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Supplementary Materials for

Optimization of Dosing for EGFR-Mutant Non–Small Cell Lung Cancer with Evolutionary Cancer Modeling

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SUPPLEMENTARY MATERIALS AND METHODS

EGFR sequencing

Genomic DNA was extracted from patient samples (≥70% tumor cells) and cell lines using standard procedures. Dideoxynucleotide sequencing of *EGFR* (exons 2-28) was performed as described (*S1*).

For 454 deep sequencing of *EGFR* exon 20, 80 ng of genomic DNA per sample was used for PCR amplification with 454 barcoded primers (F1: GCCTCCCTCGCGCCATCAGACGAGTGCGTACACTGACGTGCCTCTCCCT or F2:GCCTCCCTCGCGCCATCAGACGCTCGACAACACTGACGTGCCCTCTCCCT and R: GCCTTGCCAGCCCGCTCAGCGTATCTCCCTTCCCTGATT). PCR was performed with *Pfu* polymerase to ensure greater sequence accuracy. PCR products were separated from excess primers and dNTPs using AMPure reagent (Agencourt), as per the manufacturer's instructions. Approximately 100,000 reads per sample were generated using a 454 FLX platform (Roche). Alignment and variant detection was carried out using the ssahaSNP algorithm from the Sanger Institute (*S2*). The output of the ssahaSNP pipeline was then filtered by a set of custom written python scripts to filter on various quality metrics including total coverage, variant coverage, integrated and average Q score and frequency of variant.

Cell culture and derivation of TKI-resistant lines

EGFR mutant PC-9 cells (del E746-A750) (*S3*) or HCC827 cells (del E746-A750) were cultured in RPMI media (ATCC or Mediatech) supplemented with 10% heat

inactivated fetal bovine serum (Gemini Bio Products) and pen-strep solution (Gemini Bio Products; final concentration 100U/mL penicillin, 100µg/mL streptomycin). Cells were grown in a humidified incubator with 5% CO_2 at 37^{o}C. Resistant cells were derived as described (*S4, S5*). NR-6 and H322M cells have been previously reported (*S6, S7*).

To create EGFR TKI resistant lines, parental cells were cultured with increasing concentrations of TKIs starting with the IC_{30}. Doses were increased in a stepwise pattern when normal cell proliferation patterns resumed. Fresh drug was added every 72-96 hours. Resistant cells that grew in 500 nM BIBW-2992 and 5 µM erlotinib were derived after ~ 3 months of culturing with drug. Resistant cells were maintained initially as polyclonal populations under constant TKI selection. Clonal resistant cells were isolated by limiting dilution.

aCGH profiling

Genomic DNA from cell lines and controls was prepared, labeled, and hybridized to Agilent 244k arrays, as previously described (*S8*). Analysis was performed using DNA Analytics software (Agilent).

Immunoblotting

Cells were washed with cold PBS and lysed for 30 minutes with RIPA buffer (150mM Tris.HCl pH 7.5, 150mM NaCl, 1% NP-40 substitute, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche), 40mM sodium fluoride, 1mM sodium orthovanadate, and 1µM okadaic acid. Protein was quantified with Bradford Reagent

(Bio-Rad) and equal amounts were loaded for SDS-PAGE with 4-20% acrylamide pre-cast gels (Invitrogen), followed by transfer to PVDF membranes. Membranes were blotted with pEGFR (Y1068/1092), total EGFR (BD Biosciences), pAKT (S473), total AKT, pERK (T202/204), total ERK, and actin (Sigma-Aldrich) followed by HRP-conjugated secondary antibodies. All antibodies were purchased from Cell Signaling Technology, unless noted. Signals were detected with Western blotting detection reagents (GE Healthcare).

Thymidine Incorporation

Thymidine incorporation was determined as described previously with some modifications (*S9*). Briefly, cells were seeded at a density of 20,000 cells per well in 24-well tissue culture plates and incubated with compounds for 24 hours. ^{3}H-methyl thymidine (1 µCi/mL) was added during the last 2 hours of drug treatment. Cells were then washed three times with ice-cold 10% TCA (v/v) and then lysed in 500 µL 0.5 M NaOH (v/v) per well. The amount of labeled thymidine incorporated into DNA was determined by liquid scintillation counting. Protein concentration was determined in parallel samples and results were corrected for protein expression.

