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

Antonio Jimeno,[1] Peter Kulesza,[1,2] Erik Kincaid,[2] Nadia Bouaroud,[1] Audrey Chan,[1] Arlene Forastiere,[1] Julie Brahmer,[1] Douglas P. Clark,[2] and Manuel Hidalgo[1]

[1]The Sidney Kimmel Comprehensive Cancer Center and [2]Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland

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, 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, Valencia, CA). Concentration and time course experiments involved treatment of the cells during 24 hours with growth medium, or growth medium plus erlotinib at increasing concentrations from 1 nmol/L to 1 μmol/L and treatment of the cells during 24 and 72 hours with growth medium, or growth medium plus erlotinib at 1 μmol/L.

Note: A. Jimeno and P. Kulesza contributed equally to this work.

Requests for reprints: Manuel Hidalgo, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, 1650 Orleans Street, Room 1M89, Baltimore, MD 21231-1000. Phone: 410-502-3850; Fax: 410-614-9006; E-mail: mhidalg1@jhmi.edu.

doi:10.1158/0008-5472.CAN-05-2882

***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 phospho-EGFR (Cell Signaling Technology, Beverley, MA) and actin (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

***In vivo* growth inhibition studies.** Five groups of 6-week-old female athymic nude mice (Harlan, Indianapolis, IN) were used. A431, CAL27, HN11, HuCCT1, and Hep2 cells (1.5×10^6-5×10^6) were injected s.c. in each flank. Tumors were grown to a size of 0.2 cm^3 and mice were stratified by 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 (isofluorane) using 10-mL 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 Papanicoloau), 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.

***Ex vivo* molecular assay.** Material collected by two fine needle aspiration passes 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 EGF and erlotinib, or growth medium plus erlotinib.

Clinical samples. For this study, we used tumor materials collected from five consecutive patients treated in a clinical trial testing the biological effects of gefitinib (JHH J0315). Patients were required to be ≥18 years old and to have histologically documented metastatic or inoperable malignancy amenable to sequential biopsies, for which there was no known curative or standard palliative regimen (or failure of such regimens have occurred). Gefitinib was administered at a dose of 500 mg daily on an uninterrupted basis. The scientific review board of our institution granted protocol approval and patients were required to provide written informed consent before enrollment into the study.

RNA extraction. *In vitro* RNA extraction was done on nonconfluent cells after treatment. Wells were washed with PBS and RLT lysis buffer was added. For RNA extraction from the mice and patient samples, two passes from the fine needle aspiration were put in lysis buffer (Mini RNeasy, Qiagen) loaded onto a column, washed, and eluted into 50 μL TE (pH 8). Total RNA was extracted using the RNeasy Mini kit (Qiagen). RNA was transcribed into cDNA by reverse transcription by priming with random hexamers (M-MLTV, Promega, Madison, WI). The excess hexamers were removed using a column-based clean-up kit (Qiagen).

Quantitative real-time reverse transcription-PCR analysis. For *c-fos* determination on *in vitro* samples, fine needle aspirations from mice tumors, and fine needle aspirations from patient tumors, quantitative PCR was done on an MX3000p thermal cycler (Stratagene, La Jolla, CA) using SYBR green dye method to track the progress of the reactions with ROX dye added as reference. β-Globin DNA-specific primers were used to test DNA contamination for each sample type. Three housekeeping genes (*HPRT, UBC*, and *SDHA*) were run in parallel with test genes. The amount of change in the target gene between the control and experimental conditions was found by comparing the threshold cycle (C_t) of the target gene to the geometric mean of the threshold cycles of the housekeeping genes. The geometric mean of the C_t values of each of the housekeeping genes, and a change in threshold cycle (δC_t), between conditions were calculated as follows: $dC_{t\ \text{houskeeping}} = (C_{t\ \text{HPRT}} \times C_{t\ \text{UBC}} \times C_{t\ \text{SDHA}})_{\text{control}} - (C_{t\ \text{HPRT}} \times C_{t\ \text{UBC}} \times C_{t\ \text{SDHA}})_{\text{exp}}$. The change in threshold cycle for the target gene was calculated directly from C_t under each condition [$dC_{t\ \text{target}} = (C_{t\ \text{target}})_{\text{control}} - (C_{t\ \text{target}})_{\text{exp}}$]. The efficiency of the housekeeping genes raised to their dC_t divided by the efficiency of the target gene raised to its dC_t gave a ratio between the control and experimental conditions normalized to the housekeeping genes (ratio = $E_{\text{target}}^{dC_{t\ \text{target}}} / E_{\text{housekeeping}}^{dC_{t\ \text{housekeeping}}}$, where E is the primer efficiency).

