

Activated Epidermal Growth Factor Receptor as a Novel Target in Pancreatic Cancer Therapy

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Pancreatic cancer is one of the most fatal among all solid malignancies. Targeted therapeutic approaches have the potential to transform cancer therapy as exemplified by the success of several tyrosine kinase inhibitors. Prompted by this, comprehensive profiling of tyrosine kinases and their substrates was carried out using a panel of low passage pancreatic cancer cell lines. One of the pancreatic cancer cell lines, P196, which showed dramatic upregulation of tyrosine kinase activity as compared to non-neoplastic cells, was systematically studied using a quantitative proteomic approach called stable isotope labeling with amino acids in cell culture (SILAC). A careful analysis of activated tyrosine kinase pathways revealed aberrant activation of epidermal growth factor receptor pathway in this cell line. Mouse xenograft based studies using EGFR inhibitor erlotinib confirmed EGFR pathway to be responsible for proliferation in these tumors. By a systematic study across low passage pancreatic cancer cell lines and mice carrying pancreatic cancer xenografts, we have demonstrated activated epidermal growth factor receptor as an attractive candidate for targeted therapy in a subset of pancreatic cancers. Further, we propose immunohistochemical labeling of activated EGFR ($p\text{EGFR}^{1068}$) as an efficient screening tool to select patients who are more likely to respond to EGFR inhibitors.

Keywords: Pancreatic cancer • Epidermal growth factor receptor • Targeted therapy • Biomarker • Erlotinib • Tyrosine kinase pathways • Proteomics • SILAC • Mouse xenografts

Introduction

Pancreatic cancer is one of the most devastating and rapidly fatal of all solid malignancies.^{1,2} The major reasons for such high mortality rates are the late presentation of the disease and lack of effective therapies. The mainstay of chemotherapy for

advanced pancreatic cancer is gemcitabine.³ Therapies targeting signaling pathways that drive cancer cell proliferation are a promising strategy which is now being used to treat several cancers. The efficacy and advantage of targeted therapy against aberrantly activated tyrosine kinases was realized by the clinical success of a small molecule inhibitor, imatinib, in chronic myelogenous leukemia (CML).⁴ The target of imatinib is the constitutively active fusion protein kinase, BCR-Abl. Monoclonal antibodies and small molecules targeting tyrosine kinases to treat solid malignancies are also being tried. Examples of monoclonal antibodies include trastuzumab targeting ERBB2 in breast cancers⁵ and bevacizumab targeting vascular endothelial growth factor in metastatic renal cancers.⁶ The remarkable success of targeted therapy in solid malignancies was witnessed by the introduction of gefitinib and erlotinib, small molecule inhibitors targeting epidermal growth factor receptor (EGFR) in nonsmall cell lung carcinomas.^{7–9} The response of these tumors to EGFR inhibitors is attributed to the presence of specific mutations in the kinase domain.^{8,10}

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To identify aberrantly activated tyrosine kinase signaling molecules in the context of pancreatic cancers, we have carried out a systematic study using a panel of low passage pancreatic cancer cell lines. A careful quantitative proteomic analysis using stable isotope labeling with amino acids in cell culture (SILAC) revealed aberrant activation of epidermal growth factor receptor (EGFR) and many of its pathway substrates in a subset of pancreatic cancers. By carrying out mouse xenograft studies using EGFR inhibitor erlotinib, we demonstrate targeted therapeutic strategy as an attractive option in a subset of pancreatic cancers. Further, we establish activated EGFR as a biomarker to select for patients who are more likely to respond to EGFR inhibitors.

Materials and Methods

Cell Culture. Immortalized non-neoplastic HPDE cells were grown in defined keratinocyte-serum free medium with supplements (Invitrogen) and antibiotics. HPNE cells were grown in media preparation containing 1 vol of M3 base (Incell corp.) and 3 vol of low glucose DMEM (Invitrogen), 5% FBS, 10 ng/mL EGF and antibiotics. All low passage pancreatic cancer cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% Fetal bovine serum and antibiotics (Invitrogen) except for P196, which was grown in RPMI supplemented with 10% FBS and antibiotics (Invitrogen). All the cells were serum-starved for 16 h before lysing in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM sodium orthovanadate in the presence of protease inhibitors).

Phosphotyrosine Profiling and Antibodies. Anti-phosphotyrosine profiling of all the cell lines was carried out by immunoprecipitating tyrosine phosphoproteins from all the cell lysates followed by Western blotting. Immunoprecipitation was carried out overnight at 4 °C and tyrosine phosphoproteins were eluted using 100 mM phenyl phosphate in PBS. The eluted proteins were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and probed using HRP-conjugated anti-phosphotyrosine antibody (4G10). Agarose-conjugated and HRP-conjugated anti-phosphotyrosine antibodies (4G10) and cortactin antibody were purchased from Upstate Biotechnologies, anti-pEGFR¹⁰⁶⁸ from Cell Signaling Technologies, anti-EPS8 from Becton Dickinson and delta catenin antibody was from Santa Cruz.

