C-fos Assessment as a Marker of Anti–Epidermal Growth Factor Receptor Effect

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Abstract

Factors predicting sensitivity to epidermal growth factor receptor (EGFR) blockade are largely unknown and new strategies are being sought to individualize cancer therapy. This study evaluated the variation in the expression of the early response gene c-fos as a distal effect of EGFR inhibition and its relationship to antitumor effects. The growth-inhibitory and c-fos–modulating effects of gefitinib and erlotinib in human cancer cell lines (A431, CAL27, HN11, HuCCT1, and Hep2) were determined. Next, these cell lines were xenografted in mice and treated for 14 days with gefitinib (A431 and HuCCT1) or erlotinib (CAL27, HN11, and Hep2). Fine needle aspiration biopsy of tumors was done at baseline and after 14 days of therapy for c-fos assessment. In addition, we tested the feasibility of analyzing this marker in five paired tumor samples from a clinical trial of gefitinib in patients with solid tumors. In culture, gefitinib and erlotinib decreased c-fos mRNA levels in the susceptible cell lines A431, CAL27, and HN11; however, both drugs failed to achieve c-fos inhibition in resistant cells. Gefitinib or erlotinib abrogated the increase in c-fos expression in vivo in EGFR-sensitive A431, CAL27, and HN11 tumors but not in resistant strains. Ex vivo evaluation was feasible and predicted in vivo effects. The feasibility study in paired human tumor biopsies showed that this biomarker can be reliably measured in clinical materials. In summary, variations in c-fos expression reflect the pharmacologic actions of EGFR inhibitors in in vitro and in vivo models. (Cancer Res 2006; 66(4): 2385-90)

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is a membrane receptor with an extracellular domain, a single α-helix transmembrane domain, and an intracellular domain with tyrosine kinase (TK) activity. Ligand binding induces EGFR homodimerization and heterodimerization with other HER proteins, activation of TK activity, and autophosphorylation of the receptor. EGFR signaling ultimately increases proliferation, angiogenesis, metastasis, and decreases apoptosis. Gefitinib (Iressa; AstraZeneca, Wilmington, PA) and erlotinib (Tarceva; Genentech, San Francisco, CA) are quinazoline derivatives that reversibly inhibit the TK of EGFR, showing in vitro and in vivo activity in human cancer cell lines (1, 2). Despite the ubiquitous expression of the EGFR and the large number of patients treated in clinical trials with EGFR-targeted agents, the factors determining and predicting their efficacy are largely unknown. Recent reports have suggested that 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 (3, 4); however, the robustness and impact in clinical decision-making strategies of those retrospective observations are currently undetermined and more systematic reports do not support the initial observations (5, 6). The sole assessment of pretreatment markers may not be sufficient and even conflicting (7) and a posttreatment marker may offer a more individualized insight.

An important component in the response to proliferative signals is the rapid, transient transcriptional activation of immediate early genes, such as the c-fos proto-oncogene. C-fos expression is regulated at multiple levels by intracellular signaling events, which makes it a useful marker to identify and characterize factors that affect cancer cell growth. C-fos is a robust marker of proliferation and it has been used as a distal marker to assess EGFR activation (8) and anti-EGFR therapy (9). In this article, we tested whether variations in c-fos expression corresponded to EGFR in vitro and in vivo inhibition and whether c-fos mRNA could be developed as a biomarker to predict sensitivity to EGFR blockade using an ex vivo approach. In addition, we have tested the feasibility of measuring this biomarker in a limited number of clinical samples to determine whether it can be translated to a clinical setting.

Materials and Methods

Drugs. Gefitinib was provided by AstraZeneca. Erlotinib was provided by OSI Pharmaceuticals (Melville, NY).

