Inhibitor-Sensitive FGFR2 and FGFR3 Mutations in Lung Squamous Cell Carcinoma

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Abstract
A comprehensive description of genomic alterations in lung squamous cell carcinoma (lung SCC) has recently been reported, enabling the identification of genomic events that contribute to the oncogenesis of this disease. In lung SCC, one of the most frequently altered receptor tyrosine kinase families is the fibroblast growth factor receptor (FGFR) family, with amplification or mutation observed in all four family members. Here, we describe the oncogenic nature of mutations observed in FGFR2 and FGFR3, each of which are observed in 3% of samples, for a mutation rate of 6% across both genes. Using cell culture and xenograft models, we show that several of these mutations drive cellular transformation. Transformation can be reversed by small-molecule FGFR inhibitors currently being developed for clinical use. We also show that mutations in the extracellular domains of FGFR2 lead to constitutive FGFR dimerization. In addition, we report a patient with an FGFR2-mutated oral SCC who responded to the multitargeted tyrosine kinase inhibitor pazopanib. These findings provide new insights into driving oncogenic events in a subset of lung squamous cancers, and recommend future clinical studies with FGFR inhibitors in patients with lung and head and neck SCC. Cancer Res; 73(16); 5195–205. ©2013 AACR.

Introduction
Two goals of comprehensive next-generation sequencing of cancers are to discover novel, targetable somatic alterations, and to identify new targets for which therapies already exist. Genome-scale analyses of tumors representing many cancer types have recently been completed (1–6), enabling discoveries consistent with both goals.

Historically, targetable oncogenic alterations in cancer were discovered on an individual gene basis. This was the case for cancer-causing alterations observed in several tyrosine kinases, including EGFR and ALK in lung adenocarcinoma (7–9), FGFR2 in endometrial carcinoma (10,11), and FGFR3 in urothelial carcinoma (12). These studies and others have led to demonstrations of the successful application of targeted therapeutic agents and their superiority to conventional chemotherapy (13,14).

Lung squamous cell carcinoma (lung SCC) is a prevalent and deadly disease for which no targeted therapies are approved. Recent data reported by The Cancer Genome Atlas (TCGA) lung SCC project (4) showed that the fibroblast growth factor receptor (FGFR) tyrosine kinases are one of the most frequently altered kinase families in this disease. Amplification of FGFR1 was observed, in agreement with prior reports (15,16). Furthermore, mutations in FGFR2 and FGFR3 were reported. Although the frequency of these mutations did not reach statistical significance at the cohort size examined by TCGA, several features including recurrence, prior observation in other cancer types and congenital syndromes, and lack of other dominant oncogenic alterations in tumors with FGFR mutations, suggested they might be driving, targetable events in a subset of patients presenting with this disease.

Germline mutations in the FGFR tyrosine kinase family were first described in craniofacial and skeletal syndromes (17). Somatic point mutations identical to those germline events have also been observed in malignancies (18). The FGFR family is made up of four active members, each containing an extracellular domain (ECD) and a cytoplasmic kinase domain. Activation is stimulated by binding FGF and heparan sulfate proteoglycan (HSPG) in the ECD, and subsequent dimerization of two receptor–ligand complexes, leading to transphosphorylation of the kinase domains. This leads to phosphorylation of...
binding partner FRS2 and downstream activation of Ras/MAPK and PI3K/AKT pathways (19).

The FGF family is made up of more than 20 members, all of which retain specificities for both different FGFR family members and different isoforms of each receptor (20). In addition, tissue types vary in which receptors, isoforms, and ligands are expressed, adding further levels of complexity to the system. Dysregulation can lead to oncogenesis, as has been shown with altered expression of receptors (15, 16, 21), altered isoform expression (22, 23), and altered ligand specificity (24) driven by somatic genomic events.

Aberrant FGFR signaling has been implicated in the development of several cancer types. In addition to lung SCC, *FGFR1* amplification is observed in 10% of breast cancers (21). Point mutations in *FGFR2* are observed in 12% of endometrial carcinomas (10) and mutations in *FGFR3* are observed in more than 30% of urothelial carcinomas (12). Cell lines harboring these events have shown sensitivity to inhibition by FGFR small-molecule inhibitors, and clinical trials are now testing FGFR inhibitors in patients harboring somatic events in *FGFRs* (18).