Stable Isotope Labeling. Two independent quantitative proteomic experiments were carried out comparing HPDE/P196 and HPDE/P184. Approximately 10⁸ non-neoplastic HPDE cells and an equal number of P196 or P184 cells were used per experiment. HPDE cells were grown in defined keratinocyte-serum free medium with supplements (Invitrogen) and antibiotics; P196 and P184 were grown in custom RPMI and DMEM, respectively, supplemented with [¹³C₆]arginine (heavy arginine) and [¹³C₆]lysine (heavy lysine) that were purchased from Cambridge Isotopes, Inc. (Andover, MA). The cells were serum-starved for 16 h before lysing them with modified RIPA buffer. Following anti-phosphotyrosine immunoprecipitation, the proteins were eluted three times using 100 mM phenylphosphate. The eluate was dialyzed twice against 4 L of

distilled water at room temperature and overnight at 4 °C. After dialysis, the eluate was concentrated and resolved on SDS-PAGE.

Liquid Chromatography and Tandem Mass Spectrometry. Gel bands were excised and digested with trypsin and the extracted peptides were subjected to LC-MS/MS analysis as follows: the samples were injected onto a specially prepared trap column (i.d. 75 μm, length 5 cm) packed with C₁₈ particles (ODS-A YMC), using an autosampler (1100-microwell plate autosampler, Agilent Technologies, Palo Alto, CA). Peptides were eluted from the trap onto an analytical C₁₈ column (i.d. 75 μm, length 10 cm, Vydac, MS218) using an increasing acetonitrile gradient. The solvents were delivered by a nanoflow pump (Agilent Technologies) connected to an electrospray ion source and a Q-STAR Pulsar mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The acquired data was processed and searched using MASCOT (Matrix Science, Boston, MA) against the RefSeq database (release 26) downloaded from National Center for Biotechnology Information. The relative abundance of proteins was quantified by using open source software MSQUANT (<http://msquant.sourceforge.net/>).

Tissue Sections and Immunohistochemical Labeling. Tissue microarrays of pancreatic cancer xenografts were constructed at Johns Hopkins University tissue microarray core. Immunohistochemical labeling of phospho-EGFR¹⁰⁶⁸ was performed using antibody dilution of 1:50. Tissue microarrays were scored by a gastrointestinal pathologist (A.M.) with extensive familiarity in pancreatic cancer morphology and immunohistochemistry.

Xenograft Generation in Mice and Erlotinib Treatment. Under anesthesia with isofluorane, ~5 million cells were implanted into each side of the lower back of 5 to 6 week old female athymic mice (nu/nu) purchased from Harlan (Harlan Laboratories, Washington, DC). Once the tumors reached a size of ~200 mm³, the mice were treated with erlotinib at a dosage of 50 mg/(kg/d) i.p. Five mice were used in each group per condition and tumor size was evaluated three times a week by caliper measurements using the formula: tumor volume = [length × width²]/2. Relative tumor growth inhibition (TGI) was calculated by relative tumor growth of treated mice divided by relative tumor growth of control mice (T/C). Erlotinib treatment was stopped on 28th day and the tumors were harvested and stored at -80 °C for other assays. The animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care and the research protocol was approved by the Johns Hopkins University Animal Use and Care Committee.

Results

Identification of Pancreatic Cancers That Show Activated Tyrosine Kinase Pathways. To carry out an unbiased study to identify tyrosine kinase pathways that are activated in pancreatic cancers, we compared the phosphotyrosine profiles of 10 low-passage human pancreatic cancer cell lines with 2 pancreatic cell lines derived from non-neoplastic ductal cells, HPDE¹¹ and HPNE.¹² All low-passage cell lines were derived and maintained at Johns Hopkins Medical Institutions. All cell lines exhibited distinct tyrosine phosphorylation profiles including one cell line (P196), which showed a dramatic upregulation of tyrosine kinase signaling (Figure 1). To systematically analyze the tyrosine kinase signaling molecules and the corresponding pathways that are activated in P196, we used a quantitative proteomic strategy called "stable isotope labeling