Immunohistochemical analysis. Core biopsies from patients were processed using standard procedures (formalin fixed and paraffin embedded). Five-micrometer sections were used for Ki67 staining that was done following the instructions of the manufacturer (DAKO, Carpinteria, CA), and scored as percentage staining nuclei. Phospho-Thr^{202}/Tyr^{204} mitogen-activated protein kinase (MAPK) and phospho-Tyr^{1068} EGFR (Cell Signaling Technology) staining was done using citrate-steam recovery, followed by Catalyzed Signal Amplification kit for pMAPK, using Rabbit Link for pEGFR (DAKO).

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. 1*A*). 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* EGF-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. 1*B*). 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. 1*C*); 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. 1*D*) 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

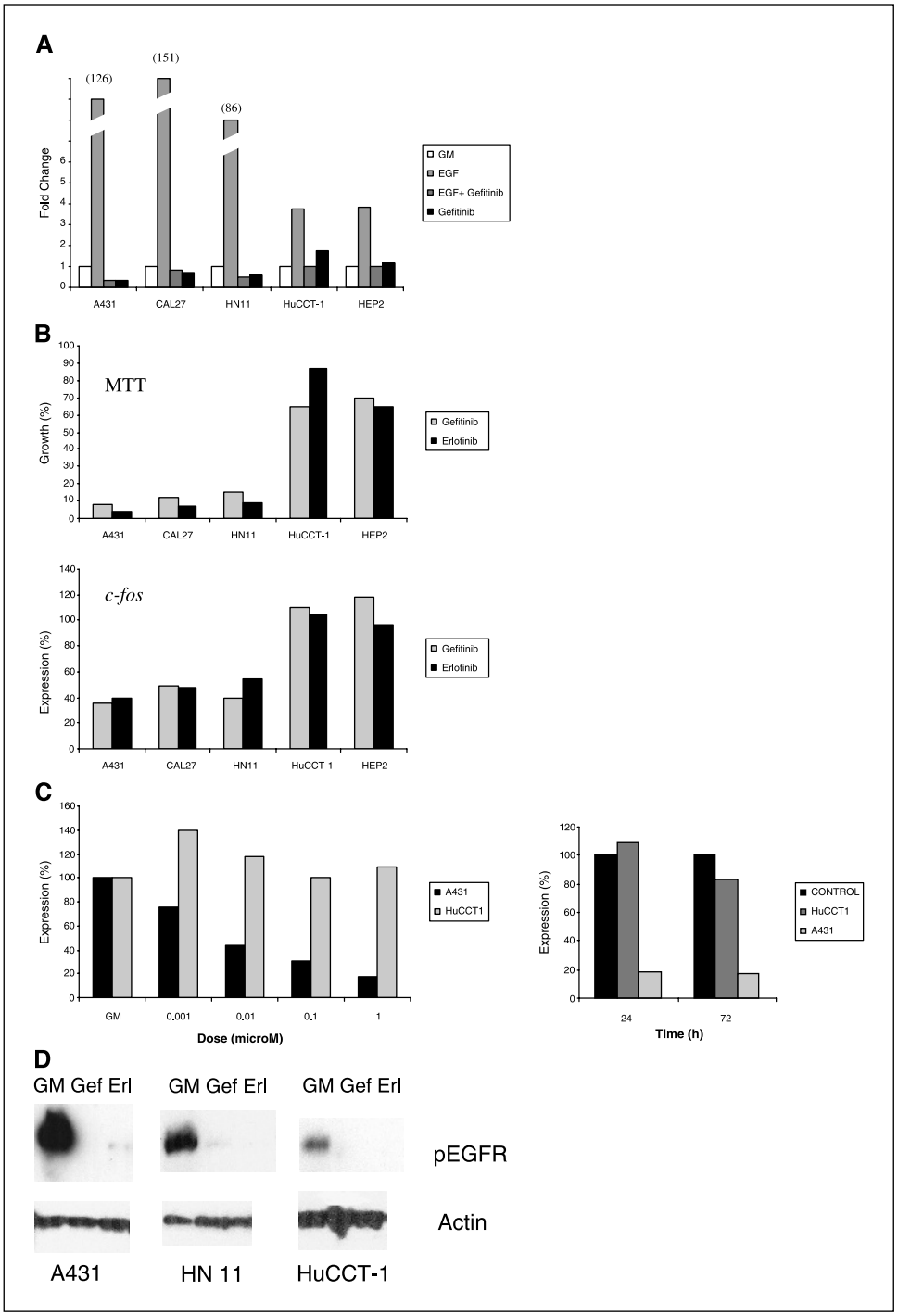

Figure 1. *A, c-fos* mRNA expression (shown as fold increase normalized to treatment with growth medium) is stimulated with a short exposure to EGF and suppressed by a 1-hour EGFR inhibition with 10 μmol/L gefitinib (in the presence or absence of EGF) in A431, CAL27, and HN11 cell lines but not in HuCCT-1 and Hep2. *B,* correlation between growth inhibition and *c-fos* behavior after exposure to both EGFR inhibitors gefitinib and erlotinib. *Top,* results of antitumor effect measured