Here, we characterize *FGFR2* and *FGFR3* mutations observed in lung SCC and show the oncogenic potential of these mutations using models of transformation and dependency. We show that cells harboring these mutations are sensitive to inhibition by several FGFR and multikinase inhibitors. In addition, we report a case of a patient with an *FGFR2*-mutated oral SCC, who responded to pazopanib, an inhibitor of multiple tyrosine kinases including the FGFR family. Together, these data identify a promising new therapeutic target for patients with lung SCC and other squamous epithelial tumors.

### Materials and Methods

#### Patient samples and genomic analysis

We manually reviewed *FGFR2* and *FGFR3* exome sequencing data generated by the TCGA research network. In addition, we queried publicly available sequencing data generated from 18 samples that were excluded from the initial TCGA report. All data were deidentified and obtained in accordance with patient protection standards set by the TCGA and were obtained from the TCGA Data Portal.

For the individual with a clinical response to pazopanib, total RNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen; #80204). Poly-adenylated mRNA was enriched from the TCGA Data Portal. From 18 samples that were excluded from the initial TCGA report, we queried publicly available sequencing data generated from the TCGA research network. In addition, we report a case of a patient with an *FGFR2*-mutated oral SCC, who responded to pazopanib, an inhibitor of multiple tyrosine kinases including the FGFR family. Together, these data identify a promising new therapeutic target for patients with lung SCC and other squamous epithelial tumors.

#### Cell lines, antibodies, ligands, and inhibitors

NIH-3T3 cells and Ba/F3 cells were obtained from the American Type Culture Collection and maintained as described previously (10, 20). Antibodies against *FGFR2* (C-8) and *FGFR3* (H-91) were purchased from Santa Cruz Biotechnology, Inc. Antibodies against *FGFR3* (C15F2), p-FGFR, p-FRS2 (Y436), AKT (C67E7), p-AKT (T308, 244F9), Erk 1/2 (137F5), p-Erk 1/2 (E10), and β-actin (8H10D10) were obtained from Cell Signaling Technology, Inc.

For FGFR stimulation experiments, the FGFR ligand was obtained from Abcam. FGF7 and FGF9 were obtained from R&D Systems. Interleukin-3 (IL-3) was purchased from VWR and heparin from STEMCELL Technologies, Inc. Ponatinib (AP24534), dovitinib (TKI258), and cediranib (AZD2171) were obtained from Selleck Chemicals. Brivanib alinate (BMS-582664) was obtained from Fischer Scientific. Pazopanib (GW786034) was obtained from Axon Medchem. AZD4547 was obtained from Active Biochem. E7080 was obtained from Evotec. BGJ398 was gifted from Novartis Pharmaceuticals Corporation.

#### Mutagenesis and cellular transfection and infection

Mutagenesis primers developed for each mutation were generated using the Agilent QuikChange Primer Design tool. *FGFR2* isoforms IIIb and IIIc, and *FGFR3* isoform IIIc were cloned into pDONR223 and mutated by site-directed mutagenesis with the QuickChange Lightning Site-Directed Mutagenesis Kit from Agilent Technologies. Sequence-verified constructs were cloned into pBabe-puro and transfected into HEK-293T cells with Fugene-6 (Promega) as described previously (10). NIH-3T3 and Ba/F3 cells were infected with the resulting virus and after 2 days the cells were selected with 2 μg/mL puromycin.

#### Western blot analysis and visualization of unreduced dimers

Cells were lysed in buffer containing 0.5% NP-40, 50 mmol/L Tris pH 8.150 mmol/L MgCl2, and phosphatase and protease inhibitors, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes via the iBlot dry transfer system (Invitrogen). Antibody binding was detected using the LI-COR Odyssey IR imaging system (LI-COR Biosciences).

