FGFR2-Amplified Gastric Cancer Cell Lines Require FGFR2 and Erbb3 Signaling for Growth and Survival

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Abstract

We have identified a critical role for amplified FGFR2 in gastric cancer cell proliferation and survival. In a panel of gastric cancer cell lines, fibroblast growth factor receptor 2 (FGFR2) was overexpressed and tyrosine phosphorylated selectively in FGFR2-amplified cell lines KatoIII, Snu16, and OCUM-2M. FGFR2 kinase inhibition by a specific small-molecule inhibitor resulted in selective and potent growth inhibition in FGFR2-amplified cell lines, resulting in growth arrest in KatoIII cells and prominent induction of apoptosis in both Snu16 and OCUM-2M cells. FGFR2-amplified cell lines also contained elevated phosphotyrosine in EGFR, Her2, and Erb3, but the elevated phosphorylation in EGFR could not be inhibited by gefitinib or erlotinib. We show that the elevated EGFR, Her2, and Erb3 phosphotyrosine is dependent on FGFR2, revealing EGFR family kinases to be downstream targets of amplified FGFR2. Moreover, shRNA to Erb3 resulted in a loss of proliferation, confirming a functional role for the activated EGFR signaling pathway. These results reveal that both the FGFR2 and EGFR family signaling pathways are activated in FGFR2-amplified gastric cancer cell lines to drive cell proliferation and survival. Inhibitors of FGFR2 or Erb3 signaling may have therapeutic efficacy in the subset of gastric cancers containing FGFR2 amplification.

[Cancer Res 2008;68(7):2340–8]

Introduction

Gastric cancer is second to lung cancer as the most lethal cancer worldwide (1) and can be classified as a well-differentiated intestinal subtype or as a poorly differentiated diffuse subtype (2). Whereas overall gastric cancer incidence has declined, the incidence remains high in Asian countries including Japan, Korea, and China (1, 3). Diffuse gastric cancer is associated with a less favorable prognosis relative to the intestinal subtype, but for gastric cancer overall, the prognosis is poor, with 5-year survival rates in the range of 10% to 15% (4, 5). An exception to this poor survival is observed in clinical practice in Japan, where a combination of screening and aggressive surgical intervention has resulted in 5-year survival rates approaching 60% (4, 6). Despite these efforts, late-stage gastric cancer continues to have a dismal prognosis, and thus there remains an urgent need for improved therapy.

Multiple oncogenic alterations have been described in gastric cancer. These include a relatively low incidence of ras mutations (7, 8), loss-of-function mutations in the E-cadherin gene CDH1 (9), as well as amplification of receptor tyrosine kinases Her2, Met, and FGFR2 (10–12). Interestingly, these alterations are associated with specific subtypes of gastric cancer. Whereas Her2 amplification (13) and ras mutations (14, 15) are found in the well-differentiated intestinal subtype, CDH1 mutation as well as Met and FGFR2 amplification occurs more frequently in the undifferentiated diffuse subtype (9, 12, 16, 17). Interestingly, in an analysis of matched primary and metastatic tumor samples, FGFR2 amplification in some cases occurred only in a metastatic lesion, suggesting a role for this kinase in the development of metastases (18).

Fibroblast growth factor receptor 2 (FGFR2) is a member of the FGFR receptor tyrosine kinase family, which consists of 4 receptors and 23 ligands (19). Ligand binding leads to FGFR2 dimerization, autophosphorylation, and activation of signaling components including Akt and Erk kinases. FGFR2 was originally identified as an amplified DNA sequence from the gastric cancer cell line KatoIII (18, 20), and subsequent efforts identified FGFR2 amplification in 3% to 10% of primary gastric cancers (12, 16, 21). A role for FGFR2 in cancer is supported by the observation that transgenic expression of the FGFR2-specific ligand FGF7 (KGF) leads to prostate hyperplasia and mammary adenocarcinoma (22). In addition, activating mutations in FGFR2 have been described in primary gastric cancer (23). Despite these observations, a role for FGFR2 in oncogenesis has not been widely accepted, and evidence also exists that FGFR2 is down-regulated and may have a growth suppressive role in some cancers (24). As well, with regard to FGFR2 amplification, it remained unclear whether FGFR2 or neighboring genes in the 10q26 locus were contributing factors for gastric cancer transformation. Recent work using multikinase small-molecule inhibitors has provided evidence that in cell lines expressing FGFR2, the kinase can be required for cancer cell proliferation (25, 26). However, the small-molecule inhibitors used in these studies inhibit multiple kinases in addition to FGFR2, raising the possibility that inhibition of a combination of kinases is required for growth inhibition.

