Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration

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Abstract
One specific limitation to the clinical development of targeted cancer therapeutics is the lack of well-validated pharmacodynamic markers. Such tools might conceivably provide a framework within which to better evaluate the selection of specific molecules as therapeutic targets. Nevertheless, the practical application of this hypothesis in clinical development remains elusive. In this study, we present a minimally invasive pharmacodynamic assay for monitoring therapy-mediated changes in the activity of target signaling pathways by using fine needle aspiration (FNA) samples and quantitative ELISA methods. To this end, we used the HuCCT-1 cholangiocarcinoma cell line treated with gefitinib (ZD1839, Iressa), a selective blocker of the epidermal growth factor receptor (EGFR), and CI-1040, a selective inhibitor of the mitogen extracellular regulated kinase [mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase 1/2]. HuCCT-1 cells were resistant to gefitinib and CI-1040 alone but susceptible to the combination of these drugs in vitro and in vivo. This effect was associated with a greater inhibition of ERK1/2 activation, a downstream mediator in the EGFR–mitogen-activated protein/ERK kinase pathway. Using this model, we sought to assess whether FNA-obtained tumor biopsies could be used to measure signaling pathway activation. Cellular extracts prepared from FNA samples yielded adequately cellular, high-quality samples to assess therapy-mediated changes in EGFR and ERK1/2 phosphorylation by Western blotting and quantitative ELISA assays. Treatment with gefitinib alone effectively inhibited EGFR activation but failed to block ERK1/2 phosphorylation and tumor growth. Blocking was achieved by the addition of CI-1040 to the treatment regimen. These results show that the combination of serial FNA sampling with highly sensitive quantitative ELISA assays permits assessment of therapy-mediated changes in signaling pathways, which correlate well with antitumor effects. This assay is simple to implement and broadly applicable to diverse tumor types in clinical studies with cancer patients and may be useful in the development of targeted anticancer agents. [Mol Cancer Ther 2006;5(7):1895–903]

Introduction
The significant advances in molecular biology of cancer have led to the elucidation of a large number of novel molecular targets in almost every pathway important for cancer development, progression, and dissemination. This has been followed by the discovery of a large number of novel anticancer agents, many of which are very specific at inhibiting cancer-relevant targets (1, 2). One of such class of targets is protein kinases. Studies have shown that protein kinases are frequently mutated in cancer and play an important role in the genesis, maintenance, and progression of such cancers (3). Tumors with mutated kinases are thought to be dependent on such kinases so that their pharmacologic inhibition results in antitumor effects (4). This is illustrated by the success of imatinib mesylate in patients with chronic myelogenous mutations and gastrointestinal stromal tumors and, more recently, epidermal growth factor (EGF) receptor (EGFR) inhibitors in patients with activating mutations in the EGFR gene (5–7).

It thus seems that targeted anticancer agents are more likely to be active in tumors in which the targeted pathway is relevant and the drugs are administered at doses and schedules that result in adequate and sustained target inhibition (2, 8, 9). There are at least two key components in this statement. One is the selection of tumors in which the target is important for tumor growth. This is illustrated by studies with trastuzumab in Her-2-positive breast cancer, with imatinib mesylate in chronic myelogenous leukemia and gastrointestinal stromal tumors with bcr/abl translocation and c-kit mutations, respectively, with gefitinib in the treatment of non–small cell lung cancer patients with EGFR mutation and perhaps with cetuximab in tumors with amplification of the EGFR gene (5, 6, 10–12). A second equally important question is whether the drug inhibits the...
putative target. It is logical that for specific agents to work, the specific target needs to be inhibited. Indeed, studies with gefitinib in EGFR-mutated cancers support the notion that these tumors are more sensitive to gefitinib because the mutant kinase is more susceptible to the agent and is inhibited at concentrations that are achievable in patients (6). Pharmacodynamic studies have the potential to be useful to prove the mechanism of action of the agent in patients, guide the dose and selection process, and eventually therapeutic monitoring. Thus far, most anticancer agents have been developed with a classic, toxicity-driven approach. Attempts to implement pharmacodynamic-driven drug development approaches have been limited by issues related to tissue collection, preservation, and assay development and validation. In fact, thus far, none of these studies have had a significant effect in the development of targeted agents (8, 13).

In this study, we aimed to show that fine needle aspiration (FNA)–obtained material is a suitable platform to assess the pharmacodynamics of anticancer agents. FNA is a minimally invasive, cost-effective, and reliable routine diagnostic procedure to diagnose neoplastic lesions. In most cases, microscopic evaluation of air-dried (AD), Diff-Quik (DQ)–stained cytologic slides prepared from tumor FNA samples can render an immediate assessment of specimen adequacy or diagnosis (14–17). The study also aimed to develop a quantitative ELISA method that can be used to assess signaling pathway inhibition cellular extracts prepared from tumor FNA samples.

