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Epidermal Growth Factor Receptor Signaling to the MAPK Pathway Bypasses RAS in Pancreatic Cancer Cells

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Abstract

Objectives—The epidermal growth factor receptor (EGFR/HER1) is overexpressed in human pancreatic cancers. However, anti-EGFR therapy does not exhibit significant therapeutic activity with oncogenic KRAS mutation. We sought to assess the signaling relationship between EGFR and mutant KRAS, which is commonly detected in pancreatic cancer.

Methods—Pancreatic cancer cells harboring mutated KRAS were treated with EGF to assess signaling from EGFR to mitogen activated protein kinase (MAPK) pathway. The role of RAS family of proteins in transducing EGFR signals was assessed using short-interfering RNA. Other components of MAPK and phosphoinositide 3-kinase (PI3K) pathways were examined for their roles in EGFR signaling.

Results—First, EGF signaling in pancreatic cancer cells occurs selectively through HER1. Second, knockdown of all RAS isoforms failed to block EGF-mediated phosphorylation of ERK. Inhibition of RAF was observed to partially abrogate ERK phosphorylation, whereas MEK inhibition resulted in complete attenuation of EGF-mediated ERK phosphorylation. Finally, inhibition of PI3K/AKT and CDC42/PAK pathways did not block EGFR signaling.

Conclusions—Our study results demonstrate that EGFR-mediated signaling in mutant KRAS pancreatic cancer cells does not follow canonical MAPK signaling. Our novel findings suggest the existence of alternate signaling pathways to downstream MAPK in the presence of mutant KRAS.

Keywords

EGFR; KRAS; MAPK; Therapeutic Resistance; Pancreatic Cancer

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INTRODUCTION

Targeted therapies have been increasingly evaluated in patients with metastatic pancreatic cancer. Erlotinib (OSI-774, Tarceva®; OSI Pharmaceuticals), a small molecule epidermal growth factor receptor (EGFR) inhibitor, is indicated in combination therapy with gemcitabine for the management of pancreatic cancer patients with locally advanced, unresectable, or metastatic disease.¹ Because nearly 60% of pancreatic cancers demonstrate EGFR overexpression,² EGFR constitutes a promising therapeutic target. Despite retrospective correlation of EGFR overexpression with poor prognosis, treatment of pancreatic cancer with erlotinib has been shown to provide only marginal survival benefit^{3,4} and must be weighed against its considerable toxicities.⁵ More recently, a multicenter trial evaluated the efficacy of cetuximab, an anti-EGFR monoclonal antibody, in combination with gemcitabine and failed to identify improved outcomes.^{6–8} Overall, the results of EGFR antagonism in patients with metastatic pancreatic cancer have been disappointing despite the success of anti-EGFR drugs in the treatment of other cancers.⁹

Extrapolating data from metastatic colorectal cancer patients treated with cetuximab may provide insight into the limited success of anti-EGFR therapies in pancreatic cancer. It is now established that failure of response to cetuximab is related to the detection of somatic KRAS mutations in colorectal cancers.^{10–13} Compared to 30–40% of colorectal cancer patients with KRAS mutations, approximately 90% of patients with pancreatic cancer harbor KRAS mutations,¹⁴ suggesting a significant proportion of patients with pancreatic cancer may demonstrate resistance to anti-EGFR therapies. Despite the correlation between anti-EGFR resistance and KRAS mutation status, the exact mechanism of resistance is not known. However, it is well-established that mutations in KRAS result in constitutive activity of the proto-oncogene, resulting in unregulated downstream signaling cascades that promote cancer development, proliferation, and metastasis.¹⁵ Recently, it has been described in murine models that EGFR is essential for pancreas tumor development induced by the endogenous KRAS oncogene^{16,17} or slow down its progression¹⁸ suggesting that EGFR activity may be involved in KRAS oncogene activity.^{16,17,19,20}

Since further investigation of the relationship between EGFR and KRAS may improve our understanding of EGFR-mediated resistance mechanisms in patients with pancreatic cancer, we sought to assess the role of KRAS in EGFR signal transduction. We previously performed KRAS knockdown and measured extracellular signal-regulated kinase (ERK) phosphorylation following EGF treatment. Despite effective knockdown of KRAS, ERK phosphorylation persisted with exposure of cells to EGF. As a control, pancreatic cancer cells were also exposed to another activator of ERK, CXCL12, which failed to increase ERK phosphorylation with KRAS knockdown.²¹ Our prior results are noteworthy because they challenge the canonical signaling pathway from receptor tyrosine kinase to KRAS to ERK.