We used both a highly specific FGFR inhibitory small molecule and FGFR2 shRNA to define a critical role for FGFR2 amplification in gastric cancer cell growth. In KatoIII, Snu16, and OCUM-2M cell lines, FGFR2 amplification results in a highly overexpressed and constitutively phosphorylated receptor. FGFR2 inhibition by shRNA or a small-molecule inhibitor induced potent and selective growth inhibition and apoptosis in these cell lines. Interestingly, elevated tyrosine phosphorylation in epidermal growth factor receptor (EGFR), Erb3, and Her2 was found in FGFR2-amplified cell lines. This elevated phosphorylation was resistant to inhibition by either gefitinib or erlotinib but was abrogated on FGFR2 kinase inhibition, revealing EGFR family members to be downstream
components of FGFR2 signaling in FGFR2-amplified cell lines. shRNA inhibition of Erbb3 inhibited growth in FGFR2-amplified lines, and thus the FGFR2-mediated activation of Erbb3 is essential for proliferation. We conclude that the activated FGFR2 kinase in FGFR2-amplified gastric cancer cells stimulates proliferation and survival through activation of multiple signaling pathways including a novel transactivation of EGFR family members. As well, our results suggest that FGFR2 inhibition may have therapeutic efficacy in the subset of poorly differentiated gastric cancers containing FGFR2 amplification.

Materials and Methods

Cell lines. KatoIII, Snu16, AGS, 23132/87, Snu1, N87, MKN45, and S5u were from American Type Culture Collection. NUGC4, OCUM-1, and IM95 were from the Health Science Research Resources Bank (Japan Health Sciences Foundation). Cells were maintained in RPMI plus 10% FCS and 100 μg/mL penicillin-strep (Sigma). IM95 medium contained 10 mg/L insulin. G1L-16 cells were a gift from S. Giordano and P.M. Comoglio (University of Tokyo Medical School, Tokyo, Italy). OCUM-2M cells were from Osaka City University (Osaka, Japan).

Quantitative PCR analysis of FGFR2 genomic amplification. Genomic DNA was purified with the DNeasy kit protocol per manufacturer’s instructions (Qiagen). Primers and probe used for FGFR2 qPCR are listed 5′ to 3′: F-CCCCCTCCACAATCATTCCT, R-ACCGGCGGCCTAGAAAAC, and VIC-TCCGTCAGGTCATGTGC; Primers and probe for single-copy reference gene RNase P were from Applied Biosystems, as was TaqMan Universal PCR reagent mix (432418). Reactions were done in quadruplicate with genomic DNA at 2.5 ng, primers at 900 μM/L, and probes at 250 μM/L under standard thermocycling conditions (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C). Data were normalized to RNase P and then to the calibrator sample (normal stomach genomic DNA, BioChain Institute D1234248).

Fluorescence in situ hybridization analysis. KatoIII gastric carcinoma cells were treated with colcemid at 0.02 μg/mL for 3 h and DNA fluorescence in situ hybridization (FISH) was done as previously described (27, 28) using bacterial artificial chromosome clones RP1-62L18 and RP1-20J15 containing FGFR2 as probes. Probes were directly labeled using Spectrum Orange dUTP and Spectrum Green dUTP (Abbott Molecular, Inc.).

shRNA production and infection. shRNA sequences were F1, GCCAACCTCGAAGACATTCAAGATCTCGTGGAGGATTGGGC; F2, GGACTTGGTGTCATGCACCTTCAAGAGAGGTGCATGACACCAAGTCC; and F3, GGAGCTGACGAGCAACCTCAGAGAGGTGATGGAGTGAAGAGCTC; VIC-TCGTCTAGGCTTTCTTTCTGTGGGC; Primers and probe for single-copy reference gene RNase P were from Applied Biosystems, as was TaqMan Universal PCR reagent mix (432418). Reactions were done in quadruplicate with genomic DNA at 2.5 ng, primers at 900 μM/L, and probes at 250 μM/L under standard thermocycling conditions (2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C). Data were normalized to RNase P and then to the calibrator sample (normal stomach genomic DNA, BioChain Institute D1234248).