Fluorescence in situ hybridization (FISH)

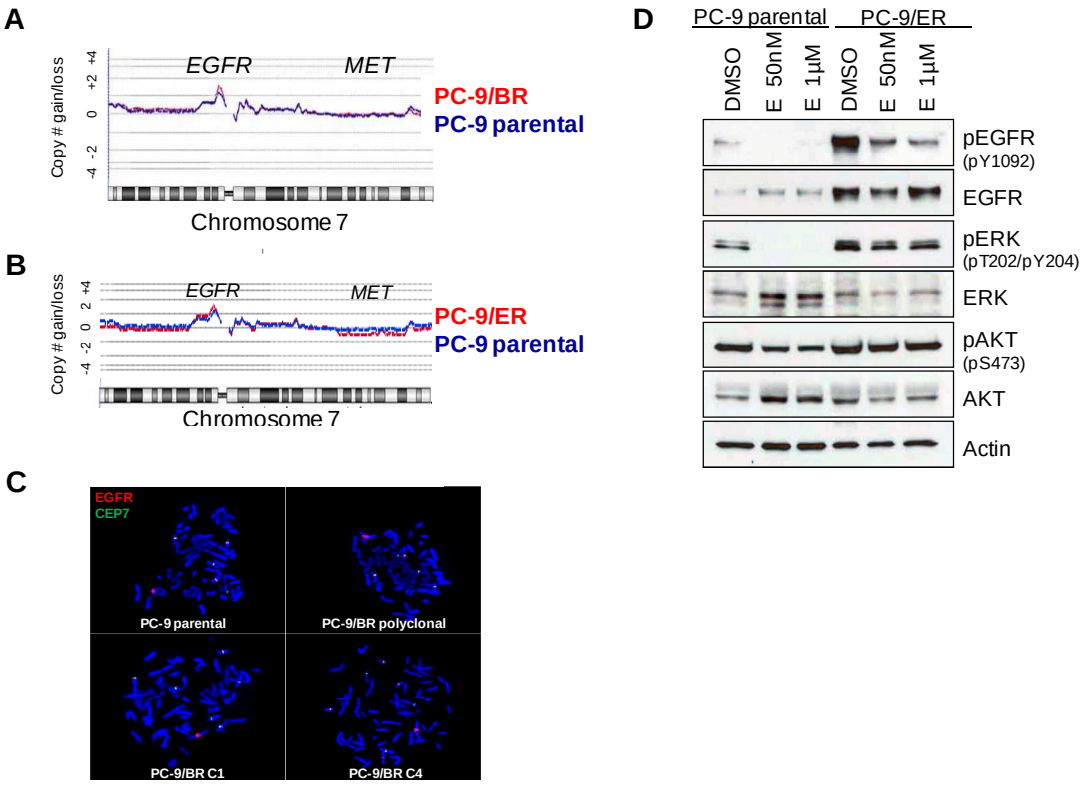
Cells were grown in RPMI 1640 with 10% FBS to ~70% confluence. One hour prior to harvest, colcemid (Invitrogen) was added into each flask containing 10 mL growth medium. Metaphase FISH slides preparation was performed as previously described (*S8*).

The EGFR/CEP7 probe set from Abbott Molecular was used for dual-color FISH. FISH was performed according to the protocol from Vysis/Abbott Molecular with a few modifications. In brief, a probe targeting the EGFR gene was labeled with SpectrumOrange (red), and a Chromosome 7 centromere probe (CEP7) was labeled with SpectrumGreen (green); nuclei were counterstained with DAPI (blue). FISH slide analysis and signal capture were performed by Fluorescence microscope (Zeiss) coupled with ISIS FISH Imaging System (Metasystems) following the manufacturer's instruction. Metaphases showing optimum hybridization signals were scored. 6 representative metaphases of each sample were captured.

Drug Sources

Cisplatin was obtained from the VICC and MSKCC pharmacies. Pralatrexate was also obtained from the MSKCC pharmacy. Erlotinib and BIBW-2992 were synthesized at the MSKCC organic synthesis core.

