Establishment and Characterization of a Model of Acquired Resistance to Epidermal Growth Factor Receptor Targeting Agents in Human Cancer Cells

Sergio Benavente, Shyhmin Huang, Eric A. Armstrong, Alexander Chi, Kun-Tai Hsu, Deric L. Wheeler, and Paul M. Harari

Abstract

Purpose: The epidermal growth factor receptor (EGFR) is recognized as a key mediator of proliferation and progression in many human tumors. A series of EGFR-specific inhibitors have recently gained Food and Drug Administration approval in oncology. These strategies of EGFR inhibition have shown major tumor regressions in approximately 10% to 20% of advanced cancer patients. Many tumors, however, eventually manifest resistance to treatment. Efforts to better understand the underlying mechanisms of acquired resistance to EGFR inhibitors, and potential strategies to overcome resistance, are greatly needed.

Experimental Design: To develop cell lines with acquired resistance to EGFR inhibitors we utilized the human head and neck squamous cell carcinoma tumor cell line SCC-1. Cells were treated with increasing concentrations of cetuximab, gefitinib, or erlotinib, and characterized for the molecular changes in the EGFR inhibitor–resistant lines relative to the EGFR inhibitor–sensitive lines.

Results: EGFR inhibitor–resistant lines were able to maintain their resistant phenotype in both drug-free medium and in athymic nude mouse xenografts. In addition, EGFR inhibitor–resistant lines showed a markedly increased proliferation rate. EGFR inhibitor–resistant lines had elevated levels of phosphorylated EGFR, mitogen-activated protein kinase, AKT, and signal transducer and activator of transcription 3, which were associated with reduced apoptotic capacity. Subsequent *in vivo* experiments indicated enhanced angiogenic potential in EGFR inhibitor–resistant lines. Finally, EGFR inhibitor–resistant lines showed cross-resistance to ionizing radiation.

Conclusions: We have developed EGFR inhibitor–resistant human head and neck squamous cell carcinoma cell lines. This model provides a valuable preclinical tool to investigate molecular mechanisms of acquired resistance to EGFR blockade.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases. Four ErbB members have been identified to date: EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). EGFR plays a critical role in the development and in the neoplastic processes of cell proliferation, apoptosis, angiogenesis, and metastatic spread. Stimulation of the receptor through ligand binding leads to receptor oligomerization at the plasma membrane. This activates the receptor tyrosine kinase and thereby promotes autophosphorylation of tyrosine residues in the cytoplasmic tail. These events lead to the activation of several signaling cascades, most notably the mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT), STAT, and phospholipase Cγ pathways that ultimately result in proliferative signals to the cell nucleus (1).

The EGFR is recognized as a central regulator of proliferation and progression in many human cancers. In general terms, tumor EGFR expression correlates inversely with clinical outcome (2). A series of potent EGFR inhibitors (cetuximab, panitumumab, erlotinib, and gefitinib) have been developed in recent years, and several thousand cancer patients have now been treated in the context of controlled clinical trials (3–6). Despite great clinical promise, with 10% to 20% of patients manifesting highly favorable response to EGFR inhibition, the majority of cancer patients show either intrinsic resistance or acquired resistance to EGFR inhibitor therapies (7–11). The identification of resistance mechanisms to EGFR inhibitors remains critical to the successful advancement of these

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Translational Relevance

The development of acquired resistance to epidermal growth factor receptor (EGFR) inhibitors is emerging as a potential treatment barrier for EGFR-targeted therapy. In the current study, we establish and characterize EGFR inhibitor – resistant tumor cells against three leading EGFR inhibitors to investigate molecular mechanisms of -acquired resistance and consider potential strategies that may help overcome resistance. Several lines of data suggest resistance mechanisms that involve the activation of alternative survival pathways, such as AKT and angiogenic signaling. These data provide a rationale for the investigation of AKT or angiogenesis targeting agents as worthy treatment approaches for tumors that manifest EGFR inhibitor resistance. In addition, results from the current study provide data regarding the combination of EGFR inhibitors with radiation. In the context of EGFR inhibitor resistance, the data suggest that it may prove most advantageous to deliver EGFR inhibitors concurrent with or immediately following radiation as opposed to prior to radiation.

promising molecular targeting agents. In this article, we present the development of cetuximab-, gefitinib-, or erlotinib-resistant head and neck tumor cells following chronic exposure to these agents. In addition, we explored the molecular mechanisms associated with the resistant phenotype by characterizing specific molecular and cellular distinctions between EGFR inhibitor – resistant and -sensitive parental cells. These studies provide valuable insight regarding molecular mechanisms of acquired resistance to EGFR-targeting agents and thereby provide a model to explore strategies to overcome acquired resistance to EGFR-targeting agents.

