Proteomic Signatures of Epidermal Growth Factor Receptor and Survival Signal Pathways Correspond to Gefitinib Sensitivity in Head and Neck Cancer

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Abstract

Purpose: Gefitinib targeting of the epidermal growth factor receptor (EGFR) has shown limited activity in clinical trials of head and neck squamous cell carcinoma (HNSCC). To investigate the underlying molecular mechanism, the proteomic signatures and responses of EGFR and downstream signals have been studied in a panel of HNSCC cell lines and tumor specimens pre- and post-gefitinib treatment.

Experimental Design: The IC50 of gefitinib for HNSCC cell lines were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay. The effects of gefitinib on activation of EGFR and downstream signaling molecules were determined by Western blot, ELISA, and reverse-phase protein microarray (RPMA). The biomarkers involved in the signaling pathways were examined in HNSCC tumor specimens from patients in a phase I gefitinib trial.

Results: In vitro, gefitinib inhibited cell proliferation with differing IC50, and suppressed activation of EGFR and downstream signaling molecules protein kinase B (AKT), extracellular signal-regulated kinase 1/2, signal transducer and activator of transcription 3 (STAT3), and nuclear factor κB. The drug sensitivity was statistically correlated with activation of phosphorylated AKT (p-AKT) and phosphorylated STAT3 (p-STAT3) detected by ELISA, and consistent with results measured by RPMA. In patient samples, a broad suppression of activation of EGFR and downstream signaling molecules was observed in a molecular responder patient, in contrast to a lack of inhibition or increased activation of biomarkers in different pathways in nonresponder patients.

Conclusions: Gefitinib sensitivity is correlated with p-AKT and p-STAT3 activation in HNSCC cell lines and tumor specimens. p-AKT and p-STAT3 could serve as potentially useful biomarkers and drug targets for further development of novel therapeutic agents for HNSCC.

Increased understanding of molecular alterations in head and neck squamous cell carcinoma (HNSCC) and other cancers has ushered in efforts to develop molecularly targeted therapies. Among these alterations, overexpression of the epidermal growth factor receptor (EGFR) has been identified in many cancers, including 80% to 100% of HNSCC (1–3), where it has also been implicated in a more aggressive phenotype, increased resistance to treatment, and poorer clinical outcome (4, 5). These observations have led to interest in the development of molecularly targeted small-molecule inhibitors of EGFR for the treatment of HNSCC. Gefitinib (Iressa), an EGFR tyrosine kinase inhibitor, has been tested in clinical trials in solid tumors, including HNSCC, as a single agent, or in the combination with other chemotherapies or radiation, but has shown limited clinical efficacy with response rates of 10% to 15% (6–9). Molecular biomarkers that could identify patients responsive to gefitinib or other EGFR inhibitors would be useful in the selection of patients for therapy.

The EGFR is a membrane-bound glycoprotein with an extracellular cysteine-rich ligand-binding domain linked by a short single transmembrane sequence to an intracellular tyrosine kinase and carboxyl-terminal scaffolding domains (1). EGFR (ErbB1) is a member of the ErbB receptor family of tyrosine kinases, which also includes ErbB2 (HER-2/neu), ErbB3, and ErbB4 (1, 4). The activation of EGFR family members may...
be either ligand-dependent or independent as a result of mutation or overexpression-induced homodimerization, or heterodimerization with other ErbB receptor family members (1). The binding by ligands such as epidermal growth factor (EGF), transforming growth factor α, amphiregulin, and heparin binding-EGF results in autophosphorylation of multiple tyrosine residues at the receptor carboxyl terminus of EGFR, which acts as scaffolding for SRC and other adaptor proteins that interact with the receptor and transduce mitogenic signals mediated by downstream molecular cascades (1, 4, 5). Ligand-independent activation of EGFR in HNSCC has been shown to result from a truncation mutation, EGFR variant III (EGFRvIII; ref. 10). Ligand-dependent and independent activation play a vital role in promoting cancer cell growth, cell migration, aberrant metabolism and differentiation, and enhanced cell survival (1, 4).

A number of pharmacologic agents that block EGFR signaling have been developed and evaluated in clinical trials. Gefitinib is the first of such molecules with oral bioavailability that inhibits aberrant metabolism and differentiation, and enhanced cell proliferation in vitro (21, 22). We found that gefitinib inhibited cell proliferation in vitro with different IC50, and suppressed activation of EGFR and downstream activation of protein kinase B (AKT), extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and nuclear factor-κB (NF-κB). Our observations indicate that these proteomic signatures to EGFR inhibitor could be useful in the development of more accurate molecular diagnosis, selection of therapy, and determinations of HNSCC prognosis. Cross-validation of results from in vitro and in vivo using RPMA by Western blot, quantitative ELISA, and immunohistochemistry provides an essential step for future application of these technologies in clinical trials.