Figure S1


A
Copy # gain/loss
+4
+2
0
-2
-4
EGFR
MET
PC-9/BR
PC-9 parental
Chromosome 7
B
Copy # gain/loss
+4
2
0
-2
-4
EGFR
MET
PC-9/ER
PC-9 parental
Chromosome 7
C
EGFR
CEP7
PC-9 parental
PC-9/BR polyclonal
PC-9/BR C1
PC-9/BR C4
D
PC-9 parental
PC-9/ER
DMSO
E 50nM
E 1μM
DMSO
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ERK
pAKT
(pS473)
AKT
Actin

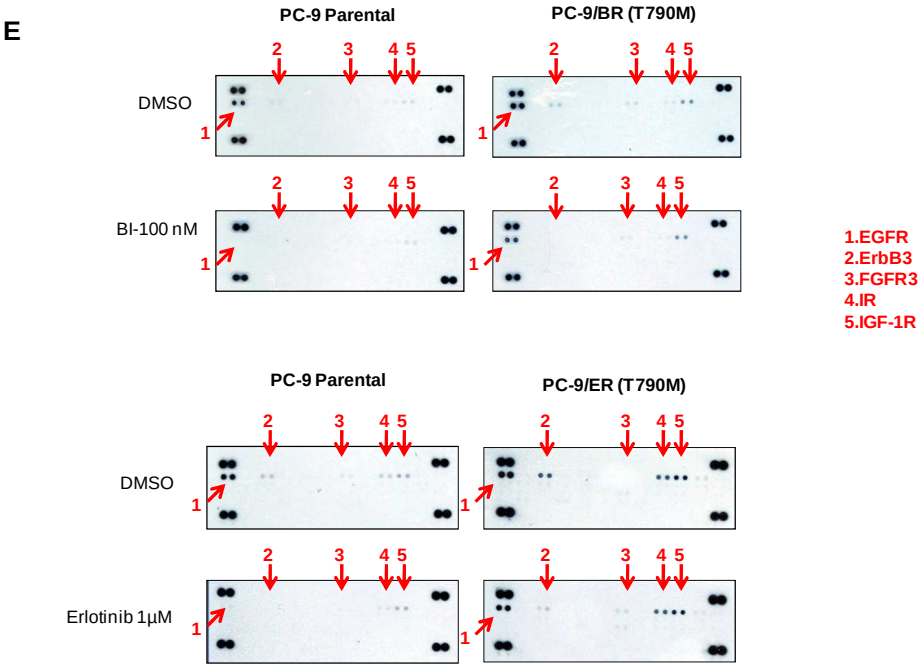

E
PC-9 Parental
PC-9/BR (T790M)
DMSO
BI-100 nM
1
2
3
4
5
1.EGFR
2.ErbB3
3.FGFR3
4.IR
5.IGF-1R
PC-9 Parental
PC-9/ER (T790M)
DMSO
Erlotinib 1μM

Figure S1. Further characterization of PC-9 TKI-resistant cell lines. (A) Array comparative genomic hybridization (aCGH) of chromosome 7 shows further amplification of *EGFR* in the BIBW-2992-resistant cells (PC-9/BR; red) and the erlotinib-resistant cells (PC-9/ER) (**B)** versus the parental cells (blue). Neither cell line, however, displayed amplification of *MET*, another receptor tyrosine kinase involved in TKI-resistance. The relative positions of *EGFR* and *MET* are labeled on the chromosome. **(C)** Fluorescence in situ hybridization (FISH) staining of metaphase cells confirms the genomic amplification of *EGFR* in the BR polyclonal cells and two resistant clones compared to parental cells. *EGFR* was not amplified on double minute chromosomes, a previously reported mechanism of resistance and TKI re-sensitization. *EGFR* is labeled in red; chromosome 7 centromere (CEP7) is labeled in green. **(D)** Parental and resistant (ER) cells were treated with vehicle (DMSO) or erlotinib (E) for 3 hours prior to protein isolation. Parental cells show decreased phosphorylation of EGFR and its downstream targets, ERK and AKT, in the presence of low (50 nM) and high (1 μM) concentrations of erlotinib. Signaling in the PC-9/ER cells remains intact, regardless of the concentration of drug. **(E)** Phospho-receptor tyrosine kinase (RTK) arrays profiling PC-9 parental, ER, and BR cells in the presence and absence of TKI do not show any dramatic differences between the T790M-harboring lines derived with two different TKIs.