Given that anti-EGFR therapies have limited efficacy in the presence of mutant KRAS and that KRAS mutations are common in pancreatic cancer, we sought to further examine the signaling relationship between EGFR and mutant KRAS. Herein, we investigate EGF signal transduction in the context of the canonical RAS/MAPK pathway (RAS-RAF-MEK-ERK)

and alternate potential crosstalk pathways that may activate mitogenic and/or oncogenic signaling.

MATERIALS AND METHODS

Cell Lines and Reagents

Established human pancreatic cancer cell lines harboring mutant KRAS (AsPC-1, MIAPaCa-2 and PANC-1) were obtained from American Type Culture Collection (Manassas, VA). The cell lines were immediately cryopreserved in liquid nitrogen after they were obtained. PANC-1 cells were grown in Dulbecco's Minimal Essential Medium (DMEM) (Mediatech; Manassas, VA) with 10% fetal bovine serum (FBS) (Omega Scientific; Tarzana, CA) and 1% penicillin/streptomycin (P/S) (Gibco; Grand Island, NY) under standard conditions: at 37 °C with 5% CO₂. MIAPaCa-2 cells were grown in as the same media as PANC-1 cells with an additional 2.5% horse serum (Omega Scientific; Tarzana, CA). AsPC-1 cells were grown in RPMI1640 (Mediatech; Manassas, VA) with 10% FBS, 1% P/S, 1mM sodium pyruvate (Gibco; Grand Island, NY) and 1% non-essential amino acids (Irvine Scientific; Santa Ana, CA). All experiments were conducted under serum-free conditions. The following reagents were utilized in these studies: epidermal growth factor (EGF) (Peprotech; Rocky Hill, NJ), pertussis toxin (PTX) (Calbiochem; San Diego, CA), erlotinib (LC Laboratories; Woburn, MA), lapatinib (GlaxoSmithKline; Philadelphia, PA), trastuzumab (Genentech; San Francisco, CA), sorafenib (Bayer Corporation; West Haven, CT), UO126 (Cell Signaling; Beverly, MA), IPA-3 (Sigma; Saint Louis, MO), ML141 (Sigma; Saint Louis, MO) and LY294004 (Cell Signaling; Beverly, MA).

Short Interfering RNA

RAS expression was inhibited in pancreatic cancer cells by 50 nM short interfering RNA (siRNA) targeting K-, N- and H-RAS (Ambion; Carlsbad, CA). Non-silencing siRNA (Thermo Scientific; Pittsburg, PA) was used as a control at the same concentrations as experimental siRNA. Cells were transfected using Lipofectamine RNAi Max (Invitrogen; Carlsbad, CA) and incubated for 120 hours prior to experimental treatments.

Immunoblotting

Protein was collected using RIPA buffer (Thermo Scientific; Pittsburg, PA) plus protease inhibitors (Thermo Scientific; Pittsburg, PA) and phosphatase inhibitors (Calbiochem; San Diego, CA) according to standard methods. 20 µg of protein was loaded on to 12% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were blocked for 1 hour with 5% non-fat dry milk and probed overnight at 4°C with primary antibodies, except for anti-GAPDH which was incubated for 45 minutes at room temperature. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, BioRad; Hercules, CA) for 1 hour. A chemiluminescent agent (Pierce; Rockford, IL) was added and membranes imaged. Antibodies used were: anti-phospho-ERK, anti-total ERK, and antiphospho-AKT (all three used at 1:1000 dilution and obtained from Cell Signaling); anti-KRAS (1:100 dilution, Genetex; Irvine, CA), anti-NRAS (1:100 dilution, Santa Cruz Biotechnology; Santa Cruz, CA), anti-HRAS (1:100 dilution, Millipore;

Billerica, MA), EGFR (1:1000 dilution, Cell Signaling), HER2 (1:1000 dilution, Cell Signaling), and anti-GAPDH (1:10,000 dilution, Advanced Immunochemical; Long Beach, CA).

