Dual mitogen-activated protein kinase and epidermal growth factor receptor inhibition in biliary and pancreatic cancer

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Abstract
This study aimed to develop rational combinations of targeted agents against biliary and pancreatic cancers. To this end, we compared the global gene expression profile of biliary cancer cell lines with different degrees of sensibility to the epidermal growth factor receptor tyrosine kinase inhibitors gefitinib and erlotinib using the Affymetrix U133A microarray platform. A set of 32 genes, including genes involved in signal transduction pathways, cell cycle regulation, and angiogenesis, was highly overexpressed in resistant cells. Five of these genes encoded proteins in the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, a finding that was confirmed by Western blot and immunohistochemistry. Gefitinib failed to inhibit the MAPK pathway in resistant cell lines. Based on these data, we explored the activity of dual treatment with gefitinib in combination with CI-1040, a MAPK inhibitor. This strategy effectively resulted in inhibition of the MAPK signaling pathway and exerted antitumor effects in vitro and in vivo in tumors resistant to each of the agents alone. To further confirm these results, we tested the combined treatment in four tumor xenografts generated from patients with resected pancreatic cancer. Combined treatment was more effective than either single agent alone in this model. This study illustrates the value of global analysis of gene expression to rationally design combinations of mechanism-based drugs. In addition, the data support the efficacy of combined epidermal growth factor receptor and MAPK inhibitors in biliary and pancreatic cancers, providing the basis to test this combination in the clinic. [Mol Cancer Ther 2007;6(3):1079–88]

Introduction
Cancer is a complex disease characterized by multiple genetic and molecular alterations affecting cell proliferation, survival, differentiation, invasion, etc. (1). Many of these alterations represent potential targets for the development of new anticancer therapeutics. Because of the enormous biological diversity of cancer, it is unlikely that attacking only one of these targets will eliminate a malignant cell. Rather, strategic combination of agents targeted against the most critical of those alterations may be needed for optimal therapeutic effect. This concept that has been empirically used for decades to develop combination regimens of cytotoxic drugs is probably applicable to biological targeted agents as well.

The epidermal growth factor receptor (EGFR) was proposed as a rational target for drug development more than 20 years ago. EGFR is a transmembrane glycoprotein that plays a crucial role in the intracellular transduction of environmental variations and the maintenance of cellular homeostasis. This receptor is dysregulated in the vast majority of human tumors of epithelial origin and is implicated in multiple cancer processes, such as cell proliferation, invasion, and survival (2). Dysregulation in the EGFR has been associated with advanced stages of disease, worse prognosis, and development of resistance to cancer treatments (3–6). The EGFR is a validated target for cancer treatment. Both monoclonal antibodies against the extracellular domain of the receptor as well as small-molecule inhibitors of the EGFR tyrosine kinase have been developed, tested in clinical trials, and approved for cancer treatment. However, single-agent response rates with these drugs are small and range from 5% to 15% (7–9). Considerable interest exists to investigate strategies that will result in better therapeutic responses. Preclinical studies suggest that factors, such as the expression of the EGFR and related members of the EGF family of receptors, activating ligands, and downstream signaling pathways, could be important factors determining the response to treatments with EGFR inhibitors (10–13). Because of the large number of new drugs being developed at the present time, methods to rationally, rather than empirically, derive combinations more likely to be efficacious are needed (9).

Cancer is a genetic disease and much of the variability in response to EGFR inhibitors is due to genetic factors (14). The development of global gene expression technologies
has enabled the generation of large-scale expression profiles of human tumors. These analyses have been used to develop disease classifications and prognostic groups and, more recently, to establish groups of patients who may be more likely to respond to certain treatment interventions (15, 16). This study aimed to develop a rational EGFR inhibitor-based combination of targeted agents capable of exerting antitumor effects in tumors resistant to EGFR tyrosine kinase inhibition using class comparison analysis of global gene expression profile in cells with different degree of susceptibility of EGFR inhibition.

Materials and Methods

Tumor Cell Lines

A panel of seven human biliary tract cancer cell lines was used for this study. The cell lines included the following: EGI-1 and TFK-1 obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), HuCCT-1 obtained from the Health Science Research Resources Bank (Osaka, Japan), SNU-308 and SNU-1079 obtained from the Korean Cell Line Bank (Seoul, Korea), and GBD-1 and GBH-3 obtained from the Saga University (Saga, Japan; refs. 17–20). SNU-308, GBD-1, and GBH-3 were derived from gallbladder carcinoma; HuCCT-1 and SNU-1079 were derived from intrahepatic cholangiocarcinomas; and TFK-1 and EGI-1 were derived from extrahepatic biliary cancers. All cell lines, except EGI-1, were grown as monolayer culture in RPMI 1640 (Life Technologies, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum and 50 IU/mL of penicillin/streptomycin. EGI-1 was grown in DMEM (Life Technologies, Invitrogen) supplemented with 10% fetal bovine serum and 50 IU/mL of penicillin/streptomycin.

