Coordinated Epidermal Growth Factor Receptor Pathway Gene Overexpression Predicts Epidermal Growth Factor Receptor Inhibitor Sensitivity in Pancreatic Cancer

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Abstract

The epidermal growth factor receptor (EGFR) inhibitor erlotinib is approved for treatment of pancreatic cancer but the overall activity is minimal, and known predictive factors for EGFR inhibitor efficacy are infrequent in this disease. We tested the hypothesis that global activation of the EGFR pathway is predictive of EGFR inhibitor efficacy. Pancreatic cancer tumors directly xenografted at surgery were treated with the EGFR inhibitors erlotinib and cetuximab and analyzed for biological features. Two of 10 tumors were sensitive, and by global gene expression profiling with gene set enrichment analysis, the EGFR pathway was highly expressed in sensitive compared with resistant tumors. The core gene components driving EGFR pathway overexpression were pathway ligands and positive effectors. In a prospective validation, the EGFR pathway-based signature correctly predicted anti-EGFR treatment response in eight additional tumors and was not predictive of response to gemcitabine and CI1040 (a MEK inhibitor). Analysis of *EGFR, KRAS*, and *PIK3CA* mutations and gene amplification by fluorescence *in situ* hybridization and multiplex ligation-dependent probe amplification showed that none of these genetic abnormalities were neither predictive nor responsible for the EGFR pathway activation. Coordinated overexpression of the EGFR pathway predicts susceptibility to EGFR inhibitors in pancreatic cancer. These results suggest a phenomenon of pathway addiction and support the value of unbiased system biology approaches in drug development. [Cancer Res 2008;68(8):2841–9]

Introduction

The epidermal growth factor receptor (EGFR) inhibitor erlotinib is approved for treatment of pancreatic cancer, but the overall therapeutic efficacy is minimal (1). There is an unmet need to identify the individual factors predicting such susceptibility. Cancer is a genetic disease (2), and accumulating data suggest that the factors determining the sensitivity to anticancer agents also have a genetic basis. The presence of acquired mutations in the catalytic domain of the *EGFR* gene increase sensitivity to anti-EGFR small-molecule inhibitors in non–small cell lung cancer (NSCLC; refs. 3, 4). Likewise, increased *EGFR* and *HER2* gene copy number detected by fluorescence *in situ* hybridization (FISH) was associated with improved gefitinib efficacy in patients with NSCLC (5, 6). In addition, there is evidence that *KRAS* mutations confer resistance to EGFR inhibition (7).

As the EGFR is a validated target in pancreatic cancer but with limited clinical activity, the identification of factors predicting drug response is a relevant question. However, several series investigating known predictive factors for EGFR inhibition, such as *EGFR* mutations or amplifications in pancreatic cancer, have failed to document a meaningful prevalence of such alterations (8, 9), and HER2 amplification assessment has rendered conflicting results (10, 11). These observations highlight the need to explore alternative explanations for pancreatic cancer aberrant EGFR pathway activation (12).

Because of existing data on other tumor types, the hypothesis driving our work was that vulnerability to EGFR-targeting agents is related to dependence on the EGFR pathway. The above negative data led us to propose that factors, other than single oncogene alterations, may be relevant in determining anti-EGFR effect. The level of complexity of common cancers may be higher than expected (13), and probably more sophisticated, integrative approaches to gather information are needed to meaningfully interrogate a tumor. Gene expression analysis has shown promise to characterize cancer (14), and recently, a diagnostic tool (MammaPrint) derived from global unbiased testing received regulatory approval for risk prognostication for breast cancer (15). From a biological perspective, it evaluates what is considered the dynamic language controlling cell processes, both normal and altered. Several computational methods have improved the ability to identify candidate genes that are correlated with a phenotype by exploiting the idea that gene expression alterations might be revealed at the level of biological pathways or coregulated gene sets, rather than at the level of individual genes (16–18).

We tested if the sensitivity to EGFR inhibitors in pancreatic cancer would be dependent on EGFR pathway alterations and whether those would predominantly be at the gene expression level. To test our hypothesis, we first targeted the EGFR with a small molecule inhibiting tyrosine kinase activity (erlotinib), a monoclonal antibody targeting the extracelular domain (cetuximab), and the combination of both in a learning set of freshly generated human pancreatic cancer xenografts (19). Then we explored gene expression–based approaches in those tumors,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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generated a predicitive signature, and prospectively queried a second cohort (validation set). The accuracy of these predictions was prospectively tested by treating those cases with erlotinib. Finally, genetic factors known to be relevant in other tumor types were explored to understand the gene expression findings, and protein expression/activation were assessed to determine the effect of differential gene expression in EGFR pathway components.

Materials and Methods

Drugs. Erlotinib (OSI Pharmaceuticals) and cetuximab (Imclone Systems) were obtained from commercially available sources.

***In vivo* growth inhibition studies.** Six-week-old female athymic nude mice (Harlan) were used. The research protocol was approved by the Johns Hopkins University Animal Care and Use Committee, and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. The xenografts were generated according to methodology published elsewhere (19). Briefly, surgical nondiagnostic specimens of patients operated at the Johns Hopkins Hospital were reimplanted s.c. to one to two mice for each patient, with two small pieces per mouse (this is the first passage of the human tumor on the mouse; thus, it constitutes the F1 generation). Tumors were let to grow to a size of 1.5 cm^3, at which point they were harvested, divided, and transplanted to another five mice (F2 generation). After a second growth, passage tumors were excised and propagated to cohorts of 20 mice or more, which constituted the treatment cohort (F3 generation). Tumors from this treatment cohort were allowed to grow until reaching ~200 mm^3, at which time mice were randomized in the following three treatment groups, with five to six mice (10 evaluable tumors) in each group: (*a*) control, (*b*) erlotinib 50 mg/kg/d i.p., (*c*) cetuximab 40 mg/kg twice a week i.p., and (*d*) erlotinib plus cetuximab at the above doses. Treatment was given for 28 d. Gemcitabine and CI1040 were given in prior experiments twice weekly for 4 wk at 100 mg/kg i.p. and twice daily for 28 d at 150 mg/kg i.p., respectively. Mice were monitored daily for signs of toxicity and were weighed thrice per week. Tumor size was evaluated twice per week by caliper measurements using the following formula: tumor volume = (length × $width^2$) / 2. Relative tumor growth inhibition was calculated by relative tumor growth of treated mice divided by relative tumor growth of control mice since the initiation of therapy [treated versus control (T/C) ratio]. Tumors with a T/C of <20% were considered sensitive.

