Identification of Oncogenic and Drug-Sensitizing Mutations in the Extracellular Domain of FGFR2

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Abstract

The discovery of oncogenic driver mutations and the subsequent developments in targeted therapies have led to improved outcomes for subsets of lung cancer patients. The identification of additional oncogenic and drug-sensitive alterations may similarly lead to new therapeutic approaches for lung cancer. We identify and characterize novel FGFR2 extracellular domain insertion mutations and demonstrate that they are both oncogenic and sensitive to inhibition by FGFR kinase inhibitors. We demonstrate that the mechanism of FGFR2 activation and subsequent transformation is mediated by ligand-independent dimerization and activation of FGFR2 kinase activity. Both FGFR2-mutant forms are predominantly located in the endoplasmic reticulum and Golgi but nevertheless can activate downstream signaling pathways through their interactions with fibroblast growth factor receptor substrate 2 (FRS2). Our findings provide a rationale for therapeutically targeting this unique subset of FGFR2-mutant cancers as well as insight into their oncogenic mechanisms.

Introduction

The discovery of oncogenic driver genes has helped identify druggable targets and the subsequent development of new therapies using small molecule tyrosine kinase inhibitors (TKI) for a broad range of cancers. Detection of epidermal growth factor receptor (EGFR) mutations or anaplastic lymphoma kinase (ALK) rearrangements in non–small cell lung cancer (NSCLC) is currently standard of care, leading to dramatically improved outcomes in patients with these genetic alterations when treated with an EGFR or ALK TKIs, respectively, as compared with systemic chemotherapy (1, 2). A number of new potentially targetable oncogenic gene alterations have been further characterized in recent years (3). However, even with recent intense research efforts, there still remain a significant proportion of cancer patients whose cancers do not harbor any known oncogenic alterations. Given the fact that cancer is the leading cause of death worldwide and accounts for 8.2 million deaths annually (4), identifying even a small subpopulation of patients who might benefit from targeted therapy could have significant clinical relevance.

Fibroblast growth factor receptor 2 (FGFR2) is a member of the FGFR family. FGFRs have a core structure containing an extracellular domain (ECD), a hydrophobic transmembrane domain, and an intracellular kinase domain. FGF ligand binding results in FGFR dimerization, followed by receptor autophosphorylation and activation of downstream signaling, playing key roles in multiple biologic processes such as tissue repair, angiogenesis, and embryonic development (5). Aside from their normal physiological roles, aberrant FGFR signaling has been implicated in a variety of diseases, including cancers (6). The FGFR signaling pathway can be activated in various ways. Amplification of FGFR1 and mutations in FGFR2 and FGFR3 have been identified in squamous cell lung carcinomas (7, 8). Recently identified FGFR gene rearrangements, involving FGFR1, FGFR2, and FGFR3, occur in diverse cancers, including in glioblastomas, cholangiocarcinomas, and cancers of the bladder, thyroid, mouth, breast, head and neck, and lung (9–11). These FGFR genetic alterations are oncogenic and sensitive to FGFR-TKIs, suggesting that a new subset of cancers might be treatable with FGFR-targeted therapies.

In this study, we identify and study novel FGFR2 extracellular domain insertion mutations. We characterize both the oncogenicity and FGFR inhibitor sensitivity of cells harboring these mutations. Finally, we evaluate the underlying mechanism supporting oncogenic signaling.

Materials and Methods

Samples and FGFR2 exon 7 mutation detection

Targeted next-generation sequencing (NGS) of the index patient’s tumor was performed in a commercial laboratory (12). Additional tumors from never smokers with lung cancer (n = 96) have been previously described (13, 14). Cancer patients
at Dana-Farber Cancer Institute (DFCI)/Brigham and Women's Hospital undergoing routine NGS (15) provided informed consent as part of an Institutional Review Board approved institutional wide sequencing effort. For detection of FGFR2 mutations in the 96 tumors from never smokers, DNA was extracted from paraffin-embedded or frozen tissues using the QIAGen DNA FFPE Tissue Kit (Qiagen) or the DNaseasy Blood and Tissue Kit (Qiagen), respectively, and was amplified via PCR. Primers used for the amplification are: forward primer 5’-ACTGACACGCCCTCCTGGAACAACAC-3’, reverse primer 5’-ACCACAGTGTGGGTACCCTTGAAG-3’.  

