EGFR Kinase Domain Duplication (EGFR-KDD) Is a Novel Oncogenic Driver in Lung Cancer That Is Clinically Responsive to Afatinib

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ABSTRACT

Oncogenic EGFR mutations are found in 10% to 35% of lung adenocarcinomas. Such mutations, which present most commonly as small in-frame deletions in exon 19 or point mutations in exon 21 (L858R), confer sensitivity to EGFR tyrosine kinase inhibitors (TKI). In analyzing the tumor from a 33-year-old male never-smoker, we identified a novel EGFR alteration in lung cancer: EGFR exon 18–25 kinase domain duplication (EGFR-KDD). Through analysis of a larger cohort of tumor samples, we detected additional cases of EGFR-KDD in lung, brain, and other cancers. In vitro, EGFR-KDD is constitutively active, and computational modeling provides potential mechanistic support for its auto-activation. EGFR-KDD-transformed cells are sensitive to EGFR TKIs, and consistent with these in vitro findings, the index patient had a partial response to the EGFR TKI afatinib. The patient eventually progressed, at which time resequencing revealed an EGFR-dependent mechanism of acquired resistance to afatinib, thereby validating EGFR-KDD as a driver alteration and therapeutic target.

SIGNIFICANCE: We identified oncogenic and drug-sensitive EGFR-KDD that is recurrent in lung, brain, and soft-tissue cancers and documented that a patient with metastatic lung adenocarcinoma harboring the EGFR-KDD derived significant antitumor response from treatment with the EGFR inhibitor afatinib. Findings from these studies will be immediately translatable, as there are already several approved EGFR inhibitors in clinical use. Cancer Discov; 5(11); 1155–63. ©2015 AACR.

INTRODUCTION

The prospective identification and rational therapeutic targeting of tumor genomic alterations have revolutionized the care of patients with lung cancer and other malignancies. Oncogenic mutations in the EGFR tyrosine kinase domain are found in an important subset of non–small cell lung cancer (NSCLC), and several large phase III clinical trials have shown that patients with EGFR-mutant lung cancer derive superior clinical responses when treated with EGFR tyrosine

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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kinase inhibitors (TKI) as compared with standard chemotherapy (1–3). Such mutations, which most commonly occur as either small in-frame deletions in exon 19 or point mutations in exon 21 (L858R), confer constitutive activity to the EGFR tyrosine kinase and sensitivity to EGFR TKIs (4). Other oncogenic alterations, including ALK and ROS1 gene rearrangements, have similarly allowed for the rational treatment of molecular cohorts of NSCLC. Unfortunately, despite these significant advances in defining clinically relevant molecular cohorts of lung cancer, the currently identified genomic alterations account for only 50% to 60% of all tumors. Additional analyses are necessary to identify therapeutically actionable molecular alterations in these tumors.

Here, we describe the case of a 33-year-old male never-smoker with metastatic lung adenocarcinoma whose tumor lacked all previously described actionable genomic alterations in this disease. Targeted next-generation sequencing (NGS)-based genomic profiling identified a novel in-frame tandem duplication of EGFR exons 18–25, the exons that encode the EGFR tyrosine kinase domain. This EGFR kinase domain duplication (EGFR-KDD) had not previously been reported in lung cancer, and there were no prediagnostic clinical or clinical evidence to support the use of EGFR inhibitors in patients whose tumors harbor the EGFR-KDD. However, the index patient was treated with the EGFR inhibitor afatinib, with rapid symptomatic improvement and significant decrease in tumor burden. Notably, upon disease progression, the patient’s tumor harbored an increase in the copy number of the EGFR-KDD, solidifying the role of this EGFR alteration as a novel driver in this disease. Through analysis of a large set of annotated tumors, we demonstrate that the EGFR-KDD is recurrent in lung, brain, and soft-tissue tumors. Overall, our data show, for the first time, that EGFR-KDD is an oncogenic and therapeutically actionable alteration.