Results

FGFR2 is overexpressed and activated in FGFR2-amplified cell lines. We assembled a panel of 10 gastric cancer cell lines and investigated FGFR2 expression and activation in relationship to FGFR2 genomic amplification. KatoIII, Snu16, and OCUM-2M have been reported to contain elevated FGFR2 gene copy (12, 31, 32). To confirm this result, we developed a quantitative genomic PCR assay that identified high-level FGFR2 gene copy in KatoIII, OCUM-2M, and Snu16 cells (Fig. 1A). Next, we determined the genomic amplification status of FGFR2 in KatoIII cells by FISH analysis using FGFR2 and chromosome 10 centromeric region specific probes. FISH analysis revealed prominent clustered, large FGFR2 signals consistent with homogeneous staining regions in both metaphase and interphase nuclei of KatoIII cells (Supplementary Fig. S1, arrowheads). This high-level FGFR2 amplification was distinct from the endogenous FGFR2 gene locus, suggesting a targeted amplification of this locus (Supplementary Fig. S1, arrow).
truncated splice variants resulting in the lower molecular weight kinase in these cells relative to Snu16 (32). Consistent with a truncation in the COOH terminus, a FGFR2 COOH-terminal reactive antibody (sc-122) does not detect FGFR2 from KatoIII and OCUM-2M cells, but does detect FGFR2 from Snu16 cells (data not shown). We similarly found very low expression of FGFR2 in the \textit{Met}-amplified cell lines MKN45, GTL-16, SNU5, and HS746T (data not shown).

High basal levels of FGFR2 activation site phosphorylation were found in the cell lines overexpressing FGFR2, although both KatoIII and OCUM-2M contained higher basal activation relative to Snu16 (Fig. 1B). The basal phosphorylation was further stimulated by FGF7 in Snu16 cells, but only marginally in KatoIII and OCUM-2M. Because the Y653/654 phosphospecific FGFR antibody cross-reacts with FGFR1, FGFR3, and FGFR4 (data not shown), we tested the FGFR2 activation status by FGFR2 immunoprecipitation and phosphotyrosine detection with 4G10. Consistent with elevated activation site phosphorylation, Fig. 1B reveals that total phosphotyrosine is highly elevated in the FGFR2-overexpressing cell lines. Similar to the Y653/654 site, total FGFR2 tyrosine phosphorylation is significantly stimulated by FGF7 addition only in the Snu16 cell line. However, the basal phosphorylation of Akt and Erk in Snu16 cells was not stimulated by FGF7 (Fig. 1C), suggesting that basal FGFR2 activation is sufficient to activate the mitogenic signaling pathways in this cell line. As well, the lack of Akt and Erk activation by FGF7 in the nonamplified cell lines is consistent with the undetectable levels of FGFR2 in these lines (Fig. 1B). \textit{Ras} was also found to be activated in \textit{FGFR2}-amplified cells, and again FGF7 did not result in further activation in Snu16 (Fig. 1D). Interestingly, \textit{ras} activation in \textit{FGFR2}-amplified cell lines can be similar to levels seen in the \textit{ras} mutant AGS and Snu1 cells (Fig. 1D and Supplementary Fig. S2). Also of note, sequencing revealed that \textit{ras} isoforms are wild-type in KatoIII, Snu16 (33), and OCUM-2M cells.