Materials and Methods

Drugs

Gefitinib was provided by AstraZeneca (Wilmington, DE) and CI-1040 was provided by Pfizer (Ann Arbor, MI). Stock solutions were prepared in DMSO and stored at −20°C. For in vitro studies, gefitinib was diluted in 5% (w/v) glucose solution, and CI-1040 was prepared in a vehicle of 10% Cremophore EL (Sigma, St. Louis, MO), 10% ethanol, and 80% water.

In vitro Studies

HuCCT-1 cells were maintained in DMEM supplemented with 10% FCS and 1% antibiotics (Life Technologies, Inc., Gaithersburg, MD). When 50% to 60% confluency was reached, they were serum starved overnight, after which they were exposed to (a) serum-free medium for 45 minutes, (b) serum-free medium for 30 minutes followed by EGF (100 ng/mL; Sigma) for 15 minutes, (c) CI-1040 (10 μM) for 30 minutes followed by CI-1040 plus EGF for 15 minutes, (d) gefitinib (10 μM) for 30 minutes followed by gefitinib plus EGF for 15 minutes, and (e) the combination of CI-1040 (10 μM) plus gefitinib (10 μM) for 30 minutes followed by CI-1040 plus gefitinib plus EGF for 15 minutes. After this, the cells were washed twice with chilled PBS, lysis buffer was added to the plates, and the cells were scraped. In vitro drug sensitivity to concentrations of gefitinib and CI-1040 ranging from 0 to 10 μM/L was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) following the instructions of the manufacturer. Briefly, cells were seeded at 5 × 104 per well in 96-well plates and grown for 24 hours before treatment with exponentially increasing concentrations of gefitinib, CI-1040, or the combination in the presence of 10% fetal bovine serum. Cell viability was assessed after 96 hours by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

DQ Cytologic Smears

HuCCT-1 collected from the different treatment groups mentioned above were smeared on glass slides. All smears were allowed to air dry and then stained with DQ stain (Baxter Healthcare, Miami, FL).

Animal 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 and animals were maintained in accordance with the guidelines of the American Association of Laboratory Animal Care. Mice were acclimatized for 1 week before injecting 2 × 106 HuCCT-1 human biliary tract cancer cells resuspended in 100 μL Matrigel (Collaborative Biomedical Products, Bedford, MA) and 100 μL PBS per mouse. After 2 weeks, when well-established tumors of ≈0.2 cm3 were detected, 10 mice each were randomly assigned to one of four groups to receive the following treatments: gefitinib, 150 mg/kg daily on days 1 to 5 and 8 to 12 administered by i.p. injection; CI-1040 (150 mg/kg) twice a day on days 1 to 14 administered by p.o. gavage; combination of gefitinib + CI-1040 at the same doses and schedule of administration; or vehicle containing 0.15 mol/L NaCl and 0.005% Pluronic. Mice were monitored daily for signs of toxicity and were weighed thrice per week. Tumor size was evaluated thrice per week by caliper measurement using the following formula: tumor volume = [length × width2]/2. Tumor growth inhibition was calculated by tumor volume of treated mice divided by tumor volume of control mice. Experiments were terminated on day 14.

FNA Technique

Fine needle aspirates were obtained from animals under general anesthesia with a 25 G needle and 10 mL syringe, passing the needle into the tumor 10 times with application of 1 to 2 mL suction. The aspirated material was expressed onto clear glass slides and smeared. Five to 10 AD/DQ–stained cytologic smears were prepared from each tumor sample. The cellular composition of each aspirate was assessed by a staff cytopathologist (S.A.) at The Johns Hopkins Hospital.