Microarray gene expression. Baseline, untreated tumors were profiled using Affymetrix U133 Plus 2.0 gene arrays in duplicate. Sample preparation and processing procedure were performed as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc.). The gene expression data have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO)[6] and are accessible through GEO series accession number GSE9599.

Gene set enrichment analysis. Gene expression levels were converted to a rank-based matrix and standardized (mean, 0; SD, 1) for each microarray. Gene set analysis was performed using the gene set enrichment analysis (GSEA) software (18) version 2.0.1 obtained from Broad Institute.[7] Genes represented by more than one probe were collapsed using the Collapse Probes utility to the probe with the maximum value. The gene sets database was compiled from the KEGG database (May 29, 2007 version; ref. 20). The KEGG gene sets database contains 197 human pathways that include metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases. One hundred sixty-five gene sets passed the gene set size filter criteria (min, 10; max, 500). *P* values for the gene sets were computed by permuting the genes 1,000 times in this study.

Core gene expression classifier. The core gene expression classifier was built by the logistic regression model using LogitBoost implemented in the WEKA machine learning package version 3.4 (21). The default variables were used in this study.

DNA mutation analysis. Mutations of the *KRAS* oncogene were determined as previously described (22). PCR amplifications of exons 18, 19, and 21 of *EGFR*; exon 11 of *BRAF*; and exons 9, 10, and 20 of *PI3KCA* were performed as described (3, 23, 24). The primers used are available upon request. Sequencing in the forward and reverse direction was performed using an ABI 3730XL Sequencer in the Genetics Resource Core Facility, Johns Hopkins University School of Medicine.

FISH assessment. Paraffin-embedded sections were submitted to dual-color FISH assays using the EGFR SO/CEP7 SG probe set and PathVysion DNA kit (HER2 SO/CEP17 SG; Vysis/Abbott Laboratories). Initially, the slides were incubated for 2 h at 60°C, deparaffinized in Citro-Solv (Fisher) and washed in 100% ethanol for 5 min. The slides were incubated in 2 × SSC at 75°C for 10 to 18 min and digested in 0.25 mg/mL proteinase K/2 × SSC at 45°C for 11 to 18 min. Then, the slides were washed in 2 × SSC for 5 min and dehydrated in ethanol. Probes were applied according to the manufacturer's instructions to the selected hybridization areas. DNA denaturation was performed for 15 min at 80°C, and the slides were incubated at 37°C for 20 h. Posthybridization washes were performed with 1.5 urea/0.1 × SSC at 45°C for 35 min. Then, the slides were washed in 2 × SSC for 2 min and dehydrated in ethanol. Chromatin was counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.3 μg/mL in Vectashield; Vector Laboratories). Analysis was performed on epifluorescence microscope using single interference filter sets for green (FITC), red (Texas red), and blue (DAPI), as well as dual (red/green) and triple (blue, red, green) band pass filters, and was done in the areas correspondent to the areas previously microdissected.

According to the frequency of cells with specific number of copies of the EGFR gene and chromosome 7 centromere, the areas were classified into six FISH categories with ascending number of copies of the EGFR gene per cell [(*a*) disomy (≤2 copies in >90% of cells), (*b*) low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of cells, ≥4 copies in <10% of cells), (*c*) high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells), (*d*) low polysomy (≥4 copies in 10–40% of cells), (*e*) high polysomy (≥4 copies in ≥40% of cells), and (*f*) gene amplification], defined by the presence of tight EGFR gene clusters, a ratio of gene/chromosome per cell of ≥2 or ≥15 copies of EGFR per cell in ≥10% of analyzed cells. FISH scores 1 to 4 classify the specimen as FISH negative (FISH−); scores 5 and 6 classify the specimen as FISH positive (FISH+).

Multiplex ligation-dependent probe amplification. For multiplex ligation-dependent probe amplification (MLPA) analysis of DNA copy number changes, a specific probe mixture with 48 subtelomeric probe sets for all chromosomes was used according to the manufacturer's recommendations (Salsa P036, MRC-Holland B.V.). In short, ~100 ng of DNA in 5 μL were denaturated at 98°C for 5 min and subsequently hybridized overnight with a mix of subtelomeric probe pairs, each consisting of two oligonucleotides (hemiprobes) that recognize adjacent DNA sequences. On day 2, the adjacently hybridized hemiprobes were ligated. After denaturation, PCR was performed with two universal PCR primers, amplifying all probe pairs in one reaction. Experiments for both test and reference samples were carried out in triplicate. Analysis of the MLPA PCR products was performed on an ABI model 3100 16-capillary sequencer (Applied Biosystems).

Immunohistochemical analysis. Five-micron sections were used for Ki67 staining that was performed following the manufacturer's instructions (DAKO) and scored as percentage staining nuclei. Phosphorylated mitogen-activated protein kinase (MAPK; Cell Signaling Technology) staining was performed using citrate steam recovery, followed by catalyzed signal amplification (DAKO).

