very similar *in vitro* to WZ4002 (14-16).

Remarkably, models harboring an *EGFR* activating mutation alone that develop resistance

through L718Q, L844V, or C797S retain sensitivity to quinazoline based EGFR inhibitors

including both gefitinib and afatinib (Figures 4A ,S4C and 5A). This has immediate clinical

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implications as gefitinib and afatinib are approved EGFR inhibitors and could be clinically effective in *EGFR* mutant EGFR TKI naive patients who develop these drug resistance mutations following initial treatment with either AZD9291 or CO-1686. Alternatively, combinations of mutant selective and quinazoline based EGFR inhibitors could be used together as initial treatment for *EGFR* mutant NSCLC, as the two classes of EGFR inhibitors are effective against non-cross resistant mutations and together prevent the emergence of resistant clones (Figures 2F and 5C). Our findings suggest that the sequential and combination approaches should be tested in future clinical trials assuming these tertiary mutations will also arise in patients treated with CO-1686 and AZD9291.

In the presence of *EGFR* T790M, the tertiary mutations (L718Q, L844V and C797S) cause resistance to all current EGFR inhibitors (Table S2). In only the *EGFR* L858R/T790M/C797S model cetuximab still retains some activity (Figures 6D-E and S7A-B). Additional studies are needed to determine if cetuximab or cetuximab based combinations are effective *in vivo* or clinically in *EGFR* L858R/T790M patients that develop the C797S mutation. Furthermore, additional strategies to inhibit EGFR in the presence of these tertiary mutations, and T790M, are needed and our findings can help guide such strategies. Given the increased ATP affinity imparted by the T790M, it may not be possible to identify a non-covalent EGFR inhibitor with sufficient affinity to inhibit EGFR in the presence mutations in C797 and hence an alternative approach may be necessary.

Our study is performed *in vitro* using model systems and hence has limitations. It is not known whether all of the mutations identified in our study will also occur in patients. However, ENU

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mutagenesis studies have been predictive of clinical drug resistance mutations in several prior studies (14, 24). Additional pre-clinical studies have also identified activation of MAPK signaling and IGF1R signaling as well as epithelial to mesenchymal (EMT) transition as potential resistance mechanisms to WZ4002 and CO-1686 (15, 19, 20). However, as ENU exposure leads to point mutations, mechanisms of drug resistance that involve genomic gains or losses are unlikely to be identified using ENU mutagenesis. Studies of tumors from NSCLC patients that have responded and subsequently developed acquired resistance to CO-1686 and AZD9291 are needed in order to determine whether the predominant mechanism of resistance involves mutations in *EGFR* itself or activation of downstream or bypass signaling pathways. The preclinical studies are useful in describing the spectrum of potential mechanisms of acquired drug resistance thus allowing the development of potential treatment strategies that can implemented clinically.

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Author Contributions

Conception and Design: D.E., N.S.G. and P.A.J.

Acquisition of Data: D.E., H.G.C., C.-H.Y., T.X., and M.C.

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Writing, review, and/or revision of the manuscript: D.E., M.J.E., N.S.G. and P.A.J.

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References

1.   Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009;361:947-57.

2.   Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004;304:1497-500.

3.   Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129-39.

4.   Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012;13:239-46.

5.   Sequist LV, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol. 2013;31:3327-34.

6.   Wu YL, Zhou C, Hu CP, Feng J, Lu S, Huang Y, et al. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-Lung 6): an open-label, randomised phase 3 trial. Lancet Oncol. 2014;15:213-22.

7.   Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med. 2010;362:2380-8.

24

8.   Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, et al. Erlotinib versus chemotherapy

as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung

cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study.

Lancet Oncol. 2011;12:735-42.

9.   Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib

versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations

of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial.

Lancet Oncol. 2010;11:121-8.

10.   Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, et al. Analysis of

tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with

EGFR-mutant lung cancers. Clin Cancer Res. 2013;19:2240-7.

11.   Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The

T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc

Natl Acad Sci U S A. 2008;105:2070-5.