**Materials and Methods**

**Cell lines and reagents.** HNSCC cell lines UMSSC-6, -9, -11A, and -11B were kind gifts from Dr. T.E. Carey (University of Michigan, Ann Arbor, MI), and their characteristics were previously described (23). The UMSSC cell lines were cultured in MEM (Invitrogen) with 10% fetal bovine serum and penicillin/streptomycin, and maintained in a humidified incubator at 37°C with 5% CO2. Gefitinib was provided for studies by AstraZeneca, under a Materials Cooperative Research and Development Agreement and Clinical Trials Agreement with the National Cancer Institute (NCI), and was dissolved in DMSO if there was a relationship between these biomarkers and the anticancer effects in HNSCC cell lines and patients’ specimens. In addition, we cross-validated the biomarkers identified by several methods, including Western blot analysis, ELISA, and reverse-phase protein microarray (RPMA), a newly developed proteomic platform that is capable of measuring numerous specific phosphorylated proteins that are involved in important signaling pathways from a small tissue biopsy or a few thousand cells (21, 22). We found that gefitinib inhibited cell proliferation in vitro with different IC50, and suppressed activation of EGFR and downstream activation of protein kinase B (AKT), extracellular signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and nuclear factor-κB (NF-κB). Our observations indicate that these proteomic signatures to EGFR inhibitor could be useful in the development of more accurate molecular diagnosis, selection of therapy, and determinations of HNSCC prognosis. Cross-validation of results from in vitro and in vivo using RPMA by Western blot, quantitative ELISA, and immunohistochemistry provides an essential step for future application of these technologies in clinical trials.

_5_ Van Waes C, et al., Int J Rad Onc Biol Phys, in press.
-11A, and -11B cells were plated in triplicate at 5 × 10^3 cells per well onto 96-well plates. The cells were cultured overnight and then exposed to gefitinib at varying concentrations. Cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Kit (Roche Diagnostics). The optical density was measured by a microplate reader at a wavelength of 570 nm. The IC50 of the drug was calculated using MTT data at day 5.

Sequencing and reverse transcription-PCR analysis of mutations in EGF coding regions. RNA was isolated from UMSCC cells, and cDNA was reverse-transcribed and sequenced for coding regions of EGF exon 18 to 21 bidirectionally by SeqWright Inc., according to the hot spots identified in the literature (24). The EGFRevIII deletion mutation was analyzed by reverse transcription-PCR, using the primers described by Dr. Grandis’ group (10).

Whole cell extraction and Western blot analysis. Cultured UMSCC cells at 75% confluence were treated with 1 or 10 μmol/L gefitinib for 4 hr and then 10 ng/ml recombinant EGF (R&D systems) were added into half of the dishes for each treatment condition for 1 hr. Cultured cells were washed with ice-cold PBS, lysed, and harvested with ice-cold cell extraction buffer (Invitrogen). The whole cell lysates were incubated on ice on a rocking platform for 30 min and vortexed every 10 min and then spun at 13,000 g at 4°C for 10 min. The supernatants were collected and the protein concentrations were quantified by BCA protein assay kit (Pierce).

For Western blot analysis, whole cell lysate was mixed with an SDS protein gel loading solution (Quality Biological, Inc.) and heated at 85°C for 2 min. The samples were loaded onto Tri-glycine precast gels (Invitrogen), and electrophoresis was completed at 125V for 105 min. The Invitrogen iBlot apparatus was used to complete the transfer (Invitrogen). The membranes were preblocked in a preblocking solution composed of 1% Tween-20 in TBS mixed with either 7.5% bovine serum albumin or 5% nonfat dry milk. Primary antibodies were diluted in either 5% nonfat powdered milk or 5% bovine serum albumin prepared from Tween-20 in TBS for: phospho-EGFR (Tyr845) and phospho-EGFR (Tyr1068), 1:1000 (Cell Signaling Technology); and phospho-EGFR (Tyr1173), phospho-EGFR (Tyr1148), and total EGFR, 1:1000 (Invitrogen). Each blot was incubated with Pierce Super Signal West Pico Substrate (Pierce) and exposed to Kodak X-Omat film.

ELISA assays. ELISA assays that were used to quantify total EGF, phospho-EGFR (Tyr 1173), total STAT3, phospho-STAT3 (Tyr 705), total ERK1/2, phospho-ERK1/2 (Tyr185/187), total AKT, and phospho-AKT (Ser 473), were done according to the manufacturer’s protocol (Invitrogen). The ELISA assays used to detect phospho-EGFR Tyr 1068, phospho-EGFR Tyr 845, and NF-κB phospho-p65 (Ser 536) were done according to the manufacturer’s protocol (Cell Signaling Technologies). The whole cell lysates were isolated as described for the Western blot analysis for all ELISA assays, except that nuclear extracts were used for testing NF-κB-p-p65 (Ser 536; Active Motif). Samples were measured for each condition against a respective standard provided by the kits. The optical density was measured by a microplate reader at a wavelength of 450 nm, and the data were calculated and presented as mean plus SD from triplicate assays.

Real time reverse transcription-PCR. RNA isolation and cDNA synthesis were done as previously described (25). Real Time reverse transcription-PCR for EGF and 18S rRNA was achieved with Assays-on-Demand Gene Expression Assay from Applied Biosystems. Amplification conditions were as follows: activation of enzymes for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 15 sec at 95°C and 1 min at 60°C. Thermal cycling and fluorescence detection was done using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). EGF values were normalized to 18S rRNA. Each condition was assayed in triplicate and data were presented as mean plus SD.