To visualize receptor dimers formed by ECD mutations to cysteine residues, NIH-3T3 cells expressing the appropriate mutations were serum-starved for 8 hours in the presence of PBS or FGF1 and heparin, washed with PBS containing 10 mmol/L iodoacetamide, and lysed in lysis buffer containing 1% Triton, 10% glycerol, 50 mmol/L Tris pH 7.4, and 10 mmol/L iodoacetamide. Two 100 μl aliquots of each protein sample were prepared, one with reducing agent and one without. Electrophoresis was carried out using 4% to 12% Tris–glycine SDS-PAGE gels (Invitrogen).
To confirm loss of phosphorylation of relevant kinases in the presence of inhibitor, NIH-3T3 cells expressing mutated FGFR2 or FGFR3 were washed with PBS, serum-starved for 4 hours in the presence of indicated concentrations of inhibitor, and ligand-stimulated with FGF1 for 30 minutes before lysis. A total of 5 × 10^3 cells were suspended in 330 μL media and mixed with 770 μL 0.5% Select agar and then plated onto the solidified bottom layer in triplicate. Plates were incubated for 3 weeks, photographed using QuickCapture (Logitech), and quantified via ImageJ for colony formation. Statistical comparison was conducted using the Student’s t test.

To evaluate the effect of clinical inhibitors on soft agar colony formation, the above protocol was conducted with the Student’s t test. To confirm loss of phosphorylation of relevant kinases in the presence of inhibitor, NIH-3T3 cells expressing mutated FGFR2 or FGFR3 were washed with PBS, serum-starved for 4 hours in the presence of indicated concentrations of inhibitor, and ligand-stimulated with FGF1 for 30 minutes before lysis.

Soft agar colony formation assays

Two milliliter of 0.5% Select agar (Gibco) and media were plated to each well of a non-tissue culture–treated 6-well plate and allowed to solidify. A total of 5 × 10^3 cells were suspended in 330 μL media and mixed with 770 μL 0.5% Select agar and then plated onto the solidified bottom layer in triplicate. Plates were incubated for 3 weeks, photographed using QuickCapture (Logitech), and quantified via ImageJ for colony formation. Statistical comparison was conducted using the Student’s t test.

To evaluate the effect of clinical inhibitors on soft agar colony formation, the above protocol was conducted with the following alteration: 5 × 10^5 cells were suspended in 330 μL media plus relevant inhibitor before addition of 0.5% agar solution and plating.

Xenograft studies

All animal experiments were carried out according to the institutional guidelines about animal safety. Immunocompromised mice were injected with NIH-3T3 cells stably expressing exogenous FGFR2-IIIb wild-type (WT), W290C-, S320C-, or K660N-mutant isoforms. Cohorts of 7 mice were injected at three sites for each cell type with 2 million cells per site, and mice were observed until tumor volume reached 200 to 300 mm^3. Mice were then treated with BGJ-398 at 15 mg/kg or DMSO controls was calculated and plotted in Prism (GraphPad Software, Inc.).

Ba/F3 dependency and inhibitor studies

Ba/F3 cells expressing each mutation construct were selected in media containing IL-3 and puromycin. To establish cells dependent on FGFR signaling, 3 million cells were washed twice with PBS and seeded into 2 mL of media containing FGF7 (for FGFR2 IIIb) or FGF9 (for FGFR2 IIIc) and heparin. These cells were maintained until IL-3 and puromycin. To test anchorage-independent growth of NIH-3T3 cells

FGFR2 and FGFR3 mutations drive anchorage-independent growth of NIH-3T3 cells

To determine whether the mutations identified in lung SCC were oncogenic, we established NIH-3T3 cells stably expressing each mutation to assess anchorage-independent growth in soft agar. We observed colony formation in cells expressing the majority of observed FGFR2 and FGFR3 mutations (Fig. 2A). We determined that ECD mutations W290C and S320C in FGFR2, and R248C and S249C in FGFR3, significantly increased colony formation compared with WT FGFR2 or FGFR3, as did kinase domain mutations K660E and K660N in FGFR2 (P < 0.05 by Student’s t test). In contrast, FGFR2 mutations E471Q and E471K, and FGFR3 mutations S435C and K717M did not form colonies above WT. Robust formation of colonies was observed in NIH-3T3 cells expressing an activating EGFR insertion.
FGFR2 and FGFR3 mutations were generated in both common isoforms of FGFR2 with similar results obtained for all assayed mutations with the exception of FGFR2 T787K, which was very modestly transforming only in isoform IIIc (Fig. 2A).