Cell culture and reagents  
NIH-3T3 cells were purchased from the ATCC in 2010. Authentication of the cell line was performed using karyotyping at the Brigham and Women's CytoGenomics Core Laboratory in May 2015. The cells with or without each gene alteration were maintained in DMEM medium with 10% fetal calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. BGJ398 was obtained from ChemieTek and ponatinib from Selleck Chemicals. FGF-1 ligand and Endo Hf were purchased from R&D Systems and New England Biolabs, respectively.  

Expression constructs and lentiviral infection  
Full-length FGFR2 cDNA from DF/HCC DNA Resource Core was cloned into pDONR-Dual (BD Biosciences) as described previously (16). To generate FGFR2 mutants, A266_S267ins, A266_S267ins K517R, 290_291WI was cloned into pDNR-Dual (BD Biosciences) as described previously. FGFR2 wild-type and (ii) FGFR2 A266_S267ins, A266_S267ins K517R, five mice per group. After 2 weeks of implantation, tumors were monitored twice weekly and volumes were calculated with the formula: (mm³) = length x width x width x 0.5. Standard deviations were calculated using Graphpad Prism (GraphPad Software, Inc.).  

Cell surface protein isolation and immunoprecipitation  
Cell surface protein isolation was done using a biotinylation-based assay using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). Flow-through lysates were examined by immunoprecipitation as previously described (18) using an anti-Flag-tag antibody.  

Immunofluorescence  
Cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100. The cells were then incubated in the indicated primary antibodies for 1 hour, followed by incubation with fluorescent conjugated secondary antibodies for 1 hour. Nuclei were counterstained with 4’,6-diamidino-2-phenylindole. Cells were visualized and photographed on a Nikon ECLIPSE 80i using NIS-Elements AR software (Nikon Instruments Inc.).  

Results  
Novel FGFR2 gene alterations detected in solid tumors  
We first studied the tumor of a 37-year-old male never smoker with advanced NSCLC. The patient received two cycles of cisplatin and etoposide as first-line systemic therapy but developed progressive disease. While sequencing was being performed, he was treated with second-line docetaxel chemotherapy. The patient had a dramatic response to docetaxel lasting over 8 months. A biopsy of a bone metastasis revealed NSCLC with neuroendocrine features. The tumor contained prominent glandular differentiation with mucin production and positive staining by IHC for CD56 and TTF-1 and was negative for synaptophysin and chromogranin (Supplementary Fig. S1 and data not shown). Further analysis by targeted NGS revealed the presence of a novel insertion in FGFR2. This insertion mutation, A266_S267insSTTVGD, is a duplication of 21 bp in the ECD of FGFR2 (Fig. 1A and B) and is somatic as determined by analyzing saliva extracted DNA (data not shown). No other genomic alteration was detected in this
FGFR2 mutations of BGJ398, an FGFR tyrosine kinase inhibitor, blunted colony formation in anchorage-independent conditions as compared with wild-type FGFR2 (Fig. 2A), whereas this effect was not observed in cells expressing FGFR2 T730S or those with V755I (Fig. 2A). BGI398, an FGFR tyrosine kinase inhibitor, blunted colony formation of FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 cells in a dose-dependent manner (Fig. 2B). We further evaluated the effect of BGI398 on signal transduction in these cells. Immunoblot analysis revealed greater phosphorylation of the FGFR2 substrate FRS2 in cells expressing FGFR2 A266_S267ins, FGFR2 290_291WI>C, or FGFR2–TACC3. BGI398 inhibited phospho-FRS2, accompanied by a concomitant inhibition of AKT and ERK1/2 phosphorylation in these cells. On the contrary, FRS2 activation was hardly observed in cells expressing wild-type FGFR2, T730S, or V755I (Fig. 2C). A pan-FGFR inhibitor AP24534 (ponatinib) also had significant inhibitory effects on phospho-ERK1/2 and phospho-AKT as well as phospho-FRS2 in FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 cells, but not in FGFR2 wild-type cells, T730S or V755I cells (Supplementary Fig. S2). These data suggest that cells expressing FGFR2 A266_S267ins, FGFR2 290_291WI>C, and those with FGFR2–TACC3 are dependent on FGFR signaling. Consistently, cells expressing the kinase dead version of FGFR2 A266_S267ins, FGFR2 A266_S267ins K517R, formed fewer colonies (Fig. 2D). We further confirmed that FGFR2 A266_S267ins is sufficient to induce tumor formation in vivo using xenografts, whereas cells expressing FGFR2 A266_S267ins K517R had significantly smaller tumors (Fig. 2E), additionally confirming FGFR kinase-dependent oncogenicity.