by the MTT assay after 72 hours of exposure to growth medium plus either gefitinib or erlotinib at a concentration of 10 μmol/L; *bottom, c-fos* mRNA expression in parallel experiments with both agents at the same dose. All values are shown in percentage normalized to control treated with growth medium only (100%). The three cell lines that showed marked increase in *c-fos* after EGF stimulation resulted to be sensitive to EGFR inhibition; in addition, *c-fos* down-regulation after treatment was confirmed in these same cell lines. Treatment with both gefitinib and erlotinib of HuCCT1 and HEP2 showed no antiproliferative effect and no changes in *c-fos* mRNA levels. *C,* in A431, erlotinib showed dose-dependent *c-fos* inhibition in A431 from 1 nmol/L to 1 μmol/L for 24 hours although there was no effect on *c-fos* in HuCCT1. The effect of 1 μmol/L erlotinib on *c-fos* was equivalent at 24 and 72 hours. No significant effects were seen in HuCCT1 at any of the time points assessed. *D,* the proximal effect on the phosphorylation of the target was assessed by immunoblot. A similar degree of phopho-EGFR inhibition was seen in sensitive versus resistant cell lines, suggesting than EGFR inhibition (proximal end point) was not accurate predicting outcome. Values are normalized to control. *GM,* growth medium; *Gef,* gefitinib; *Erl,* erlotinib.

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. 2*B*); 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