Western Blot Analysis and ELISA Assays

Protein extracts were prepared from AD/DQ–stained cytologic smear samples prepared from cell lines or tumor FNA biopsies and from in vitro cell cultures by scraping cells off the stained slides or culture plates into 100 μL cell lysis buffer (Cell Signaling Technology, Beverly, MA) in the presence of protease and phosphatase inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Cell lysates were centrifuged, protein concentrations were
measured, and cellular proteins were electrophoresed on 7% or 10% (w/v) SDS-polyacrylamide gels. After electrophoresis, transfer to Immobilon-P membranes (Millipore, Bedford, MA), membranes were blocked at room temperature using SuperBlock (Pierce, Rockford, IL) for 1 hour. The primary antibodies (Cell Signaling Technology) against phospho-EGFR, total-EGFR, phospho-extracellular signal-regulated kinase 1/2 (ERK1/2), total ERK1/2, phospho-Akt, and total Akt were diluted at 1:1,000 and the membranes were incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase–conjugated secondary antibodies, rabbit IgG-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA), or mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) at a final dilution of 1:3,000. After washing thrice with TBS [10 mmol/L Tris-HCl (pH 7.5), 0.5 mol/L NaCl, and 0.05% (v/v) Tween 20], antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography. The expression levels and phosphorylation status of EGFR and ERK1/2 proteins were analyzed on Western blot by using 15 μg of total cell lysates. The results obtained from smear samples were compared with control cell extracts. Total and phospho-ERK1/2 proteins were quantified by sandwich ELISA kits (BioSource International, Camarillo, CA) as described in the protocols of the manufacturer. The reaction was read at 450 nm in an ELISA plate reader. Studies using patient material were approved by the Joint Committee on Clinical Investigations, Johns Hopkins University School of Medicine (Baltimore, MD). Archival smear slides were reviewed by a cytopathologist (S.A.). Once the slides were selected, all patient identification numbers were erased and the slides were labeled with a reference number. After removal of coverslips, proteins were isolated off the slides as described above.

**Immunohistochemical Studies**

After harvesting, an aliquot of tissue xenograft was fixed in formalin for 24 hours and then embedded in paraffin. For immunohistochemical studies, 5 μm sections of the tissue array were deposited onto positively charged glass slides. Slides were deparaffinized and rehydrated in graded concentrations of alcohol by standard techniques before antigen retrieval in citrate buffer (pH 6.0) for 20 minutes. The slides were then cooled for 20 minutes before they were washed in 1× TBST (DAKO Corp. Carpinteria, CA). All stainings were done at room temperature using a DAKO Autostainer. Slides were incubated in 3% H2O2 for 10 minutes, followed by the primary monoclonal antibody phospho-ERK1/2 (Thr202/Tyr204; E10; Cell Signaling Technology) in 1:50 dilution for 60 minutes. Tris-HCl [0.2 mol/L (pH 7.5); Quality Biological, Inc., Gaithersburg, MD] was used as the antibody diluent solution. Negative controls were incubated for 60 minutes with the antibody diluent solution [0.2 mol/L Tris-HCl (pH 7.5); Quality Biological]. Staining was developed using the DAKO LSAB+ System (DAKO) using the following conditions: biotinylated link for 10 minutes, streptavidin for 10 minutes, and substrate-chromogen solution (3,3′-diaminobenzidine, DAKO Liquid DAB+ Substrate-Chromogen System) for 5 minutes. Slides were washed using 1× TBST after incubation with each reagent and with distilled H2O following incubation with 3,3′-diaminobenzidine.

**Statistical Analysis**

Values are expressed as mean ± SD. Where appropriate, an unpaired *t* test was conducted to analyze for differences between treatments. Statistical significance was determined at *P* < 0.05.

**Results**

**In vitro and In vivo Effects of Gefitinib and CI-1040 in HuCCT1 Cell Line**

To have a suitable platform to investigate whether FNA-based tissue sampling could be used for pharmacodynamic studies, we determined the effects of gefitinib, CI-1040, and their combination on HuCCT-1 cell growth *in vitro* by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method and *in vivo* in xenograft mice. As shown in Fig. 1A and B, these cells were resistant to gefitinib and CI-1040 used at conventional doses and schedule of administration but were markedly susceptible to their combination. Tumor growth was inhibited by ~60% with gefitinib and CI-1040 combination therapy over the 14-day treatment period. In contrast, treatment with gefitinib or CI-1040 alone caused only 4% and 11% decrease in tumor volume, respectively. To gain insight into the potential mechanisms underlying this observation, we determined the activation status of EGFR, ERK1/2, and Akt under conditions of serum starvation and stimulation with EGF in the presence and absence of drugs. Figure 1C shows that HuCCT-1 cells have constitutive activation of ERK1/2 and Akt. Gefitinib effectively blocked EGFR and Akt activation but had a minimal effect on ERK1/2 activation. CI-1040 inhibited ERK1/2 phosphorylation alone, whereas the combination of the two agents significantly blocked activation of EGFR, ERK1/2, and Akt. These results show that inhibition of ERK1/2 activity is required to achieve growth inhibition in HUCTT-1 cells. However, it is not clear whether simultaneous inhibition of EGFR and/or Akt activity is needed for antitumor effects of gefitinib in this model. Immunohistochemical assessment of ERK1/2 activation in tumor samples collected 14 days after treatment of xenograft mice showed maximum inhibition of ERK1/2 in the combined treatment group. CI-1040 alone only inhibited ERK1/2 partially *in vivo* (Fig. 1D). Overall, these data provided us with a suitable model to investigate if an FNA-based tissue collection and processing method could be used to measure therapy-induced changes in signaling pathways *in vivo*. Because ERK1/2 inhibition seems to be essential for the therapeutic response of HUCTT-1 cells to these agents, we focused subsequent efforts in measuring ERK1/2 activity in FNA samples.