RESULTS

Case Report

A 33-year-old male never-smoker was diagnosed with stage IV lung adenocarcinoma after presenting with cough and fatigue. His tumor biopsy was sent for genomic profiling using an extensively validated hybrid-capture–based NGS diagnostic assay (FoundationOne; ref. 5). The patient’s tumor was found to be negative for any previously described actionable alterations in lung cancer, including negative for the presence of previously described EGFR alterations, such as L858R, G719A/C/S, and L861Q point mutations, exon 19 deletion/insertion, and exon 20 insertion. Interestingly, however, the patient’s tumor was found to harbor an intragenic alteration in EGFR resulting in the tandem duplication of exons 18 to 25 (Supplementary Fig. S1A). The presence of this alteration was confirmed by direct sequencing (data not shown) and by an independent clinical NGS assay (MSK-IMPACT; ref. 6; Supplementary Fig. S1B). Because exons 18 to 25 of EGFR encode the entire tyrosine kinase domain, this alteration results in an EGFR protein that has an in-frame kinase domain duplication (Fig. 1A and Supplementary Fig. S2). Notably, this EGFR alteration had not previously been reported in lung cancer; the EGFR-KDD (as duplication of exons 18–25 or 18–26) had been reported only in isolated cases of glioma to date (7–11). There were no data regarding the frequency of this alteration in tumor samples, nor were there data regarding the efficacy of EGFR-targeted agents against the EGFR-KDD.

Frequency of EGFR-KDD in Lung and Other Cancers

To determine the frequency of the EGFR-KDD in lung cancer and other tumors, we analyzed data from >38,000 clinical cases, each of which had results from FoundationOne targeted sequencing, analogous to the index patient. The EGFR-KDD was detected in 5 tumors from ~7,200 total lung cancers tested. In addition, EGFR-KDD was identified in 3 gliomas, 1 sarcoma, 1 peritoneal carcinoma, and 1 Wilms’ Tumor (Table 1).

Among samples in The Cancer Genome Atlas (TCGA), we found previously unreported cases of the EGFR-KDD in lung adenocarcinoma and glioblastoma multiforme (Table 1 and Supplementary Fig. S3A–S3D). Two additional cases were found by MSK-IMPACT sequencing (Table 1). Together, these data show that the EGFR-KDD is a recurrent mutation in lung cancer, glioma, and other human malignancies. It is important to note, however, that because most conventional (exomic) sequencing platforms would not routinely detect this particular EGFR alteration (due to its intronic breakpoints), these numbers are likely an underestimate, and the true prevalence of the EGFR-KDD remains unknown.

The EGFR-KDD Is Oncogenic

We expressed EGFR-KDD in NR6 and Ba/F3 cells (Fig. 1B–E). We observed expression of EGFR-KDD at the expected molecular weight as compared to EGFR wild-type (EGFRWT) and the well-characterized EGFRL858R mutation (Fig. 1B and D). In contrast to EGFRWT, both EGFRL858R and EGFR-KDD displayed high levels of autophosphorylation. Consistent with these data, EGFR-KDD protein is constitutively autophosphorylated in the absence of serum in A1235 cells, a glioma cell line which harbors endogenous EGFR-KDD (Supplementary Fig. S4; ref. 7). To address whether EGFR-KDD is an oncogenic alteration, we tested its ability to confer anchorage-independent growth to NR6 cells. EGFR-KDD significantly increased colony formation in soft agar as compared with both EGFRWT and the known oncogenic EGFRL858R mutation (Fig. 1C and Supplementary Fig. S5A–SSD). Expression of a kinase-dead version of the EGFR-KDD (called EGFR-KDD-dead) abrogated the growth of NR6 cells in soft agar, consistent with the requirement of kinase activity for anchorage-independent growth. In parallel, we expressed the same EGFR variants in Ba/F3 cells (Fig. 1D). EGFR-KDD, but not its kinase-dead counterpart, induced IL3-independent proliferation of Ba/F3 cells, an activity phenotype associated with the transforming function of other oncogenic tyrosine kinases (Fig. 1E; ref. 12). As previously reported, EGFRL858R, but not EGFRWT, was able to support IL3-independent growth of Ba/F3 cells (12).