Cell lines and in vitro culture conditions. Five cell lines were used in this study: A431, Cal27, HN11, HuCCT1, and Hep2. HN11 was a kind gift from Dr. David Sidranski’s laboratory at Johns Hopkins University (Baltimore, MD). A431, Cal27, HuCCT1, and Hep2 were obtained from the American Type Culture Collection (Manassas, VA). A431 is a squamous cell carcinoma: Cal27 and HN11, and Hep2 are derived from head and neck squamous carcinomas; and HuCCT1 is a cholangiocarcinoma. The sensitivity profile of these cell lines to EGFR inhibitors has previously been published (10). The cell lines were grown in six-well plates with DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. After overnight serum starvation, cells were treated either with growth medium, growth medium plus human EGF 100 ng/mL (Sigma, St. Louis, MO), growth medium plus EGF and gefitinib, or growth medium plus gefitinib. The cells were incubated for 1 hour, the medium was aspirated, and RNA was collected by direct in-well lysis with 0.5 mL of RLT (Qiagen, Wilmington, MD). A431, Cal27, HN11, and Hep2 were determined. Next, these cell lines were xenografted in mice and treated for 14 days with gefitinib (A431 and HuCCT1) or erlotinib (CAL27, HN11, and Hep2). Fine needle aspiration biopsy of tumors was done at baseline and after 14 days of therapy for c-fos assessment. In addition, we tested the feasibility of analyzing this marker in five paired tumor samples from a clinical trial of gefitinib in patients with solid tumors. In culture, gefitinib and erlotinib decreased c-fos mRNA levels in the susceptible cell lines A431, CAL27, and HN11; however, both drugs failed to achieve c-fos inhibition in resistant cells. Gefitinib or erlotinib abrogated the increase in c-fos expression in vivo in EGFR-sensitive A431, CAL27, and HN11 tumors but not in resistant strains. Ex vivo evaluation was feasible and predicted in vivo effects. The feasibility study in paired human tumor biopsies showed that this biomarker can be reliably measured in clinical materials. In summary, variations in c-fos expression reflect the pharmacologic actions of EGFR inhibitors in in vitro and in vivo models. (Cancer Res 2006; 66(4): 2385-90)
**In vitro growth inhibition studies.** In vitro drug sensitivity to concentrations of gefitinib and erlotinib ranging from 0 to 10 μmol/L was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) following the instructions of the manufacturer. Briefly, cells were seeded at 5 × 10^3 per well in 96-well plates and grown for 24 hours before treatment with exponentially increasing concentrations of gefitinib or erlotinib in the presence of 10% FBS. A431, Cal27, HN11, HuCCT1, and Hep2 were assessed by MTT for both gefitinib and erlotinib after a 72-hour exposure.

**Western blot analysis.** Following 24-hour exposure to treatment, cells were harvested. Equal amounts of protein (50 μg) were resolved on 10% polyacrylamide gels. Gels were transferred onto nitrocellulose membranes that were incubated overnight at 4°C with antibodies against phosphorylated EGFR (Cell Signaling Technology, Beverly, MA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

**Ex vivo molecular assay.** Material collected by fine needle aspiration passaged on seven CAL27 xenograft tumors at baseline was aliquoted in growth medium and treated in tissue culture by short (30-60 minutes) exposure to growth medium, growth medium plus 100 ng/mL EGF, growth medium plus gefitinib or erlotinib, or growth medium plus erlotinib and gefitinib. Tumor samples were obtained weekly and washed with PBS. RLT lysis buffer was added. For RNA extraction from the mice and patient samples, two passes of tumor volume into different groups [five to six mice (10-12 tumors) per group] that were treated with vehicle, gefitinib 100 mg/kg i.p. daily for 14 days (A431 and HuCCT1), or erlotinib 50 mg/kg i.p. daily for 14 days (Cal27, HN11, and Hep2).