Patient treatment and tumor specimens. Patients with histologically proven HNSCC provided informed consent before being enrolled into a phase I study of gefitinib, paclitaxel, and radiation for patients with locoregionally advanced HNSCC, in a protocol that was approved by the Institutional Review Board of the NCI (NCI trial 04-C-0141). Gefitinib was produced by AstraZeneca Pharmaceuticals and supplied through the NCI’s Cancer Treatment Evaluation Program. Gefitinib (250 mg orally) was administered daily for 1 wk prior to paclitaxel and radiation, so that tumor specimens pretreatment and after 1 wk of treatment with gefitinib alone could be obtained from tumors accessible by transoral biopsy.

The patients received a total of 8 wk of gefitinib, including 7 wk in combination with weekly doses of paclitaxel and 35 fractions of radiation. Biopsies of patients’ normal mucosa and tumor pretreatment and 1 wk post-gefitinib treatment were obtained from 7 of 10 patients. Specimens were immediately embedded in optimum cutting temperature media and frozen at -80°C. Frozen tissues were sectioned at a thickness of 10 μm at the largest area and placed on silanated glass slides (Histoserv) for immunostaining. The complete clinical results and analysis of correlative immunohistochemical studies for EGFR, selected phospho-proteins, proliferation (Ki-67), and apoptosis (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling [TUNEL]) markers have been described in a separate report (23).

RPMA. The cell line lysates in SDS-based buffer were quantified with BCA protein assay kit (Pierce), and adjusted to 1μg/μl. The lysates were printed onto Whatman FAST nitrocellulose coated slides (Schleicher & Schuell) using a solid-pin 2470 Arrayer (Aushon Biosystems). Lysates were printed in a serial dilution curve, in duplicate.

Immunostaining and analysis of RPMA. The antibodies used in RPMA were first tested to ensure that a single band was generated in Western blot on control HeLa cell line lysates that were treated or untreated with EGF. The rabbit polyclonal antibodies from Cell Signaling were affinity-purified against EGFR, phospho-EGFR (Tyr1068), phospho-EGFR(Tyr1148), AKT, phospho-AKT (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-ERK1/2 (Tyr185/187), NF-κB p65, phospho-p65 (Ser536), STAT3 and phospho-STAT3 (Tyr705), mitogen-activated protein kinase kinase (MEK), and phospho-MEK (Ser217/221).

Each array was incubated with a specific primary antibody, which was detected by using the catalyzed signal amplification system (11). Briefly, each slide was blocked with 1-block (Tropix) for 1 h. The slides were incubated with primary and biotin-labeled secondary antibodies, and then sequentially incubated with streptavidin-biotin complex for 15 min, biotinyl tyramide (for amplification) for 15 min, streptavidin-peroxidase for 15 min, and a fluorescence imaging system (Kodak). Stained arrays were scanned on a flatbed scanner and spot images were converted to raw pixel values using ImageQuant software (v. 5.2, Molecular Dynamics). Raw pixel values for the 1:2 dilutions of all samples were used for subsequent analysis.

For RPMA study in human patient samples, frozen sections of HNSCC or normal tissues were scraped, lysed, arrayed, and stained the same ways as described above. Total protein of each spotted lysate was used for subsequent analysis. RPMA was first tested to ensure that a single band was generated in Western blot on control HeLa cell line lysates that were treated or untreated with EGF. The rabbit polyclonal antibodies from Cell Signaling were affinity-purified against EGFR, phospho-EGFR (Tyr1068), phospho-EGFR(Tyr1148), AKT, phospho-AKT (Ser473), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-ERK1/2 (Tyr185/187), NF-κB p65, phospho-p65 (Ser536), STAT3 and phospho-STAT3 (Tyr705), mitogen-activated protein kinase kinase (MEK), and phospho-MEK (Ser217/221).

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For RPMA study in human patient samples, frozen sections of HNSCC or normal tissues were scraped, lysed, arrayed, and stained the same ways as described above. Total protein of each spotted lysate was determined using Sypro Ruby Protein Blot Stain (Molecular Probes) and a fluorescence imaging system (Kodak). Stained arrays were scanned on a flatbed scanner and spot images were converted to raw pixel values by ProteinScan/P-SCAN software [Peak quantification with Statistical Comparative Analysis, (http://abs.cit.nih.gov/pscan)], and were normalized to total protein.
Results