Computational Modeling Demonstrates That EGFR-KDD Can Form Intramolecular Dimers

To provide insight into the mechanism of activation of EGFR-KDD, we examined the structure of the EGFR. The EGFR tyrosine kinase is known to be activated either due to increased local concentration of EGFR (e.g., as a result of
Figure 1. The EGFR-KDD is an oncogenic EGFR alteration. A, schematic representation of EGFR-KDD depicting the genetic and protein domain structures. ECD, extracellular domain; TM, transmembrane domain; KD1, first kinase domain; KD2, second kinase domain; C-term, carboxyl terminus. Blue, EGFR exons 18–25 #1; green, EGFR exons 18–25 #2. B, representative Western blot of NR6 cells stably expressing indicated EGFR constructs. EGFR-KDD-dead is a kinase-dead version of EGFR-KDD. C, NR6 cells stably expressing the indicated constructs (pMSCV = vector only) were plated in triplicate in soft agar, grown for 15 days, and quantified for colony formation. D, representative Western blot of BA/F3 cells expressing indicated EGFR constructs. E, BA/F3 cells transfected with indicated constructs (pMSCV = vector only) were grown in the absence of IL3 and counted every 24 hours. F, ribbon diagram and space-filling model of the EGFR-KDD kinase domains (GLY 696 - PRO 1370) illustrating the proposed mechanism of autoactivation.
The effects of these inhibitors on the autophosphorylation generation mutant-specific EGFR TKI; ref. 16) to assess inhibitor of EGFR/HER2; ref. 15), and AZD9291 (third- and EGFR-KDD with erlotinib (first- and EGFR-KDD. We treated Ba/F3 cells expressing EGFR

tive therapeutic strategy for tumors harboring the EGFR-
further experimental data would be needed to confi-
cific (N-lobe to C-lobe) intermolecular dimers between two
EGFR proteins (13). Given the presence of two tandem in-
frame kinase domains within the EGFR-KDD structure, we
hypothesized that EGFR-KDD could form an intramolecular
dimer. To test this hypothesis, we modeled the EGFR-KDD
based on the available experimental structure of the active
asymmetric EGFR dimer (13). Conformational loop sam-
ping with Rosetta demonstrates that the linker between
the tandem tyrosine kinase domains allows for the proper
positioning of the two domains necessary for asymmetric
dimerization and intramolecular EGFR activation (Fig. 1F).
Therefore, our model suggests that EGFR-KDD is an onco-
genic variant of the EGFR likely by virtue of its ability to
form intramolecular asymmetric activated dimers. Although
modeling demonstrates that the EGFR-KDD is geometri-
cally capable of forming intramolecular asymmetric dimers,
further experimental data would be needed to confirm this
mechanism.