**Fine needle aspiration.** Fine needle aspirations on mice were done according to standard cytopathologic practice under inhaled general anesthesia (isoflurane) using 10-μL syringes and 25-gauge needles. During each fine needle aspiration procedure, the first pass was smeared onto glass slides and used for morphologic analysis (DiffQuik and Papanicolaou), and the second and third passes for RNA extraction. Eighteen A431 tumors, 14 CAL27 tumors, 18 HN11 tumors, 14 HuCCT1 tumors, and 16 Hep2 tumors were tested. Fine needle aspirations were done at baseline and after 14 days of therapy for each of the tumors. Tumor biopsies on patients were done at baseline and after 28 days of therapy following an ultrasonographic-guided, fine needle aspiration–assisted methodology, with on-site cytopathologic assessment of tissue adequacy.

**Results**

**C-fos increases selectively after exposure to EGF in TK inhibitor–sensitive cell lines.** After a brief exposure to EGF, the EGFR-susceptible A431, Cal27, and HN11 cell lines showed markedly elevated levels of c-fos mRNA (126-, 151-, and 86-fold, respectively); these EGF-induced increments were abrogated when gefitinib was subsequently added for a short, 1-hour exposure at 10 μmol/L (Fig. 1A). Gefitinib alone also decreased c-fos levels in these cell lines compared with growth medium. In contrast, the EGFR-resistant HuCCT1 and Hep2 cell lines showed lower (3.6- and 4.1-fold) c-fos increases upon exposure to EGF; gefitinib alone had no significant effect on c-fos levels compared with growth medium (although blocked c-fos EGFR-induced up-regulation).

The effect of a longer (72 hours) exposure to both gefitinib and erlotinib at a concentration of 10 μmol/L was then assessed with regard to cell growth and c-fos dynamics. Cell lines with EGF-inducible c-fos up-regulation showed high (and parallel) in vitro sensitivity to both agents (Fig. 1B). HuCCT1 and Hep2 showed a high level of resistance to inhibition (IC_{50} > 10 μmol/L), and c-fos levels minimally increased with time compared with baseline. In an experiment in A431 and HuCCT1 cells to examine dose dependency, erlotinib showed dose-dependent c-fos inhibition in A431 from 1 nmol/L to 1 μmol/L (Fig. 1C); the inhibitory effect of 1 μmol/L erlotinib on c-fos was equivalent at the different time points tested. No significant effects were seen in HuCCT1 at any of the doses or time points assessed.

To examine whether growth inhibition can be predicted by analyzing proximal EGFR signaling, a Western blot analysis was done and a significant (and identical) inhibition of EGFR phosphorylation by gefitinib and erlotinib that was unrelated to the growth inhibition ultimately achieved (Fig. 1D) was documented.

**In vivo tumor growth and c-fos modulation in response to gefitinib and erlotinib.** To confirm the molecular events described before and to determine the effect of these drugs in a model closer to a clinical context, A431, Cal27, HN11, HuCCT1, and Hep2 in vivo models were generated (Fig. 2). Gefitinib or

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erlotinib induced growth arrest in A431, CAL27, and HN11 tumors. In A431, CAL27, and HN11, the average c-fos at 14 days is 4.8-, 1.8-, and 3.2-fold compared with baseline in control mice (baseline versus day 14 for control mice, \( P < 0.05 \) in A431 and HN11, and \( P = 0.09 \) in CAL27). Gefitinib or erlotinib significantly abrogated the increase in c-fos levels observed in the control mice with time (day 14 control versus day 14 treated, \( P < 0.05 \) in A431 and HN11, \( P = 0.07 \) in CAL27). In HuCCT1 and Hep2 xenografts, no growth arrest was observed after treatment, c-fos levels did not increase significantly with time, and c-fos mRNA levels were unchanged by EGFR inhibitors.

**Ex vivo molecular assay.** The ex vivo results on fine needle aspiration–acquired tumor material from seven CAL27 xenograft tumors paralleled those obtained in cell culture, with 3- to 19-fold increase in c-fos mRNA levels upon EGF stimulation and abrogation of this response with erlotinib (Fig. 2B); these results were similar to those observed in vitro with gefitinib in terms of c-fos stimulation after EGF and stability after erlotinib, although the fold variation range in vitro was larger.