**Cytologic Preparation of Tumor Cells Provides Cellular Proteins to Measured Protein Phosphorylation**

To determine whether cell extracts prepared from tumor FNA samples can be used to assess the efficacy of...
targeted therapeutics, we first tested if fixation and staining methods commonly used in the preparation of cytologic samples adversely affect detection of phosphorylation status of signaling proteins. As illustrated in Fig. 2, in control extracts prepared directly from EGF-treated HuCCT-1 cells, phosphospecific antibodies detected increased phosphorylation of EGFR and slight increase in ERK1/2 compared with EGF unstimulated cells. EGF increased phosphorylation of EGFR and slight increase in ERK1/2 in xenograft tumors obtained from mice treated as described in Fig. 1. The antiproliferative effects of gefitinib and CI-1040 against HuCCT-1 tumors. A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth inhibition curve of HuCCT-1 cell lines exposed for 96 h to gefitinib, CI-1040, or the combination. The combination regimen consisted of increased concentration of CI-1040 and 5 μmol/L gefitinib. B, in vivo response of HuCCT-1 to gefitinib and CI-1040 alone and their combination for 2 wks. Points, tumor volume; bars, SE. C, Western blot analysis of phospho-EGFR, Akt, and ERK1/2 in HuCCT-1 cells exposed to EGF alone or in combination with gefitinib, CI-1040, or their combination at maximal concentration (10 μmol/L). D, immunohistochemical analysis of phospho-ERK1/2 in xenograft tumors obtained from mice treated with the same extracts used in the ELISA assays. As shown in Fig. 3A, treatment of cells with gefitinib alone did not affect ERK activation. CI-1040 treatment, however, decreased EGF-induced ERK1/2 activation ~40%. Interestingly, the combination of gefitinib and CI-1040 dramatically decreased (95%) ERK1/2 activity. To corroborate the ELISA results, Western blot analysis was done with the same extracts used in the ELISA assays. As shown in Fig. 3B, the combination of gefitinib and CI-1040 significantly inhibited ERK1/2 activity, whereas CI-1040 blocked stimulatory effect of EGF. No effect was observed in cells treated with gefitinib alone. These results show that the use of less than one tenth of the amount of total cellular extracts required to detect ERK1/2 on Western blot is sufficient to quantitatively analyze treatment-mediated changes in the phosphorylation status of ERK1/2 in AD/DQ–stained smears. As shown in Fig. 3A, treatment of cells with gefitinib alone did not affect ERK activity. CI-1040 treatment, however, decreased EGF-induced ERK1/2 activation ~40%. Interestingly, the combination of gefitinib and CI-1040 dramatically decreased (95%) ERK1/2 activity. To corroborate the ELISA results, Western blot analysis was done with the same extracts used in the ELISA assays. As shown in Fig. 3B, the combination of gefitinib and CI-1040 significantly inhibited ERK1/2 activity, whereas CI-1040 blocked stimulatory effect of EGF. No effect was observed in cells treated with gefitinib alone. These results show that the use of less than one tenth of the amount of total cellular extracts required to detect ERK1/2 on Western blot is sufficient to quantitatively analyze treatment-mediated changes in the phosphorylation status of ERK1/2 in cytologic samples.

FNA Samples Yield Enriched Tumor Cell Populations to Study EGFR Signaling In vivo

The above results showed that AD/DQ–stained cytologic samples yield high-quality proteins to study the activity

ELISA Assays Offer Increased Sensitivity to Detect and Quantify Treatment-Mediated Changes in the Phosphorylation of ERK1/2 Proteins

We next tested the feasibility of applying quantitative ELISA assays to the cytologic samples to increase assay sensitivity to measure the expression levels and activation status of specific signaling pathways. As a model system, we used colorimetric total (recognizes proteins independent of their phosphorylation state) and phosphospecificity (recognizes only the phosphorylated (activated) state of ERK1/2) to analyze the expression and phosphorylation of ERK1/2 proteins, respectively.