The EGFR-KDD Can Be Therapeutically Targeted
with Existing EGFR TKIs

We sought to determine whether EGFR TKIs are an effec-
tive therapeutic strategy for tumors harboring the EGFR-
KDD. We treated Ba/F3 cells expressing EGFR<sup>WT</sup>, EGFR<sup>L858R</sup>
and EGFR-KDD with erlotinib (first-generation reversible
EGFR TKI; ref. 14), afatinib (second-generation irreversible
inhibitor of EGFR/HER2; ref. 15), and AZD9291 (third-
generation mutant-specific EGFR TKI; ref. 16) to assess
the effects of these inhibitors on the autophosphorylation
(and by extension, the kinase activity) and downstream sig-
aling properties of the EGFR kinase. All three EGFR TKIs
were able to inhibit EGFR-KDD tyrosine phosphorylation in
a dose-dependent manner, albeit to different levels (Fig.
2A). Afatinib was the most potent inhibitor of EGFR-KDD
autophosphorylation, at doses similar to those required for
EGFR<sup>L858R</sup> inhibition. Activation of downstream MAPK sig-
aling was also inhibited in Ba/F3 cells expressing EGFR-
KDD, as shown by decreased ERK phosphorylation after
drug treatment. Similar results were observed in 293T cells
transfected with EGFR variants (Supplementary Fig. S6A)
and in A1235 cells which harbor endogenous EGFR-KDD
(Supplementary Fig. S6B). To determine whether inhibition
of EGFR autophosphorylation translated to inhibition of
cellular proliferation, we treated Ba/F3 cells expressing vari-
ous EGFR constructs with erlotinib, afatinib, and AZD9291.
All three distinct EGFR TKIs effectively inhibited the growth
of EGFR-KDD-Ba/F3 cells (Fig. 2B and Supplementary
Table S1). Consistent with our signaling data, the growth
inhibition observed was most pronounced with afatinib.
Analogous results were seen in A1235 cells (Supplementary
Fig. S6C). Together, these results show that the EGFR-KDD
can be potently inhibited by afatinib, leading to decreased
cell viability.

Treatment of the Index Patient with Afatinib

Because there were no data regarding the use of EGFR TKIs
or monoclonal antibodies in the setting of a tumor harboring
the EGFR-KDD, the index patient was initially treated with
standard first-line chemotherapy for stage IV lung adenocar-
cinoma (cisplatin/pemetrexed/bevacizumab). However, at the

### Table 1. The EGFR-KDD is a recurrent alteration

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NOTE: Characteristics of EGFR-KDD exons 18–25 patients from Foundation Medicine, TCGA, and Memorial Sloan Kettering Cancer Center datasets.

Abbreviation: NOS, not otherwise specified.

<sup>a</sup> index patient; <sup>b</sup> this patient’s tumor also contained high level amplification of EGFR<sup>WT</sup> and an EGFR<sup>G719C</sup> mutation. The EGFR-KDD and EGFR<sup>G719C</sup> alterations were below the level of EGFR<sup>WT</sup> amplification, and presumably reflect subclonal populations.
The EGFR-KDD can be therapeutically targeted with existing EGFR TKIs. A, Ba/F3 lines stably expressing EGFRWT, EGFRL858R, or EGFR-KDD were treated with increasing doses of erlotinib, afatinib, or AZD9291 for 2 hours and lysed for Western blot analysis with the indicated antibodies. B, Ba/F3 lines stably expressing EGFRL858R or EGFR-KDD were treated with increasing doses of erlotinib, afatinib, or AZD9291 for 72 hours. Cell titer blue assays were performed to assess cell viability. Each point represents quadruplicate replicates. Data are presented as the mean percentage of viable cells compared with vehicle control ± SD.

**Figure 2.** The EGFR-KDD can be therapeutically targeted with existing EGFR TKIs. A, Ba/F3 lines stably expressing EGFRWT, EGFRL858R, or EGFR-KDD were treated with increasing doses of erlotinib, afatinib, or AZD9291 for 2 hours and lysed for Western blot analysis with the indicated antibodies. B, Ba/F3 lines stably expressing EGFRL858R or EGFR-KDD were treated with increasing doses of erlotinib, afatinib, or AZD9291 for 72 hours. Cell titer blue assays were performed to assess cell viability. Each point represents quadruplicate replicates. Data are presented as the mean percentage of viable cells compared with vehicle control ± SD.