UMSCC cells exhibited differential sensitivity to gefitinib. A phase I clinical trial combining gefitinib with paclitaxel and radiation in HNSCC patients was carried out at the NCI, and heterogeneous clinical responses were observed in 10 patients enrolled. To investigate the possible mechanism(s) for heterogeneous clinical responses observed in this and other clinical studies of HNSCC, we selected a panel of HNSCC cell lines, UMSCC-6, -9, -11A, and -11B. The selection criteria for these cell lines were based on our previous studies, in which we found that UMSCC-11A and -11B exhibited relatively higher EGFR expression and activation, and UMSCC-6 and -9 exhibited intermediate and lower levels. In addition, multiple signal transduction pathways downstream from EGFR involving AKT, ERK, STAT3, and NF-κB have been extensively studied and characterized in these cell lines by our laboratory. The effects of gefitinib on the activation of EGFR and downstream molecules were investigated in vitro. First, we tested gefitinib drug sensitivity of these cell lines using MTT assay (Fig. 1). UMSCC-6, -9, and -11A showed similar in vitro sensitivity to gefitinib with the IC50 range of 2 to 2.5 μmol/L (Fig. 1). However, the UMSCC-11B cell line was less sensitive, with an IC50 of 17 μmol/L, or about 10-fold more than that observed in the other cell lines (Fig. 1). In addition, a lower dose of gefitinib (0.5-1.0 μmol/L) showed a paradoxical stimulation of UMSCC-11B cell growth (Fig. 1).

EGFR mutations have been found to be rare among HNSCC from the United States or Europe, but to rule out the possibility that the gefitinib sensitivity is due to EGFR mutation, we sequenced EGFR exon 18 to 21, which code for the hot spots of EGFR mutations of ATP domains identified in lung cancer. We found a polymorphism in EGFR exon 18, which substitutes A for T at nucleotide 2133. This is the third codon for a threonine (aa# 629), where the T to A transversion is a silent change (Supplemental Fig. S1). The transversion was found to be homozygous in control HEKA and UMSCC-9 cells, T/A heterozygosity was found in UMSCC-6 and -11A cells, whereas the wild-type T residue presented in GENBANK was found only in UMSCC-11B cells. There was no sequence evidence for the previously identified in-frame deletion in exon 19 of the EGFR gene, which was reported in HNSCC of the Korean population. In addition, we did not find the deletion of EGFR exon 2 to 7 (EGFR vIII, PCR data not shown), which results in a truncated extracellular domain and constitutive tyrosine kinase activation.

Gefitinib significantly inhibited EGF-induced EGFR phosphorylation. Gefitinib was designed to inhibit tyrosine kinase activity of EGFR. To test the specific phosphorylation sites of EGFR that could be affected by gefitinib in the HNSCC cell lines, we did a series of experiments to identify the status of known EGFR phosphorylation sites and elucidate the drug effects by ELISA (Fig. 2A) and Western blot analysis (Fig. 2B). The cultured cells exhibited relatively low basal levels of
activated p-EGFR sites tested by both ELISA and Western blot, and gefitinib had little if any significant effect on the constitutive EGFR activation (Fig. 2). Upon EGF stimulation, the cell lines showed different responses in terms of phosphorylation of EGFR at different sites. UMSCC-6 and -11B exhibited relatively high levels of phosphorylation of p-EGFR at Tyr854; UMSCC-9 exhibited the highest level of phosphorylation of p-EGFR at Tyr1068; and UMSCC-11B exhibited the highest levels of p-EGFR at Tyr1173 (Fig. 2). Gefitinib at 1 to 10 times the pharmacologic serum concentration achieved with standard daily dosages of 250 to 500 mg orally (~1 and 10 μmol/L; ref. 32) showed completely inhibitory effects on each of the different EGFR phosphorylation sites in all cell lines, regardless of the drug sensitivity detected by MTT assay (Fig. 1). For total EGFR protein expression, both UMSCC-11A and -11B cells showed a relatively high level of expression when compared with UMSCC-6 and -9 cells (Fig. 2A). Neither EGF nor gefitinib significantly affected the total EGFR protein expression levels in any of the cell lines (Fig. 2).

Gefitinib significantly inhibited EGFR-regulated downstream pathways. Next, we tested the effects of gefitinib on downstream pathways potentially regulated by EGFR, such as AKT,

![Fig. 2](image_url)

**Fig. 2.** Gefitinib effects on phosphorylated and total EGFR expressed by HNSCC cell lines. UMSCC cell lines were cultured in the log growth phase and treated with different doses of gefitinib for 4 h. Then the cells were treated with 10 ng/mL of recombinant EGF for 1 h followed by harvesting of the cell lysates. Phosphorylated and total EGFR were quantified by ELISA (A) or tested by Western blot analysis (B). The data for ELISA were calculated from triplicates from one of repeated experiments, and presented as the mean plus SD. The statistical differences are shown for comparisons with NoTx control (*), with EGF treatment (**), and with 1 μmol/L gefitinib and EGF (#, *P* < 0.05, Student’s t-test).
ERK, and STAT3, by assessing the level of the total and phosphorylated forms of these proteins (Fig. 3). These pathways were constitutively activated, with varying levels of basal phosphorylated and total proteins observed in the different cell lines. Gefitinib sensitivity of cell lines UMSCC-6, -9, and -11A corresponded with elevated basal levels of phospho- and total AKT relative to gefitinib-resistant UMSCC-11B cells. By contrast, UMSCC-11B cells showed the highest basal levels of phosphorylated STAT3. UMSCC-9 cells showed the highest basal phosphorylated and total ERK proteins (Fig. 3). Under EGF stimulation, UMSCC-9 cells showed the strongest induction in p-AKT (S473) and ERK1/2 (TYR185/187), and UMSCC-11B cells exhibited the largest induction in phospho-STAT3 (Y705; Fig. 3). Gefitinib significantly inhibited the basal p-AKT, as well as EGF inducible phosphorylation of AKT, ERK1/2, and STAT3 in the cell lines, in a dose-dependent fashion (Fig. 3). The levels of total proteins were not affected by either EGF stimulation or by gefitinib (Fig. 3).