We first determined the linearity of these assays by using various protein amounts (0.5–20 μg) obtained from AD/DQ–stained HuCCT-1 cytologic smears. Our results showed that protein concentrations in the range of 0.5 to 5 μg yield the most accurate and linear determination of total and phosphorylated ERK1/2 levels (data not shown). Next, we tested whether ELISA assays can detect treatment-mediated changes in the phosphorylation status of ERK1/2 in AD/DQ–stained smears. As shown in Fig. 3A, treatment of cells with gefitinib alone did not affect ERK activity. CI-1040 treatment, however, decreased EGF-induced ERK1/2 activation ~40%. Interestingly, the combination of gefitinib and CI-1040 dramatically decreased (95%) ERK1/2 activity. To corroborate the ELISA results, Western blot analysis was done with the same extracts used in the ELISA assays. As shown in Fig. 3B, the combination of gefitinib and CI-1040 significantly inhibited ERK1/2 activity, whereas CI-1040 blocked stimulatory effect of EGF. No effect was observed in cells treated with gefitinib alone. These results show that the use of less than one tenth of the amount of total cellular extracts required to detect ERK1/2 on Western blot is sufficient to quantitatively analyze treatment-mediated changes in the phosphorylation status of ERK1/2 in cytologic samples.

Figure 1. The antiproliferative effects of gefitinib and CI-1040 against HuCCT-1 tumors. A, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth inhibition curve of HuCCT-1 cell lines exposed for 96 h to gefitinib, CI-1040, or the combination. The combination regimen consisted of increased concentration of CI-1040 and 5 μmol/L gefitinib. B, in vivo response of HuCCT-1 to gefitinib and CI-1040 alone and their combination for 2 wks. Points, tumor volume; bars, SE. C, Western blot analysis of phospho-EGFR, Akt, and ERK1/2 in HuCCT-1 cells exposed to EGF alone or in combination with gefitinib, CI-1040, or their combination at maximal concentration (10 μmol/L). D, immunohistochemical analysis of phospho-ERK1/2 in xenograft tumors obtained from mice treated as described in B (day 14).
of signal transduction pathways by determining the phosphorylation status of enzymes involved in cell growth. To explore the feasibility of implementing this method in in vivo studies, we next sought to test in mouse xenografts whether FNA material obtained from tumor tissue can be used to monitor and predict therapy response in vivo.

FNA samples were obtained from tumor tissue, and AD/DQ–stained smears were prepared. Morphologic assessment of the cytologic smears showed that, on average, 90% of the cells were neoplastic with some RBC and negligible amount of connective tissue fragments in the background (Fig. 4A). After comparison with the histologic sections of the same tumors (Fig. 4B), we concluded that FNA samples yielded adequate material to represent the composition of HuCCT-1 tumor tissue.

Tumor FNA Samples Provide Adequate Quality Proteins to Analyze Therapy-Mediated Changes in the Activity of EGFR and ERK1/2 In vivo

To determine whether tumor FNA samples can be used to assess the efficacy of therapy in vivo and to explore the molecular mechanisms of tumor resistance, we examined the steady-state levels of EGFR and ERK1/2 kinases in tumor FNA samples collected from control and drug-treated mice. Following morphologic evaluation, whole cell extracts were prepared from AD/DQ–stained tumor FNA samples, which, on average, yielded 100 μg of total cellular proteins. Figure 5A shows that EGFR and ERK1/2 were constitutively activated in the HuCCT-1 tumors as measured by immunoblotting of tumor lysates with phospho-EGFR and phospho-ERK1/2 antibodies, respectively. Samples from xenografted tumors treated with gefitinib showed complete inhibition of EGFR but not ERK1/2 activity, indicating that the elevated steady-state levels of ERK activity in HuCCT-1 cells are not sustained predominantly through activation of EGFR. Interestingly, only combination treatment with gefitinib and CI-1040 dramatically lowered the level of activation of ERK1/2 proteins, whereas treatment of animals with CI-1040 alone caused only a slight inhibition in ERK1/2 activity. No difference was observed in the protein levels of EGFR and ERK1/2 proteins between vehicle and drug-treated animals (Fig. 5A). These results correlated well with immunohistochemical data and tumor response as shown in Fig. 1.

Comparison of tumor FNA samples obtained from the same tumor before (untreated) and after the treatment with gefitinib and CI-1040 for 14 days revealed no morphologic differences (Fig. 5B). Treatment with gefitinib and/or CI-1040 did not cause necrotic or apoptotic cell death, consistent with data obtained with HuCCT cells in vitro (not shown), indicating that combination therapy had cytostatic rather than cytotoxic effect on tumor cells. These results were also confirmed by H&E staining of tumor resection specimens obtained from untreated and treated xenografted tumors (data not shown).