Results

Efficacy of erlotinib, cetuximab, and combination of erlotinib plus cetuximab. Initially, 10 patient-derived tumors from our colony were tested for drug efficacy. Two tumors (198 and 410) were highly sensitive to EGFR targeting, including tumor

[6] http://www.ncbi.nlm.nih.gov/geo
[7] http://www.broad.mit.edu/gsea

regressions (Fig. 1). All other tumors were resistant with best treatment, resulting only in modest growth inhibition. Overall erlotinib showed marginally higher potency compared with cetuximab, with an average T/C ratio of 54% versus 65% when the indexes of all 10 cases were pooled together. The combined therapy had an average of 45% T/C, indicating a marginally increased efficacy in some cases, such as 253, although not meeting the preestablished efficacy criteria.

Tumors from four of the cases [both sensitive strains, one fully resistant (215) and the fully resistant to single agents wherein dual

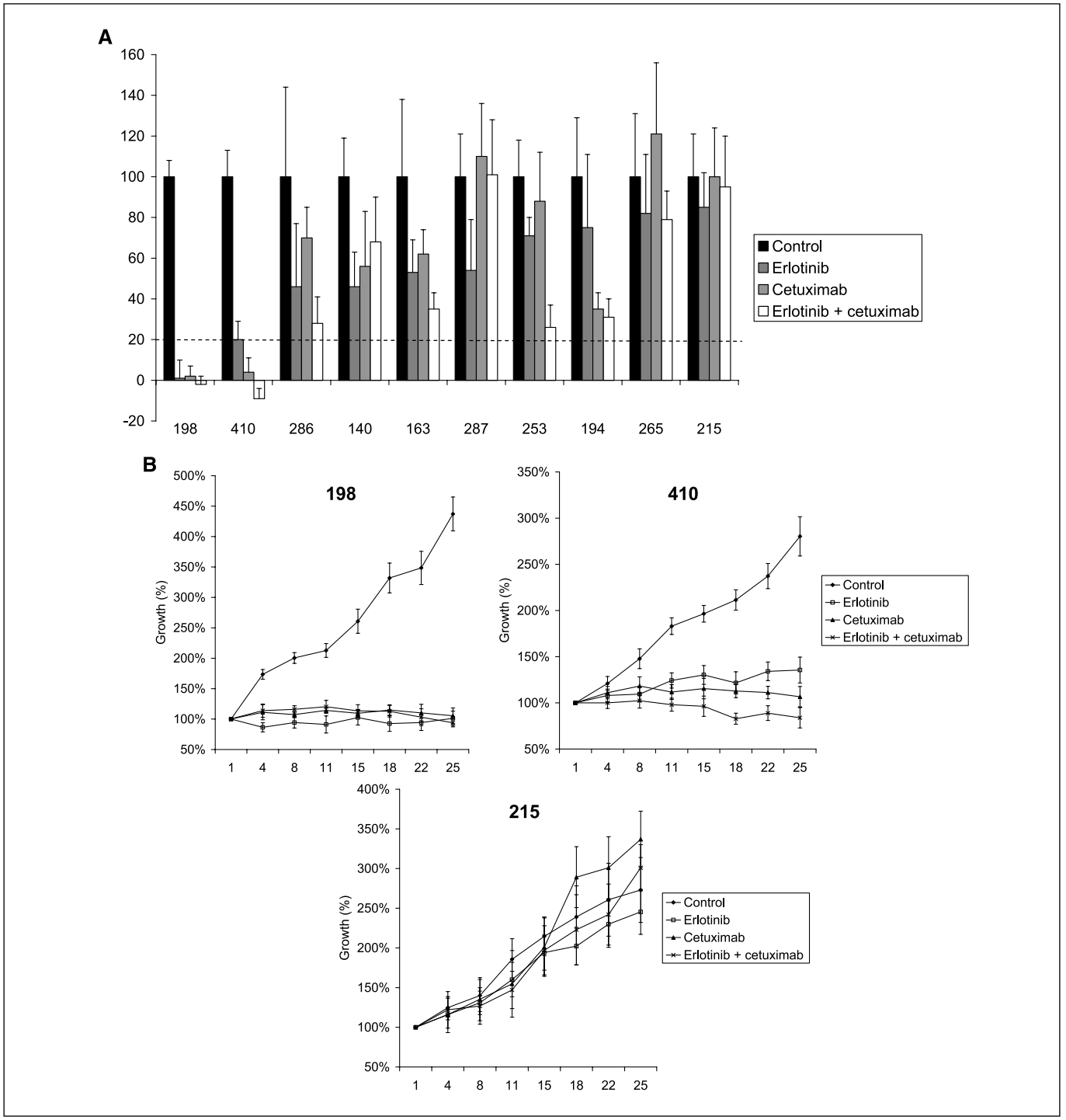


Figure 1. Efficacy of erlotinib (a small molecule inhibitor of the tyrosine kinase activity of EGFR), cetuximab (a monoclonal antibody targeting the extracellular domain), and the combination of both in pancreatic cancer xenografts. *A,* bar graph of all 10 cases. 198 and 410 were highly sensitive to either treatment modality. Overall erlotinib showed marginally higher potency compared with cetuximab, with an average T/C of 54% versus 65% when the indexes of all 10 cases were pooled together. The combined therapy had an average T/C of 45%. Within each case, the growth is normalized to the growth of the control. Cases with T/C of <20% were considered sensitive. *Bar,* SD. *B,* graph of selected cases. 198 and 410 were the tumors that showed the highest sensitivity to EGFR inhibition. 215 was resistant to all three modalities.

therapy induced a marginal effect (253)] were blindly analyzed for treatment effect (Supplementary Fig. S1), using phosphorylated MAPK and Ki67 as the most suitable and reproducible end points for immunohistochemistry assessment of anti-EGFR effect (25, 26). Phosphorylated MAPK only changed significantly in all treatment arms from 198, 410, and the 253 combination arm, wherein some degree of antitumor effect was documented. Ki67 proliferation index decreased across treatment groups in 198 and 410 but did not change in 215 or 253.