12.   Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, et al. BIBW2992,

an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models.

Oncogene. 2008;27:4702-11.

13.   Miller VA, Hirsh V, Cadranel J, Chen YM, Park K, Kim SW, et al. Afatinib versus

placebo for patients with advanced, metastatic non-small-cell lung cancer after failure of

erlotinib, gefitinib, or both, and one or two lines of chemotherapy (LUX-Lung 1): a phase 2b/3

randomised trial. Lancet Oncol. 2012;13:528-38.

14.   Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective

EGFR kinase inhibitors against EGFR T790M. Nature. 2009;462:1070-4.

25

15.   Walter AO, Sjin RT, Haringsma HJ, Ohashi K, Sun J, Lee K, et al. Discovery of a

mutant-selective covalent inhibitor of EGFR that overcomes T790M-mediated resistance in

NSCLC. Cancer Discov. 2013;3:1404-15.

16.   Cross DA, Ashton SE, Ghiorghiu S, Eberlein C, Nebhan CA, Spitzler PJ, et al.

AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR

inhibitors in lung cancer. Cancer Discov. 2014.

17.   Janne PA, Ramalingam SS, Yang JC-H, Ahn M-J, Kim D-W, Kim S-W, et al. Clinical

activity of the mutant-selective EGFR inhibitor AZD9291 in patients (pts) with EGFR inhibitor–

resistant non-small cell lung cancer (NSCLC). J Clin Oncol. 2014;32:abstract 8009.

18.   Sequist LV, Soria J-C, Gadgeel SM, Wakelee HA, Camidge DR, Varga A, et al. First-in-

human evaluation of CO-1686, an irreversible, highly selective tyrosine kinase inhibitor of

mutations of EGFR (activating and T790M). J Clin Oncol. 2014;32:abstract 8010.

19.   Ercan D, Xu C, Yanagita M, Monast CS, Pratilas CA, Montero J, et al. Reactivation of

ERK Signaling Causes Resistance to EGFR Kinase Inhibitors. Cancer Discov. 2012;2:934-47.

20.   Cortot AB, Repellin CE, Shimamura T, Capelletti M, Zejnullahu K, Ercan D, et al.

Resistance to irreversible EGF receptor tyrosine kinase inhibitors through a multistep mechanism

involving the IGF1R pathway. Cancer Res. 2013;73:834-43.

21.   Ercan D, Zejnullahu K, Yonesaka K, Xiao Y, Capelletti M, Rogers A, et al.

Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor. Oncogene.

2010;29:2346-56.

22.   Suda K, Murakami I, Katayama T, Tomizawa K, Osada H, Sekido Y, et al. Reciprocal

and complementary role of MET amplification and EGFR T790M mutation in acquired

resistance to kinase inhibitors in lung cancer. Clin Cancer Res. 2010;16:5489-98.

26

23.   Ku JL, Kim KH, Choi JS, Jeon YK, Kim SH, Shin YK, et al. Establishment and characterization of six human lung cancer cell lines: EGFR, p53 gene mutations and expressions of drug sensitivity genes. Cell Oncol (Dordr). 2011;34:45-54.

24.   Bradeen HA, Eide CA, O'Hare T, Johnson KJ, Willis SG, Lee FY, et al. Comparison of imatinib mesylate, dasatinib (BMS-354825), and nilotinib (AMN107) in an N-ethyl-N-nitrosourea (ENU)-based mutagenesis screen: high efficacy of drug combinations. Blood. 2006;108:2332-8.

25.   Engelman JA, Janne PA, Mermel C, Pearlberg J, Mukohara T, Fleet C, et al. ErbB-3 mediates phosphoinositide 3-kinase activity in gefitinib-sensitive non-small cell lung cancer cell lines. Proc Natl Acad Sci U S A. 2005;102:3788-93.

26.   Takezawa K, Pirazzoli V, Arcila ME, Nebhan CA, Song X, de Stanchina E, et al. HER2 Amplification: A Potential Mechanism of Acquired Resistance to EGFR Inhibition in EGFR-Mutant Lung Cancers That Lack the Second-Site EGFRT790M Mutation. Cancer Discov. 2012;2:922-33.