Drugs

Gefitinib (ZD1839, Iressa) was provided by AstraZeneca (Wilmington, DE). CI-1040 was a kind gift from Pfizer (Ann Arbor, MI). Erlotinib (OSI774, Tarceva) was provided by OSI Pharmaceuticals (Melville, NY). Stock solutions were prepared in DMSO and stored at −20°C for the in vitro experiments. The drugs were diluted in fresh medium immediately before each experiment. In all the experiments, the final DMSO concentration was <0.1% and did not modify response of cells to the different treatments. For the in vitro experiments, gefitinib was diluted in 5% (w/v) glucose solution, erlotinib in a vehicle of 10% DMSO and 90% PBS with 0.005% pluronic, and CI-1040 was prepared in a vehicle of 10% Cremophore EL (Sigma, St. Louis, MO), 10% DMSO, and 80% water.

Cell Viability Assay

In vitro drug sensitivity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye conversion assay (21). Briefly, cells were trypsinized, seeded at 5 × 10⁴ per well in 96-well plate, and allowed to grow for 24 h before treatment with exponential increasing concentrations of drugs (gefitinib, erlotinib, CI-1040, and the combination) in the presence of 10% fetal bovine serum. After a 96-h period of treatment, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (5 mg/mL in PBS; Sigma) were added to each well and the plates were then incubated for 3 h at 37°C. The medium was then replaced with 100 μL DMSO per well. Plates were shaken and the absorbance was measured at 570 nm using a multiwell plate reader (Model 550, Bio-Rad, Inc., Hercules, CA). Each experiment was done in triplicate for each drug concentration and carried out independently at least thrice.

Western Immunoblot

Western blot analysis was done as described (22). Briefly, cells were serum starved for 18 h, treated for 24 h with the different concentrations of drugs, and subsequently harvested in standard protein lysis buffer. Equal amounts of cell extract protein (50 μg) were resolved on polyacrylamide gels. Proteins were then transferred onto nitrocellulose membranes (Osmonics, Inc., Minnetonka, MN), which were incubated overnight with antibodies against phosphorylated and total mitogen-activated protein kinase (MAPK), phosphorylated and total Akt, total EGFR (Cell Signaling Technology, Beverly, MA), and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with appropriate secondary antibodies, and the immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ).

RNA Extraction and Hybridization

Microarray hybridization was done on the Affymetrix U133A gene array, containing ~22,000 unique human transcripts. Sample preparation and processing procedure were done as described in the Affymetrix GeneChip Expression Analysis Manual (Affymetrix, Inc., Santa Clara, CA). Briefly, the RNeasy protocol for human cell lines was directly used for extraction of total RNA. The integrity of total RNA was confirmed in each case using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Using 5 to 40 μg of total RNA, double-stranded cDNA was synthesized following SuperScript Choice System (Invitrogen). T7-(dT24) oligomer was used for priming the first-strand cDNA synthesis. The resultant cDNA was purified using Phase Lock Gel and phenol/chloroform extraction and precipitated with ethanol. The cDNA pellet was collected and dissolved in appropriate volume. Using cDNA as template, cRNA was synthesized using a T7 MEGAscript in vitro transcription kit (Ambion, Austin, TX). Biotinylated 11-CTP and 16-UTP ribonucleotides (Enzo Diagnostics, Inc., Farmingdale, NY) were added to the reaction as labeling reagents. In vitro transcription reactions were carried out at 37°C for 6 h, and the labeled cRNA obtained was purified using RNeasy columns (Qiagen, Inc., Valencia, CA). The cRNA was fragmented in fragmentation buffer [40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L potassium acetate, 30 mmol/L magnesium acetate] for 35 min at 94°C. Fragmented cRNA (10–11 μg/probe array) was used to hybridize to human U133A GeneChip array at 45°C for 24 h in a hybridization oven with constant rotation (60 rpm). The chips were washed and stained using Affymetrix fluidics stations. Staining was...
done using streptavidin-phycerothrin conjugate (Molecular Probes, Eugene, OR) followed by the addition of biotinylated antibody to streptavidin (Vector Laboratories, Burlingame, CA) and finally with streptavidin-phycerothrin conjugate. Probe arrays were scanned using fluorometric scanners (Hewlett-Packard Gene Array Scanner, Hewlett-Packard Corp., Palo Alto, CA).