Acquired Resistance to Afatinib

The index patient developed acquired resistance to afatinib after 7 cycles of therapy (Fig. 3A). This duration of response is in line with the typical responses observed in other EGFR-mutant lung cancers treated with EGFR TKIs (1–3). Molecular profiling was performed on the afatinib-resistant tumor biopsy sample, and this testing uncovered significant amplification of the EGFR-KDD allele as the only genomic alteration that differed from his pretreatment tumor sample (Fig. 3B). This sequencing result was confirmed via EGFR FISH (Fig. 3C). Amplification of the mutant EGFR allele has been reported as a mechanism of acquired resistance in the context of canonical EGFR mutations (e.g., exon 19 deletion, L858R) in lung cancer (18). Therefore, amplification of the EGFR-KDD in this post-treatment sample suggests an EGFR-dependent mechanism of resistance, thereby further validating this EGFR alteration as a driver and therapeutic target in patients.

**DISCUSSION**

Although much progress has been made over the past several decades, lung cancer remains the leading cause of
The discovery of oncogenic EGFR mutations that sensitize lung cancers to EGFR TKIs heralded the dawn of molecularly targeted therapy in this disease (20–22). Indeed, numerous phase III studies have now documented that patients with EGFR-mutant tumors derive significant clinical and radiographic benefit from treatment with EGFR TKIs, such as gefitinib, erlotinib, and afatinib (1–3). The majority of previously described activating mutations in EGFR are a series of small deletions in exon 19 or leucine-to-arginine substitutions at position 858 (L858R) in exon 21 (23). However, because mutations historically have been interrogated by “hot-spot” PCR-based methods, most EGFR mutations are biased to fall between exons 18 and 21.

Here, we report the EGFR-KDD for the first time in lung cancer. This EGFR alteration contains an in-tandem and in-frame duplication of exons 18 to 25, which encode the entire EGFR kinase domain. We demonstrate that the EGFR-KDD is an oncogenic and constitutively activated form of the EGFR. We provide a structural model whereby the EGFR-KDD can be activated by virtue of asymmetric intramolecular dimerization and improved functional status seen with afatinib therapy provides important clinical validation for the EGFR-KDD as an actionable alteration in lung cancer. Overall, the index patient derived a partial response to afatinib for 7 cycles, after treatment with EGFR TKIs, such as gefitinib, erlotinib, and afatinib (1–3). The majority of previously described activating mutations in EGFR are a series of small deletions in exon 19 or leucine-to-arginine substitutions at position 858 (L858R) in exon 21 (23). However, because mutations historically have been interrogated by “hot-spot” PCR-based methods, most EGFR mutations are biased to fall between exons 18 and 21.

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which there was progression of disease. His tumor was rebiopsied and found to contain amplification of the EGFR-KDD in this post-treatment sample—suggesting an EGFR-dependent mechanism of resistance and validating this EGFR alteration as a driver and therapeutic target in patients.

This case also reinforces the need to functionally validate and discern the therapeutic “actionability” of genomic alterations as increasingly sophisticated methods of NGS-based assays are being brought to the forefront of clinical diagnostics. Notably, the EGFR-KDD would not have been recognized by the “hot-spot” PCR-based methods for EGFR mutational analysis described above. Therefore, it is not surprising that this EGFR alteration had not previously been detected. In fact, the EGFR-KDD in the index patient’s tumor was identified because of a fortuitous intronic breakpoint that lay close to the exonic probes of the NGS diagnostic assay (Supplementary Fig. S1A). Therefore, we hypothesize that the EGFR-KDD may have gone and may continue to go undetected in other tumors because standard (exomic) sequencing platforms do not target this particular alteration due to its large intragenic repeat and intronic breakpoint. Thus, although our data show that the EGFR-KDD is recurrent in multiple tumor types, this alteration would not be detected with currently approved PCR-based methods and is difficult to detect using standard exomic sequencing, consequently making our reported frequency a likely underestimate. Future design of tumor sequencing platforms should incorporate intronic probes for EGFR in order to more reliably detect the EGFR-KDD.