27.   Penzel R, Sers C, Chen Y, Lehmann-Muhlenhoff U, Merkelbach-Bruse S, Jung A, et al. EGFR mutation detection in NSCLC--assessment of diagnostic application and recommendations of the German Panel for Mutation Testing in NSCLC. Virchows Archiv : an international journal of pathology. 2011;458:95-8.

28.   Locatelli-Sanchez M, Couraud S, Arpin D, Riou R, Bringuier PP, Souquet PJ. Routine EGFR molecular analysis in non-small-cell lung cancer patients is feasible: exons 18-21 sequencing results of 753 patients and subsequent clinical outcomes. Lung. 2013;191:491-9.

29.   Liu Y, Wu BQ, Zhong HH, Hui P, Fang WG. Screening for EGFR and KRAS mutations in non-small cell lung carcinomas using DNA extraction by hydrothermal pressure coupled with

27

PCR-based direct sequencing. International journal of clinical and experimental pathology. 2013;6:1880-9.

30.   Munfus-McCray D, Cui M, Zhang Z, Gabrielson E, Askin F, Li QK. Comparison of EGFR and KRAS mutations in primary and unpaired metastatic lung adenocarcinoma with potential chemotherapy effect. Human pathology. 2013;44:1286-92.

31.   Taron M, Ichinose Y, Rosell R, Mok T, Massuti B, Zamora L, et al. Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor are associated with improved survival in gefitinib-treated chemorefractory lung adenocarcinomas. Clin Cancer Res. 2005;11:5878-85.

32.   Godin-Heymann N, Bryant I, Rivera MN, Ulkus L, Bell DW, Riese DJ, 2nd, et al. Oncogenic activity of epidermal growth factor receptor kinase mutant alleles is enhanced by the T790M drug resistance mutation. Cancer Res. 2007;67:7319-26.

33.   Godin-Heymann N, Ulkus L, Brannigan BW, McDermott U, Lamb J, Maheswaran S, et al. The T790M "gatekeeper" mutation in EGFR mediates resistance to low concentrations of an irreversible EGFR inhibitor. Mol Cancer Ther. 2008;7:874-9.

34.   Janjigian YY, Smit EF, Groen HJ, Horn L, Gettinger S, Camidge DR, et al. Dual Inhibition of EGFR with Afatinib and Cetuximab in Kinase Inhibitor-Resistant EGFR-Mutant Lung Cancer with and without T790M Mutations. Cancer Discov. 2014.

35.   Cho J, Chen L, Sangji N, Okabe T, Yonesaka K, Francis JM, et al. Cetuximab response of lung cancer-derived EGF receptor mutants is associated with asymmetric dimerization. Cancer Res. 2013;73:6770-9.

Author Manuscript Published OnlineFirst on May 6, 2015; DOI: 10.1158/1078-0432.CCR-14-2789

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

36.   Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired

EGFR C797S mediates resistance to AZD9291 in advanced non-small cell lung cancer harboring

EGFR T790M. Nat Med. 2015:in press.

37.   Woyach JA, Furman RR, Liu TM, Ozer HG, Zapatka M, Ruppert AS, et al. Resistance

mechanisms for the Bruton's tyrosine kinase inhibitor ibrutinib. N Engl J Med. 2014;370:2286-

94.

Figure Legends

Figure 1. Identification of novel drug resistance but not oncogenic *EGFR* mutations. **A.**

Summary of *EGFR* secondary mutations identified from Ba/F3 cells following ENU treatment

and selection in the presence of WZ4002. **B.** *EGFR* L718Q and L844V Ba/F3 cells do not grow

in the absence of EGF or IL-3. **C.** EGFR L718Q and L844V Ba/F3 cells retain sensitivity to

irreversible quinazoline EGFR inhibitors. Cells were treated with different drugs at the indicated

concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to

untreated controls. For Western blots, 3T3 cells expressing the respective constructs were treated

with different drugs at indicated concentrations for 16 hours. Cell extracts were immunoblotted

to detect the indicated proteins. **D.** EGFR Del 1/L718Q Ba/F3 cells have a growth

disadvantage. Equal number of cells were seeded in the presence of or absence of EGF or IL-3.