Serial FNA Sampling Permits Monitoring and Prediction of Treatment Response to EGFR and Mitogen-Activated Protein/ERK Kinase Inhibitors In vivo

We next tested whether FNA samples obtained from tumor tissue at the early stage of therapy can be used to predict tumor response. For this purpose, FNA was done on the tumor of the same animal before, during (6 hours and 5 days), and at the end (2 weeks) of gefitinib and/or CI-1040 therapy. Expression and phosphorylation levels of ERK1/2 were analyzed on Western blot by using extracts prepared from AD/DQ–stained FNA samples. As shown in Fig. 5C (top), as early as 6 hours after the first administration of gefitinib and CI-1040, a dramatic loss in ERK1/2 activity was observed, which was sustained over the 2-week course of treatment. Consistent with data described above, neither of these agents alone caused...
inhibition in ERK1/2 phosphorylation after 6 hours or 5 days of treatment (data not shown). These effects were not due to alteration of ERK1/2 expression in treated animals because no change was observed in total levels of ERK1/2 proteins in tumor samples obtained before and after the treatment (Fig. 5C, bottom). These data show that FNA sampling at the early stage of therapy permits prediction of tumor response in vivo.

Combination of FNA with a Quantitative ELISA Increases the Sensitivity and Accuracy to Detect Therapy-Mediated Changes in ERK1/2 Activity In vivo

Western blot analysis of protein samples is a conventional method for phosphoprotein analysis, but is limited in throughput, quantitative precision, and requires large sample amounts. ELISA assays offer alternatives to Western blot with higher throughput and increased sensitivity.

Having established above that AD cytologic samples can successfully be used in ELISA assays to analyze ERK1/2 phosphorylation in vitro, we next tested to see if this approach could be used to increase assay sensitivity and to quantify treatment-mediated changes in the phosphorylation of ERK1/2 in HuCCT-1 xenograft animals in vivo. For this purpose, the tumor FNA samples, which were analyzed on Western blot above (Fig. 5A), were used to assess the expression and phosphorylation of ERK1/2 proteins by the total and phosphospecific ERK1/2 ELISA assays, respectively.

As shown in Fig. 5D (top), treating the animals with a combination of gefitinib and CI-1040 dramatically decreased the phosphorylation levels of ERK1/2, as detected by phospho-ERK1/2 ELISA. Gefitinib treatment alone did not cause any significant change in ERK1/2 phosphorylation, whereas CI-1040 led to a slight inhibition in ERK1/2 phosphorylation. The amounts of phosphorylated ERK1/2 were normalized for the total content of these proteins in each sample group and therapy-mediated changes in their phosphorylation status were quantified. Figure 5D (bottom) illustrates that treatment of animals with combination of gefitinib and CI-1040 caused a 97% inhibition of ERK1/2 phosphorylation. Gefitinib alone did not cause any significant change in ERK1/2 activity, whereas treatment with CI-1040 led to a 20% (P = 0.003) decrease in ERK1/2 activity. These results are in general consistent with the Western blot data, shown earlier (Fig. 5A), and show that the use of <1 μg of whole cell lysate is sufficient to quantitatively analyze therapy-induced changes in the enzymatic activity of ERK1/2 by ELISA in tumor FNA preparations.

Detection of ERK1/2 Activity in Archival Tumor FNA Samples

We next tested if tumor FNA samples provide adequate material to analyze the activity of signaling proteins in clinical samples. For this purpose, we used archival AD/DQ–stained tumor FNA samples previously obtained under ultrasound guidance for diagnostic purposes from three pancreatic cancer patients. After removal of coverslips, proteins were isolated off the slides and Western blot analyses were done to detect expression levels of total and phospho-ERK1/2. As shown in Fig. 6, proteins isolated from 1-year-old archival FNA smears yielded sufficient amount of cellular proteins to assess the expression and activity of ERK1/2 proteins in tumor samples obtained before and after the treatment (Fig. 5C, bottom). These data show that FNA sampling at the early stage of therapy permits prediction of tumor response in vivo.