In summary, we have identified a recurrent, oncogenic, and drug-sensitive EGFR-KDD in a subset of patients with lung cancer, glioma, and other cancer types. Our findings provide a rationale for therapeutically targeting this unique subset of EGFR-KDD–driven tumors with EGFR tyrosine kinase inhibitors, many of which are already FDA approved. Therefore, findings from our studies are expected to be rapidly translated into the clinic as they provide a new avenue for precision medicine in these difficult-to-treat malignancies.

METHODS

Cell Culture

The human lung adenocarcinoma cell line, II-18, has been previously described and was verified to harbor the EGFRL858R mutation by cDNA sequencing (26). A1235 cells were a kind gift from Drs. R. Fenstermaker and M. Ciesielski (Roswell Park Cancer Institute, Buffalo, NY; ref. 7). The 293T cells were purchased from ATCC. Ba/F3 cells were maintained in RPMI 1640 medium (Mediatech, Inc.). A1235, 293T, and NR6 cells were maintained in DMEM (Gibco). Media were supplemented with 10% heat-inactivated FBS (Atlanta Biologicals) and penicillin–streptomycin (Mediatech, Inc.) to final concentrations of 100 U/mL and 100 mg/ µg/mL, respectively. The Ba/F3 cell line was supplemented with 1 ng/mL murine IL3 (Gibco). The Plat-GP cell line was cultured in the presence of 1 µg/mL blasticidin (Gibco). All cell lines were maintained in a humidified incubator with 5% CO₂ at 37°C and routinely evaluated for Mycoplasma contamination. Besides verifying the status of EGFR mutations in cell lines, no additional cell line identification was performed.

Compounds

Erlotinib, afatinib, and AZD9291 were purchased from Selleck Chemicals.

EGFR Plasmid Construction

A CDNA encoding the EGFR-KDD (exon 18–25 tandem duplication) was synthesized by Life Technologies based on the consensus coding sequence (Supplementary Fig. S2). The pMSCV-puro vector backbone (Clontech) was used to construct all retroviruses. Assembly of pMSCV-puro-EGFRL858R and pMSCV-puro-EGFRL858R-EGFRL858R was previously described (28). The EGFR-KDD was subcloned from the pMA synthesis vector (Life Technologies) into the HpaI site of pMSCV-puro using blunt end ligation. The pCDNA1 vector was used for transient expression experiments in 293T cells. Assembly of pcDNA-EGFRL858R and pcDNA-EGFRL858R-EGFRL858R was previously described (22). EGFR-KDD was subcloned from the pMA-derived vector (Life Technologies) into the pcDNA3.1/V5 vector (Life Technologies) using Gateway cloning (Life Technologies). All plasmids were sequence verified in the forward and reverse directions. EGFR-KDD-dead was constructed using multisite-directed mutagenesis (Agilent) of the catalytic lysines (K745 and K1096) to methionines using the following primer:

KM-F: 5′-AAATGTTAATACCTCCCGTCCATCTAGGAAATAGA-3′

The plasmids were fully resequenced in each case to ensure that no additional mutations were introduced.

Bo/F3 and NR6 Cell Line Generation

The empty pMSCV-puro retroviral vector or pMSCV-puro vectors encoding EGFR (EGFRWT, EGFRL858R, EGFR-KDD, or EGFR-KDD-dead) were transfected, along with the envelope plasmid pCMV-VSV-G (CellBioLabs), into Plat-GP packaging cells (CellBioLabs). Viral media were harvested 48 hours after transfection, spun down to remove debris, and supplemented with 2 µg/mL polybrene (Santa Cruz Technology). A total of 2.5 × 10⁶ Ba/F3 cells (or 1 × 10⁶ NR6 cells) were resuspended in 10 mL viral media. Transduced cells were selected for 1 week in 2 µg/mL puromycin (Intronov), and Ba/F3 and NR6 cells were selected for an additional week in the absence of IL3. Stable polyclonal populations were used for experiments and routinely tested for expression of EGFR constructs.