Data Filtering and Analysis

The .CEL file generated by the Affymetrix Microarray Suite version 5.0 was converted into .DPC files using dCHIP as described previously (23). The .DPC files were normalized, and raw gene expression data were generated using the dCHIP system of model-based analysis. For comparison of the global gene expression profiles, cell lines were grouped as “susceptible” (SNU-308, GB1-D, and GBH-3), “intermediate” (SNU-1079, TFK-1, and EGI-1), and “resistant” (HuCCT-1). Genes that were differentially overexpressed ≥3-fold in HuCCT-1 versus the sensitive three cell lines were then identified by defining the appropriate filtering criteria in the dCHIP software (mean resistant / mean susceptible >3; mean resistant / mean susceptible ≥100; P < 0.05, t test). The same procedure was followed for the comparison of the global gene expression profiles between intermediate cell lines and HuCCT-1 or between sensitive and intermediate groups.

In vivo Studies

Four- to 6-week-old female athymic (nu+/nu+) mice were purchased from Harlan Laboratories (Washington, DC). The research protocol was approved by the Johns Hopkins University Animal Care Committee. For xenografts generated from cell lines, 2 × 10^6 HuCCT-1 or GB1-D human biliary tract cancer cells resuspended in 100 μL Matrigel (Collaborative Biomedical Products, Bedford, MA) and 100 μL PBS were injected s.c. in each side of each mouse. For the heterotransplanted human pancreas xenografts, we obtained pancreatic cancer tissues from four surgical specimens from patients operated at the Johns Hopkins Hospital as reported previously (24). Briefly, nondiagnostic tumor samples from the pancreatectomy are implanted s.c. in nude mice and expanded in three consecutive generations to obtain a total of 40 mice bearing two tumors (one per flank) each. Tumors were allowed to grow until reaching ~200 mm³, at which time mice were randomized in groups of 10 tumors (5–6 mice) as follows: (a) control group, treated with vehicle i.p.; (b) 150 mg/kg/d gefitinib i.p. (biliary cell line–derived xenografts) or 50 mg/kg/d erlotinib i.p. (pancreatic xenografts); (c) 150 mg/kg CI-1040 i.p. every 12 h; or (d) the combination of gefitinib or erlotinib plus CI-1040 at the above concentrations. Mice with cell line xenografts were treated daily during 21 days. Tumors generated from human pancreas xenografts were treated daily during 28 days. Tumor size was evaluated thrice weekly by caliper measurements using the following formula: tumor volume = [length × width^2] / 2, as reported previously. Tumor growth inhibition was calculated by tumor volume of treated mice divided by tumor volume of control mice (T/C). Susceptibility was defined as T/C < 42%. Mice were monitored daily for signs of toxicity and weighed thrice weekly. One tumor was removed in each group on days 1 and 7 to do biochemical and immunohistochemical analysis.

Ras and EGFR Mutation

Mutations of the K-Ras oncogene were determined as previously described by sequencing of exon 1 (25). Exons 18, 19, and 20 of the EGFR were sequenced as described to search for EGFR mutations (26, 27).

Cell Line and Tissue Preparation for Immunohistochemistry

Immunohistochemical analysis was done on tissue arrays of cell line pellets and blocks from tumor xenografts. Tissue arrays were constructed using a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD). To generate cell line pellets, cells were suspended in their respective growth medium, spun at 5,000 rpm for 3 min, washed in PBS, and spun at 10,000 rpm for 10 min. Pellets were preserved in formalin and paraffin embedded for placement into a tissue microarray. Xenografted tissues were harvested and fixed in formalin for 24 h. Then, they were paraffin embedded and 5-μm sections were cut onto positively charged glass slides for immunohistochemistry. Immunohistochemical analysis was done to validate the differential expression of selected genes as well as to determine the pharmacodynamic effects of the drug in the targeted pathway. For immunohistochemical staining, slides were deparaffinized and rehydrated in graded concentrations of alcohol by standard techniques before antigen retrieval in citrate buffer (pH 6.0) for 20 min. Next, the slides were cooled for 20 min before washing in 1 × TBS-Tween 20 (DAKO Corp. Carpinteria, CA). All staining was done using a DAKO Autostainer at room temperature. Slides were incubated in 3% H₂O₂ for 10 min followed by the appropriate dilution of primary antibody for 60 min. The primary antibodies used for immunohistochemistry were the following: EGFR (Zymed, South San Francisco, CA) diluted at 1:50, phosphorylated EGFR (Calbiochem, San Diego, CA) at 1:25, Akt (Santa Cruz Biotechnology) at the appropriate dilution of primary antibody for 60 min. The primary antibodies used for immunohistochemistry were the following: EGFR (Zymed, South San Francisco, CA) diluted at 1:50, phosphorylated Akt (Cell Signaling Technology) at 1:50, MAPK (Th202/Tyr204; E10) monoclonal antibody (Cell Signaling Technology) at 1:50. Staining was developed using the DAKO LSAB+ System (DAKO) using the following conditions: biotinylated link for 10 min, streptavidin for 10 min, and 3,3′-diaminobenzidine solution (DAKO Liquid DAB+ Substrate Chromogen System) for 5 min. Slides were washed using 1 × TBS-Tween 20 after incubation with each reagent and with distilled water following incubation with 3,3′-diaminobenzidine. Both the intensity (0, 1+, 2+, 3+) and the percentage (0–100%) of cells positive were considered. For statistical analyses, an index of intensity × percentage was calculated.