Figure 1. FGFR2 amplification leads to FGFR2 overexpression and activation in Snu16, KatoIII, and Ocum2M cells and results in activated downstream signaling. \textbf{A}, FGFR2 is highly amplified in Snu16, KatoIII, and Ocum2M cell lines. Quantitative PCR was done on genomic DNA from the indicated cell lines as described in Materials and Methods. RNase P was used as an internal reference and signals were standardized to normal stomach DNA. \textbf{B}, cells (5 \times 10^5) in six-well plates were untreated (--) or treated (+) with FGF7 (100 ng/mL, 5 min) and 60-\textmu g protein lysate (prepared according to Materials and Methods) was subjected to SDS-PAGE and Western blotting with antibodies recognizing FGFR2, FGFR2 activation loop phosphorylation Y653/654, and GAPDH. For phospho-tyrosine detection, lysates were first immunoprecipitated with MAB6841 as described in Materials and Methods, separated by SDS-PAGE, and Western blotted for phosphotyrosine with the 4G10 antibody. \textbf{C} and \textbf{D}, downstream signaling is activated in KatoIII, Snu16, and Ocum2M cells. \textbf{C}, lysates used in \textbf{B} were analyzed by Western blotting for the indicated phosphorylated or total proteins. \textbf{D}, Ras activation was done on separate samples as described in Materials and Methods. Representative of three experiments. (OCUM-2M was abbreviated to Ocum2M in the figures for space considerations.)
FGFR2 kinase activity is required for proliferation of FGFR2-amplified cell lines. Although FGFR2 is highly activated, it remained to be determined if FGFR2 kinase activity is required for gastric cancer cell growth. A Merck multikinase inhibitor compound in clinical development inhibited multiple serine/threonine and tyrosine kinases (including FGFR2) and inhibited the growth of FGFR2-amplified gastric cancer cell lines (data not shown). However, it was not clear whether a combined inhibition of multiple kinases was required for growth inhibition or whether specific FGFR2 inhibition alone was sufficient. Therefore, we used the FGFR-selective inhibitor PD173074 for further experiments. This compound was found to inhibit FGFR kinases in vitro at an IC_{50} of 3.6 nmol/L for FGFR1, 3.3 nmol/L for FGFR2, and 5.3 nmol/L for FGFR3 (data not shown). To confirm selectivity for FGFR, we found that this compound significantly inhibited only FGFR1, FGFR2, and FGFR3 (but not FGFR4) of 224 kinases tested (Supplementary Fig. S3). We note that inhibition of the CAMKII isoform was not linear.

Having established PD173074 as a potent and selective inhibitor of FGFR activity in vitro, we found that this compound also potently inhibited FGFR2 phosphorylation in cells (Fig. 2A), with IC_{50} values from 7 to 13 nmol/L (Fig. 2B). PD173074 inhibition of FGFR2 phosphorylation was correlated with a strikingly potent and selective growth inhibition in the FGFR2-amplified cell lines (Fig. 2B). The average IC_{50} for growth inhibition of three FGFR2-amplified lines was 18 nmol/L, whereas the average for 10 nonamplified lines was 4,200 nmol/L, a difference of x200-fold in potency. Importantly, the similar IC_{50} for biochemical inhibition of FGFR2 phosphorylation and growth inhibition strongly implicates FGFR2 phosphorylation as the activity required for cell growth. These results reveal that the overexpressed and highly activated FGFR2 kinase is the key driver of proliferation in FGFR2-amplified cell lines.

We were interested to determine if the FGFR2-amplified cell lines might be nonspecifically sensitive to a variety of receptor tyrosine kinase or chemotherapeutic inhibitors. However, gefitinib and erlotinib were not potent growth inhibitors of FGFR2-amplified cell lines, with IC_{50} values ranging from 6,000 to 8,900 nmol/L in KatoIII cells (Supplementary Fig. S4), and combination treatment of PD173074 and gefitinib did not improve the potency of either compound treatment alone (data not shown). Gefitinib function was confirmed by inhibition of the NUGC4 cell line at an IC_{50} of 125 nmol/L (data not shown), similar to previously reported inhibition (36). Regarding chemotherapeutic compounds, paclitaxel and 5-fluorouracil had relatively similar potency against FGFR2-amplified cells in comparison with non-FGFR2-amplified cells (Supplementary Fig. S5).