Cell number was evaluated in triplicate at the indicated time points.

Figure 2. Impact of *EGFR* L718Q and L844V WZ4002 binding and sensitivity to quinazoline

based EGFR inhibitors. **A.** Crystal structure of EGFR T790M in complex with WZ4002 (drawn

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from PDB Code 3IKA). Both L718 and L844 directly contact the inhibitor, which forms a covalent bond with C797. **B.** Structural model illustrating the effects of the L718Q and L844V resistance mutations. The glutamine side chain at position 718 is expected to sterically interfere with binding of WZ4002, while the shorter valine substitution at position 844 will alter hydrophobic contacts with the inhibitor. **C.** EGFR L718Q and L844V reduce WZ4002 binding. Cell extracts from NIH-3T3 cells expressing different EGFR mutations following affinity labeling with increasing concentrations of TX 2-30 were immunoblotted using an anti-EGFR antibody. **D.** Del/T790M/L718Q and Del/T790M/L844V Ba/F3 are resistant to all known EGFR kinase inhibitors. **E.** Del/L718Q and Del/L844V double mutant Ba/F3 cells retain sensitivity to quinazoline based EGFR inhibitors. **F.** Summary of *EGFR* secondary mutations identified from Ba/F3 cells following ENU treatment and selection in the presence of gefitinib alone, WZ4002 alone or with the combination. **G.** Combination of WZ4002 and gefitinib is effective in EGFR L858R/L718Q Ba/F3 cells.

Figure 3. Comparison of irreversible pyrimidine EGFR inhibitors and with afatinib. **A.** Afatinib is more effective at inhibiting WT EGFR than irreversible pyrimidine inhibitors. Cells were treated with different drugs at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. For Western blots, 3T3 cells expressing the respective constructs were treated with different drugs at indicated concentrations for 16 hours. Cell extracts were immunoblotted to detect the indicated proteins. **B.** AZD9291 is most potent inhibitor in PC9 GR4 (Del 19/T790M) cells and the efficacy is mirrored be effects on phosphorylation of EGFR and downstream signaling.

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Figure 4. WZ4002, CO-1686 and AZD9291 have differential effects against EGFR tertiary mutations. **A.** IC$_{50}$ comparisons in *EGFR* mutant Ba/F3 cells with EGFR activating mutations alone or in the presence of a concurrent *EGFR* T790M. Both afatinib and AZD9291 are effective in cell lines with an EGFR activating mutation together with either L718Q or L844V. Only AZD9291 is effective in EGFR L858R or Del 19/T790M/L844V cells. **B.** NIH-3T3 cells expressing EGFR Del 19/L844V were treated with different drugs at indicated concentrations for 16 hours. Cell extracts were immunoblotted to detect the indicated proteins. **C.** NIH-3T3 cells expressing EGFR Del 19/T790M/L718Q or Del 19/T790M/L844V were treated with different drugs at indicated concentrations for 16 hours. Cell extracts were immunoblotted to detect the indicated proteins. **D.** Summary of *EGFR* secondary mutations identified from Ba/F3 cells following ENU treatment and selection in the presence of WZ4002, CO-1686 or AZD9291 at either 100 nM or 1 μM.