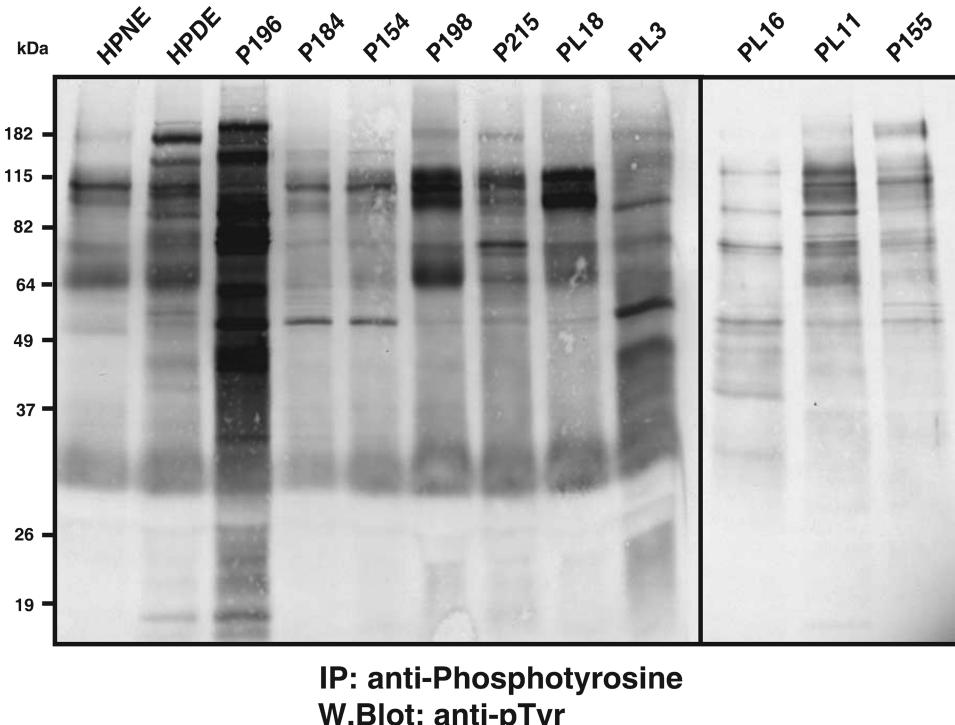


Figure 1. Anti-phosphotyrosine profiling of low-passage pancreatic cancer cell lines. Anti-phosphotyrosine profiling was carried out by immunoprecipitating and Western blotting using a phosphotyrosine specific antibody. Phosphotyrosine profile of 2 non-neoplastic pancreatic ductal cell lines and 10 low-passage pancreatic cancer cell lines is shown. Molecular weight markers are shown on the left.

with amino acids in cell culture" (SILAC).¹³ SILAC is a simple *in vivo* labeling strategy in which heavy amino acids are metabolically incorporated into proteins, allowing the relative abundance of proteins to be quantified by mass spectrometry. We compared the P196 pancreatic cancer cell line, which showed significantly higher phosphotyrosine content signifying activation of tyrosine kinase signaling pathways, with the non-neoplastic ductal cell-derived cell line, HPDE, using SILAC. HPDE was chosen as control because of concerns regarding the ductal epithelial origin of HPNE cells.^{12,14}

Briefly, HPDE was grown in a medium containing naturally abundant isotopic forms of amino acids with ¹²C and P196 was grown in a medium containing heavy ¹³C-labeled arginine and lysine. Cell lysates from HPDE and P196 were then mixed and tyrosine phosphoproteins were immunoprecipitated using phosphotyrosine specific antibodies. The phosphoproteins were eluted and resolved by SDS-PAGE and the resulting protein bands were digested using trypsin and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The labeling of proteins from one cell population using heavier amino acids allowed us to specifically identify those proteins that are highly tyrosine phosphorylated in pancreatic cancer-derived cells relative to non-neoplastic pancreatic duct epithelial cells.

Activation of Epidermal Growth Factor Receptor (EGFR) and Its Pathway Substrates in P196. The unbiased proteomic approach identified hyperphosphorylation of several tyrosine kinases and their substrates in P196 as compared to the non-neoplastic pancreatic duct epithelial cells (HPDE) (Supplementary Tables 1 and 2 in Supporting Information). A careful analysis based on the data from NetPath (<http://www.netpath.org/>), a curated resource of signal transduction pathways revealed hyperphosphorylation of EGF receptor along

with several of its downstream signaling intermediates in P196. Figure 2 shows the intensity ratios of EGFR, epidermal growth factor receptor substrate 8 (EPS8) and cortactin that were increased in P196 cells as compared to the HPDE cells. Activation of a subset of these EGFR pathway substrates (EGFR, EPS8, cortactin and delta catenin 1) identified in the proteomic screen was further validated by immunoblotting (Figure 3a). This led us to hypothesize EGF receptor signaling as a potential pathway driving cancer cell proliferation in P196. We did not see this activation of the EGFR pathway in another pancreatic cancer cell line, P184, using the same SILAC method (Supplementary Tables 3 and 4 in Supporting Information). Phosphorylation of EGFR itself was downregulated in P184 as compared to HPDE. Fold increase in the phosphorylation of some of the well-described EGFR pathway substrates identified in HPDE/P196 screen are shown in Table 1. Figure 3b shows a snapshot of EGFR pathway where some of the proteins identified as hyperphosphorylated in P196 are highlighted in red along with the corresponding fold increase in phosphorylation.