**Feasibility assessment in paired patient tumor samples.** The paired tumor material from five randomly selected, consecutive patients was used for this feasibility analysis. A detailed summary
of the clinical and pharmacologic results of the complete trial will be reported separately. The patients (henceforth numbered 1-5) had colorectal (1 and 3), non–small-cell lung (2), breast (4), and neuroendocrine (metastatic carcinoid; 5) carcinomas, and received 2, 4, 2, 2, and 5 + 1–month cycles of gefitinib. Best responses to therapy were stable disease in patients 2 and 5, and progressive disease in patients 1, 3, and 4. Patients 1 to 3 showed marked increases in $c$-$fos$ after 28 days (to 2,600%, 610%, and 910% of baseline values). In two patients, $c$-$fos$ decreased (to 56% and 37% of baseline values; Fig. 3). Tumors in which treatment blocked $c$-$fos$ increase had also evidence of MAPK inhibition and cell proliferation arrest as determined by the Ki67 (Fig. 3).

Discussion

There is an increasing interest in examining determinants of response to anticancer agents as tools to prospectively tailor therapy to individuals more likely to benefit from the drugs. This strategy is intuitive and appealing from both a clinical and a financial standpoint (11). This is especially evident for novel targeted therapies, and proof-of-principle pilot analyses are increasingly being embedded into clinical protocols. The aims of this study were to examine whether assessment of $c$-$fos$ dynamics could predict the activity of EGFR TKI and whether this marker could be developed as an ex vivo tool that can be incorporated in clinical studies. As $c$-$fos$ is downstream of the EGFR pathway, it was expected that it would show a wider range of dynamic range upon EGFR pathway modulation by both ligand stimulation and drug inhibition and it was hypothesized that $c$-$fos$ expression would predict EGFR TKI effects. Our group has previously reported that in the selected cell lines, $egfr$ mutational status (all wild type), $egfr$ amplification by fluorescence in situ hybridization, and EGFR protein content as assessed by ELISA (10) are not related to anti-EGFR activity.

The first conclusion of this report is that $c$-$fos$ levels increased after EGF stimulation and that this effect was inhibited by anti-EGFR agents in vitro in cell lines that are naturally sensitive to EGFR inhibitors, but not in those intrinsically resistant. C-fos levels
increase and correlate with tumor growth in untreated control
tumors corresponding to EGFR TKI–sensitive cell lines, and c-fos mRNA
dynamics correlates with tumor response to gefitinib and erlotinib in a xenograft model in both sensitive and resistant cell
lines. In the current experiments, the assessment of a proximal end
point (EGFR phosphorylation) was a less specific indicator of
efficacy than a distal end point (c-fos down-regulation). This
underscores the importance and likely superiority of functional
assays using proximal and distal end points in pharmacodynamic
studies. Although proximal end points (i.e., target inhibition) may
be more useful for dose and schedule selection, distal end points
may likely be associated with antitumor effects. An intriguing
aspect of this report is the increase in c-fos expression with time
seen in xenografts of untreated mice. We are unable to ascertain
whether this may relate to EGFR dependence or have a component
of tumor growth–driven stimulation. In three of the five patients,
a marked increase of c-fos was seen with time; this may be related
to tumor growth and be paralleled to the c-fos increase in
untreated xenografts. However, as we do not have sequential
tsamples of untreated patients, we cannot define the natural
evolution of c-fos levels in patient tumors and whether this effect
may be related to gefitinib treatment. Interestingly c-fos levels
have been found to be similar between normal and tumor tissue in head
and neck squamous cell carcinoma patient samples (12), but signifi-
cantly higher in tumor tissue in esophageal cancer patients (13).