RESULTS

Activation of EGFR results in ERK phosphorylation

PANC-1, MIAPaCa-2, and AsPC-1 showed increased ERK and AKT phosphorylation following stimulation with EGF (100 ng/ml). To confirm that pathway activation was a direct result of EGFR activation, pancreatic cancer cells were incubated with erlotinib, an EGFR-specific inhibitor, prior to exposure to EGF. Pretreatment with erlotinib (12 μ M) abrogated EGF-mediated ERK and AKT phosphorylation in all cell lines (Figure 1A). A dual EGFR and HER2 inhibitor, lapatinib (1 μ M), also inhibited expression of phospho-ERK and phospho-AKT (Figure 1B). However, treatment with trastuzumab (1 μ M), a HER2-specific inhibitor, did not affect phosphorylation levels of ERK or AKT. Similar ERK phosphorylation by EGF and inhibition by erlotinib was found in Hs-766T cell line which has w/t KRAS (Supplementary Figure S1). Endogenous expression of EGFR and HER2 in the selected pancreatic cancer cell lines was also verified (Supplementary Figure S2).

ERK phosphorylation is dependent on RAF and MEK

In order to verify EGFR-mediated activation of the RAF/MEK/ERK pathway, pancreatic cancer cells were pretreated with sorafenib (15 μ M), a RAF inhibitor, prior to stimulation with EGF. Partial inhibition of phospho-ERK expression was observed (Figure 2A). The cells were then pretreated with UO126 (10 μ M), a MEK inhibitor. Following addition of EGF, complete inhibition of phospho-ERK expression was observed in the cells treated with UO126 (Figure 2B). Inhibition at RAF and MEK demonstrates that activation of ERK is partially dependent on the MAPK pathway in pancreatic cancer cells. Similar MEK dependence on EGF-mediated ERK phosphorylation was observed in Hs-766T cell line with w/t KRAS (Supplementary Figure S1).

EGF-mediated ERK activation bypasses RAS

The RAS oncogene has a well-established role in cell growth and regulation. The selected pancreatic cancer cell lines express all three known human RAS isoforms (NRAS, HRAS, and KRAS) (Supplementary Figure S2).²² Our previous work has shown that silencing KRAS does not interfere with EGFR-mediated ERK phosphorylation.²¹ Therefore, we next examined EGF-mediated signaling in pancreatic cancer cells when all three RAS isoforms were individually silenced. Despite effective RAS knockdown in all cell lines, ERK phosphorylation was only slightly reduced (Figure 3A), indicating that EGFR-mediated ERK phosphorylation is mostly RAS-independent. To further examine the importance of RAS in the MAPK pathway, expression of all three RAS isoforms together was blocked prior to EGF treatment. Interestingly, the triple RAS knockdown also did not effectively inhibit ERK phosphorylation either (Figure 3B). Our results suggest that EGFR signaling in pancreatic cancer can activate downstream MAPK effectors through a RAS-independent mechanism.

EGF-mediated ERK activation is independent of G_i proteins and PI3K and CDC42/PAK pathways

To further characterize EGFR-mediated signal transduction, we investigated alternative pathways by which ERK may be activated. Reports have suggested the presence of crosstalk between receptor tyrosine kinases (*i.e.*, EGFR) and G-protein coupled receptors (GPCRs).²³ To assess whether G_i proteins mediate EGFR activation of ERK, we treated cells with PTX (800 ng/ml) prior to EGF stimulation. PTX failed to block EGF-mediated ERK phosphorylation (Figure 4A). However, PTX effectively attenuated CXCL12-mediated ERK and AKT phosphorylation, confirming its ability to inhibit G_i proteins (data not shown). Because prior publications have shown that crosstalk can also occur between the phosphoinositide 3-kinase (PI3K) and MAPK pathways,^{24,25} we hypothesized that the PI3K pathway may transduce EGF-mediated signals resulting in ERK phosphorylation. After pretreating cells for 1 hour with the PI3K inhibitor LY294002 (15 μ M), we evaluated changes in ERK phosphorylation. In contrast to RAF and MEK inhibition, PI3K inhibition did not show any inhibitory effect on ERK phosphorylation (Figure 4B). Hs-766T cell line (w/t KRAS) has shown similar independence on PI3K and MEK inhibition (Supplementary Figure S1). p21-activated kinase 1 (PAK1)/CDC42 signaling, another putative crosstalk with the MAPK pathway was also tested. EGF-driven activation of ERK was also not inhibited by the PAK1 inhibitor IPA-3 or the CDC42 inhibitor ML141 (Figure 4C and 4D, respectively). Altogether these results demonstrate that EGF-mediated ERK phosphorylation is independent of G_i proteins and that EGFR mediated crosstalk between the MAPK pathway and the PI3K or PAK1/CDC42 pathways does not occur.