Figure S2

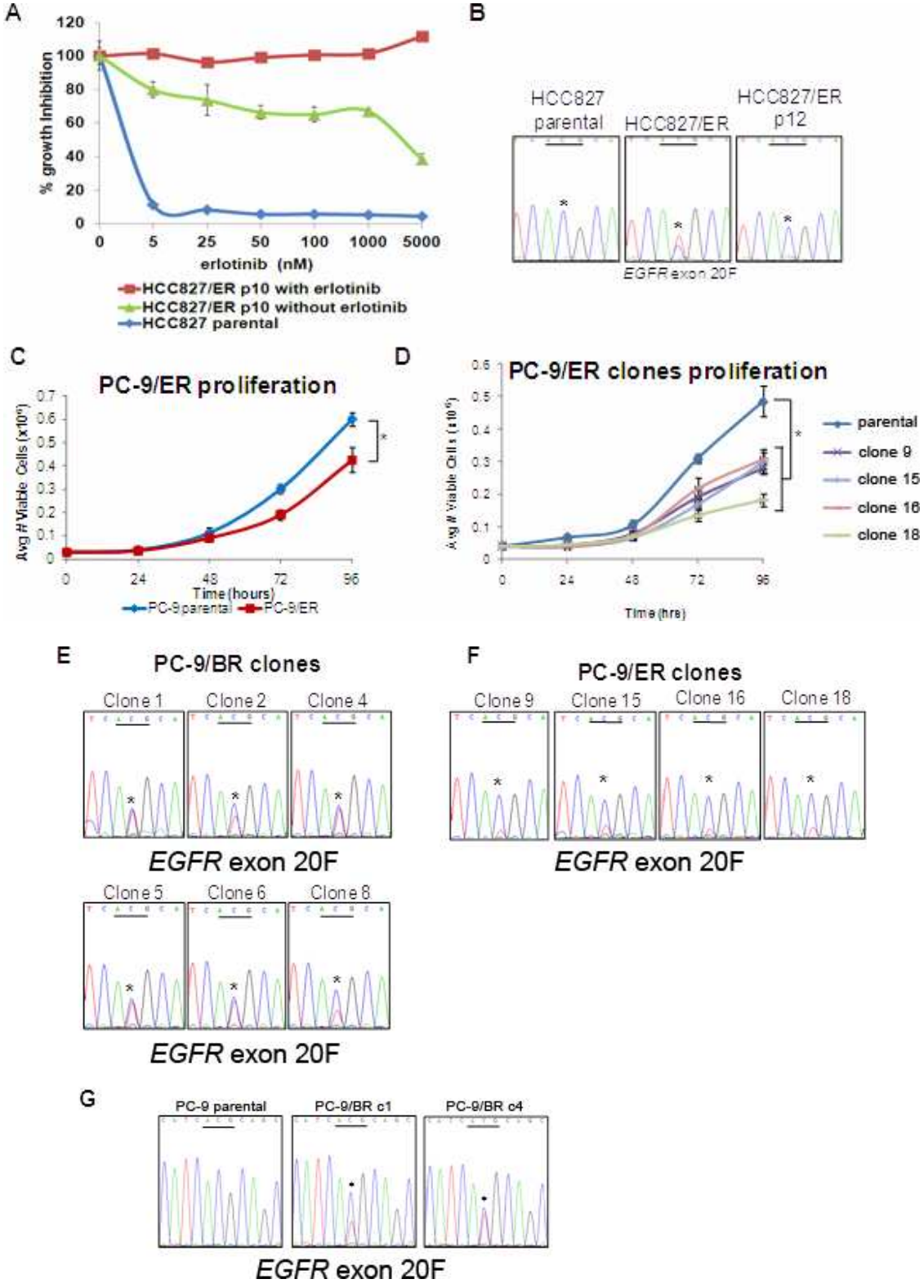

A
% growth inhibition
erlotinib (nM)
HCC827/ER p10 with erlotinib
HCC827/ER p10 without erlotinib
HCC827 parental
B
HCC827 parental
HCC827/ER
HCC827/ER p12
EGFR exon 20F
C
PC-9/ER proliferation
Avg # Viable Cells (x10⁴)
Time (hours)
PC-9 parental
PC-9/ER
D
PC-9/ER clones proliferation
Time (hrs)
parental
clone 9
clone 15
clone 16
clone 18
E
PC-9/BR clones
Clone 1
Clone 2
Clone 4
EGFR exon 20F
Clone 5
Clone 6
Clone 8
EGFR exon 20F
F
PC-9/ER clones
Clone 9
Clone 15
Clone 16
Clone 18
EGFR exon 20F
G
PC-9 parental
PC-9/BR c1
PC-9/BR c4
EGFR exon 20F