Automated Scoring of Samples

Immunohistochemical slides from the tissue microarray were scored using the ChromaVision ACIS II System
(ChromaVision Medical Systems, Inc., San Juan Capistrano, CA) as reported previously (28). The ACIS II system (version 2.3.3) used for the analysis consists of a computer-controlled bright-field microscope coupled to a CCD camera capable of simultaneously detecting levels of hue (color), saturation (density), and luminosity (darkness). The learn-by-example tool in the ACIS software package was used to train the system to separately recognize brown pixels (immunoreactivity) and blue pixels (counter stain). For homogeneous samples, such as cell line pellets, ACIS II scoring was done at $10^5$ using an overlay grid technique included in the software in which each sample on the array is analyzed in totality and scored. Scoring was done directly by or under the supervision of a gastrointestinal surgical pathologist (C.I-D.).

### Results

#### Differential Effects of Treatment with EGFR Inhibitors on Cell Growth in the Panel of Biliary Tract Cancer Cell Lines

We first tested the susceptibility to gefitinib and erlotinib at concentrations ranging from 0.1 to 10 μmol/L in a panel of seven human biliary tract cancer cell lines using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cells exhibited different sensibilities to the drugs, with SNU-308, GBH-3, and GBD-1 being sensitive to both such treatments (IC$_{50}$ <1 μmol/L), and the rest of cell lines showing a variable degree of resistance, which was high for HuCCT-1 (IC$_{50}$ not reached) and intermediate for the other three (Table 1). To explore whether differences in susceptibility could be related to variations in the amount of EGFR, we did an ELISA in the panel of cell lines and did not find differences in EGFR expression between sensitive and resistant cell lines (data not shown). Furthermore, none of these cells had mutations in the EGFR gene that could explain the differences observed.

#### Differential Global Gene Expression Profile in Resistant Cell Line

Human oligonucleotide (Affymetrix U133A) cDNA microarrays containing individual cDNAs were hybridized with cDNAs prepared from all seven biliary tract cancer cell lines. We then compared the global gene expression patterns of these biliary tract cancer to search for genes that might provide insights into the response of these cells to EGFR inhibition. Table 2 lists genes found to be up-regulated at least 3-fold in the resistant cell line HuCCT-1 compared with the susceptible and intermediate susceptible cells. A total of 32 genes reflecting a diversity of function, such as cell cycle control (cyclin-dependent kinase 2), photolytic processing (matrix metalloproteinase-2), growth control (insulin-like growth factor-I), angiogenesis (vascular endothelial growth factor C), and mitogenic activity (MAPK kinase 2 and MAPK3), was identified as being of interest. The complete list of genes found to be up-regulated can be found as Supplementary File 1.

#### Pathway Profiling in Biliary Cancer Cell Lines

Five of the genes overexpressed in the resistant cell line coded proteins of the Ras-Raf-MAPK pathway (MAPK kinase 2, MAPK kinase 12, MAPK-activated protein kinase 2, MAPK-activated protein kinase 3, and v-raf-murine leukemia viral oncogene homologue 1). Because MAPK pathway is one of the main downstream mediators of EGFR signaling, these results suggest that the differences observed in this pathway might be responsible, at least in part, of the resistance to EGFR tyrosine kinase inhibitors in these cell lines. To evaluate this hypothesis, we tested whether the EGFR and/or MAPK pathways were differentially expressed and/or activated. We used a cell line tissue microarray constructed with pellets of each of the cell lines at baseline and stained with relevant antibodies (Fig. 1A). Sensitive cell lines presented less MAPK activation, but phosphorylated Akt, phosphorylated EGFR, and EGFR levels showed no apparent correlation with sensitivity to EGFR inhibitors.