Epidermal Growth Factor Receptor as a Potential Target for Treating Pancreatic Cancers. To test whether EGFR signaling was indeed driving proliferation in P196, we turned to an *in vivo* pancreatic cancer model utilizing mouse xenografts. P196 and P184 cells were implanted into athymic nude mice. In this experiment, we also evaluated two other primary pancreatic cancer xenografts, P410 and P420, that were xenografted directly from surgically resected specimens. P410 was positive for activated EGFR (pEGFR¹⁰⁶⁸), while P420 was negative as assessed by immunoblotting (data not shown). Groups of mice harboring these pancreatic cancers were treated with erlotinib. Tumor size was evaluated 3 times a week by caliper measurements and average tumor volumes were recorded (Supplementary Table 5 in Supporting Information).

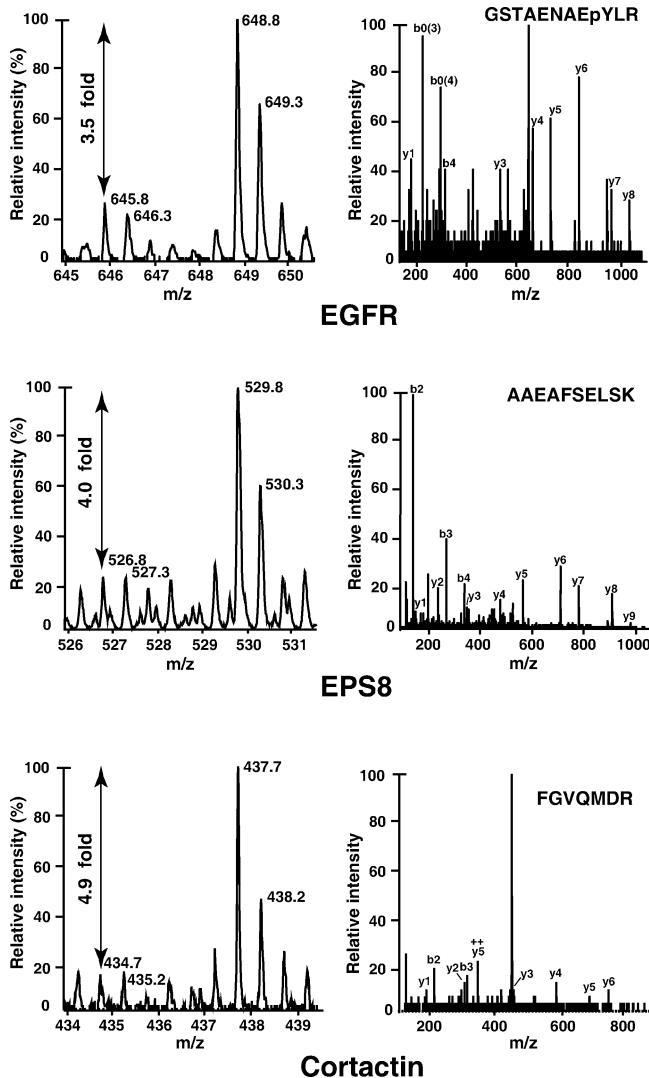


Figure 2. Activation of EGF receptor pathway substrates detected by quantitative mass spectrometry. MS spectra of one representative peptide each from EGFR, EPS8 and cortactin are shown on the left. The fold change is determined by the difference in the height of the peaks representing relative intensities. The panels on the right show the corresponding MS/MS spectra along with the peptide sequence.

Consistent with their having activated EGFR, P196 and P410 showed a dramatic response to erlotinib as evidenced by near complete reduction of tumors. However, pancreatic cancer xenografts P184 and P420 did not respond to erlotinib treatment consistent with their lack of EGFR activation (Figure 4a). Sequencing EGFR in these tumors did not show any known activating mutations in the kinase domain (data not shown). This is also reflected in a recent study which sequenced EGFR in 31 pancreatic cancer clinical specimen and 9 pancreatic cancer cell lines and found no activating mutations.¹⁵ This scenario is somewhat analogous to what has been observed in non-small cell lung carcinoma therapy where a subset of patients respond to EGFR inhibitor therapy although no mutations can be detected.¹⁰ In such cases, the reason for activation of the receptor is unknown but could occur because of aberrant activation of a phosphatase that negatively regulates EGFR, upregulation of EGFR ligands or modulation of hitherto unknown negative regulators of proximal EGF receptor signaling. Regardless of the mechanism of activation, activated EGF

receptor (phosphorylated EGFR) could be a molecular determinant of sensitivity to erlotinib. However, because tandem mass spectrometry is not feasible in the clinical setting, we determined if immunohistochemical labeling of phosphoEGFR could be used to identify pancreatic cancers that would most likely respond to erlotinib.