A second relevant aspect of this work is the feasibility evaluation
of measuring c-fos sequentially on patient-derived material;
this was preliminarily tested in a series of five unselected patients
receiving gefitinib and undergoing pretherapy and posttherapy fine
needle aspiration–guided tumor biopsies. It is of interest that c-fos
level could be reliably measured in clinical materials, that there was
a range of values obtained, and that the expression of this marker
related to signaling inhibition and cell proliferation as measured
by MAPK activation and Ki-67 proliferative changes. Because of
the low number of patients treated and the lack of patients with
objective responses, no conclusions can be extracted with regard to
the predictive value of this biomarker. We could not get adequate
phospho-EGFR staining and although this may be related to the
little amount of tissue available, other technical issues cannot be
ruled out. The use of this technique has resulted in paradoxical
results in other studies (14). The ultimate goal of these studies is,
obviously, to implement this marker in clinical trials and to assess
if variations in c-fos relate to clinical outcome. The data presented
here suggest that this would be a feasible question for future
clinical trials. A potential advantage of the reverse transcription-
PCR (RT-PCR) c-fos assessment over immunohistochemical
variables is the fully quantitative measurement, the higher
reproducibility of RT-PCR, and the lower amount of tissue needed
(fine needle aspiration aspirate versus core biopsy). However,
for future studies, a combined analysis may be contemplated if
the amount of tissue is sufficient. It needs to be stressed, however,
that this small set of clinical samples does not provide the statis-
tical power to draw any firm conclusion and has to be considered
exploratory.

The third significant facet of this report is that fine needle
aspiration has been shown to be a robust and safe method to
acquire tumor material in sufficient quantities to assess pharma-
codynamic end points in a serial manner. In addition, preliminary
evidence suggests that fine needle aspiration can be efficiently used
in procuring tissue to reproduce in vitro conditions and develop an
ex vivo molecular sensitivity and resistance assay. This approach
has drawn considerable interest and the outcome and ultimate
significance of a number of these studies has been the subject of
two recent reviews (15, 16). Most studies analyzed whether cells
derived from a sample of viable tumor tissue show a response when
exposed to selected therapeutic agents under in vitro conditions.
Typically, cloning and proliferation assays are used for this purpose,
which suffer from many disadvantages, such as setup complexities,
and the necessity for some growth of lesional tissue under in vitro
conditions. Consequently, lack of reproducibility has prevented
these appealing strategies from being widely incorporated to the
clinical practice. However, if a robust correlation can be established
between a given pharmacodynamic effect and outcome in
preclinical models and pilot clinical studies, molecular testing
has several advantages when compared with proliferation assess-
ment: (a) it requires a lower amount of tumor cells, (b) ex vivo
proliferation is not a requirement (although cells have to maintain
viability), and (c) short-term exposure, as opposed to long-term
treatment, is sufficient to elicit an assessable response.

The potential clinical relevance of the above findings is 2-fold.
First, evaluating the dynamic behavior of c-fos mRNA levels may
prove useful early in the course of treatment before clinical and
radiologic evidence of response to therapy can be reliably sought.
It is unlikely that examining tumor features at baseline only will
be sufficiently informative and assays looking at distal mark-
ers are needed. Second, an ex vivo molecular assay can be useful
before treatment to prospectively determine the potential level
of responsiveness of a patient to EGFR inhibitors, taking a step
forward in the development of individualized approaches to cancer
therapy.

Despite the encouraging findings, the present study has a number of
limitations. It is still to be defined whether the c-fos response on
EGF stimulation is a solid predictor of EGFR responsiveness per se,
as in resistant cell lines where there is a modest but consistent increase in c-fos. The combined assessment of both the response to EGF and to EGFR inhibition seems more robust. The ex vivo paradigm needs confirmation in a wider tumor population, but more importantly needs standardization of conditions to attempt a prospective clinical validation. In particular, the clinical correlate presented has to be interpreted with extreme caution, first considering the exploratory intent and the low number of patients, but especially because there were no true objective responses. Although Ki67 is a robust indicator of drug activity in general (and anti-EGFR therapy in particular), it cannot substitute validated efficacy end points.

In summary, the evaluation of c-fos predicted response to EGFR inhibitors in an in vitro and in vivo model. In addition, in vitro conditions may be reproducible to interrogate tumor material in an ex vivo manner.

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