Given that both FGFR2 A266_S267ins and FGFR2 290_291WI>C alterations reside in the Ig3 ligand-binding domain of ECD, we further evaluated the effect of FGF stimulation in FGFR2 A266_S267ins compared with cells expressing wild-type FGFR2. In FGFR2 A266_S267ins cells, elevated basal FRS2 phosphorylation levels precluded further increases following FGF stimulation as compared with cells expressing wild-type FGFR2 (Supplementary Fig S3A). We also observed greater colony formation with FGF stimulation in FGFR2 wild-type cells, whereas FGF had little additional effect in cells with FGFR2 A266_S267ins,
consistent with the FRS2 phosphorylation results. Taken together, these data suggest that FGFR2 A266_S267ins leads to FGF-independent oncogenic transformation with little, if any, additional effect from exogenous FGF.

FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 form dimers

To identify the mechanistic basis for the oncogenicity of these mutant FGFR2 proteins, we examined whether the receptors dimerize in a ligand-independent fashion. Immunoblot analysis in nonreducing condition revealed that FGFR2 A266_S267ins and FGFR2 290_291WI>C form dimers as detected using either an anti-FLAG antibody or an anti-FGFR2 antibody (Fig. 3). FGFR2 wild-type proteins only form dimers following FGF ligand exposure (Fig. 3). In cells expressing FGFR2–TACC3, dimer bands were observed only with the anti-FLAG antibody, but not with the anti-FGFR2 antibody as this fusion protein lacks the C-terminal portion of FGFR2 that is recognized by this FGFR2 antibody. These data suggest that FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 are oncogenic through ligand-independent dimerization.

FGFR2 A266_S267ins, FGFR2 290_291WI>C primarily exist as partially glycosylated forms

FGFR2 can be detected on a SDS-PAGE gel in two distinct forms: a fully glycosylated, 120-kDa form of the receptor (upper band), and a partially glycosylated, 110-kDa form of the receptor (lower band; ref. 20). Cells harboring FGFR2

Figure 2. FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 are FGFR-TKI-sensitive driver gene alterations. A, soft-agar colony formation assay of NIH-3T3 cells stably expressing the indicated gene alterations after 21 days. Data are means of triplicates from representative experiments and expressed as fold over the numbers of colonies observed in cells expressing FGFR2 wild-type. B, soft-agar assay with increasing concentrations of BGJ398. After 21 days, the numbers of colonies were counted. Data are expressed relative to the corresponding value for nontreated cells and are means ± SE from three independent experiments. C, cells with the indicated gene alterations were serum starved overnight and then treated with BGJ398 at the indicated concentrations for 6 hours. Cell extracts were immunoblotted to detect the indicated proteins. D, results of colony formation assay after 21 days with cells expressing FGFR2 A266_S267ins, a kinase dead version (A266_S267ins K517R) or FGFR2 wild-type. Data are means of triplicates from representative experiments. E, tumor volume measurements from mouse subcutaneous xenograft models. Each curve represents means from five to ten mice per group.
A266_S267ins and FGFR2 290_291WI>C were found to show higher expression levels of the lower band (open arrow) detected by either an anti-FGFR2 antibody or an anti-FLAG antibody, compared with FGFR2 wild-type and the other mutations or translocations evaluated in this study (Fig. 4A). To prove that the higher expressed lower band in cells with ECD mutations was due to a partially processed receptor, cell lysates were digested with endoglycosidase (Endo Hf), which is specific for the high mannose N-linked oligosaccharides characteristic of proteins that have not completed Golgi-mediated maturation of glycosylation. Endo Hf digestion results in a shift in molecular mass from a primarily 110-kDa to a primarily 85-kDa band (open arrow) in both FGFR2 A266_S267ins cells and FGFR2 290_291WI>C cells (Fig. 4B), suggesting that FGFR2 receptors with those ECD mutations mainly exist as partially glycosylated forms.

FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 express in cytoplasm and colocalize with Golgi and ER markers

To determine whether the FGFR2 mutations lead to altered cellular localization of FGFR2 proteins, we evaluated the cellular localization using an immunofluorescence assay. In FGFR2 wild-type cells, immunofluorescence signals detected by both an anti-FGFR2 and anti-Flag antibodies were detected primarily in the plasma membrane and also perinuclearly to a lesser degree (Fig. 5A). However, in cells with FGFR2 A266_S267ins, FGFR2 290_291WI>C, or FGFR2–TACC3, the staining was localized primarily in a perinuclear pattern (Fig. 5A). Given this cytoplasmic distribution pattern, we next performed a co-immunofluorescence assay using antibodies against the ER marker, calnexin, and the Golgi marker, GM130. The FGFR2 immunofluorescence performed in cells with FGFR2 A266_S267ins, FGFR2 290_291WI>C, and FGFR2–TACC3 demonstrated colocalization with calnexin (Fig. 5B) and GM130 (Fig. 5C), suggesting that these mutant FGFR2 proteins predominately localize in the ER/Golgi. In contrast, colocalization with calnexin and GM130 was not observed in cells expressing wild-type FGFR2. We next used biotin labeling as a complementary method to determine the cellular location of wild-type and mutant FGFR2 proteins. Biotinylation and the subsequent isolation of cell surface proteins revealed that FGFR2 wild-type is mainly present at the cell surface, whereas FGFR2 A266_S267ins and FGFR2 290_291WI>C in the cytoplasm (Fig. 6A). The specificity of cell surface labeling by biotinylation was confirmed by the immunobLOTS for the cytoplasmic protein, HSP90. Cells with FGFR2–TACC3 translocation also showed cytoplasmic expression of FGFR2 (Fig. 6A). We finally evaluated whether these ER/Golgi-located mutant FGFR2 proteins are functional and can initiate signaling. We performed immunoprecipitation with the flow-through lysates from the cell surface protein isolation assay, which contain...
cytoplasmic proteins but not cell surface proteins. FLAG-tagged FGFR2 was immunoprecipitated from FGFR2 wild-type, FGFR2 A266_S267ins, FGFR2 290_291W=C, and FGFR2–TACC3 cell lysates, and the resulting precipitates were subjected to immunoblot analysis with antibodies to FRS2. We found that FGFR2 A266_S267ins, FGFR2 290_291W=C, and FGFR2–TACC3, which localize in the cytoplasm, coprecipitated with FRS2 (Fig. 6B), suggesting that these proteins are in complex with FRS2 and hence could initiate signaling from intracellular compartments.

Figure 6.
A, wild-type FGFR2 expresses on the cell surface, whereas FGFR2 A266_S267ins, FGFR2 290_291W=C, and FGFR2–TACC3 express primarily in the cytoplasm. Cell surface proteins were biotinylated and precipitated with streptavidin beads (M). Streptavidin precipitates and the flow-through corresponding to the intracellular compartment (I) were immunoblotted and detected with FGFR2, Flag, and HSP90 (control for cytoplasmic proteins) B, FGFR2 A266_S267ins, FGFR2 290_291W=C, and FGFR2–TACC3 interact with FRS2 in cytoplasm. Flow-through lysates from A were immunoprecipitated with antibodies to Flag antibody. The resulting precipitates were subjected to immunoblot analysis with antibodies against FRS2 and those against Flag.
Discussion

Aberrant FGFR signaling has been implicated in the development of several cancer types, including FGFR1 amplification in lung squamous cell carcinoma (7, 21). FGFR3 mutations in urothelial carcinomas (22), and recently reported fusions of FGFR family kinases with TACC or other genes in several cancer types (9, 11, 23, 24). In this study, we identify novel, albeit rare, FGFR2 genetic alterations that are oncogenic and can be targeted with FGFR inhibitors in solid tumors, including lung cancer.

FGFR2 point mutations have been well described in studies of different diseases, such as craniosynostosis syndrome, gastric cancer, and endometrial carcinomas (6, 25). Other cancers, such as craniosynostosis syndrome, gastric cancer, and endometrial carcinomas (6, 25). Recent studies suggest that FGFR2 might define a molecular subset in other cancers as well, including NSCLC (8, 10, 19). FGFR2 A266S267insSTVV found in an NSCLC patient and FGFR2 290_291W1>C found in a cholangiocarcinoma patient localize to the ECD of FGFR2. Although mutations in the ECD do not seem to promote direct activation of FGFR2 kinase activity, they could be oncogenic by changing the affinity to ligand (28, 29), or lead to ligand-dependent dimerization and subsequent activation of FGFR2 signaling (8, 30, 31). In this study, we detected elevated basal phosphorylation of FGFR and that of FRS2 in cells expressing these ECD mutations accompanied by dimer formation. Together with the in vivo results, our data indicate that FGFR2 A266S267ins and FGFR2 290_291W1>C are oncogenic mutations through dimer formation as a possible mechanism, but in a ligand-independent manner.