DISCUSSION

In 2007, the National Cancer Institute of Canada Clinical Trials Group published the results of their international phase III trial (NCIC CTG PA.3) evaluating the combination of the anti-EGFR agent erlotinib and gemcitabine in patients with unresectable or metastatic pancreatic cancer.⁷ Although improvements in overall survival and progression-free survival were reported, the clinical importance of adding erlotinib was debatable, resulting in limited impact for patients with metastatic pancreatic cancer. More recently, the results from the Southwest Oncology Group multicenter phase III trial (S0205) failed to demonstrate a survival advantage from the addition of another anti-EGFR agent to gemcitabine in patients with locally advanced, unresectable or metastatic pancreatic cancer.⁸ Together, these two trials suggest that prior strategies to capitalize on EGFR as a therapeutic target have been suboptimal.

In order to further investigate potential bypass mechanisms that may be involved in the failure of anti-EGFR therapies, we examined the HER2 receptor as an alternate pathway to MAPK activation, as well as the role of RAS in EGFR-mediated signaling. Because HER2 is also expressed on pancreatic cancer cells²⁶ and can form heterodimers with EGFR, it was important to ascertain if HER2 has a role in EGF-mediated activation of MAPK. We observed that although EGFR may activate ERK and AKT, selective inhibition of HER2 did not block this effect. Our results are consistent with a report by Larbouret *et al* in 2007,

which reported similar limited efficacy of HER2 inhibition in blocking EGF-mediated activation of ERK and AKT in pancreatic cancer cells.²⁷

Because RAS mutations are among the most commonly detected genetic alterations in human cancers, strategies to target RAS have become the subject of intense investigation. Despite significant advances in the understanding of RAS protein function and its tumorigenic mechanisms, there has not been significant progress in the development of therapeutically effective compounds against RAS.^{28,29} Because RAS proteins are direct molecular switches for EGFR-mediated cellular activities, KRAS may be the key to understanding the failure of EGFR antagonism. In colorectal cancer, large retrospective analyses have revealed that cetuximab is ineffective when tumors bear select KRAS mutations.^{30–32} Over 90% of patients with pancreatic cancer harbor similar somatic mutations in KRAS, which result in constitutively active protein product.³³ Signals from upstream receptor tyrosine kinases are transmitted by KRAS to downstream MAPK pathway effectors. However, gain of function KRAS mutations may activate these MAPK effectors independent of receptor-mediated signaling, which may be the route for resistance to anti-EGFR therapies.

In our current study, we confirmed that RAF and MEK play key roles in EGF-mediated MAPK signal transduction in pancreatic cancer cells. Interestingly, we discovered that ERK activation is independent of RAS signaling. Of the three RAS isoforms, KRAS is more frequently mutated than HRAS or NRAS. Mutations in the 3 genes appear to be largely mutually exclusive, with specific RAS mutations occurring in certain human cancers. For example, KRAS mutations are most often found in pancreatic and colon cancers, while NRAS mutations are common in melanoma and hematopoietic cancers, and HRAS mutations are common in bladder and cervical cancers.³⁴ The reason behind this observed tissue specificity in the RAS mutations is currently unclear. While mutations of the NRAS gene are relatively infrequent,³⁴ activating mutations in this gene may also affect response to anti-EGFR therapies.¹² However, our triple RAS knockout studies did not demonstrate impairment of EGF-mediated ERK phosphorylation when all three isoforms were silenced. This finding suggests that pancreatic cancer cells may have an alternative RAS-independent pathway capable of activating the MAPK pathway in response to EGFR stimulation.

