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

Jean-Nicolas Gallant1,2, Jonathan H. Sheehan3,4, Timothy M. Shaver2,3, Mark Bailey5, Doron Lipson5, Raghu Chandramohan6, Monica Red Brewer2,7, Sally J. York2,7, Mark G. Kris8, Jennifer A. Pietenpol2,3, Marc Ladanyi6, Vincent A. Miller5, Siraj M. Ali5, Jens Meiler4,9, and Christine M. Lovly1,2,7

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.

INTRODUCTION
The prospective identification and rational therapeutic targeting of tumor genomic alterations have revolutionized the care of patients with lung cancer and other malignancies.
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 preclinical data 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 against 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.

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 EGFRKDD 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.

Key
Blue = Kinase domain #1
Green = Kinase domain #2
Red = Linker
Yellow asterisks = Active sites
Table 1. The EGFR-KDD is a recurrent alteration

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Identification #</th>
<th>Age</th>
<th>Gender</th>
<th>Reported diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foundation Medicine</td>
<td>FM-1</td>
<td>52</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>FM-2</td>
<td>33</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>FM-3</td>
<td>53</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>FM-4</td>
<td>57</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>FM-5</td>
<td>29</td>
<td>Female</td>
<td>Lung NSCLC (NOS)</td>
</tr>
<tr>
<td></td>
<td>FM-6</td>
<td>53</td>
<td>Female</td>
<td>Brain astrocytoma</td>
</tr>
<tr>
<td></td>
<td>FM-7</td>
<td>49</td>
<td>Male</td>
<td>Brain glioblastoma</td>
</tr>
<tr>
<td></td>
<td>FM-8</td>
<td>54</td>
<td>Male</td>
<td>Brain glioblastoma</td>
</tr>
<tr>
<td></td>
<td>FM-9</td>
<td>2</td>
<td>Female</td>
<td>Kidney Wilms' tumor</td>
</tr>
<tr>
<td></td>
<td>FM-10</td>
<td>63</td>
<td>Female</td>
<td>Peritoneal serous carcinoma</td>
</tr>
<tr>
<td></td>
<td>FM-11</td>
<td>27</td>
<td>Female</td>
<td>Soft tissue sarcoma (NOS)</td>
</tr>
<tr>
<td>TCGA</td>
<td>TCGA-49-4512</td>
<td>69</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>TCGA-12-0821</td>
<td>62</td>
<td>Female</td>
<td>Brain glioblastoma</td>
</tr>
<tr>
<td>MSKCC</td>
<td>MSKCC-1</td>
<td>33</td>
<td>Male</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>MSKCC-2</td>
<td>67</td>
<td>Female</td>
<td>Lung adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>MSKCC-3</td>
<td>53</td>
<td>Male</td>
<td>Brain glioblastoma</td>
</tr>
</tbody>
</table>

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.

*index patient; †this patient’s tumor also contained high level amplification of EGFRWT and an EGFRG719C mutation. The EGFR-KDD and EGFRG719C alterations were below the level of EGFRWT amplification, and presumably reflect subclonal populations.

ligand binding or overexpression), mutations in the activation loop (e.g., L858R), or through formation of asymmetric (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 sampling 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 oncogenic variant of the EGFR likely by virtue of its ability to form intramolecular asymmetric activated dimers. Although modeling demonstrates that the EGFR-KDD is geometrically 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 effective therapeutic strategy for tumors harboring the EGFR-KDD. We treated Ba/F3 cells expressing EGFRWT, EGFRL858R, 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 signaling 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 EGFRL858R inhibition. Activation of downstream MAPK signaling 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 various 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 adenocarcinoma (cisplatin/pemetrexed/bevacizumab). However, at the
time of disease progression on this treatment regimen, the patient was treated with afatinib. Immediately after beginning afatinib, the patient reported feeling markedly better, with improvements in his symptoms of cough and fatigue. After two cycles of afatinib, the patient showed a partial radiographic response (~50% tumor shrinkage) per RECIST criteria (ref. 17; Fig. 3A). This clinical activity is consistent with our in vitro studies and provides a rationale for further clinical investigation.

**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...
After 2 cycles of afatinib, many of which are already FDA approved. In addition, 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, as opposed to the typical asymmetric intermolecular dimerization between adjacent EGFR molecules. Furthermore, we demonstrate that the EGFR-KDD can be therapeutically targeted with EGFR TKIs, many of which are already FDA approved. In addition, we establish that the EGFR-KDD alteration is recurrent not only in lung cancer but also in gliomas and other tumor types.