**FGFR2 and FGFR3 mutations drive tumor formation in xenograft models**

NIH-3T3 cells expressing transforming FGFR2 mutations or WT were injected into nude mice. Tumors reached approximately 200 to 300 mm³ in all mice injected with mutant cells by day 13 and began treatment with a pan-FGFR inhibitor, BGJ398 (28), or vehicle, with ECD mutations driving particularly strong tumor formation (Fig. 2B, solid lines). Tumors formed by cells expressing WT FGFR2 grew more slowly, and began treatment on day 16 (Fig. 2B).

Tumors treated with BGJ398 slowed or reversed their growth compared with vehicle (Fig. 2B, dashed lines), so that by the end of the study, tumor burden in vehicle-treated versus BGJ398-treated mice was noticeably distinct (Fig. 2C and Supplementary Fig. S2).

**Extracellular domain mutations form ligand-sensitive intermolecular disulfide bonds**

A common mechanism of activation of the FGFR2 and FGFR3 kinases is through the formation of covalently bound receptor dimers (29, 30). Although WT receptor tyrosine kinases maintain extracellular structure required for ligand binding and receptor dimerization through intramolecular disulfide bonds, mutant receptors can form intermolecular disulfide dimers through a novel cysteine residue created by the mutation itself.
or through instability created by a mutated residue near a structural intramolecular disulfide bond (29). This mechanism was previously established for FGFR3 mutations that we have observed in lung SCC, R248C, and S249C (30).

To assess whether mutations in the ECD of FGFR2 and FGFR3 lead to covalent dimerization, and whether dimerization could be increased by ligand stimulation, we serum-starved cells in the presence of PBS or 5 nmol/L FGF1 and 2 mg/mL heparin for 8 hours, or 5 nmol/L FGF1 and 2 mg/mL heparin for 30 minutes, followed by washing with PBS and serum-starving in the presence of PBS for the remaining 7.5 hours followed by electrophoresis in both reducing and non-reducing conditions. FGFR2 ECD mutations were sufficient to drive covalent dimerization in the absence of ligand, but dimerization was increased in the presence of even 30 minutes of ligand stimulation (Supplementary Fig. S3A). In FGFR3 mutations, on the other hand, dimerization was observed but not increased under ligand-stimulation conditions (Supplementary Fig. S3B). As has been shown previously (31), FGFR proteins typically form highly glycosylated folded protein products. Although FGFR2 W290C seems to undergo a glycosylation defect contributing to its lower molecular weight, this mutant form still retains the capacity to dimerize.

We then seeded the same cells into soft agar in the presence of PBS, 2 mg/mL heparin alone, or 5 nmol/L FGF1 and 2 mg/mL heparin. After 3 weeks, we observed greater colony formation in response to FGF1 and heparin treatment than in heparin alone or PBS-treated cells (Supplementary Fig. S3C).

FGFR2- and FGFR3-driven cellular transformation is blocked by clinically relevant FGFR inhibitors

Having established that FGFR2 and FGFR3 mutations in lung SCC drive anchorage-independent growth in NIH-3T3 cells, we asked whether this transformation could be blocked by small-molecule inhibitors of FGFRs. NIH-3T3 cells were seeded into soft agar in the presence or absence of the multikinase inhibitor AP24534 (ponatinib), which targets imatinib-resistant BCR-ABL (32), and has activity against FGFR family members.
Colony formation was inhibited in the presence of ponatinib in cells harboring activating FGFR2 or FGFR3 mutations, but not in cells harboring an activating EGFR insertion (Fig. 3A, left). All ECD mutations in FGFR2 and S249C in FGFR3 lost colony-forming potential when exposed to 100 nmol/L of drug, whereas kinase domain mutations lost colony-forming potential at 10 nmol/L of drug. Exceptions were FGFR2 K660E expressed in the IIIc isoform, which behaved similarly to the FGFR2 ECD mutations, and FGFR3 R248C, which had a 10-fold higher inhibitory concentration than any other mutation, at 1 μmol/L. Colony formation driven by EGFR was not lost until cells were exposed to 10 μmol/L of drug.

To determine whether ponatinib was inhibiting colony formation driven by mutant FGFR2 and FGFR3, we assessed phosphorylation of several proteins in the FGFR signaling pathway. Levels of phospho-FGFR, phospho-FRS2, and phospho-Erk all decreased in response to increasing concentrations of ponatinib (Fig. 3B), suggesting that colony formation was lost due to a decrease in FGFR-mediated signaling.

