Drug-sensitive *FGFR2* mutations in endometrial carcinoma

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Oncogenic activation of tyrosine kinases is a common mechanism of carcinogenesis and, given the druggable nature of these enzymes, an attractive target for anticancer therapy. Here, we show that somatic mutations of the fibroblast growth factor receptor 2 (*FGFR2*) tyrosine kinase gene, *FGFR2*, are present in 12% of endometrial carcinomas, with additional instances found in lung squamous cell carcinoma and cervical carcinoma. These *FGFR2* mutations, many of which are identical to mutations associated with congenital craniofacial developmental disorders, are constitutively activated and oncogenic when ectopically expressed in NIH 3T3 cells. Inhibition of *FGFR2* kinase activity in endometrial carcinoma cell lines bearing such *FGFR2* mutations inhibits transformation and survival, implicating *FGFR2* as a novel therapeutic target in endometrial carcinoma.

endometrial cancer | fibroblast growth factor receptor 2 | oncogene | targeted therapy | tyrosine kinase

Tyrosine kinases play a major role in transduction of proliferative signals and can become oncogenic when deregulated by somatic mutation (1). Somatic altered tyrosine kinases have proven to be tractable therapeutic targets in several tumor types; examples of successfully targeted tyrosine kinases include *ABL1* in chronic myeloid leukemia (2), *KIT* in gastrointestinal stromal tumors (3), *ERBB2* in breast cancer (4), and *EGFR* in non-small-cell lung cancer (5–7). The tyrosine kinase family has not been exhaustively studied in human cancer, and it is likely that additional tyrosine kinase therapeutic targets remain to be discovered.

The fibroblast growth factor receptor (*FGFR*) tyrosine kinase family, which is comprised of four kinases that differentially respond to 18 FGF ligands (8, 9), has long been implicated in cancer. Translocations involving *FGFR3*, and activating somatic mutations in *FGFR3*, have been identified in multiple myeloma patients (10, 11), and translocations of *FGFR1* have been found in patients with 8p11 myeloproliferative syndrome (12). Isolated cases of a missense mutation of *FGFR4* in a lung adenocarcinoma patient and missense mutations of *FGFR2* in a lung squamous cell carcinoma patient and gastric cancer patient have also been reported (13, 14). In addition to these documented examples of somatic mutation of *FGFR* family members in cancer, a germ-line polymorphism in the second intron of *FGFR2* was found to be associated with breast cancer in genome-wide association studies (15, 16).

FGFR1–FGFR3 are characterized by alternative splicing of the mRNA encoding the third Ig-like repeat in the extracellular ligand-binding domain. This differential splicing determines ligand specificity such that isoforms expressed primarily in epithelial cells (IIIb) preferentially bind FGF ligands expressed

by mesenchymal cells, and isoforms expressed primarily in mesenchymal cells (IIIc) preferentially bind FGF ligands expressed by epithelial cells (17–19). Alteration of this restricted expression pattern can lead to oncogenic transformation (20). Mutations in *FGFR2* and *FGFR3* can also alter ligand specificity (21, 22); such gain-of-function mutations have been found in patients with the congenital craniofacial malformation disorders, Apert and Crouzon syndromes, characterized by craniosynostosis and syndactyly of hands and feet (23, 24). Moreover, these mutations are directly correlated with development of Apert syndrome in mouse models (25–27).

In a search for novel druggable therapeutic targets, we have undertaken sequencing of tyrosine kinase genes in a variety of tumor types. Endometrial cancer, the most common gynecological cancer in industrialized countries, with no curative treatment for patients with nonresectable disease (28), was examined in early discovery experiments. Here, we report somatic mutations of *FGFR2* in endometrial cancer, with additional instances found in lung squamous cell carcinoma and cervical carcinoma.