FGFR2 shRNA inhibits cell growth. Although PD173074 is highly selective for FGFR1, FGFR2, and FGFR3, we additionally treated cells with shRNA directed to FGFR2 to confirm its role in cell growth. We infected FGFR2-amplified cell lines with FGFR2 shRNA using lentiviral vectors and achieved efficient FGFR2 knockdown in OCUM-2M and KatoIII cells with shRNAs F2 and F3, but not with F1 and F4 (Fig. 3A). By contrast, we were unable to decrease FGFR2 protein in Snu16 cells. Using lentivirally expressed green fluorescent protein, we found that Snu16 cells were not efficiently infected even at much higher multiplicity of infection.
In OCUM-2M and KatoIII cells, FGFR2 knockdown by F2 and F3 was accompanied by growth inhibition, whereas the F1 and F4 shRNAs and a luciferase shRNA were not growth inhibitory (Fig. 3B). The non–FGFR2-amplified cell line AGS was not growth inhibited with F1, F2, F3, F4, and luciferase shRNAs. To address the poor infectivity of Snu16 cells, we selected for stable expression of FGFR2 shRNA with blasticidin. Stable lines could be established with all FGFR2 shRNA constructs, but Western blotting revealed no decrease in FGFR2 protein (data not shown), and thus shRNA treatment was uninformative for FGFR2 function in Snu16 cells. However, KatoIII and OCUM-2M show growth inhibition with F2 and F3 shRNAs, confirming the inhibition seen with PD173074.

**FGFR2-amplified cell lines undergo growth arrest or apoptosis after inhibition of FGFR2.** To understand the mechanisms of growth inhibition in PD173074-treated cells, we analyzed the cell cycle distribution in KatoIII, OCUM-2M, and Snu16 by flow cytometry. Cells were treated with 100 nmol/L PD173074, and the cell cycle distribution was determined by flow cytometry. Western blotting was performed to detect cleaved PARP, a marker of apoptosis.

**Figure 3.** FGFR2 shRNA decreases FGFR2 protein levels and inhibits growth of KatoIII and OCUM-2M cells. FGFR2 protein down-regulation by shRNA inhibits growth. A, cells were treated with four shRNAs targeting FGFR2 (F1–F4) or luciferase (L), or were untreated (0), as described in Materials and Methods. Western blotting for FGFR2 was done with MAB6841. B, cell growth after treatment with shRNA. Vialight reagent was used to measure relative cell growth. Columns, average percent inhibition relative to untreated cells from three independent experiments; bars, SD.

**Figure 4.** PD173074 results in a loss of S phase in KatoIII and apoptosis in OCUM-2M and Snu16 cells. A, cell cycle profile of drug-treated cells. KatoIII, Snu16, and OCUM-2M cells (1 × 10^6) treated with 100 nmol/L PD173074 or DMSO at the indicated time points were processed for propidium iodide staining and fluorescence-activated cell sorting analysis as described in Materials and Methods. Tabular representation of cell cycle profiles as determined by ModFit software. B, duplicate cells treated as in A were isolated at the indicated time points and lysed for Western blotting analysis with cleaved PARP. β-Actin was used as a loading control (bottom). The position of full-length and cleaved PARP is indicated. Representative of two independent experiments.
PD173074, and cell cycle distribution was analyzed at 24, 48, and 72 hours after drug addition. In all cell lines, 24 hours of drug treatment resulted in a strong (3- to 4-fold) decrease in S phase (Fig. 4A). In addition, both OCUM-2M and Snu16 revealed a 4-fold increase in the sub-G1 cell population. This sub-G1 fraction, which is suggestive of apoptosis, increased further by 72 hours to include >50% of the cell population (Fig. 4A). By contrast, even at the 72-hour time point, KatoIII cells had only a minor sub-G1 population. Visual inspection of Snu16 and OCUM-2M cells after 72 hours of drug treatment revealed a profound loss of cell integrity in OCUM-2M and Snu16 cells, whereas KatoIII cells remained intact (Supplementary Fig. S6 and data not shown). Treatment of OCUM-2M with shRNAs F2 and F3 also resulted in a similar loss of cell integrity (data not shown).