Figure 5. *EGFR* L718Q and L844V cause resistance in *trans* in *EGFR* mutant NSCLC cell lines. **A.** PC9 GR4 (Del 19/T790M) cells were retrovirally infected to express either GFP, *EGFR* Del 19/T790M/L718Q or EGFR Del 19/T790M/L844V. Cells were treated with different drugs at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls. **B .*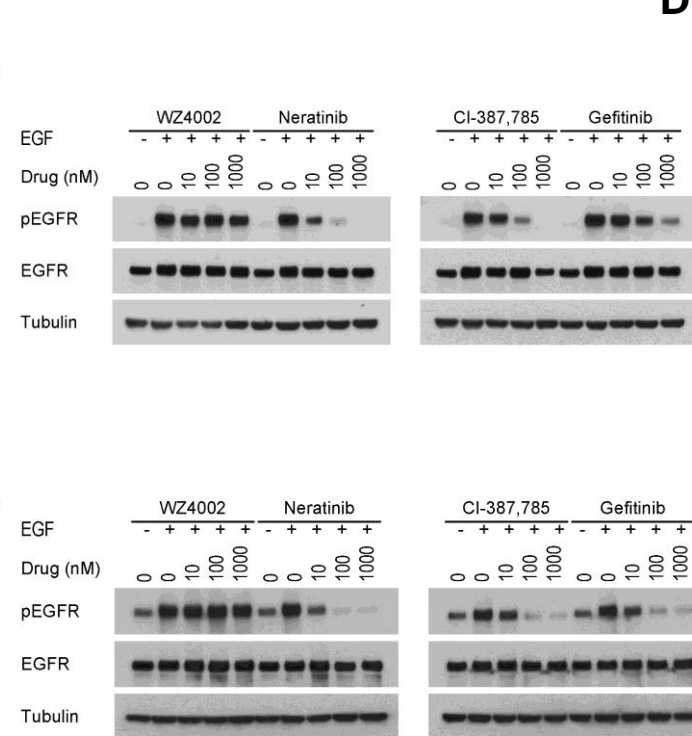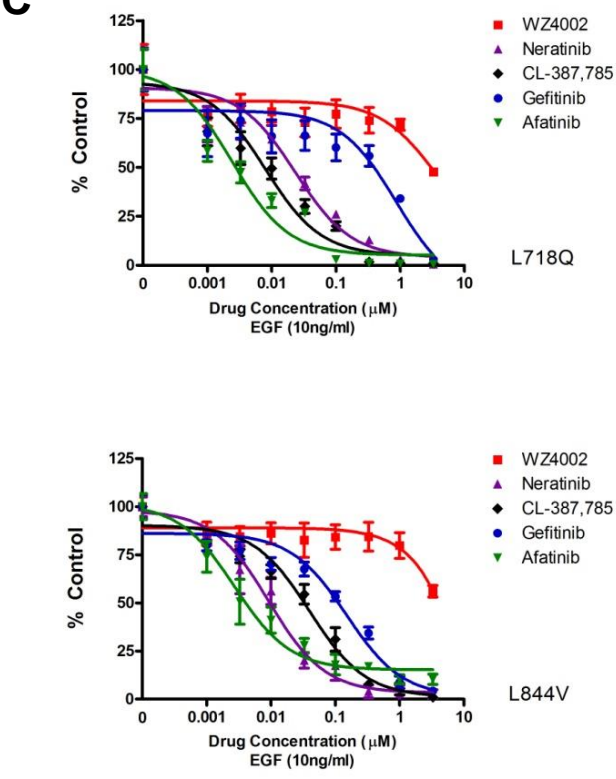* Ce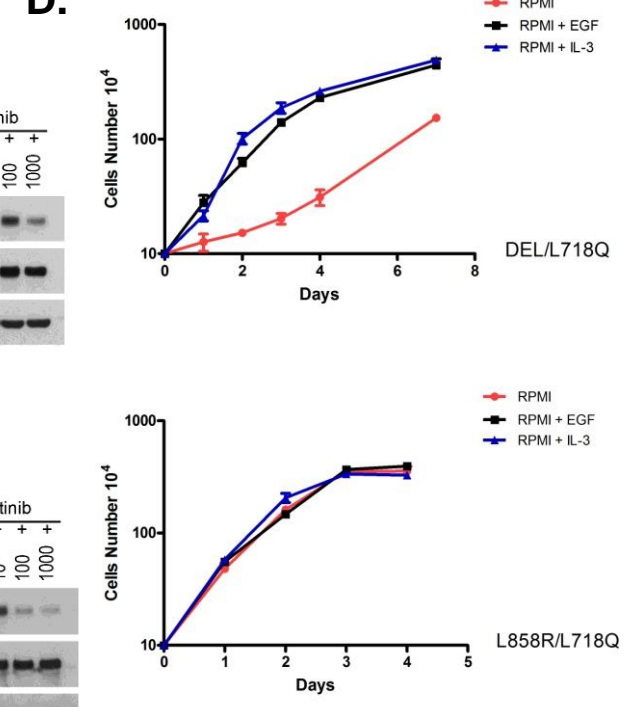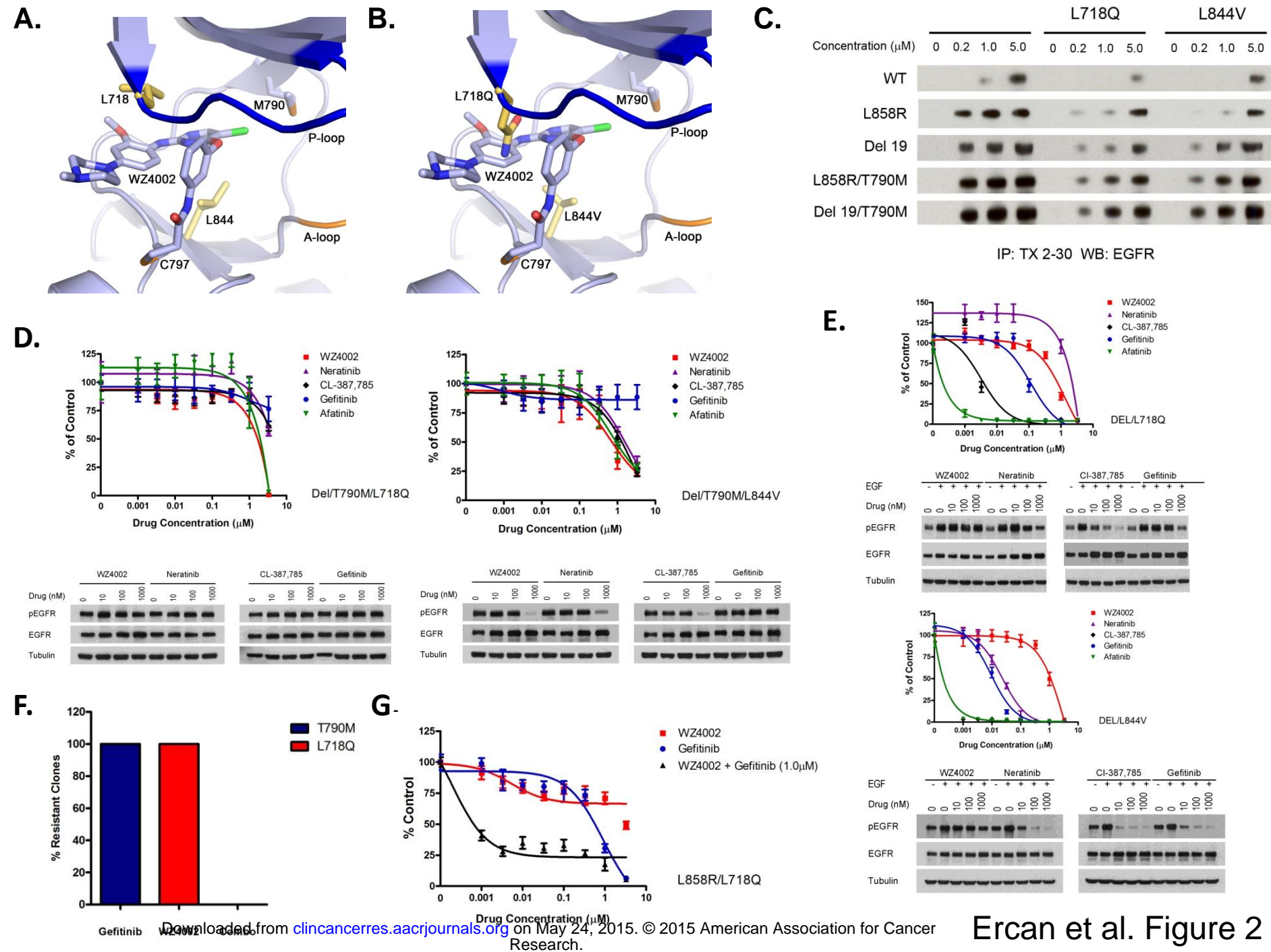lls from A. were treated with indicated drugs for 16 hours. Cell extracts were immunoblotted to detect the indicated proteins. **C.** PC9GR cells expressing different EGFR constructswere plated on agar with indicated drugs for 8 days, after which the absorbance was measured according to the manufacturer's protocol. Data are means +/- SD of triplicates from an experiment that was repeated 3 times with similar results. D.