Activated EGF Receptor as a Biomarker of Cancers That Are Likely to Respond to Erlotinib. Although EGF receptor is phosphorylated on several sites which involve tyrosine and serine/threonine residues, there is limited understanding on the downstream effects of each one of these sites. We decided to use pY¹⁰⁶⁸ on EGFR as a surrogate for activated form of the receptor. Y¹⁰⁶⁸ on EGF receptor has been well-characterized as an autophosphorylation site that acts as a docking site for Grb2 and an antibody against this phosphorylated residue that performs well in paraffin-embedded sections is commercially available.¹⁶ Immunohistochemical labeling of tissue sections was carried out using an antibody that specifically recognizes EGFR phosphorylated at tyrosine residue 1068. The xenografts P196 and P410 which were sensitive to erlotinib showed strong positive labeling for pEGFR¹⁰⁶⁸, while P184 and P420 did not show any labeling for phosphoEGFR in accordance with the erlotinib sensitivity (Figure 4). Further, immunohistochemical labeling of P196 tissue sections from untreated and erlotinib treated tumors provided direct evidence for tumor shrinkage due to loss of EGFR activity. While the sections from untreated tumor showed intense staining for pEGFR, the erlotinib-treated tumor did not show any labeling due to loss of EGFR signaling (Supplementary Figure 1 in Supporting Information). These results prompted us to further validate if immunohistochemical labeling of pEGFR¹⁰⁶⁸ could be used as a molecular predictor of a tumors response to EGFR inhibitors.

We therefore, immunohistochemically labeled a panel of 12 pancreatic cancer xenografts that were previously tested for response to erlotinib,¹⁷ using the antibody against pEGFR¹⁰⁶⁸. Of these tumors, only P196 responded to erlotinib, while all other xenografts did not show any significant response (Figure 5a). PhosphoEGFR staining revealed that P196 was positive for pEGFR¹⁰⁶⁸, while all others were negative (Figure 5b). By combining proteomic analysis with immunohistochemistry, we have shown activated EGFR as a novel target in a subset of pancreatic cancers. Three of the three tumors that responded to erlotinib stained positive for pEGFR¹⁰⁶⁸ as compared with zero of eleven that did not indicating pEGFR¹⁰⁶⁸ positivity is significantly associated with erlotinib sensitivity (*p*-value = 0.000031 from Fisher's Exact test). Thus, we have demonstrated immunohistochemical labeling of activated EGFR is a significant predictor of pancreatic cancers that are likely to respond to EGFR inhibitors. As all our studies have been carried out in mouse xenografts, further studies involving cancer specimens from pancreatic cancer patients participating in clinical trials would provide significant insights. To find the prevalence of pEGFR in pancreatic cancers, we immunohistochemically labeled tissue microarrays having tumor sections derived from 250 pancreatic cancer patients. About 33% of pancreatic cancer patients showed positive staining for pEGFR¹⁰⁶⁸. Immunohistochemical images of a subset of pancreatic adenocarcinomas with no labeling for pEGFR and adenocarcinomas with intense labeling for pEGFR are provided in Supplementary Figure 2 in Supporting Information. This emphasizes the need for a systematic analysis of activated EGFR in pancreatic cancers and its possible utility as a biomarker for predicting erlotinib sensitivity. One of the recent studies on pulmonary adenocar-

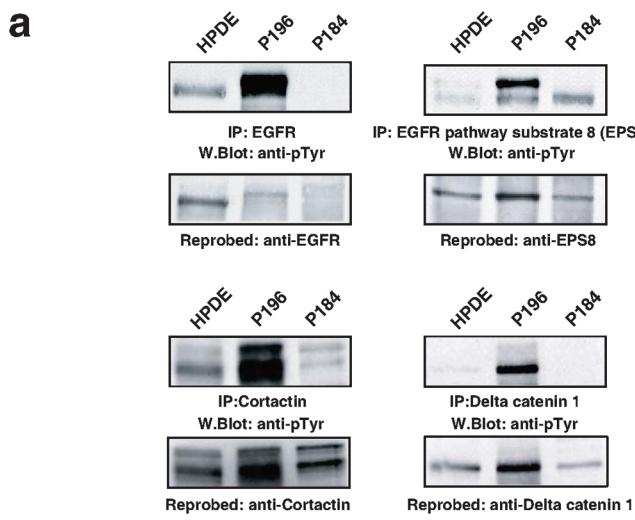
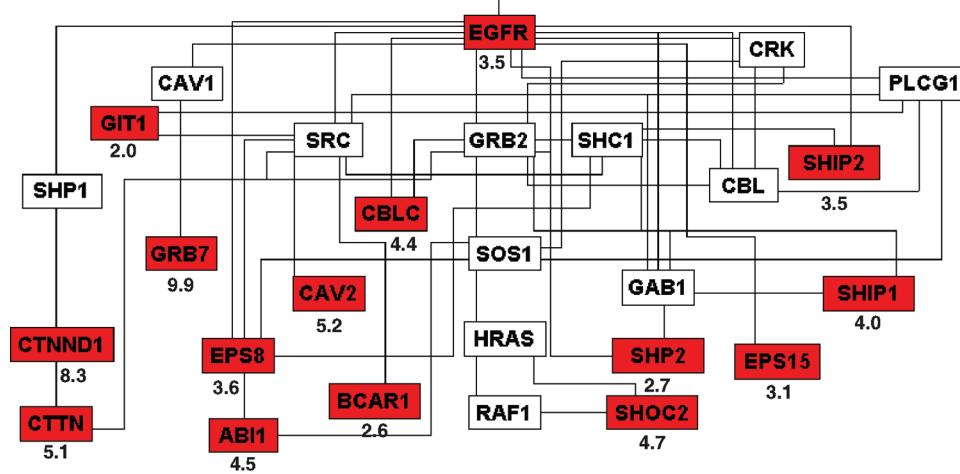
**b**