As a further test for apoptosis, we probed for cleaved PARP, a marker of caspase activation associated with apoptosis (Fig. 4B). Prominent induction of cleaved PARP could be seen as early as 24 hours after addition of PD173074 in both Snu16 and OCUM-2M cells. By contrast, KatoIII cells showed no induction of cleaved PARP, consistent with flow cytometry results. Thus, FGFR2 inhibition can result in dramatically different growth arrest phenotypes.

The growth suppression resulting from FGFR2 inhibition was likely due to loss of multiple signaling pathways after inhibitor treatment. To test this, we analyzed Erk and Akt phosphorylation as well as activation of Ras after treatment of cell lines with PD173074 (Supplementary Fig. S7). Erk and Akt phosphorylation and Ras activation were blocked in KatoIII, OCUM-2M, and Snu16 cells at increasing drug concentrations after 2 hours of treatment. Akt has been reported to become reactivated after EGFR inhibitor treatment of Her2-amplified cells at later time points (37). However, Akt phosphorylation remained inhibited in KatoIII cells at 48 and 72 hours of treatment with 40 and 100 nmol/L of PD173074 (data not shown). Whereas Ras activity remained elevated in KatoIII cells after 2 hours of drug treatment (Supplementary Fig. S7), at 6 and 24 hours, loss of Ras activation was similar to that seen in Snu16 and OCUM-2M cells (data not shown).

FGFR2 activates EGFR family members in FGFR2-amplified cell lines. Met amplification in lung and gastric cancer cell lines results in activation of EGFR family (29, 30). A receptor tyrosine kinase array revealed that FGFR2-amplified lines also contain activated EGFR family members (Supplementary Fig. S8). We immunoprecipitated EGFR and ErbB3 from our cell line panel.
and probed for phosphotyrosine (Fig. 5A), confirming activated EGFR and Erbb3 in FGFR2-amplified cell lines as well as in Her2-amplified (N87) or gefitinib-sensitive (NUGC4) cell lines. Interestingly, EGFR and Erbb3 tyrosine phosphorylation was elevated similarly in FGFR2-amplified cells and the Her2-amplified cell line N87. Using EGFR phosphospecific antibodies, we found that the Y845 activation site is constitutively phosphorylated, whereas the Y1173 docking site remains ligand inducible (data not shown). As well, Erbb3 Y1289 is phosphorylated in KatoIII and OCUM-2M, but not in Snu16 (data not shown). We tested Her2 activation and found that only KatoIII contained significant phosphotyrosine in Her2, whereas phosphotyrosine was not recovered from Erbb4 in any cell line (Fig. 5B and data not shown). A striking observation was that phosphorylation of Erbb3 and EGFR in Snu16 cells was stimulated by FGF7. Because FGF7 is a dedicated ligand for FGFR2 (19), this result suggested that FGF7 indirectly activated Erbb3 and EGFR via activation of FGFR2.

If EGFR family members are components of amplified FGFR2 signaling, loss of FGFR2 would result in loss of EGFR family phosphorylation. This was confirmed as PD173074 treatment inhibited EGFR, Erbb3, and Her2 phosphorylation at concentrations similar to those required to inhibit FGFR2 phosphorylation (Fig. 5B and C). shRNA inhibition of FGFR2 also resulted in loss of Tyr1289 phosphorylation in Erbb3 (Supplementary Fig. S10). We conclude that EGFR family members are downstream targets of the amplified and highly activated FGFR2 kinase.

Erbb3 is required for proliferation of FGFR2-amplified cell lines. We treated cells with gefitinib to test EGFR function in FGFR2-amplified cell lines. Surprisingly, 10 μmol/L gefitinib did not inhibit basal EGFR phosphorylation in these three cell lines (Fig. 5D). Erlotinib at 10 μmol/L also failed to block EGFR phosphorylation (data not shown). Gefitinib activity was confirmed by its ability to block the dramatic EGF-mediated stimulation of EGFR in Snu16 cells (Fig. 5D, compare lanes EGF and I/EGF). As discussed above, gefitinib and erlotinib are poor inhibitors of KatoIII, OCUM-2M, and Snu16 proliferation, which is consistent with the observed lack of EGFR phosphorylation inhibition. Although EGFR was highly activated by EGF addition to Snu16 cells, both OCUM-2M and KatoIII had only a minor increase in EGFR phosphorylation, suggesting that the basal EGFR phosphorylation in KatoIII and OCUM-2M represents a near-maximal receptor activation.