To evaluate whether ponatinib was acting by specific inhibition of FGFR kinases, these assays were also conducted with BGJ398, a selective FGFR kinase inhibitor (28) as well as pazopanib (GW786034; ref. 34) and dovitinib (TKI-258; ref. 35), two multikinase inhibitors with specificity for FGFR family members. Colony formation was inhibited by at least 50% in the presence of 10 nmol/L BGJ398 for all cells expressing FGFR mutations, whereas cells expressing the activating EGFR insertion did not lose the capacity for colony formation until 1 μmol/L BGJ398 (Fig. 3A, right), and WT phosphorylation was lost at 10 nmol/L under ligand stimulation conditions (Supplementary Fig. S4A). Dovitinib also inhibited colony formation in cells expressing mutant FGFR compared with activated EGFR, but with less uniformity across mutations. FGFR2 ECD mutations lost 50% colony formation between 100 nmol/L and 1 μmol/L of drug.

Figure 3. Anchorage-independent colony formation is abrogated in the presence of anti-FGFR inhibitors. A, NIH-3T3 cells expressing each transforming mutation were seeded in the presence of increasing concentrations of ponatinib (AP24534; left) and BJG398 (right). B, cells were serum-starved and exposed to the indicated concentrations of ponatinib for 4 hours and then ligand-stimulated for 30 minutes with FGF1, after which cells were lysed and probed via immunoblot. These experiments were carried out with several other clinical inhibitors; those results are documented in Supplementary Fig. S4.
1 μmol/L dovitinib. In contrast, colony formation was inhibited by 50% between 10 and 100 nmol/L for FGFR2 kinase domain mutations excluding K660E IIle, which behaved similarly to the FGFR2 ECD mutations. Cells expressing FGFR3 R248C and S249C were sensitive between 10 and 100 nmol/L. Again, cells transformed by mutant EGFR did not lose colony formation until exposed to 10 μmol/L drug (Supplementary Fig. S4B, left). Mutant EGFR-expressing cells had sustained phosphorylation at AKT T308 up to 10 μmol/L dovitinib, as detected by immunoblot, whereas detectable AKT phosphorylation was lost by 100 nmol/L to 1 μmol/L dovitinib in cells expressing FGFR mutations (Supplementary Fig. S4C). Pazopanib similarly inhibited colony formation in cells expressing all FGFR2 and FGFR3 mutations at concentrations of 100 nmol/L to 1 μmol/L drug, whereas cells expressing mutant EGFR formed colonies even in the presence of 10 μmol/L drug (Supplementary Fig. S4B, right). Consistently, biochemical studies revealed sustained AKT T308 phosphorylation in mutant EGFR cells exposed to 10 μmol/L pazopanib, whereas detectable AKT phosphorylation was lost at 100 nmol/L to 1 μmol/L pazopanib (Supplementary Fig. S4D).

In NIH-3T3 cells expressing the ECD mutations of both FGFR2 and FGFR3 and in the kinase domain mutation FGFR2 K660E IIle, we observed that low concentrations of ponatinib (10 nmol/L) conferred a growth-promoting phenotype above control, which was abrogated at higher concentrations (Fig. 3A, left). This could be due to the multikinase inhibitory properties of ponatinib, which may inhibit a second kinase that could impact FGFR2 or FGFR3 signaling. This phenomenon was also observed when these experiments were carried out with the two other multikinase inhibitors with anti-FGFR activity, pazopanib and dovitinib (Supplementary Fig. S4B), but not with BGJ398, a more selective FGFR kinase inhibitor (Fig. 3A, right).

**Analysis of FGFR2 and FGFR3 inhibition in IL-3–independent Ba/F3 cells**

To test whether cellular transformation driven by mutated FGFR2 could be abrogated in a second system by small-molecule FGFR inhibitors and to test the relative efficacy of these compounds, we generated Ba/F3 cells expressing the FGFR2 mutations that had shown significant colony formation in the NIH-3T3 anchorage-independent assay. These cell lines were dependent on FGFR signaling in the presence of FGF and heparin, and in the absence of IL-3. Phosphorylation of the FGFR kinase domain and FRS2 were measured by immunoblot, and interestingly, cells expressing FGFR2 K660E IIle showed a greater degree of phosphorylation of both molecules despite similar expression levels as compared with cells expressing other mutations (Fig. 4A).