Next we tested if there were correlations between IC50 values and EGFR downstream molecules tested. The IC50 were statistically and negatively correlated with levels of basal p-AKT ($R^2 = -0.93; P = 0.022$) and EGF-induced p-AKT ($R^2 = -0.89; P = 0.037$), and positively correlated with EGF-induced p-STAT3 ($R^2 = 0.98; P = 0.008$). The other correlations, such as basal and EGF-induced p-ERK, as well as basal p-STAT3, did not reach statistical significance, possibly due to the smaller sample sizes.

**Gefitinib significantly inhibits the EGFR-regulated NF-$\kappa$B pathway.** In addition, we also tested the effects of the drug on the NF-$\kappa$B pathway, because our laboratory has shown that NF-$\kappa$B promotes HNSCC pathogenesis, and EGFR can contribute to aberrant activation of NF-$\kappa$B in HNSCC cell lines (26),

[Fig. 3. Gefitinib effects on phosphorylated and total proteins of signal pathways downstream of EGFR. UMSCC cells were treated and the whole cell lysates were harvested the same way as indicated in Fig. 2. The phosphorylated and total proteins were quantified using ELISA and the data were calculated from triplicates from one of repeated experiments with the mean plus SD. The statistical differences are shown for comparisons with No Tx controls (*), with 1 $\mu$mol/L gefitinib (††), EGF treated with No Tx conditions (‡), EGF treated with 1 $\mu$mol/L gefitinib and EGF-treated conditions (§), and between different doses of gefitinib when both treated with EGF (##), $P < 0.05$ (Student’s $t$-test).]
which was inhibited in the tumor specimen of the molecular responder to gefitinib. We tested the effects of EGF and gefitinib on the NF-κB subunit p65 (S536), implicated as the most important site for p65 transactivation by IκB kinase α of the canonical IκB kinase α/β/γ complex (33). EGF induced significant activation of NF-κB p65 (S536) in 3 of 4 cell lines (Fig. 4). Gefitinib partially inhibited constitutive and significantly inhibited EGF-induced activation of NF-κB p65 (S536) in 2 of 3 of the gefitinib-sensitive lines, UMSCC-6 and -11A (Fig. 4). In general, EGF and gefitinib did not show inhibitory effects on total p65 protein expression levels.

Cross-comparison of the results from RPMA with those detected by ELISA and Western blot analyses. RPMA is a recently developed proteomic microarray platform that is capable of simultaneously quantifying concentrations of numerous proteins and posttranslational modifications such as phosphorylation from small amounts of tissue or cells using validated antibodies (21, 22). To compare the results obtained by RPMA with results previously obtained using the classic Western blot and ELISA methods, RPMA was done with the same whole cell and nuclear lysates as used in Figs. 2–4. We observed similar trends of EGF induction and gefitinib inhibition of the activation of EGFR and downstream molecules in UMSCC-9 and -11B cells (Fig. 5), although there was some variability in results among RPMA, Western blot, and ELISA. More significant alteration in phosphorylation of signaling proteins than in abundance of total signaling proteins was observed, consistent with the findings observed by the classic methods.

Identification of potential biomarkers in HNSCC patients’ samples from a phase I clinical trial using RPMA. A phase I clinical trial combining gefitinib with paclitaxel and radiation in HNSCC patients was concluded at the NCI, and heterogeneous clinical responses were observed in 10 patients enrolled (Fig. 6A and B). The details of clinical responses, and the selection and results of the immunohistochemistry biomarker studies will be presented elsewhere. In biopsies obtained from patients on treatment day 8 of gefitinib alone, significant inhibition of EGFR, multiple-signal phospho-proteins, and proliferation marker Ki-67 was observed, which was accompanied with increased apoptosis (TUNEL) staining in 1 of 7 subjects. This patient was one of the five patients who had a complete response to the combination (patient 3; Fig. 6A and B) and was identified as a molecular responder. Of the remaining six patients defined as molecular nonresponders to gefitinib based on the biomarkers detected by immunohistochemistry, most of the patients responded to standard modalities included in the combined therapy of gefitinib, paclitaxel, and radiation. As expected for a chemotherapy and radiation therapy combination of known activity for previously untreated patients, the 6 of 10 (60%) complete clinical response observed is close to that seen with paclitaxel and radiation without gefitinib in our previous trial (34). It is expected that the response to paclitaxel and radiation exceeded the molecular response to gefitinib, because clinical responders received 8 weeks of gefitinib, including 7 weeks in combination with weekly doses of paclitaxel and 35 fractions of radiation, whereas the patient samples collected for the biomarker study to define the molecular responders were obtained after receiving gefitinib alone for a run-in period of only 7 days, as indicated in Fig. 6A and B.