Results

Identification of Cancer-Associated Mutations in Endometrial Carcinoma. We performed DNA sequence analysis of all exons of 89 tyrosine kinase genes and 19 additional known oncogenes and tumor suppressor genes in 40 primary endometrial carcinoma tumor DNA samples. Nonsynonymous candidate mutations detected by sequencing of tumor DNA were confirmed and determined to be somatic or germ-line by mass spectrometric genotyping of the tumor and matched normal DNA. Somatic mutations in genes previously demonstrated to be altered in endometrial carcinoma, including *KRAS*, *CTNNB1*, *PIK3CA*, *PTEN*, and *TP53*, were found at the expected frequencies [supporting information (SI) Fig. S1]. One sample was deemed to be hypermutated because of the presence of an unusually large number of somatic mutations in the genes sequenced and was analyzed separately (Table S1). Somatic mutations in 19 additional genes were detected in the nonhypermutated samples,

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Fig. 1. Schematic diagram of novel *FGFR2* mutations. Black diamonds indicate each instance of mutations found in endometrial samples; red diamonds indicate squamous lung; blue diamonds indicate cervical carcinoma. Asterisks indicate mutations demonstrated to be oncogenic (data in Table S3). K310R and A389T were not transforming; the remaining mutants without asterisks were not tested. Ig, Ig-like repeat. TM, transmembrane domain.

most frequently in the known tumor suppressor gene *VHL* (Table S2 and Fig. S1).

Among the remaining mutated genes, we found that two tumor DNA samples harbored identical mutations in *FGFR2*, S252W (Table S2 and Fig. S2). Using a combination of sequencing and mass spectrometric genotyping, we identified 13 more samples with *FGFR2* mutations among an additional 73 endometrial carcinoma samples and 9 endometrial cancer cell lines (Fig. 1 and Table S3), for a total of 15 samples with *FGFR2* mutations of 122 endometrial carcinoma DNAs tested (12.3%). We also found *FGFR2* mutations in two of 46 cervical carcinoma DNA samples and two of 42 lung squamous cell carcinoma DNA samples (Fig. 1 and Table S3). No mutations in *FGFR2* were detected in other tumor types in which we systematically sequenced tyrosine kinase genes, including lung adenocarcinoma, renal cell carcinoma, glioblastoma, and prostate cancer (data not shown).

The somatic *FGFR2* mutations include the S252W and P253R alleles, autosomal dominant mutations associated with the congenital developmental disorder Apert syndrome, and N549 and K659 mutations associated with a second congenital developmental disorder, Crouzon syndrome. These latter kinase domain

residues have recently been shown to participate in a “molecular brake” regulating *FGFR2* kinase activity (24, 29).

Mutant *FGFR2* Is Oncogenic. Although Apert syndrome-associated *FGFR2* mutants have been shown to exhibit increased kinase activity, the putative transforming activity of such *FGFR2* mutants is only tangentially referred to in the literature (30). We therefore transduced NIH 3T3 fibroblasts with retroviruses encoding isoform IIIb WT *FGFR2*, *FGFR2* S252W, and *FGFR2* S252W in combination with the kinase-inactivating D626A substitution. Ectopic expression of the Apert syndrome-associated S252W mutant in pooled NIH 3T3 cells conferred anchorage-independent growth, forming 8-fold more colonies in soft agar than cells expressing WT *FGFR2* (Fig. 2A), despite higher expression levels of WT *FGFR2* (Fig. 2B). Transformation was accompanied by elevated phosphorylation of the *FGFR2* substrate FRS2 (Fig. S3). NIH 3T3 cells expressing *FGFR2* S252W in the context of an inactivating D626A mutation did not form colonies in soft agar, indicating that enzymatic activity is required for transformation (Fig. 2A). Other endometrial cancer-derived *FGFR2* mutations were found to be similarly oncogenic (Table S3).

Mutant *FGFR2* Is Required for Tumor Cell Survival. To determine whether expression of mutant *FGFR2* is required for tumor cell survival, we tested a series of shRNA constructs in endometrial tumor cell lines expressing WT *FGFR2* (Hec-1B) or mutant *FGFR2*

Fig. 2. Ectopic expression of *FGFR2* S252W in NIH 3T3 cells supports anchorage-independent growth. (A) Pooled NIH 3T3 cells stably expressing the empty vector or the WT, S252W, or kinase-dead S252W/D626A isoform IIIb *FGFR2* constructs were assessed for colony formation in soft agar. *n* = average number of colonies counted in three sets of 10 fields. (B) *FGFR2* expression was confirmed by immunoblotting using actin as a loading control. wt, WT *FGFR2*.