Gene expression analysis. We approached gene expression analysis by seeking a tool that would enable group interrogation. To rationally explore this hypothesis in a reproducible fashion, we used GSEA and pathway analysis, an approach that offers an unbiased global search for genes that are coordinately regulated in predefined pathways (in this case, as per the KEGG database; ref. 20) rather than interrogating expression differences of single genes. Overall, 98 gene sets were enriched in the sensitive cases, but only eight gene sets had a nominal P value of < 0.01 (Fig. 2*A*). Out of these eight gene sets, four of them have a false discovery rate (FDR) of <0.10. One of these four was the EGFR signaling pathway that, according to the KEGG database annotation, consists of 87 genes. Of these, the 25 genes that contributed most to the enrichment result were defined as the core enrichment genes (enrichment plot illustrated in Fig. 2*B*; list of genes in Supplementary Table S1). These include seven ligands (*EGF, HB-EGF, TGFα, BTC, EPR, NRG2*, and *NRG4*), and pathway genes, such as *MAPK8-10, Akt3, NRAS, PIK3CA, STAT5*, and *p27*, were up-regulated in the sensitive tumors. The heatmap of these core enrichment genes, shown in Fig. 2*C* and *D*, illustrates the location of these core enrichment genes in the EGFR signaling pathway. These results suggest that global increase in the expression and activation of pathway-related genes is linked to drug susceptibility.

Prospective prediction in the validation set. Thus, we hypothesized that, by querying the EGFR pathway, we could predict the response to EGFR inhibitors and used an independent set of eight tumors, of which no efficacy results were known. We tested whether the expression profiles of the core gene members in the EGFR signaling pathway could be used as discriminative features for prediction. We built a logistic regression classifier from these core gene features based on these 10 cases (learning set). Next, we collected gene expression profiles of eight independent cases (159, 185, 219, 247, 281, 294, 354, and 420) and used the core gene classifier to predict their drug response to EGFR inhibition. The classifier identified 219 as sensitive and the rest to be resistant to EGFR inhibition. To test this prediction, we conducted drug efficacy testing on the eight tumors with erlotinib, the EGFR inhibitor that is approved for use in pancreatic cancer patients. The tumor 219 was sensitive (T/C, 3%), and the other seven predictions were also accurate (global GSEA prediction χ^2 test, $P < 0.001$), as those cases were uniformly resistant to erlotinib.

Specificity of the signature. To exclude the possibility that these tumors were inherently sensitive/labile to any treatment, erlotinib efficacy was correlated with the response in these cases to gemcitabine and CI1040, a cytotoxic agent and a signal transduction inhibitor with similar level of efficacy (3 of 15), respectively. No correlation existed between the responses to these three treatments, indicating that each tumor's response depends on inherent features. The EGFR core signature is not indicative of response to these drugs, and by GSEA, the EGFR pathway is not differentially up-regulated in the gemcitabine or CI1040-responsive tumors (Table 1). On the other hand, some of the EGFR-sensitive tumors (198 and 247 with T/C below 10%) were sensitive to temsirolimus, a mammalian target of rapamycin (mTOR) pathway inhibitor (19). This is significant, as components in this pathway were also represented in the EGFR pathway signature. However, cases resistant to erlotinib, such as 215, were also sensitive to temsirolimus (T/C, 16%), so the overlap between sensitivities was again partial and not explained by the EGFR pathway overexpression.

Gene mutation, FISH, and MLPA analysis. Next, we explored factors that have been related to EGFR sensitivity in other disease types. *EGFR, KRAS, BRAF,* and *PIK3CA* mutation and amplification profile of *EGFR* and *HER2* by FISH were explored in these tumors (Table 2). No mutations or deletions were found in exons 18, 19, or 21 of the *EGFR* or in exon 11 of *BRAF*. One sensitive tumor had a mutation in the *PIK3CA* gene. *KRAS* mutations were prevalent, but both mutated and wild-type cases were sensitive to EGFR inhibition. *EGFR* gene amplification was not detected, but in seven tumors, there was high polysomy (Fig. 3*A*). *HER2* gene amplification was found in two specimens. There was no correlation between the mutation or amplification profile and sensitivity to EGFR inhibitors. Thus, these best candidates to explain differential response to the drugs were not informative, further reinforcing the value of the EGFR signature as the best predictor of efficacy.

We then conducted an MLPA analysis of selected genes in the EGFR pathway to investigate whether changes in gene dosage could explain pathway activation by using a novel, high-throughput quantitative method (ref. 27; Fig. 3*B*). Sensitive tumors had copy gains of key EGFR pathway genes, such as *AKT1, NRAS, PIK3CA*, and *SRC*, and no change in *HER4*, but multiple resistant tumors (247, 420) showed similar profiles.

Analysis of pathways activated by underlying genetic abnormalities. To further investigate whether the presence of the EGFR signature was related to any of the above individual genetic factors, we determined the GSEA signatures of the cohort of 18 tumors stratifying by each of the variables. The strata was mutated versus nonmutated for *KRAS* and *PIK3CA*, a score of 5 to 6 versus 1 to 4 for *EGFR* and *HER2* FISH, and increased copy number versus no increase in each of the eight genes of the MLPA analysis (Supplementary Table S2). The EGFR pathway was not present in the top scoring pathways of the cases with neither mutations nor increased copy number/dosage compared with the normal state tumors, indicating that none of these individual features was causing per se the EGFR pathway overexpression.