We found that FGFR2 A266S267ins and FGFR2 290_291W1>C have altered receptor subcellular localization. Increased expression levels of the incompletely glycosylated receptor support this finding since the full N-glycosylation of FGFR2 is considered to be involved in normal trafficking of the receptor to the cell surface (32). Immunofluorescent costaining for ER or Golgi markers showed that these mutated receptor forms are predominantly located within these intracellular compartments, whereas such colocalization was not detectable in cells expressing wild-type FGFR2. Similar to our observation, other FGFR2 ECD mutations have been reported to lead to diminished FGFR2 glycosylation, accompanied with altered localization of FGFR2 (32, 33). A crystal structure study revealed that FGFR2 ECD mutation G271E can cause local structure perturbations and destabilization, which could interfere with dimers or receptor mislocalization by interfering with the correct receptor maturation and trafficking (33). A previous study has shown that incompletely glycosylated FGFR2 ECD mutations are able to dimerize (8) and activate signal transduction in the ER/Golgi (32), consistent with our finding. Furthermore, we showed that FGFR2-TACC3 fusions localize primarily in the cytoplasm, and more precisely in the ER/Golgi. Although the papers reporting FGFR–TACC fusions are still limited, it has been shown that FGFR3–TACC3 fusion results in enhanced expression of this fusion gene (23) and that FGFR3–TACC3 localizes in the cytosol, in mitotic spindle poles (9). Conventional cell signaling models involve initiation of signaling cascades of receptor tyrosine kinases by ligand binding at the cell surface; however, recent findings suggest that some of these signaling pathways can be initiated within internal compartments (34, 35). Thus, an altered expression or localization pattern of FGFR2 protein could be involved in oncogenic mechanisms in addition to forming dimers. Taken together, our data suggest that these novel FGFR2 ECD mutations, A266S267ins and 290_291W1>C, form dimers in a ligand-independent fashion and activate signaling possibly through diminished glycosylation of the receptor and altered localization in the ER/Golgi. However, it remains to be elucidated whether increased proportion of incompletely glycosylated FGFR2 results either from less efficient trafficking to the cell surface or from more rapid endocytosis and degradation after trafficking to the cell surface. Moreover, the mechanism by which glycosylation is involved in receptor trafficking remains unclear, with further investigations needed.

In summary, we have identified and characterized several drug-sensitive FGFR2 gene alterations, providing a rationale for FGFR-targeted therapy in solid cancer. Reports of FGFR2 gene alterations, especially in NSCLC, are still limited. There are 314 FGFR2 mutations from 87 published studies in cbioportal for cancer genomics and one W290C mutation has been reported in a lung squamous cell carcinoma patient (36). Furthermore, recent study also reported W290C mutation in lung squamous cell carcinoma (8). These data support a rationale to investigate the frequency of these ECD alterations together with the related clinic-pathologic features. Given the number of cancer patients all over the world, identifying even a small subpopulation of patients who may respond to targeted therapy can have significant therapeutic relevance.

Disclosure of Potential Conflicts of Interest

P.S. Hammerman is a consultant/advisory board member for Astra Zeneca and Janssen. G.R. Oxnard is a consultant/advisory board member for Novartis, Clovis, AstraZeneca, Boehringer-Ingelheim, and Genentech. P.A. Jänne is a consultant/advisory board member for Astra Zeneca, Boehringer Ingelheim, Pfizer, Genentech, Clovis Oncology, MerckMack Pharmaceuticals, Chugai Pharmaceuticals, and Sanofi. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Dr. Mohammadi for helpful discussions and suggestions on this article.

Grant Support

This work is supported by The Uehara Memorial Foundation (J. Tanizaki), the Cammarata Family Foundation Research Fund (M. Capelletti and P.A. Jänne), the Nirenberg Fellowship at the Dana-Farber Cancer Institute (M. Capelletti and P.A. Jänne), and by the National Cancer Institute (RO1 CA163677; P.S. Hammerman). P.S. Hammerman is supported by a V Foundation Scholar Award.

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Received December 22, 2014; revised March 22, 2015; accepted April 28, 2015; published OnlineFirst June 5, 2015.
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Cancer Res  Published OnlineFirst June 5, 2015.