Figure S2. Characterization of *EGFR*-mutant TKI-resistant cells. (A) Polyclonal HCC827/ER cells, harboring *EGFR* T790M, were derived after ~150 days in culture with increasing concentrations of erlotinib. Once resistant, cells were then maintained in erlotinib or drug was removed from the culture. Following 10 passages without drug (P12), the cells regained intermediate sensitivity to erlotinib, as shown in growth inhibition assays. By contrast, cells passaged in parallel with erlotinib for 10 passages remained resistant. **(B)** Direct sequencing chromatograms from *EGFR* exon 20 show the presence of T790M (starred) in the HCC827/ER cells and a decrease of the mutant allele in DNA from cells that had been passaged 12 times in the absence of erlotinib (p-12). **(C)** The T790M-containing PC-9/ER cells proliferate more slowly than their parental counterparts. PC-9 parental and ER cells were counted in triplicate over 72 hours in the absence of inhibitor, and the average number of viable cells was plotted +/- standard deviation. **(D)** PC-9/ER clonal cells (derived from polyclonal PC-9/ER cells) also proliferate more slowly than the parental cells. Cells were counted and plotted as described above. (* $p < 0.01$). **(E)** Direct sequencing chromatograms from *EGFR* exon 20 show all BR clones contain the T790M mutation (starred). **(F)** T790M is also observed in clones derived from PC-9/ER cells, at slightly lower levels. **(G)** Direct sequencing chromatograms from *EGFR* exon 20 with DNA extracted from PC-9 parental, PC-9/BR c1, and PC-9/BR c4 xenograft tumors (see **Figure 2G**). The presence of the mutant T790M is starred in c1 and c4. Note that mice with parental tumors had to be sacrificed after 34 days post implantation due to tumor size, while mice with c1 and c4 tumors were not sacrificed until 78 days post implantation.

Figure S3

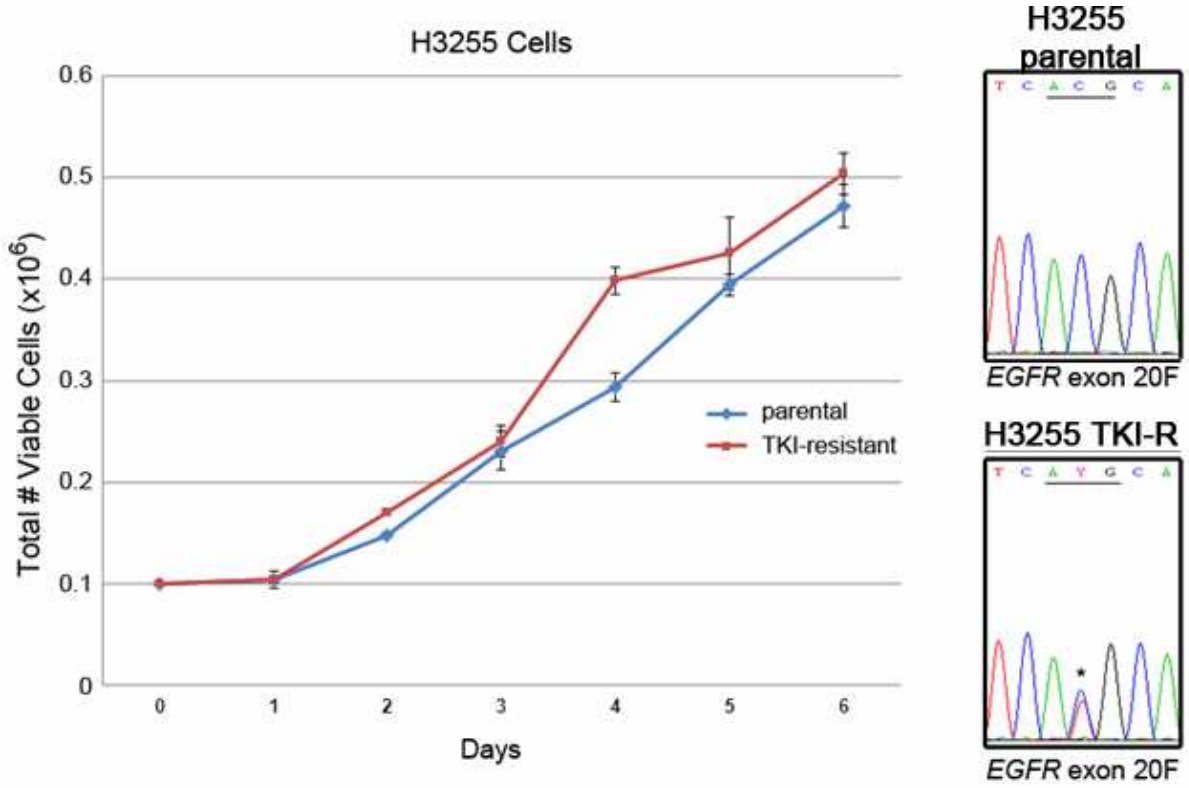


Figure S3. Characterization of H3255 TKI-resistant cells. H3255 parental and TKI-resistant cells were counted daily in the absence of drug. The TKI-resistant cells harbor the T790M-mutation, as shown by direct sequencing of EGFR exon 20 (right panel). Unlike the PC-9 cells, the H3255 TKI-R did not exhibit a growth disadvantage compared to their parental counterparts.