Then, we tested the profile and dynamics of the MAPK pathway before and after treatment in a resistant cell line

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Table 1. Sensitivity of biliary cell lines to EGFR inhibitors (gefitinib and erlotinib) and a MAPK/ERK kinase inhibitor (CI-1040)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Gefitinib 1 μmol/L (%)</th>
<th>Gefitinib 10 μmol/L (%)</th>
<th>Erlotinib 1 μmol/L (%)</th>
<th>Erlotinib 10 μmol/L (%)</th>
<th>CI-1040 1 μmol/L (%)</th>
<th>CI-1040 10 μmol/L (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuCCT-1</td>
<td>81</td>
<td>78</td>
<td>101</td>
<td>96</td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td>SNU-1079</td>
<td>76</td>
<td>55</td>
<td>66</td>
<td>52</td>
<td>78</td>
<td>63</td>
</tr>
<tr>
<td>TFK-1</td>
<td>69</td>
<td>45</td>
<td>64</td>
<td>55</td>
<td>97</td>
<td>78</td>
</tr>
<tr>
<td>EGI-1</td>
<td>47</td>
<td>42</td>
<td>90</td>
<td>60</td>
<td>65</td>
<td>62</td>
</tr>
<tr>
<td>GBD-1</td>
<td>43</td>
<td>44</td>
<td>90</td>
<td>40</td>
<td>64</td>
<td>53</td>
</tr>
<tr>
<td>GBH-3</td>
<td>29</td>
<td>17</td>
<td>45</td>
<td>30</td>
<td>80</td>
<td>77</td>
</tr>
<tr>
<td>SNU-308</td>
<td>32</td>
<td>13</td>
<td>35</td>
<td>21</td>
<td>85</td>
<td>58</td>
</tr>
</tbody>
</table>

**NOTE:** Growth is expressed as percentage normalized to an untreated control. Both EGFR inhibitors showed consistency in their antiproliferative effect. HuCCT-1 showed a high level of resistance to both EGFR inhibitors, and GBD-1, GBH-3, and SNU-308 had IC$_{50}$ <1 μmol/L for both agents. The rest of cell lines showed an intermediate behavior. CI-1040 did not show significant antitumor activity as a single agent.

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4 Supplementary material for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
### Table 2. Variation in expression of selected, relevant genes in resistant versus susceptible and of intermediate susceptibility cells