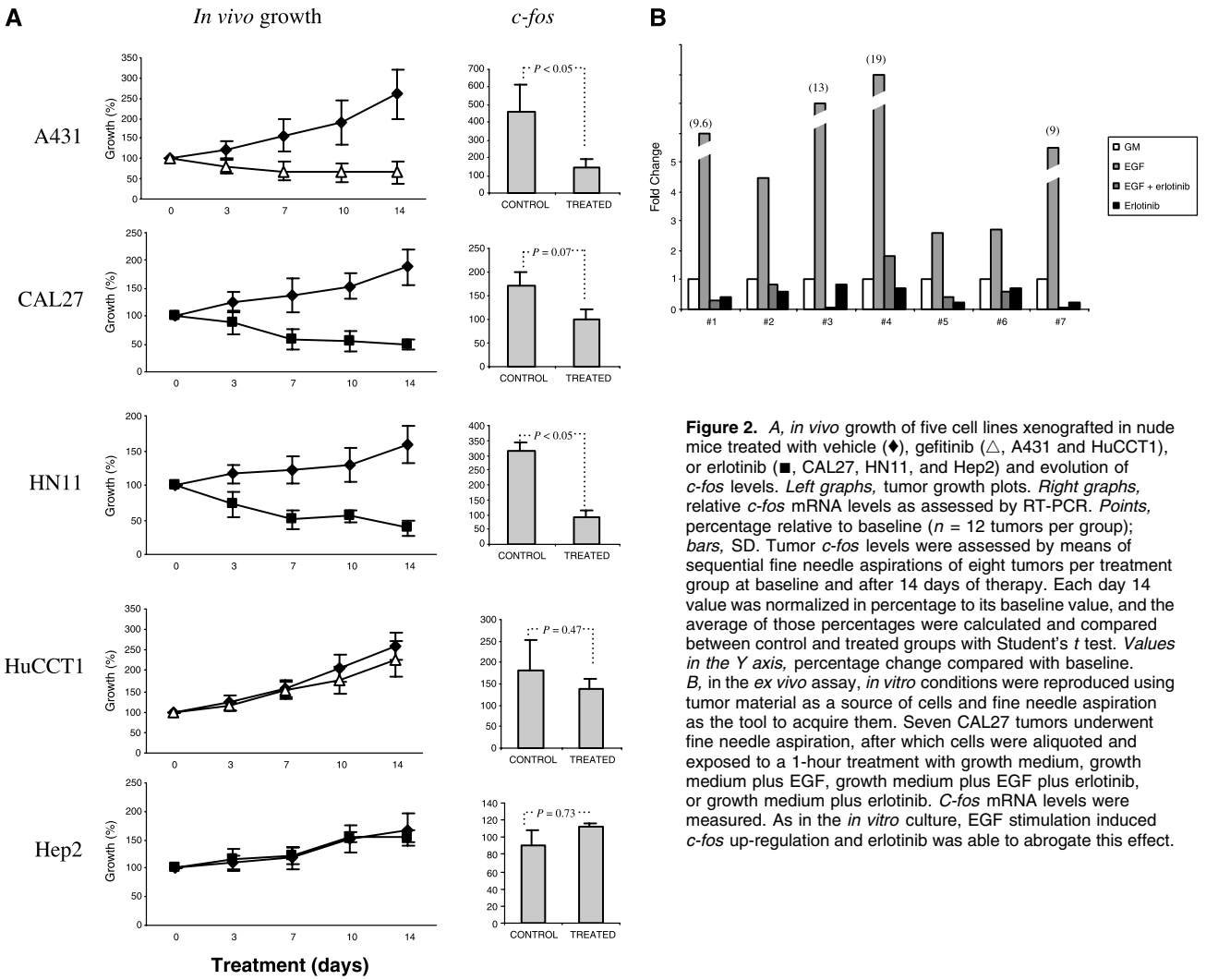

Figure 2. *A, in vivo* growth of five cell lines xenografted in nude mice treated with vehicle (♦), gefitinib (△, A431 and HuCCT1), or erlotinib (■, CAL27, HN11, and Hep2) and evolution of *c-fos* levels. *Left graphs,* tumor growth plots. *Right graphs,* relative *c-fos* mRNA levels as assessed by RT-PCR. *Points,* percentage relative to baseline (n = 12 tumors per group); *bars,* SD. Tumor *c-fos* levels were assessed by means of sequential fine needle aspirations of eight tumors per treatment group at baseline and after 14 days of therapy. Each day 14 value was normalized in percentage to its baseline value, and the average of those percentages were calculated and compared between control and treated groups with Student's t test. *Values in the Y axis,* percentage change compared with baseline. *B,* in the *ex vivo* assay, *in vitro* conditions were reproduced using tumor material as a source of cells and fine needle aspiration as the tool to acquire them. Seven CAL27 tumors underwent fine needle aspiration, after which cells were aliquoted and exposed to a 1-hour treatment with growth medium, growth medium plus EGF, growth medium plus EGF plus erlotinib, or growth medium plus erlotinib. *C-fos* mRNA levels were measured. As in the *in vitro* culture, EGF stimulation induced *c-fos* up-regulation and erlotinib was able to abrogate this effect.

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),[3] *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

[3] Unpublished data.

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 samples 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 significantly 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