Fig. 3. Knockdown of mutant *FGFR2* expression with shRNA inhibits transformation and survival of endometrial cancer cell lines. Infection with two independent hairpins (shFGFR2#1 and shFGFR2#4) inhibited cell survival as assessed by WST-1 cell survival assay (A and B) and anchorage-independent growth as assessed by colony formation in soft agar (C and D) in the MFE-280 cells harboring an S252W mutation, but not the Hec-1B cells, which express WT *FGFR2*. shGFP, a hairpin specific for green fluorescent protein, was used as a negative control. All results are normalized to survival or colony formation by cells infected with empty vector.

Whole-Genome Amplification. Primary tumors and matched normals were whole-genome-amplified for sequencing and genotyping. Genotype validation of candidate mutations was performed on an independent whole-genome amplification. Forty nanograms of genomic DNA was used for whole-genome amplification using the REPLI-gTM kit (Qiagen), yielding $\approx 40 \mu\text{g}$ of total DNA.

Sequencing. PCR primers were designed with which to amplify each exon of interest (Table S4). PCRs for each exon and flanking intronic sequences contained 5 ng of whole-genome-amplified DNA, $1 \times$ HotStar buffer, 0.8 mM dNTPs, 2.5 mM MgCl_2 , 0.2 units of HotStar Enzyme (Qiagen), and 0.25 μM forward and reverse primers in a 6- or 10- μl reaction volume. PCR cycling parameters were: one cycle of 95°C for 15 min; 35 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 1 min; followed by one cycle of 72°C for 3 min. The resulting PCR products were sequenced by using bidirectional dye-terminator fluorescent sequencing with universal M13 primers. Sequencing fragments were detected via capillary electrophoresis with an ABI Prism 3730 DNA Analyzer (Applied Biosystems). Sequencing traces were analyzed by using an automated pipeline consisting of SNPCompare (described below) and Mutation Surveyor 2.51 (SoftGenetics), followed by manual review of candidate mutations. Base coverage was determined by using SNPCompare. An exon was considered covered if 80% of the bases were covered in 80% of the samples. Minimum exon coverage was 80%. PCR and sequencing of MFE-280 and Hec-1B were additionally performed at Agencourt Bioscience, Beverly, MA.

SNPCompare. SNPs were called by using PolyPhred 6.02b (37, 38) followed by a postprocessing pipeline we refer to as SNPCompare. In brief, we retain all SNPs called by PolyPhred with a confidence of 99. SNPs with a confidence ≥ 95 are kept if the average local read quality is at least 30, whereas those with confidence ≥ 90 are kept if average local read quality is at least 40. For SNPs with PolyPhred confidence ≥ 60 but not meeting the preceding criteria, we check traces with an independent SNP detection method, PolyDhan (D. Richter, unpublished data; see below). PolyDhan has relatively poor sensitivity but very low false discovery, so we retain low confidence PolyPhred SNPs also seen with PolyDhan in the same sample marked heterozygous by PolyPhred. We define local read quality as the average Phred (39, 40) quality score in a “notched” window of length 11 centered on the putative SNP but excluding the SNP base itself and the immediate flanking base on either side (whose quality is expected to be low if the base is a true heterozygote). Average local read quality at a site is computed by averaging the local read quality of all reads aligning to reference. If any sample at an alignment position is called nonreference according to the above criteria, we consider the site to be variant and accept PolyPhred’s genotype for all samples at that site without further quality assessment. Briefly, PolyDhan compares the processed trace signal for all reads on a given strand at a given site in an alignment and determines whether some subset of traces show evidence of a nonreference signal in excess of the average level of background signal in that channel at that base. Both strands are examined independently, and a SNP is called if either strand shows evidence. As used in SNPCompare, PolyDhan is required to confirm one or more heterozygous calls by PolyPhred only at sites not meeting other quality filtering criteria. We do not require PolyDhan to confirm every heterozygous call once the site is accepted as variant.