Because gefitinib was unable to block EGFR phosphorylation, we tested a functional role for EGFR pathway activation in KatoIII, OCUM-2M, and NUGC4 cells using shRNA to Erbb3, a required partner for EGFR family signaling. Each of three shRNAs directed to Erbb3 resulted in efficient knockdown of Erbb3 protein (Fig. 6A). The FGFR2 phosphorylation was reduced after Erbb3 knockdown, and this reduction was strongest with shRNA1 (Fig. 6A), confirming activated FGFR2 phosphorylation was reduced after Erbb3 knockdown. Because Erbb3 shRNA did not induce cell death in OCUM-2M cells, as was observed with PD173074 treatment or FGFR2 knockdown (Fig. 6B, and data not shown), it is possible that signal transduction through activated Erk or FGFR2 accounts for survival in Erbb3-deficient OCUM-2M cells. In contrast to OCUM-2M, in KatoIII cells FGFR2 phosphorylation was reduced after Erbb3 knockdown, and this reduction was strongest with shRNA1 (Fig. 6A). As well, Erk phosphorylation was consistently inhibited with shRNA1. The FGFR2 phosphorylation that remains after shRNA2 and shRNA3 treatment may be sufficient to maintain Erk phosphorylation. The increased potency

![Figure 6. Erbb3 shRNA decreases Erbb3 protein levels and inhibits proliferation. A, the indicated cell lines were treated with three separate shRNAs directed to Erbb3 (1, 2, and 3), a scrambled shRNA sequence (S), or vector (V), or left untreated (O), and were subjected to Western blotting 24 h after virus removal and probed for Erbb3, Y653/654 FGFR2, total FGFR2, Ser473 Akt (pAkt), Akt Thr202, Tyr204 pErk, and Erk, with GAPDH as a loading control. Representative of two experiments; B, cells in 96-well plates were treated with the indicated shRNAs and allowed to grow for 6 d. Medium was replaced on day 3. Cell growth was quantitated from duplicate wells in three independent experiments and, using Vialight reagent and untreated control values, were normalized to 100% growth. F2 indicates that FGFR2 shRNA was included as a control for FGFR2 inhibition. Columns, average growth (percentage of untreated cell growth) from three experiments; bars, SD.](cancerres.aacrjournals.org)
of shRNA1 to inhibit Erk and FGFR2 phosphorylation is currently not explained. As well, the molecular mechanism that results in reduction of FGFR2 phosphorylation in KatoIII cells relative OCUM-2M cells remains to be identified. However, we conclude that Erbβ3 is a functional component of FGFR2-amplified cell growth.

Discussion

A key finding of this work is that FGFR2-amplified gastric cancer cell lines are dependent on the overexpressed and activated FGFR2 kinase for growth. Interestingly, in Snu16 and OCUM-2M cells, loss of FGFR2 resulted in apoptosis, whereas in KatoIII cells the loss of FGFR2 signaling resulted in growth arrest without apoptosis. Withdrawal of 100 nmol/L PD173074 from KatoIII cells after 7 days of treatment resulted in rapid outgrowth of cells (data not shown), confirming that compound-treated KatoIII cells are viable and capable of cell cycle reentry. In each of the FGFR2-amplified cell lines, there was a loss of Erk, Akt, and ras activation after FGFR2 inhibition. Recent work suggested that Akt may become reactivated at later time points following receptor tyrosine kinase inhibitor treatment, possibly contributing to drug resistance (37). However, Akt remained inhibited in KatoIII cells after 48 and 72 hours of PD173074 treatment, and thus the survival pathways that are active in KatoIII cells after FGFR2 inhibition remain to be identified.