Immunohistochemistry assessment. Finally, to determine the effect of EGFR pathway gene overexpression at the protein level, we determined the baseline expression of selected elements in the EGFR by immunohistochemistry. Sensitive cases had a globally activated EGFR pathway profile (high EGFR, phosphorylated MAPK, and phosphorylated Akt positivity), but resistant cases (215, 265, 185) did stain too for these individual markers (Table 3). So, it can be concluded that pathway activation by immunohistochemistry is necessary, but not sufficient, to confer sensitivity to anti-EGFR therapy.

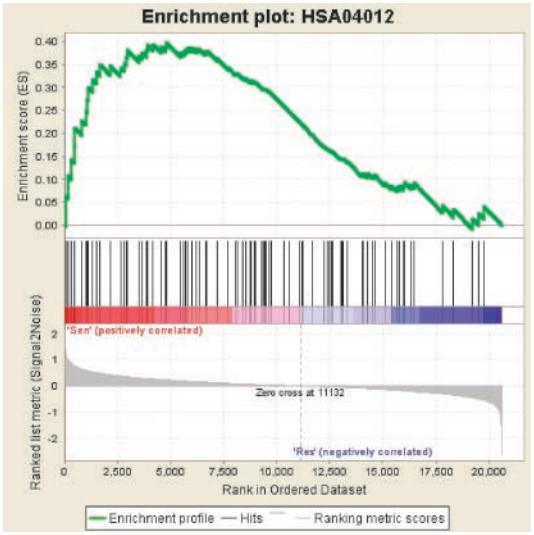
Discussion

Cancer is a complex disease characterized by multiple genetic and molecular alterations affecting cell proliferation, survival, differentiation, and invasion among others (28). The concept that gene abnormalities induce the cancer phenotype and drive tumor

A

GENE SETS ENRICHED IN THE "SEN" CASES	KEGG MAP	SIZE	ES	NES	NOM *p*-val	FDR *q*-val
Melanoma	SA05218	71	0.51	1.87	0.000	0.036
Glioma	SA05214	64	0.50	1.83	0.000	0.042
ABC transporters - general	HSA02010	45	0.58	1.75	0.002	0.064
ErbB signaling pathway	HSA04012	87	0.40	1.70	0.008	0.092
Chronic myeloid leukemia	HSA05220	76	0.37	1.54	0.010	0.268
Small cell lung cancer	HSA05222	86	0.36	1.52	0.000	0.226
Cell communication	HSA01430	117	0.41	1.52	0.000	0.217
MAPK signaling pathway	HSA04010	255	0.29	1.37	0.000	0.275

B



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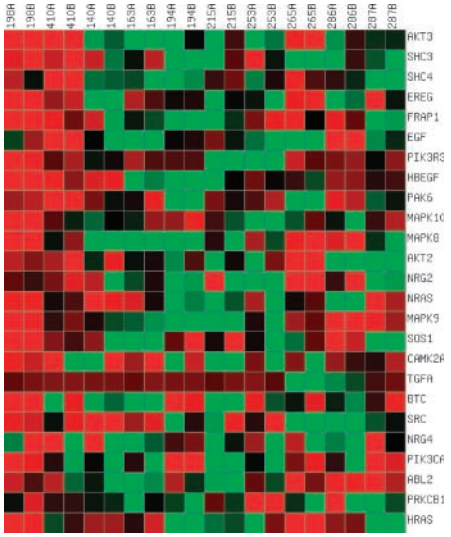

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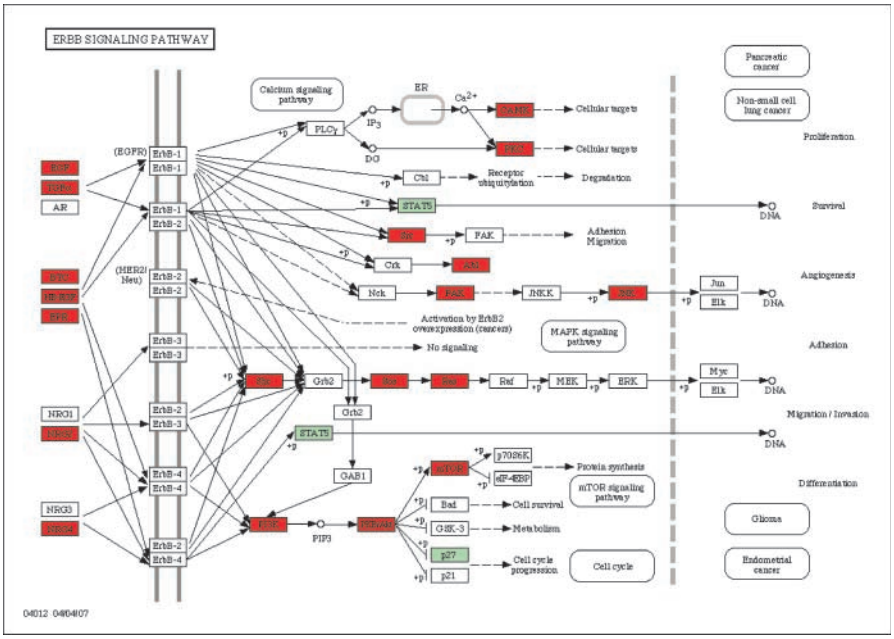


Figure 2. *A,* list of the top eight gene sets enriched in the erlotinib-sensitive cases with nominal *P* value of < 0.01. The gene list is sorted in descending order by NES score. The EGFR signaling pathway is highlighted (*yellow*). According to the KEGG database annotation, the EGFR signaling pathway consists of 87 genes, and 25 of these genes that contribute most to the enrichment result were defined as the core enrichment genes (Supplementary Table S1); *size,* number of genes in the gene set; *ES,* enrichment score; *NES,* normalized enrichment score; *NOM p-val,* nominal *P* value; *FDR q-val,* FDR. *B,* enrichment plot for the EGFR signaling pathway. *Top,* the running enrichment score for the gene set as the analysis walks down the ranked list. The score at the peak of the plot is the enrichment score for the gene set. *Middle,* the members of the gene set appear in the ranked list of genes. *Bottom,* the value of the ranking metric along the list of ranked genes. *C,* heatmap of the core enrichment genes. *Rows,* gene; *columns,* sample array. The expression level for each gene is normalized across the samples, such that mean is 0 and SD is 1. Genes with expression levels greater than the mean are colored in red, and those below the mean are colored in blue. *D,* EGFR signaling pathway. The core enrichment genes were colored in red according to the KEGG map annotation.