Ba/F3 cells expressing WT and mutated FGFR2 transgenes were first seeded into media containing increasing concentrations of ponatinib. We observed that ponatinib inhibited IL-3–independent proliferation of Ba/F3 cells expressing the FGFR2 mutations at about 10 nmol/L of drug treatment, but cells expressing an EGFR-activating insertion or parental Ba/F3 cells grown in the presence of IL-3 were only inhibited by 10 μmol/L of drug (Fig. 4B, left). IC50 values for Ba/F3 cells expressing each mutant were also calculated and plotted (Fig. 4C, left). These assays were also conducted on cells seeded into media containing BGJ398, and similarly, cells expressing FGFR mutations, but not the EGFR insertion or parental Ba/F3 cells, were inhibited at about 10 nmol/L inhibitory concentrations of drug (Fig. 4B, right and Fig. 4C, right). Interestingly, insensitive controls in the presence of ponatinib seemed to gain a growth advantage in the presence of drug at concentrations in the range of 10 to 100 nmol/L (Fig. 4B), similar to our observations in the anchorage-independent colony formation assay (Fig. 3A and Supplementary Fig. S4B).

To further assess the potency of small-molecule FGFR kinase inhibitors in the Ba/F3 system, we assembled a panel of FGFR kinase inhibitors described in the literature (refs. 28, 32–39; Supplementary Table S2) and tested the Ba/F3 inhibitory response in the presence of each. Each of these inhibitors showed similar trends to those seen for ponatinib and BGJ398: a multi-log increase in drug sensitivity in cells expressing FGFR mutations compared with controls (Supplementary Fig. S5). IC50 values for each mutation in the presence of each drug were also calculated (Supplementary Fig. S5). Strikingly, FGFR2 K660E expressed in the IIle isoform (in yellow) repeatedly exhibited a 5- to 10-fold higher IC50 concentration as compared with the IIib isoform and either isoform of the K660N mutation in the FGFR2 kinase domain (Supplementary Fig. S5). This observation was consistent with the concentrations at which anchorage-independent growth observed for FGFR2 K660E IIle was lost in the presence of several inhibitors (Fig. 3A and Supplementary Fig. S4B).

**Case report of a head and neck SCC patient responding to an FGFR inhibitor**

We identified an individual with SCC of the head and neck who was found to harbor an extracellular FGFR2 mutation (p.P253R) in a biopsy specimen (Fig. 5A). This mutation was initially identified in RNA sequencing data and then confirmed by Sanger sequencing in a CLIA-certified laboratory (Fig. 5B). FGFR2 mutations have previously been observed at low frequencies in head and neck cancer (40, 41), and confirmed by initial reports from TCGA where seven mutations were observed in exome sequencing data of 279 individuals as of October 1, 2012 (data obtained from TCGA Data Coordinating Center). FGFR2 P253R has previously been observed in endometrial carcinoma (10). Cellular and biochemical analysis of the FGFR2 P253R mutation suggest that this event is transforming and sensitive to targeted therapies in our assays, similar to the events observed in lung SCC (Supplementary Fig. S6).

The patient was diagnosed with locally advanced (T2N1M0, stage III) SCC of the right tongue in 2008 at the age of 52 years. He had no history of tobacco use or alcohol abuse and was treated with a right hemiglossectomy and postoperative radiotherapy. He subsequently developed recurrences in the right and left neck over a period of 3 years and was treated with surgery, two additional courses of radiotherapy and multiple courses of chemotherapy including carboplatin, paclitaxel, cisplatin, and cetuximab. In 2012, he had further progression in the right neck and left axilla. He began daily treatment with 800 mg pazopanib starting on April 12, 2012. At this time, he had gross disease in the right neck (Fig. 5C, left). A follow-up therapy. He subsequently developed recurrences in the right and left neck over a period of 3 years and was treated with surgery, two additional courses of radiotherapy and multiple courses of chemotherapy including carboplatin, paclitaxel, cisplatin, and cetuximab. In 2012, he had further progression in the right neck and left axilla. He began daily treatment with 800 mg pazopanib starting on April 12, 2012. At this time, he had gross disease in the right neck (Fig. 5C, left). A follow-up
visit 12 days later showed a marked reduction in tumor size (Fig. 5C, right). He continued on pazopanib for 2 months, when he presented with a right carotid hemorrhage. Pazopanib was discontinued at that time, and the patient remains alive as of March 15, 2013 under hospice care. This correlative observation does not definitively identify FGFR2 as the target of pazopanib, but we believe that this result provides compelling rationale to continue to pursue treatment of FGFR2-mutated tumors with anti-FGFR-targeted therapies.