Figure 3. (a) Immunoblotting shows activation of EGFR pathway substrates. Immunoprecipitation of EGFR, EPS8, cortactin and Delta catenin 1 from HPDE, P196 and P184 followed by Western blotting with anti-phosphotyrosine antibody (top panels); bottom panels show reprobing to show corresponding protein expression. (b) A snapshot of EGFR pathway with the downstream substrates identified in the screen. The proteins that were found to be hyperphosphorylated in pancreatic cancer cell line P196 as compared to non-neoplastic ductal epithelial cell line HPDE are colored red. The corresponding fold changes are marked below the colored rectangles.

cinoma has carried out immunohistochemical evaluation of pEGFR and its correlation to the tumors response to gefitinib. Using an antibody against pEGFR⁹⁹², this study has demonstrated pEGFR⁹⁹² reactivity to be significantly correlated with clinical responsiveness to gefitinib.¹⁸

Discussion

One of the most important outcomes of cancer research in the recent years is our understanding of the role of signaling pathways in driving cancer cell proliferation. This has significantly changed our approach to cancer therapeutics. Traditionally, cytotoxic chemotherapy has been the mainstay for treating cancers for a long time, but with recent understanding on molecular pathways that drive cancers, targeted therapy is now being used as an alternate approach in treating subset of cancers. Among solid tumors, non-small cell lung cancers have been extensively studied with respect to targeted therapies

Table 1. A Partial List of EGFR Pathway Substrates That Were Identified in the Screen

protein name	fold change
Epidermal growth factor receptor	3.5
GRB7	9.9
Delta catenin 1	8.3
Caveolin 2	5.2
Cortactin	5.1
SHOC2	4.7
ABL1 interactor	4.5
CBL3	4.4
SHIP1	4.0
Epidermal growth factor receptor substrate 8	3.6
SHIP2	3.5
STAP2	3.5
Epidermal growth factor receptor substrate 15	3.1
SHP2	2.7
Breast cancer anti-estrogen resistance 1	2.6
G protein-coupled receptor kinase interactor 1	2.0