Table 1. List of the top eight gene sets enriched in the gemcitabine-sensitive and CI1040-sensitive tumors with nominal *P* value of ≤0.05

Name	KEGG pathways enriched in sensitive tumors	Size	ES	NES	NOM p-val	FDR q-val
Gemcitabine						
HSA00591	Linoleic acid metabolism	35	0.58	1.59	0.021	1.00
HSA00920	Sulfur metabolism	13	0.64	1.59	0.027	0.84
HSA00603	Glycosphingolipid biosynthesis	14	0.58	1.56	0.041	0.69
HSA04740	Olfactory transduction	31	0.50	1.55	0.020	0.58
HSA00360	Phenylalanine metabolism	29	0.51	1.53	0.029	0.54
HSA03320	PPAR signaling pathway	66	0.46	1.51	0.026	0.52
HSA00590	Arachidonic acid metabolism	52	0.53	1.51	0.050	0.45
HSA00340	Histidine metabolism	39	0.44	1.46	0.045	0.56
CI1040						
HSA05211	Renal cell carcinoma	69	0.47	1.82	0.004	0.114
HSA05040	Huntington's disease	31	0.52	1.76	0.006	0.146
HSA00740	Riboflavin metabolism	16	0.60	1.62	0.026	0.398
HSA05220	Chronic myeloid leukemia	76	0.41	1.59	0.006	0.387
HSA05212	Pancreatic cancer	73	0.41	1.57	0.029	0.377
HSA05210	Colorectal cancer	85	0.42	1.53	0.030	0.439
HSA04520	Adherens junction	79	0.36	1.50	0.038	0.484
HSA04910	Insulin signaling pathway	135	0.29	1.33	0.040	0.903

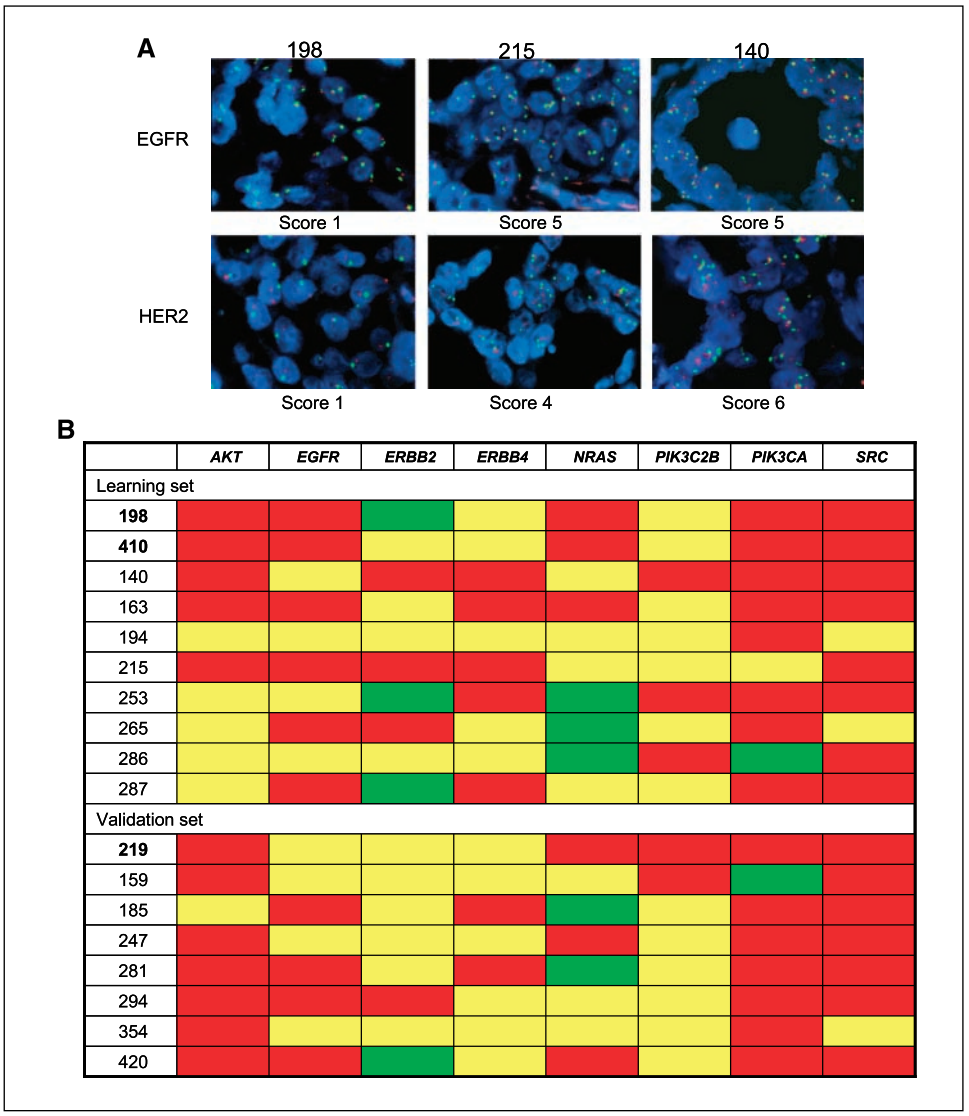
NOTE: The gene list is sorted descending with NES score. In the gemcitabine table, it is noteworthy the lower statistical significance compared with Fig. 3*A*.
Abbreviation: size, number of genes in the gene set; ES, enrichment score; NES, normalized enrichment score; NOM p-val, nominal *P* value; FDR q-val, FDR.