In a previous study, we discovered crosstalk between the MAPK and PI3K pathways. Inhibition of PI3K prior to treatment with CXCL12, a chemokine known to activate both pathways, led to a more robust increase in ERK phosphorylation than seen with CXCL12 stimulation alone.²⁴ Here we find that although we do see some influence of PI3K on basal ERK phosphorylation, the inhibition of PI3K did not meaningfully influence EGF-mediated ERK phosphorylation. Additional crosstalk has been reported between GPCRs and EGFR in some cancers.^{23,35} To rule out this condition, we blocked GPCR signaling using PTX and found no inhibition of EGF-mediated ERK or AKT phosphorylation. The CDC42/PAK pathway was another potential crosstalk mechanism to alternatively regulate the MAPK pathway and contribute to cancer growth.^{36–38} However, inhibition of CDC42 and PAK did not result in suppression of ERK phosphorylation.

Studies have suggested that gain-of-function KRAS mutations confer resistance to EGFR inhibitors because constitutively active KRAS is able to overcome EGFR antagonism, resulting in downstream ERK activation independent of upstream extracellular signals.³⁹ Others have attributed lack of response in KRAS mutant colorectal cancer to the paradigm that EGFR-mediated signaling is regulated by KRAS through a single convergent pathway; thus, inhibition of KRAS rather than EGFR is necessary for effective therapy.⁴⁰ However, attempts to develop therapeutic approaches directly targeting KRAS have been unsuccessful. Our discovery that EGFR may have a secondary signaling pathway independent of KRAS highlights the importance of subsequent studies to further characterize this potential alternate signaling pathway and its key molecules (Figure 5). In summary, we demonstrate that EGFR-mediated signaling in KRAS mutant pancreatic cancer cells phosphorylate ERK in a RAS-independent fashion, bypassing G proteins and RAS signaling. Our novel findings suggest that antagonism of KRAS alone is likely to be insufficient to inhibit EGFR-mediated growth signals. Given that potential escape pathways may exist, strategies targeting only EGFR or only KRAS in patients with mutant KRAS are likely to be ineffective. Further elucidation of alternative MAPK-activating pathways may result in the identification of additional molecular targets to serve as novel therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. EGF-induced ERK phosphorylation is abrogated by EGFR inhibition, but not by HER2 inhibition

PANC-1, MIAPaCa-2 and AsPC-1 cells were pretreated with (A) erlotinib (12 μ M), and (B) lapatinib (1 μ M) and trastuzumab (1 μ M) for 4 hours followed by 5 minute incubation with EGF (100 ng/ml). Protein was collected for western blotting for phosphorylated ERK. GAPDH was used as loading control.

Figure 2. EGF-mediated ERK phosphorylation is blocked by specific inhibitors of the MAP kinase pathway

PANC-1, MIAPaCa-2 and AsPC-1 cells were pretreated with (A) sorafenib (15 μM) and (B) UO126 (10 μM) for 1 hour followed by 5 minute incubation with EGF (100 ng/ml). Protein was collected for western blotting for phosphorylated ERK. Total ERK used as loading control.

Figure 3. EGF-mediated ERK phosphorylation is independent of K-, H- and NRAS
PANC-1, MICPaCa-2 and AsPC-1 cells were transfected with K-, H- and N-RAS siRNA (50 nM) separately (A), and together (B) for 120 hours prior to treatment with EGF (100 ng/ml) for 5 minutes. Protein was collected for western blot. Membranes were probed for H-, K-, N-RAS and phospho-ERK.

Figure 4. EGF-mediated ERK phosphorylation is independent of G_i-proteins, PI3 kinase pathway and CDC42/PAK pathway

PANC-1, MIAPaCa-2 and AsPC-1 cells were pretreated with (A) PTX (800 ng/ml, Left), (B) Ly294002 (15 μM, Right), (C) IPA-3 (15 μM) for 1 hour, or (D) ML141 (10 μM) for 24 hours followed by 5 minute incubation with EGF (100 ng/ml). 18 Protein was collected for western blotting for phosphorylated ERK. Total ERK used as loading control.

Figure 5. EGFR signaling in pancreatic cancer

PI3K and CDC42 pathways are not involved in ERK phosphorylation. Alternate pathways that bypass oncogenic RAS in the activation of EGFR-mediated MAPK pathway may be present in pancreatic cancer cells.