**Figure 5.** A, tumor FNA samples detect therapy-mediated changes in the phosphorylation of EGFR and ERK1/2 in vivo. Cell lysates were prepared from AD/DQ FNA slides and protein phosphorylation (P-) and total expression (T-) levels of EGFR and ERK1/2 proteins were determined on Western blot analysis. Results were correlated with therapy-induced changes in tumor size. B, combination therapy with gefitinib and CI-1040 does not affect the morphologic features of tumor cells. Tumor FNA samples were done from the same animals before (untreated) and 14 d after treatment with gefitinib and CI-1040. AD/DQ–stained cytology smears were prepared and evaluated under light microscopy. C, early FNA sampling can predict tumor response in vivo. Protein extracts were prepared from FNA slides and phosphorylation and expression levels of ERK1/2 proteins were determined on Western blot analysis. D, detection and quantification of total and phospho-ERK1/2 in tumor FNA samples. Cell extracts were prepared from FNA smears (day 14) and analyzed with the ERK1/2 [pTyr185/187] phospho-ELISA and ERK1/2 (total) ELISA kits (top, raw data from one representative experiment; bottom, the average normalized data from three independent experiments); bars, SD. *P* values are calculated relative to tumors obtained from vehicle-treated control animals.
phosphorylation levels of ERK1/2 in these patients. These data show that tumor FNA samples can be used to detect phosphorylation of signaling proteins in vivo and indicate that FNA-based approach may allow assessment of the efficacy of targeted therapeutics in cancer patients.

Discussion

Some of the major challenges in the development of molecular targeted agents are the demonstration of target inhibition in patient samples, definition of effective doses and schedule of administration, and the prediction of which patients will more likely respond to treatment. The central hypothesis underlying these issues is that targeted agents will be more effective in patients whose tumors are dependent on that particular target and in whom the target is properly inhibited upon treatment. Therefore, proper development of targeted anticancer agents may require pharmacodynamic assays (8, 13). These studies can be relevant to prove the mechanism of action of the agent in patient tumors, to optimize dose and schedule of drug, and to predict patient outcome. The current study tested whether cytologic specimens obtained by FNA biopsies are suitable for pharmacodynamic monitoring. To this end, we used a human cholangiocarcinoma model and inhibitors of the EGFR and mitogen-activated protein/ERK kinase pathways as an experimental platform. The results show that FNA samples can be used to perform pharmacodynamic studies to assess the efficacy of gefitinib and CI-1040 in vivo. These data provide the rational to implement this methodology in clinical trials with these drugs as well as to expand it to other anticancer agents.

We based our analysis in the HuCCT-1 cholangiocarcinoma model. In this cell line, the Akt and mitogen-activated protein/ERK kinase pathways are active in serum-free conditions. Inhibition of EGFR with gefitinib abrogates serum-induced activation of the EGFR, blocks Akt, and partially inhibits ERK1/2 but is not associated with antitumor effects. We have recently shown that EGFR blockade failed to modulate EGFR-dependent genes in this cell line as well (18). Immunohistochemical analysis of Akt did not perform optimally as a surrogate end point for drug efficacy in our model system to obtain reliable results (data not shown). For this reason, we have focused our studies on ERK1/2 as the proximal end point in the signaling cascade. The specific mechanism why ERK1/2 is not modulated by EGFR inhibition in this cell line is not known. HuCCT-1 has mutant KRAS and that may explain why ERK is not inhibited by upstream blockage of EGFR (19). CI-1040, a specific mitogen-activated protein/ERK kinase 1/2 inhibitor, inhibited ERK1/2 phosphorylation in vivo but not in vitro and did not result in tumor growth inhibition (20). It should be noted that this agent has poor pharmacologic properties in vitro, which may explain why, despite being used at the recommended doses, it failed to exert pharmacodynamic effects (21). Indeed, the clinical development of CI-1040 has been halted for this reason. The combination of the two agents profoundly inhibited ERK1/2 in xenografted tumors and resulted in tumor growth inhibition. The mechanistic basis for this cooperative effect is not fully elucidated and could be linked to a greater ERK1/2 inhibition or simultaneous inhibition of Akt and ERK1/2. Additional studies using more specific methodologies, such as small interfering RNA targeting of these genes, are needed to fully elucidate the underlying mechanism of this observation. In addition, further studies should be conducted to determine if this cooperative effect are also evident in other EGFR-resistant tumors. Published studies suggest that this can indeed be the case and could support testing this combination strategy in the clinical setting. The differential response to the agents couple with differences in ERK1/2 activation, however, suggested this model could be a suitable platform to develop FNA-based pharmacodynamic monitoring.

Thus far, the implementation of pharmacodynamic studies in clinical trials with novel anticancer agents has been limited. Most studies have been based on collection of tumor core biopsies before and after treatment. Although some reports note a high success rate with this approach, in our own experience, only 50% to 60% of blind tumor biopsies contain viable cancer tissue (22). Furthermore, core biopsy and surgical resection specimens are composed of large number of heterogeneous tissue, including normal epithelial cells, fibroblasts, adipocytes, infiltrating leukocytes, and vascular components in addition to cancer cells. Therefore, protein lysates prepared from this type of specimen are not ideal to perform Western blot and ELISA assays, as they may not represent the biological features of tumor cells, and are useful only for immunohistochemical studies. In addition, these biopsies are not practical to be done in a serial manner as may be needed for real-time monitoring of pharmacodynamic effects.