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PC9GR cells expressing indicated constructs were treated with afatinib alone, cetuximab alone or with the combination. Cell extracts were immunoblotted to detect the indicated proteins.

Figure 6. *EGFR* C797S containing cells remain sensitive to current EGFR inhibitors in some genomic contexts. **A.** PC9 (Del 19) cells were retrovirally infected to express either GFP, or *EGFR* Del19/C797S. Cells were treated with different drugs at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls **B.** Cells from A. were treated with indicated drugs for 16 hours. Cell extracts were immunoblotted to detect the indicated proteins. **C.** Summary of *EGFR* secondary mutations identified from *EGFR* L858R Ba/F3 cells following ENU treatment and selection in the presence of gefitinib alone, AZD9291 alone or with the combination. **D.** *EGFR* L858R/T790M Ba/F3 cells or those harboring a concurrent L718Q, C797S or L844V mutation were treated with cetuximab for 16 hours. Cells were incubated to nonpermeable cross-linker bis (sulfosuccinimidyl) substrate. Cell extracts were immunoblotted to detect the indicated proteins. D, dimer; M, monomer. **E.** Cells from D were treated with cetuximab at the indicated concentrations, and viable cells were measured after 72 hours of treatment and plotted relative to untreated controls.

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Ercan et al. Figure 1

A.

L718 · M790 · WZ4002 · L844 · C797 · P-loop · A-loop

B.

L718Q · M790 · WZ4002 · L844V · C797 · P-loop · A-loop

C.

Concentration (μM)   L718Q   L844V

0 0.2 1.0 5.0   0 0.2 1.0 5.0   0 0.2 1.0 5.0

WT
L858R
Del 19
L858R/T790M
Del 19/T790M

IP: TX 2-30 WB: EGFR

D.

% of Control vs Drug Concentration (μM)

- WZ4002
- Neratinib
- CL-387,785
- Gefitinib
- Afatinib

Del/T790M/L718Q

Del/T790M/L844V

Drug (nM): 0 10 100 1000 — WZ4002 · Neratinib · CL-387,785 · Gefitinib

pEGFR
EGFR
Tubulin

E.

- WZ4002
- Neratinib
- CL-387,785
- Gefitinib
- Afatinib

DEL/L718Q

EGF: − + + + +   Drug (nM): 0 0 10 100 1000
WZ4002 · Neratinib · CI-387,785 · Gefitinib
pEGFR
EGFR
Tubulin

DEL/L844V

WZ4002 · Neratinib · CI-387,785 · Gefitinib
pEGFR
EGFR
Tubulin

F.

% Resistant Clones

- T790M
- L718Q

Gefitinib   WZ4002

G.

% Control vs Drug Concentration (μM)

- WZ4002
- Gefitinib
- WZ4002 + Gefitinib (1.0μM)

L858R/L718Q

Ercan et al. Figure 2

A. Wild Type

B. PC9GR4

Ercan et al. Figure 3

A.

B.

C.

D.

Ercan et al. Figure 4

A.

B.

C.

D.