<table>
<thead>
<tr>
<th>Affitag no.</th>
<th>Gene name</th>
<th>Fold change vs susceptible</th>
<th>Fold change vs intermediate</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>204854_at</td>
<td>CD44 antigen (homing function and Indian blood group system)</td>
<td>6.7</td>
<td>10.25</td>
<td>Cell surface antigen</td>
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<td>205098_at</td>
<td>Chemokine (C-C motif) receptor 1</td>
<td>18.3</td>
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<td>Cell receptor</td>
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<tr>
<td>211804_s_at</td>
<td>Cyclin-dependent kinase 2</td>
<td>3.5</td>
<td></td>
<td>Cell cycle regulator</td>
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<tr>
<td>204422_s_at</td>
<td>Fibroblast growth factor 2 (basic)</td>
<td>5.5</td>
<td></td>
<td>Growth factor</td>
</tr>
<tr>
<td>205110_s_at</td>
<td>Fibroblast growth factor 13</td>
<td>5.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>210973_s_at</td>
<td>Fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)</td>
<td>10.8</td>
<td>6.3</td>
<td>Growth factor receptor</td>
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<tr>
<td>210495_x_at</td>
<td>Fibronectin 1</td>
<td>8.8</td>
<td>14.0</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>210735_at</td>
<td>Hepatocyte growth factor (hepatopoietin A; scatter factor)</td>
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<td>Growth factor</td>
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<tr>
<td>201393_s_at</td>
<td>Insulin-like growth factor 2 receptor</td>
<td>5.7</td>
<td>3.6</td>
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<td>205302_at</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>32.4</td>
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<td>Insulin-like growth factor binding protein 3</td>
<td>149.0</td>
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<td>Insulin-like growth factor binding protein 7</td>
<td>24.0</td>
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<tr>
<td>201601_x_at</td>
<td>IFN-induced transmembrane protein 1 (9-27)</td>
<td>283.0</td>
<td></td>
<td>Transmembrane protein</td>
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<tr>
<td>218656_s_at</td>
<td>Lipoma HMGIC fusion partner</td>
<td>17.3</td>
<td>3.6</td>
<td>Fusion protein</td>
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<td>201069_at</td>
<td>Matrix metalloproteinase-2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)</td>
<td>43.0</td>
<td>57.5</td>
<td>Angiogenesis, metastasis</td>
</tr>
<tr>
<td>221762_s_at</td>
<td>Matrix metalloproteinase-9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)</td>
<td>3.7</td>
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<td>202827_s_at</td>
<td>Matrix metalloproteinase-14 (membrane inserted)</td>
<td>89.0</td>
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<td>209087_x_at</td>
<td>Melanoma cell adhesion molecule</td>
<td>6.2</td>
<td>4.8</td>
<td>Adhesion</td>
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<td>213490_s_at</td>
<td>MAPK kinase 2</td>
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<td>211688_s_at</td>
<td>Plasminogen activator, urokinase</td>
<td>23.9</td>
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<td>14.0</td>
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<td>203131_at</td>
<td>Platelet-derived growth factor receptor, α polypeptide</td>
<td>4.6</td>
<td>3.6</td>
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<td>209465_x_at</td>
<td>Pleiotrophin (heparin-binding growth factor 8, neurite growth-promoting factor 1)</td>
<td>26.7</td>
<td>46.0</td>
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<td>206631_at</td>
<td>Prostaglandin E receptor 2 (subtype EP2), 53 kDa</td>
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<td>Protein tyrosine phosphatase, receptor type, R</td>
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<td>Phosphatase receptor</td>
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<td>Rho GDP dissociation inhibitor α</td>
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<td>Adhesion</td>
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<td>212667_at</td>
<td>Secreted protein, acidic, cysteine rich (osteonectin)</td>
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<td>25.1</td>
<td>Adhesion</td>
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<td>4.5</td>
<td>Transcription factor</td>
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<td>205131_x_at</td>
<td>Stem cell growth factor; lymphocyte-secreted C-type lectin</td>
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<td>3.1</td>
<td>Growth factor</td>
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<td>210513_s_at</td>
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<td>5.8</td>
<td>15.0</td>
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<td>209946_at</td>
<td>Vascular endothelial growth factor C</td>
<td>7.7</td>
<td>5.9</td>
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NOTE: In gray, the genes that change in both comparisons.
(HuCCT-1) in comparison with two susceptible cell lines (GBD-1 and SNU-308) by Western blot studies. As shown in Fig. 1B, MAPK is constitutively activated in the resistant cell lines. Interestingly, exposure to gefitinib completely inhibited MAPK in the susceptible cell lines but not in HuCCT-1. These data indicate that the activation of MAPK is not regulated by EGFR in these resistant cell lines. It is relevant to note the correlation between the studied variables using both techniques.

**Combination of Gefitinib and CI-1040 In vitro**

To test the hypothesis that modulation of MAPK pathway could reverse the resistance to EGFR inhibitors in this model, we exposed these cells to CI-1040, a selective inhibitor of MAPK/ERK kinase that is orally active and has been tested in clinical development (29). As a single agent, CI-1040 did not inhibit growth in these cell lines, although it inhibited MAPK activation in both susceptible and resistant cells (Fig. 1A). The combination of CI-1040 with gefitinib, however, inhibited the growth of the EGFR tyrosine kinase inhibitor–resistant cell lines as shown in Fig. 1C, an effect that was paralleled by inhibition of the MAPK phosphorylation in these cells with the combined treatment.

**In vivo Studies**

To determine whether the observed results in vitro extended to the in vivo setting, we tested the antitumor and pharmacodynamic effects of the different drugs either alone or in combination in one resistant cell line (HuCCT-1) and one susceptible cell line (GBD-1) grown as xenografts in nude mice. Treatment with gefitinib or CI-1040 was ineffective in the HuCCT-1 model. Tumors from animals treated concurrently with CI-1040 and gefitinib had markedly reduced mean tumor volumes, evidencing a synergistic effect of the combination. Pharmacodynamic studies in tumors collected 6 h after treatment commencement showed that MAPK was not inhibited in tumors treated with gefitinib, and CI-1040 alone resulted in incomplete inhibition of MAPK. The combination, however, resulted in maximal target inhibition, which correlated with tumor growth inhibition (Fig. 2B). As expected based on the in vitro data, CI-1040 alone had modest efficacy in this model and the response of GBD-1 tumors to treatment with gefitinib alone (T/C index < 40%) was similar to the combined treatment (Fig. 2C). Parallel pharmacodynamic studies showed target inhibition in treatments associated with antitumor effects as in the previous model (Fig. 2D). No significant toxicity was observed in any group. Overall, the concordance seen between results in Figs. 1C and 2 validated our in vitro findings.