A striking finding is that FGFR2-amplified cell lines contain elevated EGFR and Erbβ3 phosphorylation (and in KatoIII cells, Erbβ2 phosphorylation) that is dependent on FGFR2 kinase activity. Cross talk between FGFR2 and EGFR family kinases is also supported from our finding that the FGFR2-specific ligand FGF7 stimulated EGFR and Erbβ3 phosphorylation in Snu16 cells. Importantly, EGF-stimulated phosphorylation of EGFR in Snu16 cells is not inhibited by PD173074 (Supplementary Fig. S9), and thus only the elevated basal phosphorylation in EGFR family members is FGFR2 dependent. Therefore, the dramatic phenotype associated with FGFR2 inhibition is a result of the combined loss of FGFR2 and EGFR family signaling.

shRNA inhibition of Erbβ3 revealed that EGFR family signaling is required for cell growth in KatoIII and OCUM-2M cells. The FGFR2-EGFR interaction described here parallels recent reports that amplified Met can coactivate EGFR family signaling in lung cancer (29, 30). We find similar EGFR activation in Met-amplified cell lines (data not shown). Together these results reveal that Met- and FGFR2-amplified cell lines activate EGFR family proteins for cell transformation. The functional interaction between FGFR2 and EGFR family kinases could be mediated by a physical interaction, and thus we carried out communoprecipitation experiments in KatoIII, Snu16, and OCUM-2M cells. We used NH2- and COOH-terminal antibodies under varying degrees of stringency, immunoprecipitating with either FGFR2 or Erbβ3 and blotting for the converse protein. However, we were unable to show a specific interaction. Thus, a physical relationship between FGFR2 and Erbβ3 remains to be identified.

The inability of gefitinib or erlotinib to block basal phosphorylation in EGFR is also consistent with a model in which amplified FGFR2 activates EGFR family members. FGFR2 is not inhibited by gefitinib and is therefore able to maintain elevated EGFR phosphorylation in the presence of gefitinib. This result also cautions against the use of phosphorylated EGFR to stratify gastric or lung cancer patients for EGFR small-molecule inhibitor therapy because, in a subset of these patients, EGFR is activated by FGFR2 or Met and will be resistant to EGFR inhibitor therapy. Furthermore, because Met amplification activates an EGFR signaling network that is resistant to gefitinib (29, 30), FGFR2 amplification may be yet another mechanism of clinical gefitinib resistance.

Because Met, FGFR2, and Her2 amplification and ras mutations are exclusive of each other in gastric cancer cell lines, it is likely that each oncogene can independently activate transformed growth. The genetic defect in these cell lines can correlate with the specific inhibitor required to inhibit cancer cell growth. For example, Met-amplified cell lines SNU5, GTL-16, and MKN45 are insensitive to FGFR2 inhibition but are potently inhibited by Met inhibition (data not shown; ref. 38). Conversely, FGFR2-amplified cell lines are insensitive to Met-specific small-molecule inhibitors (data not shown) but are sensitive to FGFR2 inhibition. Finally, the NUG4 (EGFR/Erbβ3 amplified; Fig. 6) and Her2-amplified N87 cell lines (39) are selectively inhibited by EGFR inhibitors or Herceptin, respectively (36, 40). It remains to be tested whether gastric cancers harboring these genetic alterations will be similarly responsive in a clinical setting. If this is the case, these studies along with those of others are beginning to define a rational treatment strategy for the subset of gastric cancers with activated/amplified receptor tyrosine kinases.

Acknowledgments

Received 9/7/2007; revised 1/2/2008; accepted 2/6/2008.

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We thank Bo Pan and Wei Lu for discussions and Nancy Kohl and Victoria Richon for manuscript review; Chris Dinsmore, Jason Katz, Jonwon Lim, James Jewell, Kerrie Burgess, Michelle Machacek, Alon Nothrup, David Guerin, and Jon Young for medicinal chemistry support; and M. Sekiguchi and J.G. Park for isolating the KatoIII and Snu16 cell lines, respectively.

References

Correction: FGFR2 Dependence of Gastric Cancer Cell Lines

In the article on FGFR2 dependence of gastric cancer cell lines in the April 1, 2008 issue of Cancer Research (1), the names of the Erbb3 shRNAs in PLKO-1 from Open Biosystems in the Materials and Methods should have been as follows: 1, TRCN0000040108; 2, TRCN0000040109; 3, TRCN0000040110.

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