In contrast, FNA is a rapid, cost-effective, and minimally invasive procedure that provides a nearly pure tumor cell population. Therefore, FNA sampling has advantages over tissue biopsy and has been found to be a viable alternative to conventional surgical biopsy to perform prognostic and experimental studies. Furthermore, FNA is well tolerated by patients and can be done on multiple occasions (14, 15, 17). The data presented here show that FNA is an ideal tissue procurement technique and

**Figure 6.** Detection of expression and phosphorylation status of ERK1/2 in tumor FNA samples prepared from archival cytologic slides of pancreatic cancer patients. Proteins were isolated from the archival AD/DQ-stained cytologic slides prepared from tumor FNA samples obtained by ultrasound guidance during diagnostic procedures. Protein levels were determined and 15 μg of whole cell extract were analyzed by Western blot.
provides suitable material to assess pharmacodynamic markers that were predictive of antitumor effects in this model. Furthermore, FNA obtained from three patients showed the feasibility of this method in the clinical studies. The results also showed that AD slides with tumor FNA samples can be held at least 1 year at room temperature before proteins are extracted for biochemical analysis (data not shown). This would allow storage of smear samples for an extended period of time for further analysis and easy shipment of samples to distant laboratories to analyze tumor response, if needed.

An important limitation of most pharmacodynamic studies is that they have relied on immunohistochemical methods to assess pathway activation. This is in part due to the need to analyze neoplastic cells in blind biopsies of tumor tissue, where tissue components are very diverse. It is well accepted that these methods are not particularly robust, in general not quantitative, and have poor reproducibility as shown in published data by the disparate results in applying this methodology. This fact is best illustrated by a close analysis of changes in EGFR signaling in skin biopsies that have shown markedly inconsistent results in studies conducted by different groups, including our own (23–26). The FNA materials can be used for Western blot analysis but also for other methods such as ELISA. The advantage of ELISA is that it needs minute quantities of proteins and is fully quantitative. Because the sample obtained is composed of pure tumor cells, there are no concerns about contamination with normal cells. This platform can also be used for other methods. We have recently shown the suitability of FNA materials to obtain RNA for PCR analysis (18). Recent early clinical trials with novel anticancer agents, including CI-1040 itself, routinely incorporate tumor biopsies for pharmacodynamic evaluations (21, 27). These studies do show the ability of the drug to inhibit their target but have not been particularly useful in the process of dose and schedule selection for the reasons mentioned above. The data presented here suggest that FNA-based tissue collection coupled with quantitative assessment of target inhibition may be a more versatile approach for pharmacodynamic studies.

An important issue in the development of novel anticancer agents is the definition of factors that may be predictive of tumor response. Studies have focused on analysis of static determinants of tumor response, such as receptor expression, gene amplification, and target mutations among others in archival tumor materials (12, 28, 29). There are important limitations with these studies, including ignoring potential changes in these factors over time and the heterogeneity of tumors with regard to these features. Studies with the EGFR illustrates this problem as there is an important variability in receptor expression from primary to metastatic tumors (29). Thus, there is accumulating evidence that the tumoral expression of the drug target may be insufficient to predict tumor response to targeted drugs, especially because the anticancer activity of specific signaling inhibitors may largely depend on alterations in signaling both upstream and downstream of their target proteins (30, 31). A given drug will be effective in tumors in which its target is not only biologically relevant but also in which the drug is able to achieve target inhibition at realistic serum concentrations. The relevance of this point is suggested by the finding that cells with EGFR mutations, shown to be more sensitive to EGFR antagonists, seem to obtain target inhibition at relatively lower drug concentrations (6). It can be proposed then that the ultimate predictor of activity where host, cancer, and drug factor converge is target inhibition. Thus, pharmacodynamic monitoring has the potential to be useful to monitor and predict patient response. To this end, tissue collection methods of low morbidity and complexity that can be applied to patient tumors in a serial manner are needed. The FNA approach presented here is indeed a potential approach to solve this problem because low morbidity and easy applicability.

In summary, we have shown that tumor FNA samples can effectively be used to assess therapy-induced changes in the expression and activity of signal transduction pathway components. Thus, this approach provides a means to perform serial FNA sampling of tumor tissue before and during therapy to monitor early tumor response, which may ultimately allow selection of optimal dose and schedule to design a patient-tailored rational therapy for cancer.

References


Assessment of gefitinib- and CI-1040-mediated changes in epidermal growth factor receptor signaling in HuCCT-1 human cholangiocarcinoma by serial fine needle aspiration

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