**Antitumor Effects in Freshly Generated Human Pancreatic Cancer Xenografts**

To confirm the results obtained with existing cell lines, we established pancreatic cancer xenografts directly from tissue obtained at the time of the surgical resection from four patients. Because of clinical considerations, erlotinib was chosen as the EGFR inhibitor of choice. All of these tumors had mutant K-Ras. As shown in Fig. 3A, these tumors are resistant to treatment with EGFR inhibition alone. Two tumors (Panc 185 and 294) had intermediate susceptibility to CI-1040. The combined treatment was effective in all four xenografts, however, an effect that was paralleled by a decreased in MAPK phosphorylation in these tumors with the combined treatment but not with erlotinib alone (Fig. 3B).
Discussion

The clinical development of highly specific targeted agents, such as inhibitors of the EGFR for cancer treatment, is complicated by the fact that these agents are predicted to work only in a subpopulation of patients whose tumor growth and/or survival are driven either by the target or target-dependent processes. This concept has been shown with several new drugs, such as imatinib mesylate (Gleevec, Novartis, Basel, Switzerland) in the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors (30–32), anti-erbB2 monoclonal antibody trastuzumab (Herceptin, Genentech, San Francisco, CA) in erbB2-overexpressing breast tumors (26, 27), or, more recently, gefitinib and erlotinib in tumors with somatic mutations of the EGFR gene (33, 34). However, most cancers have multiple dysfunctional pathways and accumulate new oncogenic mutations as they progress. One potential strategy to overcome this problem is to combine drugs against several relevant targets. Currently, it is estimated that more than 1,800 new drugs are being studied for cancer treatment, suggesting that the possible combination of regimens is endless and that strategies to define such combinations are needed (35).

Global analysis of gene expression permits the interrogation of thousand of genes and is being used for molecular classification, prognostication, and prediction of response to treatments and for target discovery in oncology (36–40). This study aimed to use global gene expression profile to rationally develop combinations of targeted agents with antitumor effects in EGFR tyrosine kinase inhibitor–resistant models. We have compared the global gene expression of cells with different susceptibility to gefitinib and erlotinib, two specific inhibitors of the EGFR. The results show that this approach permitted the identification of candidate genes that are differentially expressed in resistant cells compared with cells susceptible to the drugs. This strategy allowed the selection of potential targets for combination studies. Specifically, we found that genes in the MAPK pathway are overexpressed in an EGFR tyrosine kinase inhibitor–resistant cell line and that targeting both the EGFR and the MAPK pathway results in antitumor effects in vitro and in vivo. It is important to mention that, although the work presented here was focused on EGFR tyrosine kinase inhibitor–resistant models, this strategy can certainly be applied to other drugs and therapeutic interventions.

One of the limitations of using global gene expression profile methods is that it can result in a very large number of genes being differentially expressed. In this study, we limited the number of relevant genes to 32 by focusing on...
cells with very different response to the drug and similar levels of wild-type EGFR expression and selecting genes up-regulated >3-fold with a significant P value. The genes identified were genes involved in very specific cellular functions, such as growth factor-growth factor receptor interactions, signal transduction, angiogenesis, and cell cycle regulation, which represent potential pathways to escape from EGFR blockade. Preliminary reports from similar studies in other tumor types that compare the gene expression in cells, in which resistance to the drug has been induced by prolonged drug exposure, have actually identified some of the genes here described, such as the platelet-derived growth factor receptor and fibroblast growth factor receptor genes (41). It is possible that this list of relevant genes can be further reduced by expanding the number of cell lines tested using more strict criteria to select the key genes, excluding inducible genes, by combining the expression profiles before and after exposure to the drug, and by combining data from cell with intrinsic and acquired resistance. These strategies may help not only to reduce number of targets identified but also to recognize the ones more likely to be relevant.

We elected to focus the subsequent work in genes involve in the MAPK signaling pathway. Several reasons contributed to that decision, including (a) 5 of the 32 up-regulated genes coded for proteins involved in this pathway; (b) physiologically, the MAPK pathway is one of the principal pathways activated by the EGFR and the notion that autonomous up-regulation of a downstream pathway is able to abort the effects of inhibition of an upstream receptor is logical; (c) recent studies have shown that persistent activation of this pathway is associated with resistance to gefitinib (42–44); and (d) inhibitors of the MAPK are currently in clinical development, increasing the possibilities that these findings can be rapidly translated to the clinic (45).