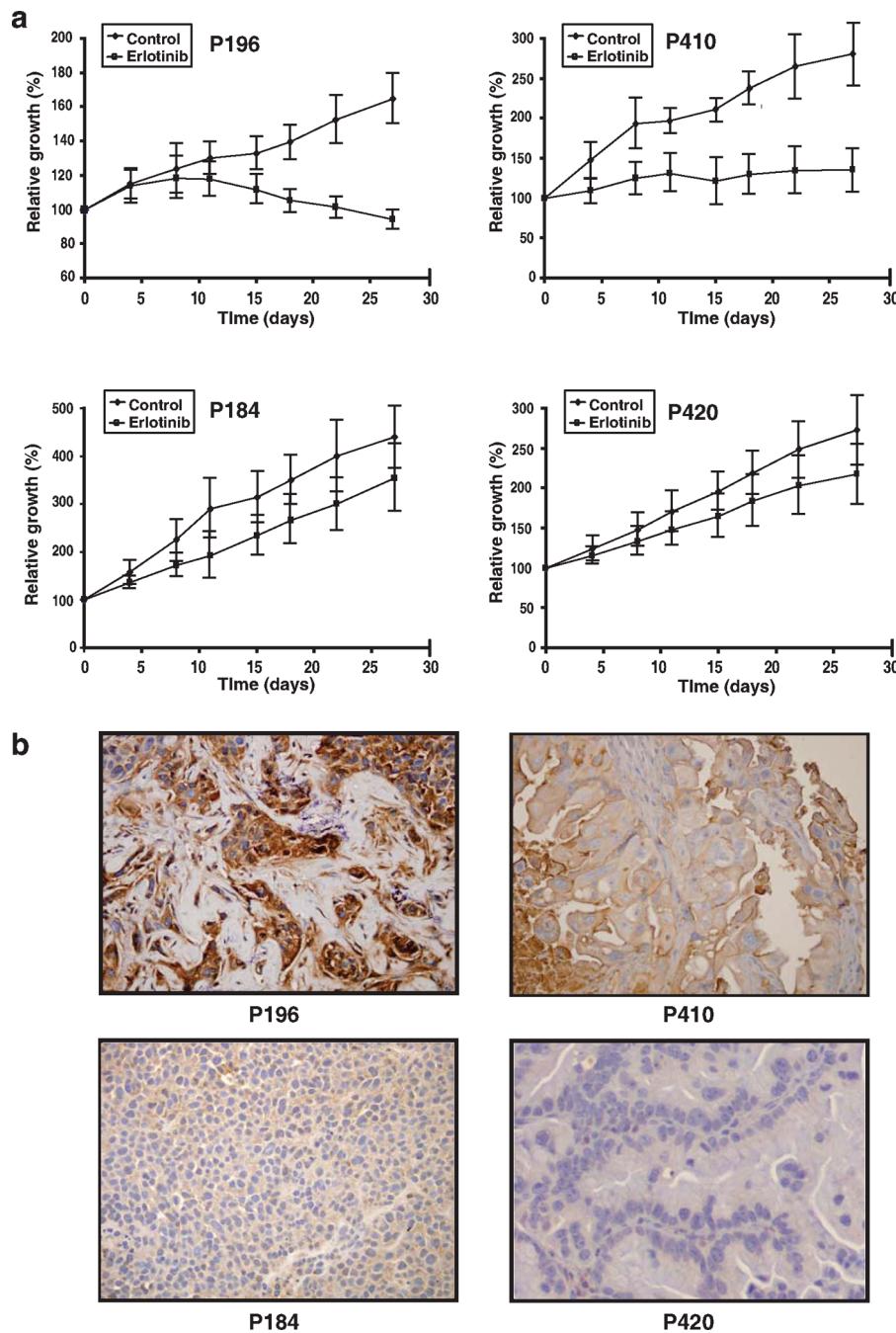


Figure 4. (a) Response of pancreatic cancer xenografts to EGFR inhibitor, erlotinib. Mice bearing pancreatic cancer xenografts P196, P410, P184 and P420 were either treated with the EGFR inhibitor, erlotinib, or left untreated. Tumor sizes were evaluated thrice a week by caliper measurements. (b) EGFR pathway activation status in pancreatic cancer xenografts. The tumor sections from P196, P410, P184 and P420 were immunohistochemically labeled with phospho EGFR (pY1068) antibody. The brown staining marks positive staining for phospho EGFR.

involving epidermal growth factor receptor, a member of ERBB family of receptor tyrosine kinases. Erlotinib, a small molecule EGFR inhibitor, is now the treatment of choice in subset of non-small cell lung cancer patients. This drug in combination with gemcitabine is now approved by FDA for treatment of locally advanced or metastatic adenocarcinoma of the pancreas.

Through phosphoproteomic profiling and quantitative proteomics, we have identified a subset of pancreatic cancers that show activation of epidermal growth factor receptor pathway. By carrying out mouse xenograft studies involving EGFR inhibitor erlotinib, we have established that these tumors

respond to targeted therapy. While looking for activating mutations and overexpression of the receptor is one way of determining possible role of EGFR in driving cancers, finding activation status of the receptor takes us one step further. By choosing to study phosphorylated proteins in pancreatic cancers, we have been successful in identifying subset of cancers that are driven by the EGFR pathway. While this is the first step in identifying molecular pathways that are potential targets for targeted therapy, it is also important to identify candidate markers that would aid in identifying these tumors that are likely to respond to such therapy.