growth has been validated clinically by developing therapies specifically targeting the products of those altered genes (29–31). These have generally consisted of single-gene events, such as gene rearrangements (32), gene copy gains (33), or activating mutations (4). There is increasing evidence that the overexpression of groups of genes that define a phenotype is associated to sensitivity to specific drugs, such as in head and neck and lung cancers, where the presence of an epithelial or mesenchymal phenotype by gene array was predictive of sensitivity or resistance to the EGFR inhibitor gefitinib (34). In this case, the connection between the

Table 2. Mutation patterns identified for the *EGFR, KRAS*, and *PI3KCA* genes and gene amplifications in *EGFR* and *HER2* in pancreatic cancer tumors

Cases	*EGFR*	*KRAS*	*BRAF*	*PIK3CA*	*EGFR* FISH	*HER2* FISH
Learning set						
198	wt	mut	wt	wt	1	1
410	wt	wt	wt	mut	5	5
286	wt	mut	wt	wt	2	2
140	wt	mut	wt	wt	5	6
163	wt	mut	wt	wt	4	4
287	wt	wt	wt	wt	5	5
253	wt	mut	wt	wt	4	4
194	wt	mut	wt	wt	4	2
265	wt	mut	wt	wt	2	4
215	wt	mut	wt	wt	5	4
Validation set						
219	wt	mut	wt	wt	2	3
294	wt	mut	wt	wt	4	4
281	wt	mut	wt	wt	5	6
247	wt	mut	wt	wt	5	5
185	wt	mut	wt	wt	5	4
159	wt	mut	wt	wt	4	1
354	wt	wt	wt	wt	2	2

NOTE: Sensitive cases are shown in bold.
Abbreviations: wt, wild type; mut, mutant.



	AKT	EGFR	ERBB2	ERBB4	NRAS	PIK3C2B	PIK3CA	SRC
Learning set								
198								
410								
140								
163								
194								
215								
253								
265								
286								
287								
Validation set								
219								
159								
185								
247								
281								
294								
354								
420								

Figure 3. *A, EGFR* and *HER2* FISH of selected cases. *B,* MLPA analysis. Both sensitive tumors had a very similar distribution of dosage by MLPA with the exception of *HER2* and were the cases with the highest similitude. Half or more of the cases had gains of *EGFR, PIK3CA*, and *Akt1*. There was a poor correlation between *EGFR* FISH and MLPA. Cases with low number of *HER2* copies by FISH tended to have either no change (286, 194) or a loss (198) by MLPA, but there was no good correlation between *HER2* by both techniques in cases scoring 4 or more by FISH. There was no pattern between *KRAS* mutations and MLPA *NRAS* profile. Red and green indicate gain and loss of the chromosomal area where the gene resides, respectively; yellow notes no change.

targeted and the overexpressed pathways was not direct. In other cases, the presence of coordinated alterations of complex gene sets responsible for a function has been indicative of both the relevance of such phenotype and the potential for direct pharmacologic intervention (35). Here, we present evidence of a similar phenomenon in pancreatic cancer, where we find the targeted pathway (EGFR) among those being overexpressed at the transcription level in cases sensitive to its modulation.

Although the EGFR is a validated target in pancreatic cancer, as shown by the fact that erlotinib has been the first agent to increase survival when added to gemcitabine compared with gemcitabine alone (1), the improvement is modest. As expected, in our model, EGFR inhibitors had a significant activity only in a subset of pancreatic cancer tumors. This sensitivity was not modality-specific, as both monoclonal antibody–mediated receptor blockage and tyrosine kinase activity inhibition exerted similar effect. There seemed to be a ceiling of activity, as dual EGFR inhibition only modestly augmented the antitumor effect, unlike in prior reports in other models (36). This was consistent with true target dependence and effective pathway shutdown with any intervention, as long as it is directed against the relevant target.

Our working hypothesis was that the efficacy of EGFR inhibitors in this disease had to be related to an alteration or increased predominance of the targeted pathway. The reports exploring in pancreatic cancer alterations in the EGFR pathway that are known to determine the sensitivity to EGFR inhibitors in other tumor types have been uniformly negative; no *EGFR* mutations (8) and modest low-level *EGFR* amplification frequencies (9) have been communicated. Thus, we focused on gene expression and used gene GSEA, which is a tool that helps identify growth-promoting pathways in cancer (35). By GSEA analysis, the EGFR pathway was among the highest expressing of the 197 pathways, on which the 55,000 transcripts are distributed. The gene classifier built using the relevant genes in the EGFR pathway was capable of correctly predicting the susceptibility of eight additional prospective cases and then the whole cohort of 18 cases (3 as sensitive, 15 as resistant; $P < 0.001$). Interestingly, the MAPK pathway was also among the top scoring sets. This highlights the plausibility of the findings as both pathways are interconnected.