We did RPMA from tumor lysates obtained pre-gefitinib and after 7 days of gefitinib alone and compared the results with the immunohistochemistry biomarker analysis done on the same biopsy specimen. Protein microarrays were generated from tissue lysates of patient biopsy samples, and 13 biomarkers related to EGFR and its downstream pathways were tested. In patient 3 (Fig. 6B), who was determined to be a molecular responder based on effects on EGFR signaling, apoptosis, and proliferation biomarkers by immunohistochemistry, we also observed consistent molecular responses by RPMA.
Consistent with immunohistochemistry data, a broad decrease in RPMA staining of EGFR and downstream signal molecules (10 of 13 biomarkers) was observed in this patient after gefitinib treatment, including the molecules involved in the AKT, ERK, STAT3, and NF-κB pathways (Fig. 6C, left panel). In addition, the only molecular responder identified in this study did show significantly higher levels of total AKT, p-AKT (Ser473), and total STAT3 in the pretreated samples, but did not show higher levels of total and p-EGFR, p-AKT (Ser308), and total and p-ERKs and MEKs (data not shown). An increased staining in cleaved caspase 3 was only observed in specimen from this patient, consistent with the increase in apoptosis detected by TUNEL assay in the same tumor specimen.5 In contrast, in a representative sample of a molecular nonresponder (Fig. 6C, right panel), there was only a ~40% decrease of total EGFR and p-AKT, a 10% to 20% decrease of total AKT and MEK, but ~60% increase of p-MEK, ~100% increase of STAT3, ~40% increase of p-STAT3, and a slight increase of p-NF-κB. No increase in cleaved caspase 3 was observed in this or other RPMA nonresponder samples. In addition, an increase of either total or phospho-proteins involved in each of the ERK/MEK, STAT3, or NF-κB pathways.

Fig. 5. Reverse phase protein array detection of phosphorylated and total proteins in UMSCC cell lines. Reverse phase protein array was done using the same whole cell and nuclear extracts as used in the previous experiments. The images of immunohistochemical staining were scanned, and data were extracted by ImageQuant5.2 software from replicated slides. * indicates the statistical significance when compared with NoTx conditions; ** indicates the difference when compared with EGF-treated conditions (P < 0.05, Student’s t-test).
Discussion

We investigated the effect of gefitinib on EGFR and related molecular signaling pathways in HNSCC cell lines and patient tumor samples. We showed that gefitinib suppressed EGF-induced EGFR phosphorylation to basal levels at three phosphorylation sites studied, and it inhibited the activation-specific phosphorylation of the downstream signal pathway components AKT, ERK, STAT3, and NF-κB to various degrees in different HNSCC cell lines and tumors. In vitro, a dose-dependent inhibition of tumor cell proliferation was observed that corresponded to higher basal AKT phosphorylation, and was associated with EGF-dependent phosphorylation of AKT and STAT3. In a clinical trial using gefitinib in HNSCC patients, consistent results were observed from tumor specimens of seven patients where the phosphorylation of coactivated downstream pathways and a marker of apoptosis were compared by immunohistochemistry and RPMA. Thus, our study identified alterations in a panel of specific EGFR and downstream signal pathway components of known biological and clinical significance in HNSCC, which could be used as early biomarkers for molecular responsiveness in clinical trials of EGFR-targeted therapeutic agents alone, or used in combination with other agents targeting these multiple pathways.

Using a panel of four UMSCC cell lines, we examined EGFR and downstream molecular pathways activated in HNSCC that are potentially related to EGFR sensitivity. The four cell lines exhibited various levels of total EGFR and EGF-induced receptor phosphorylation (Fig. 2), which are consistent with the reported overexpression of EGFR in HNSCC (1–5, 35). The UMSCC-11A and -11B cell lines exhibited the highest total EGFR compared with UMSCC-6 and -9 by ELISA (Fig. 2A), which is consistent with our previous reports (26, 27). The constitutive activation of EGFR was low among the sites tested, and variability in inducible EGFR phosphorylation was observed (Fig. 2). However, drug sensitivity of the cell lines (IC\textsubscript{50}) could not be simply explained by the level of total EGFR, basal or EGF-induced EGFR phosphorylation, in that gefitinib induced strong suppression of all EGFR phosphorylation sites in all of the cell lines tested (Figs. 1 and 2).