Materials and Methods

Cell lines and chemicals. The human head and neck squamous cell carcinoma cell lines SCC-1 (UM-SCC1) were kindly provided by Dr. Thomas E. Carey (University of Michigan) and were cultured routinely in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum, 1 μg/mL hydrocortisone, and 1% penicillin and streptomycin. Cell culture media and supplements were obtained from Life Technologies, Inc. Gefitinib (ZD1839; Iressa) was generously provided by AstraZeneca. Erlotinib (OSI-774; Tarceva) was generously provided by Genentech, Inc. Cetuximab (C225; Erbitux) was generously provided by ImClone Systems Inc. Primary antibodies against p-MAPK (Thr202/Tyr204), p-AKT(Ser473), and STAT3 (Tyr705) were obtained from Cell Signaling Technology. Anti-p-EGFR(Tyr1173) antibodies were obtained from Santa Cruz Biotechnology Inc. and anti-α-tubulin antibody was obtained from Oncogene Research Products. Annexin V-FITC apoptosis detection kit was obtained from BD Biosciences Pharmingen. All other chemicals were purchased from Sigma.

Establishment of acquired resistance to EGFR inhibitors. Over a period of 12 mo, tumor cells in culture were continuously exposed to increasing concentrations of cetuximab, gefitinib, or erlotinib. Commencing with the IC_{50} of EGFR inhibitors for a particular tumor cell line, the exposure dose was progressively doubled every 10 to 14 d until 7 ~ 8 dose doublings had been successfully achieved. In parallel, controlled parental cells were exposed to corresponding vehicle for the drugs, i.e., PBS for cetuximab and DMSO for gefitinib and erlotinib. The established resistant cell lines were then maintained in continuous culture with the maximally achieved dose of EGFR inhibitor that still allowed cellular proliferation.

Cell proliferation assay. Exponentially growing cells were seeded in 6-well plates. Following the treatment, cells were then washed with PBS and fixed/stained with 0.5% crystal violet. Plates were air-dried overnight and dye was eluted with 0.1 mol/L sodium citrate (pH 4.2) in ethanol (1:1). Elution was transferred to 96-well plates, and the absorbance was read at 540 nm to determine cell viability.

Cell cycle analysis. Parental or resistant cells were harvested by trypsin, washed with PBS, then fixed in 95% ethanol and stored at 4°C for up to 7 d prior to DNA analysis. After the removal of ethanol by centrifugation, cells were incubated with phosphate-citric acid buffer (0.2 mol/L Na_2HPO_4, pH 7.8, 4 mmol/L citric acid) at room temperature for 45 min. After centrifugation, cells were then stained with a solution containing 33 μg/mL propidium iodide, 0.13 mg/mL RNase A, 10 mmol/L EDTA, and 0.5% Triton X-100 at 4°C for 24 h. Stained nuclei were analyzed for DNA-propidium iodide fluorescence using a Becton Dickinson FACScan flow cytometer. Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc.) for the proportion of cells in G_1, S, and G_2/M phases of the cell cycle.

Immunoblotting analysis. Following treatment, cells were lysed with Tween-20 lysis buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonylfluoride, and 10 μg/mL of leupeptin and aprotinin) and sonicated. Equal amounts of protein were analyzed by SDS-PAGE. Thereafter, proteins were transferred to nitrocellulose membranes and analyzed by specific primary antibodies as indicated in the experiment. Proteins were detected via incubation with horseradish peroxidase – conjugated secondary antibodies and ECL chemiluminescence detection system.

Assessment of apoptosis. Apoptosis was detected by flow cytometry via the examination of altered plasma membrane phospholipid packing by lipophilic dye Annexin V. Using Annexin V as a FITC conjugate in combination with propidium iodide as an exclusion dye for cell viability, this assay not only can detect apoptotic cells but also discriminate between apoptosis and necrosis. Briefly, treated cells were harvested by trypsin, washed twice with PBS, and were then resuspended in binding buffer at a concentration of 1×10^6 cells/mL according to the manufacturer's instruction. Thereafter, 5 μL of Annexin V-FITC and 5 μL of propidium iodide were added into 100 μL of cell suspension and incubated for 30 min at room temperature in the dark. After adding 400 μL of binding buffer, labeled cells were counted by flow cytometry within 30 min. All early apoptotic cells (Annexin V – positive, propidium iodide – negative), necrotic/late apoptotic cells (double positive), as well as living cells (double negative) were detected by FACSCalibur flow cytometer and subsequently analyzed by Cell Quest software (Becton Dickinson). Argon laser excitation wavelength was 488 nm, whereas emission data were acquired at wavelength 530 nm (FL-1 channel) for FITC and 670 nm (FL-3 channel) for propidium iodide.

Assay of tumor growth in athymic nude mice. Athymic nude mice (3- to 4-week-old females) were obtained from Harlan Bioproducts for science and maintained in a laminar air-flow cabinet under aseptic conditions. The care and treatment of experimental animals was in accordance with institutional guidelines. Human cancer cells ($\sim 1 \times 10^6$) were injected s.c. into the dorsal flank area of the mice. Following the establishment of tumor, cetuximab was administered i.p. at a dose of 0.2 mg twice per week for 4 consecutive wk, and gefitinib or erlotinib was given by oral gavage at a dose of 1 mg, 5 d/wk for 4 consecutive wk. Tumor volume was determined by direct measurement with calipers and calculated by the formula; $\pi/6 \times$ (large diameter) $\times$ (small diameter)^2.