Most importantly, we provide the first documentation of a clinical response to EGFR inhibitor therapy in a patient with lung cancer whose tumor harbored the EGFR-KDD alteration. In contrast with lung cancer patients with more common EGFR mutations (e.g., exon 19 deletion and L858R), prior to our study, there was no precedent to support the use of EGFR inhibitors in patients whose lung tumors harbor the EGFR-KDD alteration. Therefore, our patient was not eligible for first-line EGFR TKI therapy and was instead treated with platinum-based chemotherapy, the standard of care for metastatic lung adenocarcinoma (24). The index patient was treated with afatinib for second-line therapy because this agent is FDA approved for the treatment of EGFR-mutant NSCLC and because, interestingly, afatinib was consistently the most potent EGFR TKI against the EGFR-KDD across several different assays. This was not unexpected, as it has been shown that various EGFR mutations or truncations have differential sensitivity to EGFR TKIs due to nuanced structural differences (25). The marked tumor regression 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...
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 exonic 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 EGFR-L858R 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 Drs. R. Fenstermaker and M. Ciesielski (Roswell Park Cancer Institute, Buffalo, NY; ref. 7). The 293T cells were purchased from Invitrogen. The II-18 and A1235 cell lines were cultured 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 μg/mL, respectively. The II-18 and A1235 cell lines were cultured in a humified 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-EGFRWT and pMSCV-puro-EGFR-L858R 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 pcDNA3.1 vector was used for transient expression experiments in 293T cells. Assembly of pcDNA-EGFRWT and pcDNA-EGFR-L858R was previously described (22). EGFR-KDD was subcloned from the pMA synthesis 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:

**KMF-5’-AAAGTAAAAATCTCCGCATCATGGAATTAAGAAGACAAAC-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, EGFR-L858R, 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 (Invitrogen), and Ba/F3 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), ERLK (#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 Pharmingen. For immunoblotting, cells were harvested, washed in PBS, and lysed in RIPA buffer (50 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, 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 20-fold and counted using a Z1 Coulter Counter (Danaher). For clonogenic assays, cells were seeded at 5,000 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 quadruplicate, 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 Optronix) with identical acquisition and analysis settings.

**Transient Transfections**

The 293T cells were transfected using Lipofectamine 2000 (Life Technologies) per the manufacturer’s recommendations. Twenty-four hours after transfection, cells were treated for 2 hours, gathered for Western blot analysis, and prepared as described above.

**Structural Modeling of the EGFR-KDD**

The linker residues in the EGFR-KDD protein sequence, FSSPST-SRTPLLSSLVEPLTPS, 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. 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 (c714b1a84-dd57-40b1-83fd-d309b2d4ad1120 and c552b1e3-9158-4e4d-b02b-16f7903552) and whole-genome sequencing (27c2031a-39f1-473c-88af-9e7ad3edi004) 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 Qiagen; and is a consultant/advisory board member for Genoptix, Novartis, Pfizer, and Sequenom. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

**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. Pieterpol, 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. Pieterpol, 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. Pieterpol, 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. Pieterpol, 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

**Acknowledgments**

The authors thank the index patient and his family. They also thank Evelyn Vazquez for her assistance with the EGFR FISH.

**Grant Support**

This study was supported in part by the NIH and NCI R01CA121210 (to C.M. Lovly), R01CA129243 (to C.M. Lovly, M. Ladanyi, and M.G. Kris), and F31CA129852 (to T.M. Shaver). Other research was supported by the NIH and NCI R01CA121210.
and P01CA68485. Work in the Meiler laboratory is supported through the NIH (R01GM080403, R01GM099842, R01DK097376, R01HL122010, and R01GM073511) and the NSF (CHE1305878). C.M. Lovly was additionally supported by a Damon Runyon Clinical Investigator Award and a LUNGevity Career Development Award. J.-N. Gallant was supported by MSTP grant T32GM007347. T.M. Shaver was supported by F31CA183531.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 28, 2015; revised August 12, 2015; accepted August 13, 2015; published OnlineFirst August 18, 2015.

REFERENCES


AfatinibOncogenic Driver in Lung Cancer That Is Clinically Responsive to Afatinib


Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-15-0654

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2015/08/18/2159-8290.CD-15-0654.DC1

Cited articles
This article cites 30 articles, 12 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/5/11/1155.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/5/11/1155.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/5/11/1155.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.