Figure S4

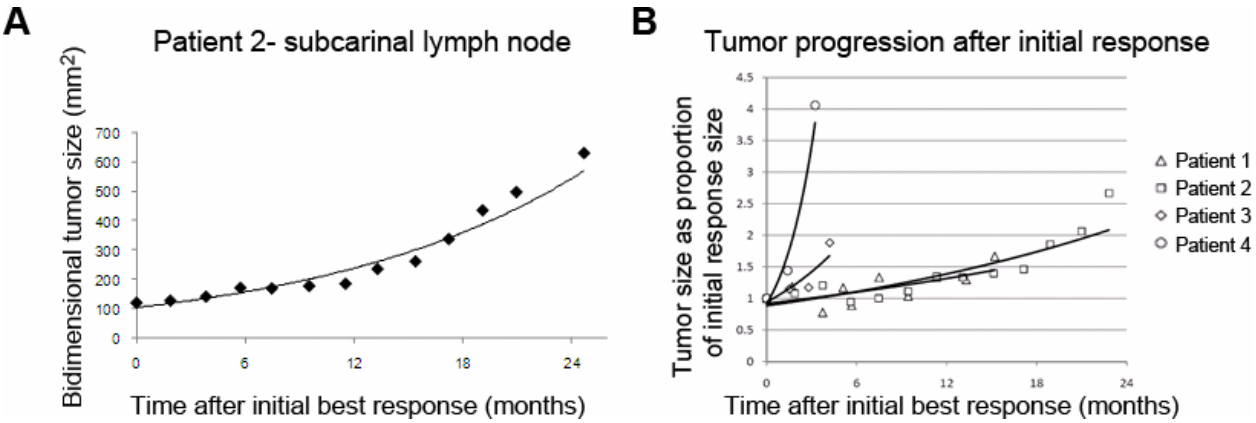
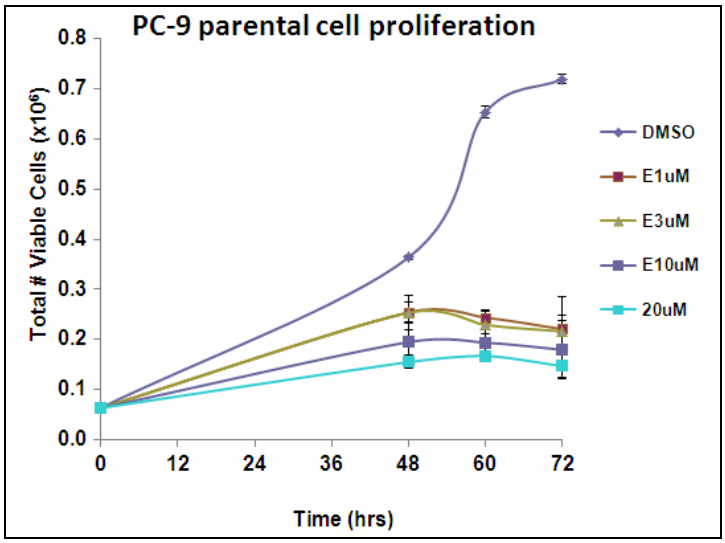
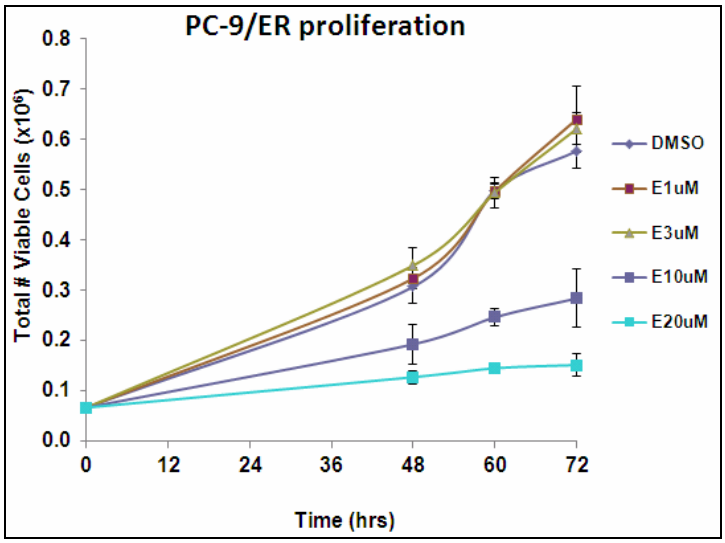


Figure S4. Rate of progression in T790M-harboring *EGFR*-mutant lung cancer.