EGFR pathway components are present in some of the other differentially up-regulated sets, such as the glioma pathway. The core gene components that drove EGFR pathway activation were

Table 3. Immunohistochemistry baseline patterns identified for the cases where efficacy data were obtained

Case	EGFR	pEGFR	PMAPK	pAkt
Learning set				
198	++	+	++	+
410	++	+	++	+
286	++	−	++	−
140	−	−	+	−
163	+	−	−	−
287	+	−	+	−
253	+	−	+	−
194	−	−	+	−
265	++	−	++	+
215	+	−	++	−
Validation set				
219	+	−	++	+
294	−	−	++	−
281	+	−	+	−
247	+	−	++	+
185	++	−	++	−
159	++	−	+	−
354	−	−	++	+
420	++	−	++	−

NOTE: Sensitive cases are shown in bold.

ligands and positive effectors, indicating an activating effect. Products from some of these genes (Akt, MAPK) were shown to have increased activation by protein analysis. Higher pathway activation by immunohistochemistry was linked with higher activity, but the reverse was not true, as the presence of protein activation did not necessarily predict an antitumor effect. This dicotomy of EGFR pathway overexpression at the mRNA and protein levels may imply that, whereas a state of EGFR pathway activation can exist due to transactivation of proteins by other transducers (and thus can be considered a secondary activation), only when this activation is the result of an increased gene expression does it indicate a primary or driving alteration. It can be hypothesized that in pancreatic cancer only primary activation states, i.e., those that start at the transcription level, will be effectively tackled by a specific anti-EGFR pharmacologic intervention. In breast cancer, initial selection strategies for trastuzumab treatment were based on protein overexpression (33), but *HER2* gene amplification has shown to be at least as good a predictor (37), and the debate is ongoing with authors advocating simplified algorithms where the primary genetic abnormality is tested up-front (38). Considering that many of the components of the EGFR signature lie downstream of other receptors, such as HER2 testing, their inhibition in the sensitive cases (either with a dual EGFR/HER2 inhibitor or with a HER2-specific compound) seems warranted.

We do not know the relative importance of each of the genes and if there are intrinsic markers that could summarized the signature. It is of interest to note that the EGFR pathway specifically predicted the response to EGFR inhibitors, as it was not predictive of response to gemcitabine and CI1040, a cytotoxic drug commonly used in pancreatic cancer treatment and a signal transduction (MEK) inhibitor. Also the correlation with an mTOR inhibitor was not substantial despite the presence of overlap between pathways. The observation that a stronger pathway association existed in targeted versus cytotoxic agents is relevant, as it supports the notion that sensitivity to the former is related to pathway expression patterns.

After analyzing these EGFR markers that are relevant in other diseases, no individual feature or alteration reliably identified the sensitive tumors to EGFR inhibition. Mutation in the *KRAS* gene, an almost universal finding in pancreatic cancer, is unlikely to be a resistant mechanism in this disease, as opposed to lung or colorectal cancer. Otherwise, the positive outcome of the pivotal trial is difficult to explain. In this work, two of the sensitive tumors were in fact *KRAS* mutant, indicating that having a mutation in *KRAS* does not necessarily preclude EGFR having a prominent role in maintaining the tumor phenotype and growth. In fact, this platform may be an ideal candidate to test the hypothesis that even after KRAS inhibition EGFR signaling independent of KRAS may drive cancer growth. This constitutes one of the future work items once a validated KRAS inhibitor becomes available. MLPA analysis indicated that the sensitive tumors had gains in pathway-related genes, such as *NRAS, PIK3CA*, and *Akt1*, but this was not a specific profile, and resistant cases presented identical patterns. We are uncertain as to the reasons of the lack of correlation between *EGFR* and *HER2* FISH and MLPA results in our samples and can include tumor heterogeneity and differences in signal/noise ratio, considering the tumor selectivity of FISH assessment. The EGFR pathway was not present in the top scoring pathways of the cases with neither mutations nor increased copy number/dosage, suggesting that none of these individual genetic abnormalities was responsible for the observed pattern. Altogether, this suggests that the mechanistic basis for higher pathway gene expression may not be related to a single genetic alteration.

For this work, we took advantage of the PancXenoBank, a collection of individual pancreas cancer tumors obtained from patients with pancreatic cancer (19). Generally, before entering clinical trials, new agents are tested against high-passage cell lines and typically a few xenografts established from these lines. It is unclear how representative those models are of the biology of pancreatic cancer, in view of the historic disconnection between preclinical and clinical results in this disease. We have shown that directly xenografted tumors retain the key features of the originator tumor, represent the heterogeneity of the disease, are easily amenable to treatment with different drugs, and offer endless source to tumors for complex biological studies (39). Indeed, in this study, we were able to conduct a large set of complex biological studies, as well as compare the activity of different agents against each individual tumor. Whereas, obviously, clinical specimens and clinical response data are more valuable, the detailed biological and therapeutic assessment conducted in this work is not possible in the clinical setting, as patients are not treated with more than two or three drugs, and available tissues are not adequate in quantity and quality for broad biological testing. We propose that this platform is useful for screening purposes, and best candidate selection after that can be tested in focused clinical studies.

Gene expression analysis has shown promise to characterize cancer, as primary genetic alterations prompting or maintaining a cancer phenotype ultimately manifested by differential expression of genes required to sustain such a state. Whereas proteomic assessment may be considered the ultimate step in these processes, current technology has not produced proteomic tools ready for use

in a clinical scenario. Recently, a gene expression platform derived from global unbiased testing received regulatory approval for risk prognostication for breast cancer (14, 15). The potential applicability of the presented findings is that the core EGFR signature could be readily incorporated to a quantitative tool, and this could be explored in the context of a clinical trial. If successful, this would represent avoiding unnecessary toxicities from inefficacious treatments and a step forward in the individualization of anticancer care.

In summary, EGFR inhibition showed activity in a subset of cases from a direct xenograft pancreatic cancer platform. This subset was characterized by EGFR pathway up-regulation by gene expression. The EGFR pathway activation only predicted response to EGFR inhibitors and not to other agents. The data suggest the presence of a global pathway activation. These results can be readily applied to clinical trials with EGFR inhibitors in pancreatic cancer and provide a framework to explore biomarkers of drug activity in this disease.

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