The gefitinib sensitivity in HNSCC lines (IC\textsubscript{50}) seemed to correlate most closely with the extent of phosphorylation of the individual EGFR sites and their mediated AKT, ERK, and STAT3 signal molecules (Figs. 2 and 3). Phosphorylation at EGFR Tyr845 of the kinase domain is involved in c-SRC activation and regulated by both EGF- and integrin-mediated EGFR activation (4, 5, 36). Tyr1068 serves as docking site for PIP3K and both sites are involved in mitogen-activated protein (MAP) kinase signaling activation (38, 39). In addition, Tyr1173 is the favored autophosphorylation site in ligand-activated wild-type EGFR and seems to be the major site of autophosphorylation in the mutant EGFRvIII (10, 40). Among these sites, UMSCC-9 cells exhibited the greatest level of activation of Tyr1068 after EGF stimulation, and also showed the highest basal ERK, as well as strong EGF-induced AKT and ERK phosphorylation (Figs. 2 and 3), consistent with the role of this EGFR site in phosphoinositide 3-kinase, AKT, and ERK pathway activation. UMSCC-9 was also the most sensitive cell line to gefitinib, with an IC\textsubscript{50} of ~ 1.09μmol/L. In addition, the statistical correlations were observed between gefitinib sensitivity with basal and EGF-induced phosphorylation of AKT across the cell lines. The data from cell lines are consistent with the data from the molecular responder in the clinical study, who retained the highest total AKT and p-AKT Ser 473 prior to treatment (data not shown). Although we did not find known mutations previously detected in lung cancer, our data are consistent with previous preclinical studies in lung cancer cells that harbor EGFR mutations (12–16, 41), in which the drug sensitivity to gefitinib is closely correlated with EGF-dependent AKT activation. However, the overexpression and activation of ERK observed did not reach statistical significance in the cell line study, and was not associated with the molecular responder in this small clinical trial. The significance of ERK activation as the biomarker for gefitinib sensitivity in HNSCC needs to be validated in a larger clinical correlative biomarker trial. Our results suggest that phosphorylation of EGFR and AKT may serve as useful early markers of EGFR activation and drug response, which may be due to different etiology.

In addition, a truncation mutation of EGFR, EGFRvIII, has been reported, which harbors an in-frame deletion that results in a truncated 150 kDa protein with constitutive phosphorylation (10, 42–44). These HNSCC with the EGFRvIII deletion showed increased proliferation and resistance to cisplatin and cetuximab treatment, but the response to gefitinib in the cells with such a mutation has not been well studied (10). In this study, however, the EGFR protein from the four UMSCC cell lines comigrate with EGFR of normal keratinocytes and recombinant EGFR protein on Western blot analysis, and sequencing and PCR analyses did not show such deletion mutation. Our data suggest that these truncated forms are unlikely to account for EGFR activation, as well as gefitinib sensitivity and resistance in these HNSCC cells. We sequenced the EGFR genes of these lines for ATM domain mutations identified in gefitinib-responsive lung cancers from Asian populations (17–19), but we did not find the mutations in the exon 18 to 21 of EGFR coding regions. We did find a polymorphism at the EGFR exon 18, but the T to A transversion is a silent sequence change which did not alter the coded amino acid (Supplemental Fig. S1). In addition, the constitutive EGFR tyrosine phosphorylations in UMSCC-9 cells were at the comparable or lower levels compared with those found in other UMSCC cells, suggesting that the gefitinib sensitivity observed in UMSCC-9 and other responsive HNSCC lines could be due to different mechanisms.

\* Pernas F, unpublished observation.
Gefitinib significantly suppressed basal AKT and ERK phosphorylation but not the basal EGFR phosphorylation, which suggests that the basal AKT and ERK activation could be due to other sites of EGFR phosphorylations or other receptor tyrosine kinases, such as hepatocyte growth factor receptor/c-Met (25). The observation is consistent with the fact that gefitinib may not be strictly specific for EGFR, but also be a competitor for the ATP site of other tyrosine kinases as well (45). We previously reported that the hepatocyte growth factor/c-Met pathway is constitutively activated in UMSCC cells, which modulates downstream ERK and PI3K pathways (28). In addition, the relatively high IC_{50} in UMSCC-11B correlated with the higher level of constitutive and EGF-induced activation of STAT3, in which gefitinib did not affect basal and only partially suppressed EGF-induced STAT3 activation (Figs. 3 and 5). As previously reported, STAT3 activation could be mediated by EGFR and interleukin-6R signaling in HNSCC (27, 46, 47), in which gefitinib does not affect interleukin-6R–activated STAT3 signal pathway (27). Consistent with this observation, the rest of the cell lines in the panel exhibited only a modest basal and increase in p-STAT3 when treated with EGF, indicating that EGF is not the major signal to induce STAT3 activation in these cells (Figs. 3 and 5). The data provided by the UMSCC cell lines are in good agreement with the data from