Ercan et al. Figure 5

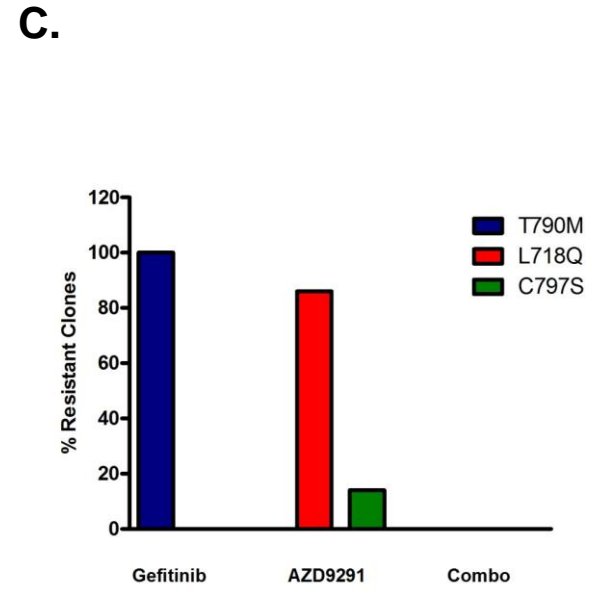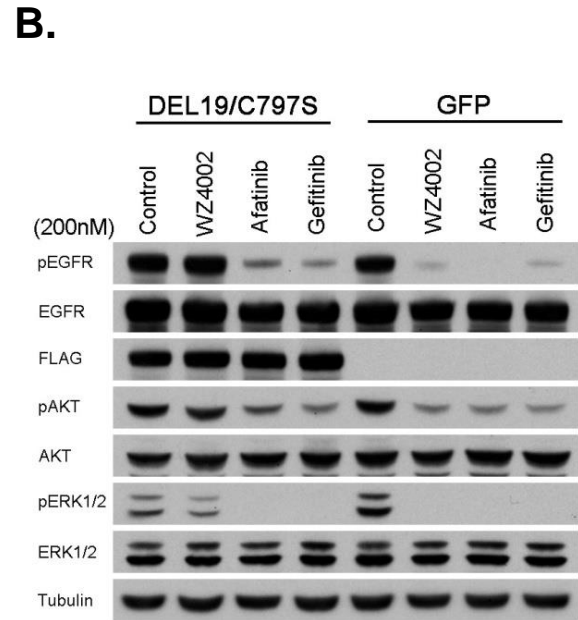Ercan et al. Figure 6

AACR   American Association
        for Cancer Research
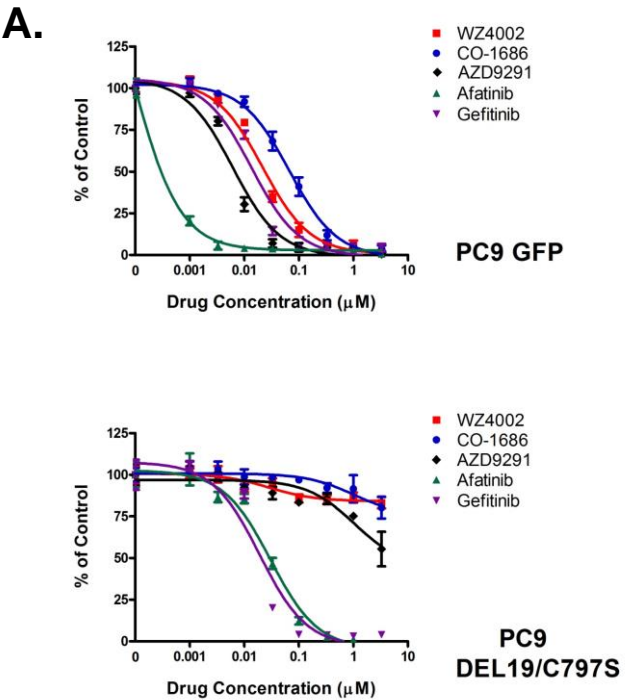
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EGFR mutations and resistance to Irreversible pyrimidine based EGFR inhibitors

Dalia Ercan, Hwan Geun Choi, Cai-Hong Yun, et al.

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