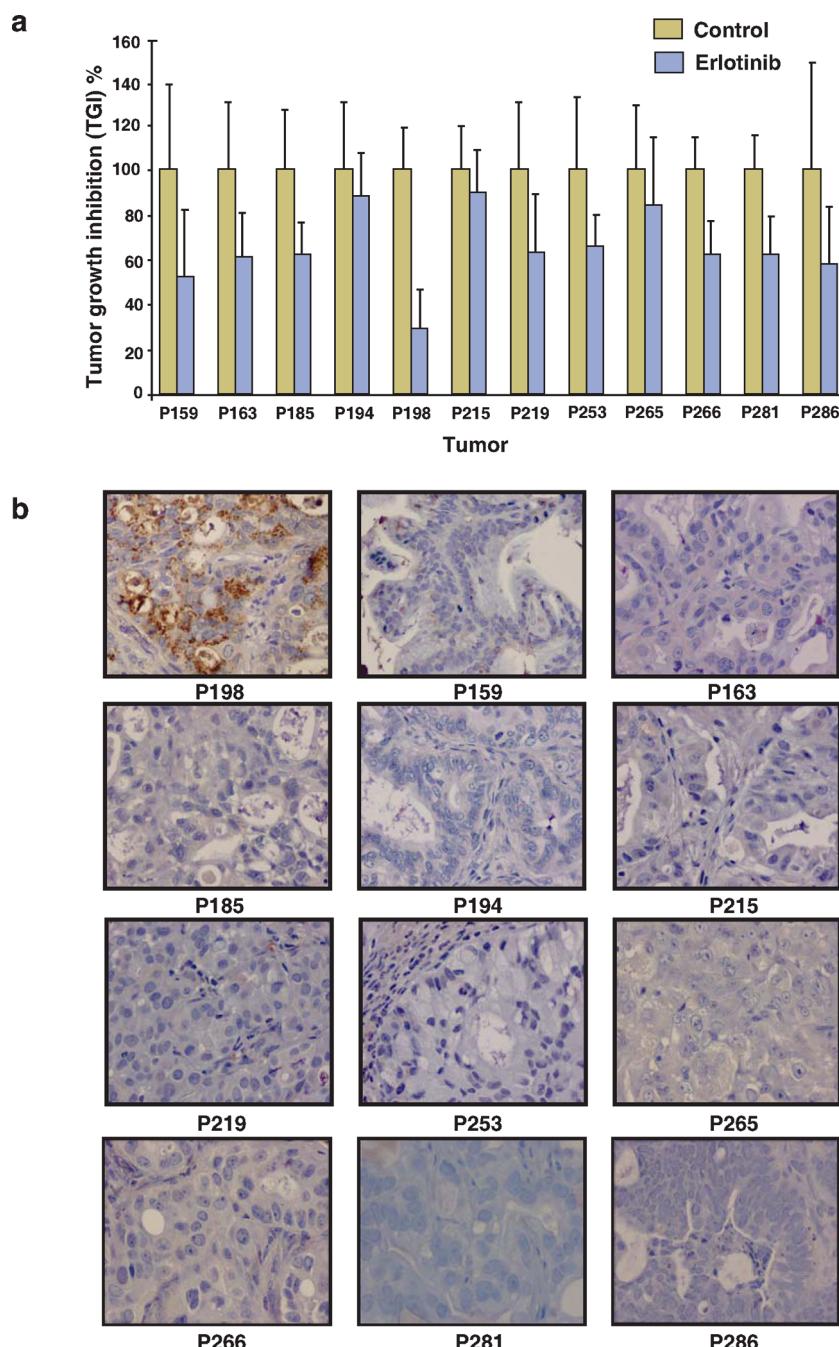


Figure 5. (a) Tumor growth inhibition by erlotinib correlates with pEGFR¹⁰⁶⁸ expression. Tumor growth inhibition (TGI) in 12 pancreatic cancer xenografted mice treated with erlotinib. Relative inhibition of tumor growth was calculated as the relative tumor growth of treated mice divided by relative tumor growth of control mice. (b) Immunohistochemical labeling of activated EGFR in a panel of pancreatic cancer xenografts. Immunohistochemical labeling of pEGFR¹⁰⁶⁸ was carried out by using an antibody specific to EGFR phosphorylated at tyrosine residue 1068. The brown staining marks positive staining for phospho EGFR¹⁰⁶⁸.

In our study, we have validated pEGFR¹⁰⁶⁸ as a promising marker for selecting the cases which are more likely to respond to erlotinib therapy. The growing list of unsatisfactory clinical trials of targeted agents has emphasized the need for markers capable of predicting clinical response to novel chemotherapeutic agents. In the case of protein targets with a catalytic activity (such as receptor tyrosine kinases), a more accurate predictor of activity of such targets is measurement of the activated form of the relevant protein, if possible. For instance, previous clinical trials that showed modest survival advantage using erlotinib and gemcitabine to treat advanced pancreatic

cancers did not use molecular predictors to predict the patients who are likely to benefit from EGFR inhibition.¹⁹ We propose that use of activated EGFR as a predictive tool for clinical response to EGFR inhibitors could lead to an improved outcome of clinical trials while sparing the large majority of the patients who might not benefit from these drugs.

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Supporting Information Available: Supplementary Table 1, peptides identified from SILAC comparison of HPDE and P196 and their corresponding fold values; Supplementary Table 2, protein quantitation from SILAC comparison of HPDE and P196; Supplementary Table 3, peptides identified from SILAC comparison of HPDE and P184 and their corresponding fold values; Supplementary Table 4, protein quantitation from SILAC comparison of HPDE and P184; Supplementary Table 5, tumor volume averages of mouse xenografts; Supplementary Figure 1, immunohistochemical labeling of P196 sections from erlotinib treated and untreated tumors; Supplementary Figure 2, (a) immunohistochemical labeling of pancreatic adenocarcinoma tissue sections showing no labeling of pEGFR (pY1068), (b) immunohistochemical labeling of pancreatic adenocarcinoma tissue sections showing intense labeling of pEGFR (pY1068). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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