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**Fig. 6.** Clinical information and reverse phase protein array detection of phosphorylated and total proteins in HNSCC patient tumor samples. Ten patients with HNSCC were enrolled in the phase I clinical trial combining gefitinib, radiation, and paclitaxel. **A,** biopsy was done prior to treatment and 1 wk after 7 doses of gefitinib treatment. Gefitinib was given weeks 2-8 concurrent with weekly paclitaxel on Monday and radiation Monday through Friday, as indicated. **B,** molecular response and clinical responses of HNSCC patients. TNM, tumor-node-metastasis staging. Molecular response (with gefitinib treatment alone, a), indicated by inhibition of phospho-signaling and increased apoptosis after treatment (yes); no consistent inhibition of signaling or apoptosis (no); not available (NA). Clinical response, determined 3 mo after treatment (gefitinib + paclitaxel + radiation). **C,** reverse phase protein array was done using the lysates from tumor tissues of this clinical trial. Left panel, data from patient 3, who was identified as the molecular responder and a complete responder clinically. Right panel, data from patient 6, who was identified as the molecular nonresponder. However, clinically, after the combination treatment, the patient showed partial response. The data presented are the percentage changes in intensity, which were calculated from the intensity ratio between the stained samples before versus after treatment.
RPMA from the HNSCC patient specimens, in that the molecular responder showed highest basal total STAT3, as well as suppressed total and phosphorylated STAT3 proteins after gefitinib treatment; however, in the molecular nonresponders, 4 of 5 patients showed increased total and phosphorylated STAT3 proteins after treatment (Fig. 1 and data not shown). These observations suggest that supplementing inhibition of EGFR with agents blocking interleukin-6 or STAT3 may have combinatorial or broader activity in patients with HNSCC.

Furthermore, we found evidence of inhibition and stimulation by EGFR of the NF-κB pathway. Previously we showed that EGFR signaling partially contributes to NF-κB activation (28), which is also inducible and important for cell survival in response to tumor necrosis factor-α, radiation, and chemotherapy (48), consistent with its known functions modulating the critical cellular processes involved in proliferation and apoptosis (48). In this study, EGFR induced and gefitinib suppressed NF-κB p65 Ser536 phosphorylation in 2 of 3 of the gefitinib-sensitive UMSSC cell lines (Fig. 4). Our data are consistent with our previous report (26), showing that EGFR signals partially contribute to NF-κB activation, and are in good agreement with the studies from other laboratories that targeting EGFR can sensitize renal cell carcinoma cell lines to NF-κB inhibition by bortezomib (49).

We compared the results from this study using the traditional ELISA and Western blot (Figs. 2–4) with newly developed RPMA technology (Fig. 5). The development and improvement of RPMA technology provide a new platform for proteomic mapping of signaling pathways in tumor specimens that can help us to simultaneously identify and characterize multiple protein biomarkers that are critical for diagnosis, prognosis, and selection of targeted agents for personalized cancer treatment (21, 50). The advantages of this technology are the small sample size required, as well as the semiquantitative and sensitive protein measurements permitted with use of high-quality validated antibodies. A 1-cm long core or punch biopsy can yield up to 100 RPMA slides, which makes it possible to screen a panel of biomarkers (21, 22, 50), in contrast to the extensive usage of tissue specimen by classic immunohistochemistry staining or other testing methods.

Secondly, this method is able to generate the quantitative and sensitive protein measurements permitted with use of high-quality validated antibodies. A 1-cm long core or punch biopsy can yield up to 100 RPMA slides, which makes it possible to screen a panel of biomarkers (21, 22, 50), in contrast to the extensive usage of tissue specimen by classic immunohistochemistry staining or other testing methods.

The successful cross-comparison of biomarkers by RPMA technology identified in vitro and in small pilot clinical studies herein shows the feasibility of applying the newly developed technology in clinical trials in which a major obstacle includes the limited clinical samples from patients. In this study, we obtained data consistent with the results using classic immunohistochemistry and the studies from UMSSC cell lines (Figs. 2–4). We observed the best molecular response was associated with the highest level of pretreatment p-AKT Ser 473, total AKT, and total STAT3 (data not shown), as well as with the strong gefitinib-suppressed p-EGFR Tyr1148, p-AKT Ser473, p-ERK Ser185/187, and NF-κB p-65 Ser536 in the only molecular and clinical responder (Fig. 6). In addition, an increase of cleaved caspase 3 activation was also observed in the responder, which is consistent with the tumor apoptosis data. In contrast, gefitinib failed to inhibit but rather induced activation of the MEK, STAT3, and/or NF-κB pathways in the rest of nonresponder patients. Our study revealed the heterogeneity of cancer as a major hurdle that limits the effective treatment. Such heterogeneity usually remains undetected by standard histologic pathologic classification and clinical grading systems. To address this problem, we recently have identified such subgroup-specific gene expression signatures in HNSCC cell lines with increased frequency of promoter binding sites for transcription factors NF-κB, AP-1, and STAT3 or p53 (23, 30), that support conclusions drawn from this study. Our study suggests that by utilizing RPMA and other proteomic technologies, it may be feasible to identify clinically useful biomarkers associated with the higher risk for HNSCC recurrence and metastasis, and provide more accurate prognosis and selection of therapy based on the proteomic signatures.

Disclosure of Potential Conflicts of Interest

The authors have received gefitinib for research and clinical trials from AstraZeneca Inc., under a cooperative research development and clinical trials agreement with the National Cancer Institute.

Acknowledgments

We thank Justin L. Ricker, MD, PhD, medical director at Abbott, Abbott Park, IL, and John Sunwoo, MD, assistant professor in the Department of Otolaryngology, Washington University School of Medicine, for reading and providing useful comments on the manuscript.

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Proteomic Signatures of Epidermal Growth Factor Receptor and Survival Signal Pathways Correspond to Gefitinib Sensitivity in Head and Neck Cancer

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