Mass Spectrometric Genotyping. Multiplex PCR was performed in 5- μl volumes containing 0.1 units of *Taq* polymerase, 5 ng of whole-genome-amplified genomic DNA, 2.5 pmol of each PCR primer, and 2.5 μmol of dNTP. Thermocycling was at 95°C for 15 min followed by 45 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 30 s. Unincorporated dNTPs were deactivated by using 0.3 unit of shrimp alkaline phosphatase followed by primer extension using 5.4 pmol of each primer extension probe, 50 μmol of the appropriate dNTP/ddNTP combination, and 0.5 unit of Thermosequase. Reactions were cycled at 94°C for 2 min, followed by 40 cycles of 94°C for 5 s, 50°C for 5 s, 72°C for 5 s. After addition of a cation exchange resin to remove residual salt from the reactions, 7 nl of the purified primer extension reaction was loaded onto a matrix pad (3-hydroxypicolonic acid) of a SpectroCHIP (Sequenom). SpectroCHiPs were analyzed with a Bruker Biflex III MALDI-TOF mass spectrometer (SpectroREADER; Sequenom).

Cell Culture and Reagents. NIH 3T3 cells obtained from ATCC were maintained in DMEM (Cellgro/Mediatech) supplemented with 10% calf serum (Gibco/Invitrogen) and penicillin/streptomycin (Gibco/Invitrogen). MFE-280 and Hec-1B cells were maintained in 40% RPMI medium 1640 + 40% MEM + 20% FBS and DMEM/F12 at 1:1 + 15% FBS, respectively. Unless otherwise noted, cells were placed in media containing 0.5% calf serum 24 h before 30 ng/ml FGF7 (Peprotech) stimulation for 20 min at 37°C . PD173074 was purchased from Calbiochem and diluted in DMSO to the indicated concentrations.

Expression Constructs. *FGFR2b* cDNA clone (IOH36372) was obtained from Invitrogen. Point mutations were made by using the QuikChange Mutagenesis XL kit (Stratagene) with the following mutation-specific oligonucleotide primers: S252W, 5'-GACGTACCTTATCTATAAGAGGACCGAC-3' and 5'-AGT-ATTCGTGGTGGTCTCTTATA-3'; K310R, 5'-TGCCCTACCTCAGGGTCTCAAGGC-3' and 5'-GCCTTGAGAACCTGAGGTAGGGCA-3'; A314D, 5'-GGTCTCAAGGACGCCGGTGTAAAC-3' and 5'-GTAAACACCGCGCTCTTGAGAAC-3'; A389T, 5'-TCTTAATCGCTATATGGTGAACAG-3' and 5'-CTGTACCACTATAGGCGATTAAGA-3'; N549K, 5'-CATAAATCTCTGGGAGCCTGCACAC-3' and 5'-GTG TGCAGGCTCCAGAAGATTATG-3'; C382R, 5'-GATAGCCATTACCGCATAGGGGTC-3' and 5'-GACCCCTATGCGGTAATGCGTATC-3'; D101Y, 5'-CGC CACGCTAGATACTCCGGCTC-3' and 5'-GAGGC-GGTAGTATCTAGGCGTGCGC-3'; P253R, 5'-TGGAGCGATCGCGTACCGGCCCATC-3' and 5'-GATGGG CCGGTGACGCGATCGTCCA-3'.