(A) Bi-dimensional measurements from a second patient with *EGFR* mutant lung cancer show an indolent rate of progression following progressive disease (as determined by RECIST criteria; see **Methods**). **(B)** As a comparison, we reviewed the imaging of disease in patients with *EGFR* wild-type tumors who responded to first-line platinum based chemotherapy on a separate prospective clinical trial. Patients 1 and 2 are the same as those shown in **Figures 4C and S4A**, respectively. T790M-harboring tumors (patients 1 and 2) displayed a slow increase in tumor size, eventually meeting criteria for progressive disease. However, *EGFR* wildtype tumors (patients 3 and 4) had a rapid increase in tumor size between the scan showing progressive disease and the previous scan.

Figure S5


PC-9 parental cell proliferation
Total # Viable Cells (x10⁶)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
Time (hrs)
DMSO
E1uM
E3uM
E10uM
20uM


PC-9/ER proliferation
Total # Viable Cells (x10⁶)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
12
24
36
48
60
72
Time (hrs)
DMSO
E1uM
E3uM
E10uM
E20uM

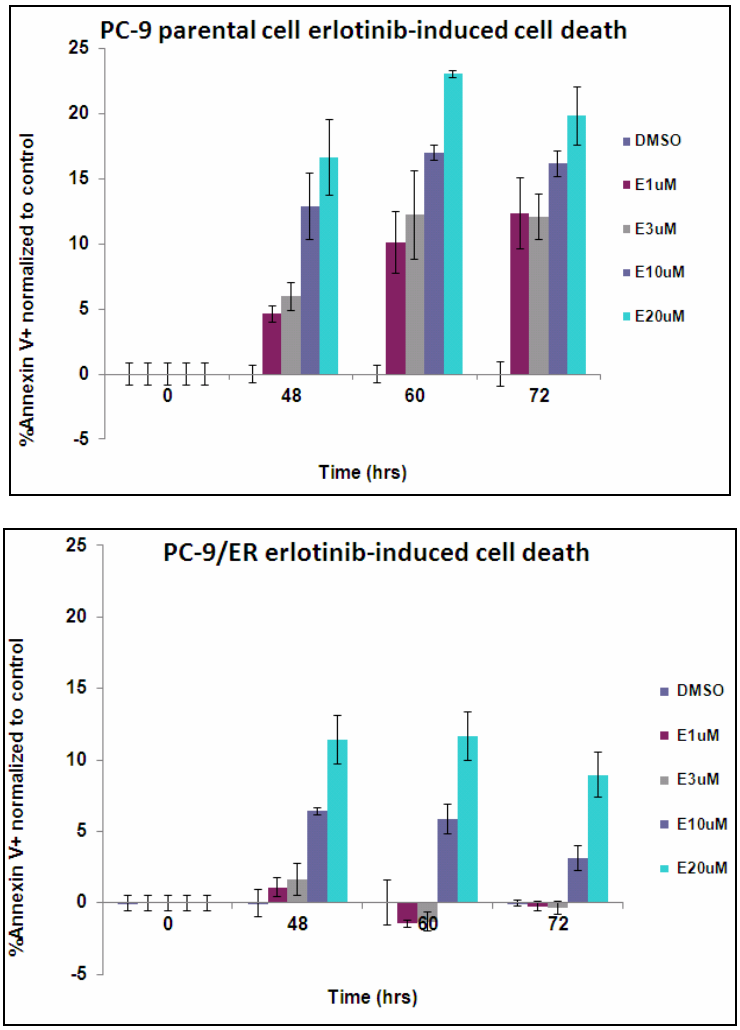


Figure S5. Derivation of mathematical parameters. Growth and death rates of PC-9 parental and PC-9/ER cells were determined by cell counting and annexin V/PI staining, respectively. Measurements were taken every 12 hours beginning 48 hours after the addition of drug. Data are plotted as the average of three replicates +/- standard deviation. Par- parental; ER- erlotinib resistant; E- erlotinib.