Resistant cell lines were characterized by constitutive activation of the MAPK pathway, which was not abrogated by EGFR inhibition. In contrast, susceptible cell lines did show inhibition of the MAPK pathway after treatment. No differences were observed in the modulation of Akt phosphorylation, the other main EGFR downstream pathway. These findings have also been observed in studies analyzing the susceptibility of EGFR-mutated cell lines to gefitinib. Although EGF-induced signaling by mutant receptors shows selective activation of downstream effectors via Akt and signal transducers and activators of transcription pathways compared with the wild-type cells, the principal differential effects of gefitinib in these cell lines were the ability of the drug to inhibit the MAPK and signal transducers and activators of transcription signaling pathways (46). This is similar to data published by Paez et al. (33) in which gefitinib inhibited the MAPK pathway in lung cancer cell line susceptible to the drug but not in resistant ones. More recently, clinical studies have linked the presence of K-Ras mutations that result in activation of MAPK as a determinant of resistance to EGFR inhibitors, similar to the observation made in this work (47–49). Collectively, these data suggest that the proper selection of patients for EGFR treatment will need to consider not only the biological features of the tumor before treatment but also the ability of the drug to inhibit the targeted pathway. Thus, incorporation of pharmacodynamic studies remains critical for proper development and administration of these agents.

From a practical perspective, the most important observation of this work is that dual targeting of the EGFR and MAPK pathway is associated with antitumoral effects in tumors with up-regulation and activation of both pathways that are resistant to each intervention separately. Similar observations have been made in other models in cells with mutations in the PTEN gene and the constitutive activation of the phosphatidylinositol 3-kinase/Akt pathway, supporting the logical notion that activation of downstream pathways aborts the antitumor effects of drugs directed to upstream targets (50, 51). We have
extended our observation not only to homogeneous cell lines but also to human pancreas xenografts with heterogeneous cell population with closer similarity to clinical tumors. This finding provides the basis to conduct phase I and phase II clinical studies of EGFR inhibitors in combination with inhibitors of the MAPK, which we are in the process of initiating. Furthermore, the data suggest that this strategy will likely be effective in tumors with expression of activated MAPK providing the basis to restrict the study to patients whose tumors display the feature in which the combined treatment more likely to be effective. In addition, because the preclinical data suggest that combined treatment is associated with inhibition of both the EGFR and MAPK provides also the basis to incorporate measurement of these markers as pharmacodynamic correlative end points in such a clinical trial.

Although the results presented here support the importance of the MAPK pathway and show that combined targeting of EGFR and MAPK results in antitumor effect in vitro and in vivo, we did not explore whether targeting some of the other genes and possibly more the two genes is also able to induce an antitumor effect. Some of them, such as the fibroblast growth factor receptor and the platelet-derived growth factor receptor, are certainly drug candidates for which inhibitors exist and are or will be soon clinically available. Strategies are therefore needed to make such selections. The first step is the validation of the expression of the gene by PCR and the coded protein by Western blot and/or immunohistochemistry so that genes which overexpression is not confirmed can be ignored. A second strategy, which is currently possible and very attractive for this purpose, is the use of RNA interference techniques to silence the expression of one or combinations of several genes and determine silencing of which genes result in reversal of resistance to the agent (52). Subsequent steps can be followed to test whether pharmacologic interventions targeting the genes identified in the small interfering RNA screen are associated with antitumor effects and can be selected for clinical studies.

In summary, the results from this article show the practical application of global gene expression profiling of tumors to rationally define combination of targeted agents. By comparing the gene expression profile of tumors with different susceptibility to EGFR tyrosine kinase inhibitors, we were able to identify a discrete set of differentially expressed genes in resistant cells, including genes in the MAPK pathway. We have shown that, in tumors susceptible to EGFR inhibition, MAPK activation is EGFR dependent. In contrast, EGFR inhibitors failed to abrogate MAPK activation in resistant tumors. Combined treatment with inhibitors of the EGFR and MAPK pathways resulted in marked antitumor effects. The data suggest that inhibitors of the EGFR pathway should be tested in combination with inhibitors of the MAPK, especially in tumors with heightened activation of the MAPK, which would likely be unresponsive to EGFR inhibition alone.

References


Molecular Cancer Therapeutics

Dual mitogen-activated protein kinase and epidermal growth factor receptor inhibition in biliary and pancreatic cancer

Antonio Jimeno, Belen Rubio-Viqueira, Maria L. Amador, et al.


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