Discussion

Lung SCC is a poorly characterized disease responsible for 40,000 new deaths per year in the United States. One of the most provocative findings from genomic analysis is that of
Figure 5. An oral SCC patient harboring a somatic FGFR2 P253R mutation shows a partial response to an FGFR inhibitor. A, a schematic shows the P253R mutation in the FGFR2 ECD. B, mRNA sequencing was carried out and a somatic mutation in FGFR2 was identified, shown in the Integrative Genomics Viewer (The Broad Institute of Harvard and MIT, Cambridge, MA). C, pre- and posttreatment images from the patient.
recurrent FGFR2 and FGFR3 mutations, which are significant given that germline FGFR mutations are known to be pathogenic (17), that somatic mutations have been described in other malignancies (18), and that focal FGFR1 amplification is known to occur in lung SCC and seems to be a therapeutic target (15, 16).

We have confirmed that a subset of observed mutations drive transformation in NIH-3T3 cells in an anchorage-independent growth assay and xenograft assays, and that this is reversible by pan-FGFR and multikinase inhibitors. Some mutations were not transforming, but given the very high somatic mutation rate in lung SCC, this observation is not surprising. We found that ECD mutations in FGFR2 are able to form ligand-sensitive covalent receptor-dimers, as has been observed in other FGFR2 ECD mutations (29) and in FGFR3 mutations that have been described previously in urothelial carcinoma, and that we also observe here in the lung SCC data (30). This finding is especially relevant given that the FGFR2 W290C mutation has been observed independently in lung SCC sequencing on two previous occasions (10, 42). It is also possible that the glycosylation deficiency that we observed in the expressed protein harboring this mutation impacts protein function, a phenomenon with precedence in this receptor family (31).

We found that the FGFR mutations also exhibited sensitivity to inhibition by FGFR inhibitors in the Ba/F3 system, which models dependency on oncogenic pathways. Many drugs in the panel of inhibitors that we tested are already approved for clinical use in other malignancies, and clinical trials are underway to test sensitivity to FGFR inhibitors in patients harboring FGFR events (NCT01004224, NCT01457846, and NCT00979134). Although we cannot infer in vivo sensitivity to these inhibitors from our models, we believe that this study provides a compelling rationale for extending trials of FGFR kinase inhibitors to patients with lung and oral SCC harboring FGFR2 or FGFR3 mutations.

This study represents one of the first functionally validated novel recurrent targets to emerge from analysis of the systematic genomic profiling of lung SCC by the TCGA Research Network. It is our expectation that these findings will continue with the publication of more genomic studies of malignancies, and that this will lead to improved treatment options for patients with this disease.

Disclosure of Potential Conflicts of Interest
M.D. Wilkerson is a consultant/advisory board member of GeneCentric. M. Meyerson has a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member of Novartis and Foundation Medicine. P.S. Hammerman is a consultant/advisory board member of ARIAD. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Ani S. Bhatt, member of the Meyerson laboratory, Pamela M. Pollock, investigator at Queensland Institute of Technology (Brisbane, Australia), and David M. Ornitz, investigator at Washington University in St. Louis (St. Louis, MO) for helpful discussion and technical support.

Grant Support
P.S. Hammerman is a recipient of a Young Investigator Grant from the National Lung Cancer Partnership and is supported by National Cancer Institute (NCI) grants 1K08CA163877 and the Stephen D. and Alice Cutler Investigator Fund. M. Meyerson is supported by Uniting Against Lung Cancer, the Lung Cancer Research Foundation, the American Lung Association, Novartis Pharmaceuticals, and NCI grant 5P01CA090578. M.D. Wilkerson is supported by a Ruth L. Kirschstein National Research Service Award Individual Fellowship from the NCI (NIH F32CA142039). T.J. Pugh is supported by a Canadian Institutes of Health Research Fellowship.

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Received October 15, 2012; revised April 16, 2013; accepted June 3, 2013; published OnlineFirst June 20, 2013.

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