Antibodies and Immunoblotting

The following antibodies were obtained from Cell Signaling Technology: phospho-EGFR tyrosine 1068 (#2234, 1:1,000 dilution), EGFR (#4267, 1:1,250 dilution), phospho-ERK threonine 202/tyrosine 204 (#9101, 1:2,000 dilution), ERK (#9102, 1:2,000 dilution), horseradish peroxidase (HRP)–conjugated anti-mouse (#7076, 1:5,000 dilution), and HRP-conjugated anti-rabbit (#7074, 1:5,000 dilution). The actin antibody (#A2066, 1:5,000 dilution) was purchased from Sigma-Aldrich. The EGFR antibody (#610017, 1:2,000 dilution) was purchased from R&D Pharmaceuticals. For immunoblotting, cells were harvested, washed in PBS, and lysed in RIPA buffer (150 mmol/L NaCl, 1% Triton-X-100, 0.5% Na-deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl, pH 8.0) with freshly added 40 mmol/L NaF, 1 mmol/L Na-orthovanadate, and protease inhibitor mini tablets (Thermo Scientific). Protein was quantified using protein assay reagent and a SmartSpec Plus spectrophotometer (Bio-Rad) per the manufacturer’s protocol. Lysates were subjected to SDS-PAGE followed by blotting with the indicated antibodies and detection by Western Lightning ECL reagent (Perkin Elmer).

Cell Viability, Counting, and Clonogenic Assays

For viability experiments, cells were seeded at 5,000 cells/well in 96-well plates and exposed to treatment the following day. At 72 hours after drug addition, Cell Titer Blue reagent (Promega) was added incubator with 5% CO₂ at 37°C and routinely evaluated on February 17, 2016. © 2015 American Association for Cancer Research.
added, and fluorescence at 570 nm was measured on a Synergy MX microplate reader (Biotek) according to the manufacturer’s instructions. For cell counting experiments, cells were seeded at 20,000 cells/well in 24-well plates in the presence or absence of 1 ng/mL IL3. Every 24 hours, cells were diluted in 20-fold and counted using a Z1 Coulter Counter (Danaher). For clonogenic assays, cells were seeded at 500 cells/well in 24-well plates and exposed to treatment the following day. Media and inhibitors were refreshed every 72 hours, and cells were grown for 1 week or until confluence in control wells. Cells were fixed with 4% v/v formalin and stained with 0.025% crystal violet. Dye intensity was quantified using an infrared imaging system (Li-COR). Viability assays were set up in triplicate, clonogenic assays were set up in triplicate, and cell-counting assays were set up in duplicate. All experiments were performed at least three independent times. Data are presented as the percentage of viable cells compared with control (vehicle only treated) cells. Regressions were generated as sigmoidal dose–response curves using Prism 6 (GraphPad) by normalizing data and constraining the top to 100.

**Soft-Agar Assays**

1.5 mL of 0.5% agar/DMEM was layered in each well of a 6-well dish. A total of 10,000 NR6 cells in 1.5 mL of 0.33% soft agar/DMEM were seeded on top of the initial agar and allowed to grow for 15 days. Each cell line was plated in triplicate. Colonies were counted using GelCount (Oxford Opptromix) with identical acquisition and analysis settings.

**Structural Modeling of the EGFR-KDD**

The linker residues in the EGFR-KDD protein sequence, FSSSPST-SRTPLSSLLIVEPTPS, were defined as those between the two kinase domains and not present in the X-ray crystal structure of EGFR in its allosterically activated dimeric form, 2GS6.pdb (13). This linker was manually built and placed into the EGFR crystal structure using PyMOL 1.5.0.3. The conformational space for the linker was then sampled using the loop-modeling functionality of Rosetta version 2015.05 (29). A total of 20,000 independent loop modeling runs were performed using kinematic closure. The best model from these runs was still lacking residues 748–750, 992–1004, 1099–1101, and 1343–1355 because these surface-exposed loops were not resolved in the experimental structure. These four loops were reconstructed using Modeller 9.14, and the model with the lowest DOPE score was selected (30). Those loops were then sampled for an additional 20,000 runs using Rosetta to generate the complete energy-minimized model of EGFR-KDD residues GLY 696–PRO 1370.