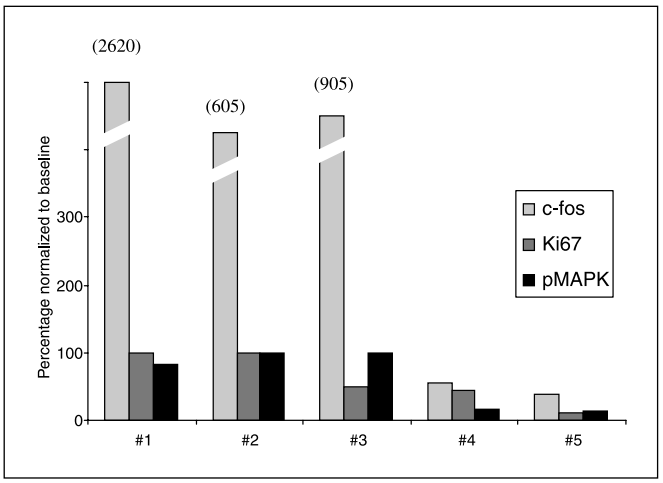

Figure 3. Relationship between changes in *c-fos*, Ki67 proliferation index, and phospho-MAPK in paired (before and after 28 days of therapy) tumor samples of patients treated with gefitinib, shown as percentage variation from baseline. None of the patients responded to therapy and we compared the evolution of *c-fos* levels to Ki67 proliferation index variability and the phosphorylation status of downstream components of the pathway. *C-fos* was assessed from mRNA extracted from a snap-frozen fine-needle aspirate pass and immunohistochemistry was done on the formalin-fixed core biopsy obtained in the same procedure. Patients 1 to 3 showed both marked increases in *c-fos* after 28 days and no change in phospho-MAPK, whereas in patients 4 and 5, *c-fos* decreased (to 56% and 37% of baseline values) as well as phospho-MAPK (to 17% and 13% of baseline values, respectively). Ki67 index was not influenced by therapy in patients 1 and 2, whereas it decreased to 52%, 42%, and 10% of baseline values in patients 3 to 5, respectively.

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 statistical 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 pharmacodynamic 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 assessment: (*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 markers 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.

Acknowledgments

Received 8/15/2005; revised 11/17/2005; accepted 12/14/2005.

Grant support: Head and neck Specialized Programs of Research Excellence grant P50 CA58184-09 and Instituto de Salud Carlos III grant BF03/00631.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Jenna Wheelhouse for her expert assistance.

References

1. Pollack VA, Savage DM, Baker DA, et al. Inhibition of epidermal growth factor receptor-associated tyrosine phosphorylation in human carcinomas with CP-358,774: dynamics of receptor inhibition *in situ* and antitumor effects in athymic mice. J Pharmacol Exp Ther 1999;291: 739–48.
2. Moyer JD, Barbacci EG, Iwata KK, et al. Induction of apoptosis and cell cycle arrest by CP-358,774, an inhibitor of epidermal growth factor receptor tyrosine kinase. Cancer Res 1997;57:4838–48.
3. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
4. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
5. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al. Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 2005;353:123–32.
6. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. N Engl J Med 2005;353:133–44.
7. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. J Natl Cancer Inst 2004;96:1133–41.
8. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat Med 2002;8:289–93.
9. Yano S, Yamaguchi M, Dong RP. EGFR tyrosine kinase inhibitor "gefitinib (Iressa)" for cancer therapy. Nippon Yakurigaku Zasshi 2003;122:491–7.
10. Amador ML, Oppenheimer D, Perea S, et al. An epidermal growth factor receptor intron 1 polymorphism mediates response to epidermal growth factor receptor inhibitors. Cancer Res 2004;64:9139–43.
11. Chabner BA. The miracle of Iressa. Oncologist 2004;9: 245–6.
12. Mangone FRR, Brentani MM, Nonogaki S, et al. Overexpression of Fos-related antigen-1 in head and neck squamous cell carcinoma. Int J Exp Pathol 2005;86: 205–12.
13. Wu MY, Zhuang CX, Yang HX, Liang YR. Expression of Egr-1, c-fos and cyclin D1 in esophageal cancer and its precursors: An immunohistochemical and *in situ* hybridization study. World J Gastroenterol 2004; 10:476–80.
14. Tan AR, Yang X, Hewitt SM, et al. Evaluation of biologic end points and pharmacokinetics in patients with metastatic breast cancer after treatment with erlotinib, an epidermal growth factor receptor tyrosine kinase inhibitor. J Clin Oncol 2004; 22:3080–90.
15. Schrag D, Garewal HS, Burstein HJ, Samson DJ, Von Hoff DD, Somerfield MR. American Society of Clinical Oncology Technology Assessment: chemotherapy sensitivity and resistance assays. J Clin Oncol 2004; 22:3631–8.
16. Samson DJ, Seidenfeld J, Ziegler K, Aronson N. Chemotherapy sensitivity and resistance assays: a systematic review. J Clin Oncol 2004;22:3618–30.

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

Antonio Jimeno, Peter Kulesza, Erik Kincaid, et al.

Cancer Res 2006;66:2385-2390.