Transfection and Infection. Replication-incompetent retroviruses were produced from pBabe-Puro-based vectors by transfection into the Phoenix 293T packaging cell line (Orbigen) by using Lipofectamine 2000 (Invitrogen). Cells were infected with these retroviruses in the presence of polybrene. Two days after infection, 2 $\mu\text{g}/\text{ml}$ puromycin (Sigma) was added, and pooled stable cell lines were selected, from which clonal cell lines were derived.

shRNA-Mediated FGFR2 Knockdown. shRNA vectors targeted against *FGFR2* and *GFP* were obtained from The RNAi Consortium. The target sequences of the *FGFR2* shRNA constructs are: FGFR2#1 (TRCN0000000367), 5'-GCCACCAACCAATACCAATCTC-3'; FGFR2#2 (TRCN0000000368), 5'-CCGAATGAAGAACACGACCAA-3'; FGFR2#3 (TRCN0000000369), 5'-CCCAACAATAGGACAGTGCTT-3'; and FGFR2#4 (TRCN0000000370), 5'-GCCAACCTCTCGAACAGTATTC-3'. The sequence targeted by the GFP shRNA is 5'-GCAAGCTGACCTGAAGTTCAT-3'. Lentiviruses were made by transfection of 293T packaging cells with these constructs by using a three-plasmid system as described (41). Target cells were incubated with lentiviruses for 6 h in the presence of 8 $\mu\text{g}/\text{ml}$ polybrene and left in their fresh respective media. Two days after infection, puromycin (2 $\mu\text{g}/\text{ml}$ for MFE-280, Hec-1B, and AN3CA and 3.0 $\mu\text{g}/\text{ml}$ for MFE-296) was added. Cells were grown in the presence of puromycin for 4 days. Fifty micrograms of total cell lysates prepared from the puro-selected cell lines was analyzed by Western blotting using anti-FGFR2 mAb (Santa Cruz Biotechnology) for MFE-296, AN3CA, and Hec-1B and anti-FGFR2 (S252W) polyclonal antibody for MFE-280 tumor cell line, obtained from Bethyl Laboratories and anti-actin mAb (sc-1615; Santa Cruz Biotechnology).

Cell Survival Assays with Tumor Cell Lines Expressing shFGFR2 and shGFP Constructs. One thousand cells for each tumor cell line expressing shRNAs targeting *FGFR2* or *GFP* along with uninfected cells were seeded in six wells on a 96-well plate. Cell viability was determined at 24-h time points for 4 consecutive days by using the WST-1 assay (Roche Applied Science). The percentage of cell viability was plotted for each cell line of readings obtained on day 3 relative to day 1.

Soft Agar Anchorage-Independent Growth Assay. FGFR2-expressing NIH 3T3 cells were suspended in a top layer of DMEM containing 10% calf serum and 0.4% Select agar (Gibco/Invitrogen) and plated on a bottom layer of DMEM containing 10% calf serum and 0.5% Select agar. PD173074 was added as described to the top agar. After 3 weeks of incubation, NIH 3T3 colonies were counted in triplicate wells from 10 fields photographed with a $\times 4$ objective. Endometrial cancer cell lines (with and without shRNA constructs) MFE-280, MFE-296, Hec1B, and AN3CA were suspended in a top layer of media described above with 0.4% Select Agar and otherwise assayed as for NIH 3T3 cells, but photographed with a $\times 2$ objective after 2 weeks of incubation. EC_{50} s were determined by nonlinear regression with Prism GraphPad software.

Cytotoxicity Assays. Endometrial cancer cell lines were treated with PD173074 1 day after plating, and cell survival was assessed 4 days later by using the WST-1 assay (Roche). Each data point represents the median of six replicate wells for each tumor cell line and PD173074 concentration. EC_{50} s were determined by nonlinear regression using Prism GraphPad software.

Immunoblotting. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, and 0.25% Igepal. Protease inhibitors (Roche) and phosphatase inhibitors (Calbiochem) were added before use. Samples were normalized for total protein content. Lysates were boiled in sample buffer, separated by SDS/PAGE on 8% polyacrylamide gels, transferred to PVDF membrane, and probed as described. Antibodies used for immunoblotting were: anti-FGFR2 (sc-6930; Santa Cruz Biotechnology), anti-phospho-FRS2 Y436

(3861; Cell Signaling Technologies), anti-phospho-FRS2 Y196 (3864; Cell Signaling Technologies), and anti-FRS2 (sc-17841; Santa Cruz Biotechnology).

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