**Tumor Biopsy Samples**

All patient tumor biopsy samples were obtained under Institutional Review Board (IRB)–approved protocols (Vanderbilt University IRB# 050644). Written informed consent was obtained from the index patient and his family. All samples were deidentified, protected health information reviewed according to the Health Insurance Portability and Accountability Act (HIPAA) guidelines, and studies conducted in accordance with the Declaration of Helsinki.

**Identifying EGFR-KDD in The Cancer Genome Atlas**

Copy-number data from the Broad Institute TCGA Genome Data Analysis Center 2015-04-02 run were visually inspected to identify samples with focal amplification of the EGFR-KDD region (exons 18–25). RNA sequencing (gse14884-dd57-b01l-83fd-d3a9f2d24d1d120 and c552b1e3-9158-4e4d-b01l-b16f79035252) and whole-genome sequencing (27c2031a-39f1-473c-88a6-97a03edf040) files were inspected to confirm the presence of tandem duplication reads. Raw data were available at doi:10.7908/C1K64H04 and doi:10.7908/C1MP525H.

**EGFR FISH**

EGFR FISH was performed by Integrated Oncology, a LabCorp specialty testing group, using the EGFR-CEP7 Dual Color DNA Probe (Vysis). A trained pathologist quantified the copies of CEP7 and EGFR in 60 nuclei per sample.

**Statistics and Data Presentation**

All experiments were performed using at least two technical replicates and at least three independent times (biologic replicates). Each figure or panel shows a single representative experiment. Unless indicated otherwise, data are presented as mean ± SD. Western blot autoradiography films were scanned in full color at 600 dpi, desaturated in Adobe Photoshop CC, and cropped in Powerpoint. EGFR-FISH images were normalized using “Match Color” in Adobe Photoshop CC. No other image alterations were made.

**Disclosure of Potential Conflicts of Interest**

M. Bailey has ownership interest (including patents) in Foundation Medicine Inc. D. Lipson has ownership interest (including patents) in Foundation Medicine Inc. M.G. Kris reports receiving commercial research grants from Pfizer and PUMA, and is a consultant/advisory board member for AstraZeneca and Roche/Genentech. V.A. Miller has ownership interest (including patents) in Foundation Medicine. S.M. Ali has ownership interest (including patents) in Foundation Medicine. C.M. Lovly reports receiving commercial research grants from AstraZeneca and Novartis; has received speakers bureau honoraria from Abbott Molecular and Qagen; and is a consultant/advisory board member for Genoptix, Novartis, Pfizer, and Sequenom. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: J.-N. Gallant, J. Meiler, C.M. Lovly Development of methodology: J.-N. Gallant, D. Lipson, J. Meiler, C.M. Lovly Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-N. Gallant, T.M. Shaver, M.G. Kris, J.A. Pietenpol, M. Ladanyi, V.A. Miller, S.M. Ali, C.M. Lovly Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.-N. Gallant, J.H. Sheehan, T.M. Shaver, M. Bailey, D. Lipson, R. Chandramohan, M.R. Brewer, M.G. Kris, J.A. Pietenpol, J. Meiler, C.M. Lovly Writing, review, and/or revision of the manuscript: J.-N. Gallant, J.H. Sheehan, M.R. Brewer, M.G. Kris, J.A. Pietenpol, M. Ladanyi, V.A. Miller, S.M. Ali, J. Meiler, C.M. Lovly Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.G. Kris, J.A. Pietenpol, C.M. Lovly Study supervision: M.G. Kris, J. Meiler, C.M. Lovly Other (involved in the initial description of the mutation, discussed experimental results with the senior author, and provided clinical care for the patient described in the study): S.J. York Other (coordinated studies): J.-N. Gallant

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