Figure S6

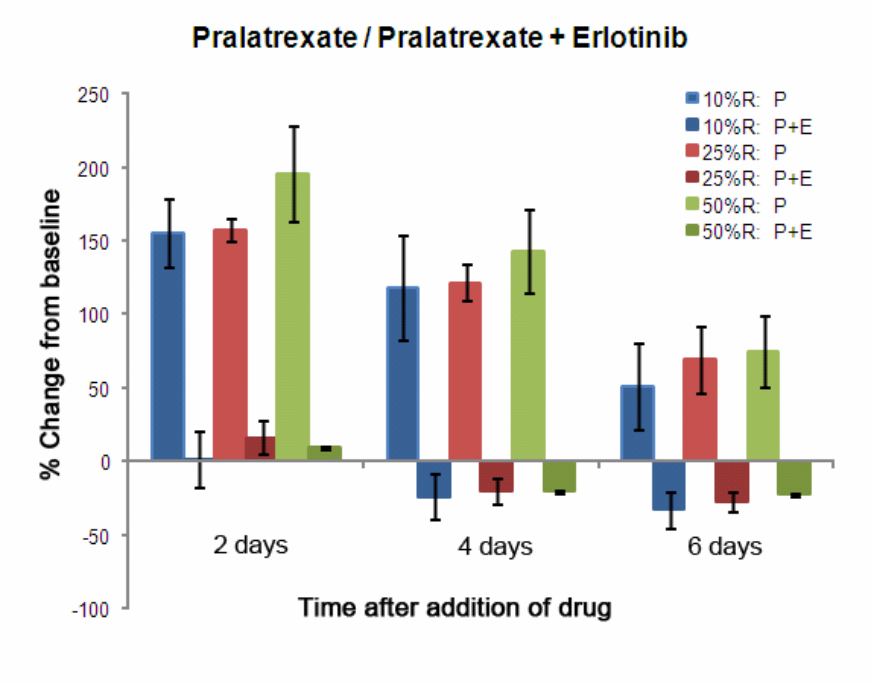


Figure S6. Continuation of TKI therapy in cell populations with T790M-harboring clones leads to better tumor cell control. PC-9/BR c1 resistant cells were diluted in parental cells at various concentrations (see **Figure 3A**) and randomized to receive chemotherapy (pralatrexate, 100 µM) or chemotherapy plus erlotinib (3 µM). In all cases, the TKI-chemotherapy combination was more efficacious at inhibiting cell growth.

Figure S7

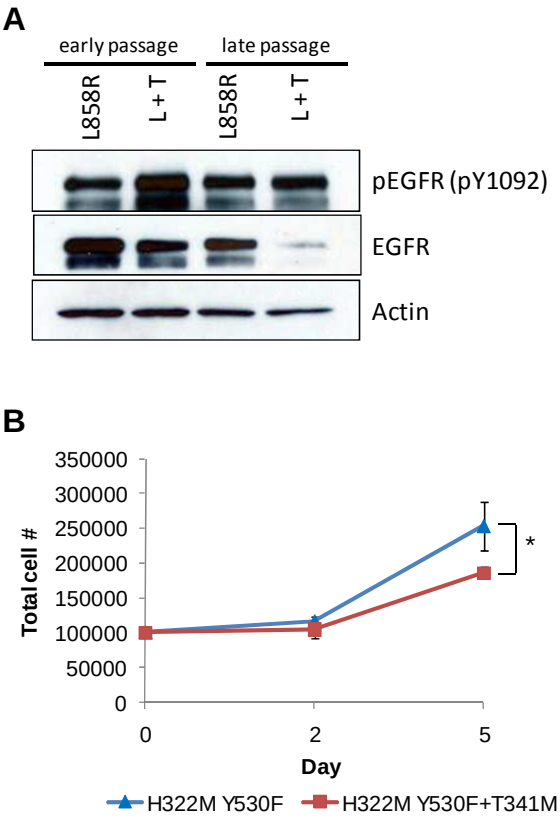


Figure S7. Toxicity of "gatekeeper" mutations in other cell models. (A) NR-6 cells transduced with either L858R or L858R in combination with T790M (L+T) spontaneously selected for lower levels of the double mutant EGFR over time, suggesting toxicity of the double mutant allele in these cells. **(B)** H322M cells were transfected with constitutively active SRC (Y530F) alone or in combination with the SRC gatekeeper mutation (Y530F+T341M). Cells with the double mutant SRC grew slower than the parental and single mutant cells. (* $p < 0.01$)

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