Tumor xenograft angiogenesis assay (Matrigel plug). Tumor angiogenesis was evaluated by Matrigel plug neovasculation assay as

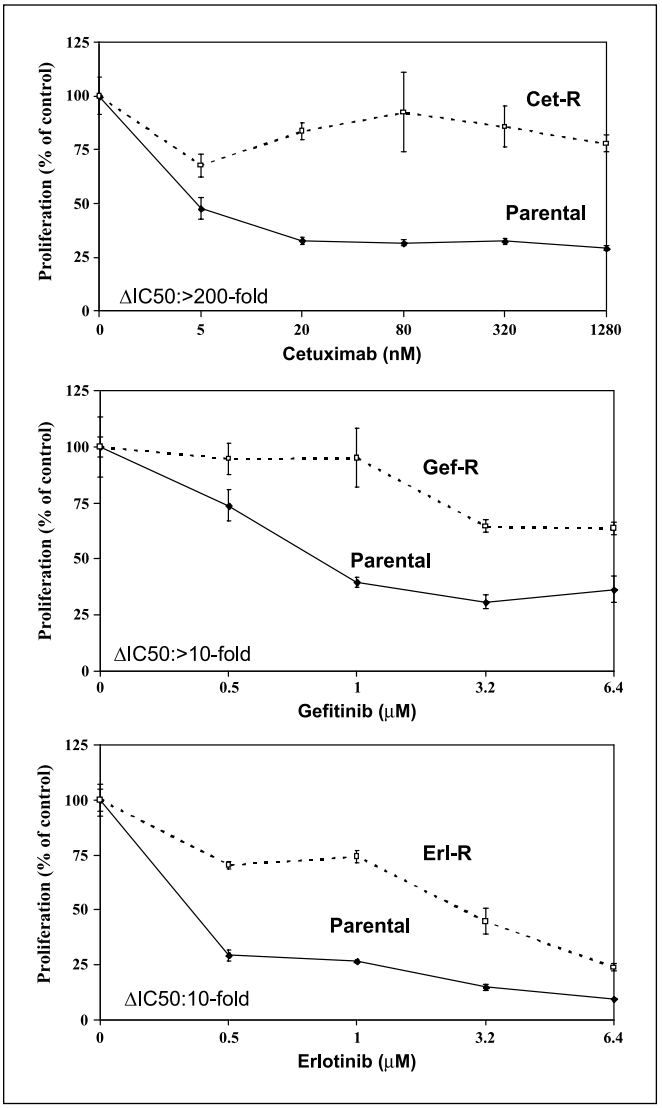

Fig. 1. Growth profile of EGFR inhibitor – resistant cells. Cet-R, Gef-R, and Erl-R cells, and their corresponding parental SCC-1 controls were treated with increasing amounts of EGFR inhibitors. Following 72 h incubation, the numbers of viable cells in each well were determined by a proliferation assay as described in Materials and Methods. Results were expressed as the percentage of cell growth relative to controls. Each point represents mean ± SD of three determinations.

described previously (12). Briefly, athymic mice were injected s.c. along the flank area with 0.5 mL Matrigel. After 24 h, a suspension of SCC-1 cells (1×10^6 in 5 μL) was soaked with a polyvinyl sponge ($2 \times 2 \times 1.5$ mm). The sponge was then introduced into a surgically created micropocket in the center of Matrigel plug formed within the mouse abdominal wall. The wound was then closed with a suture. Ten days later, mice were injected with 0.2 mL of a 50 mg/mL FITC-Dextran (MW, ~2,000 kDa) solution via the tail vein for the purpose of visualizing vessels within the Matrigel plug. After 15 min, mice were sacrificed, and the Matrigel plugs were removed and fixed in 10% formalin solution. To visualize the general layout of the Matrigel plug and the presence of perfused blood vessels, phase-contrast microscopy and fluorescence microscopy were used respectively. The intensity of fluorescence was further quantified by Adobe Photoshop software (Adobe Systems).

Radiation survival. Survival following radiation exposure was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Briefly, after exposure to radiation, cells were trypsinized, counted, and seeded for colony formation in 35-mm dishes at 50 to 5,000 cells per dish. Following incubation intervals of 14 to 21 d, colonies were stained with crystal violet and manually counted. Colonies consisting of ≥50 cells were scored, and 4 to 10 replicate dishes containing 10 to 150 colonies per dish were counted for each treatment.

Statistical analysis. The effect of EGFR inhibitors on apoptosis induction was evaluated using Student's *t* test.

Results

Development of EGFR inhibitor–resistant cells. The human head and neck squamous cell carcinoma cell line SCC-1 was used to develop resistance to the EGFR inhibitors cetuximab, erlotinib, and gefitinib. As described in Materials and Methods, treatment started at the IC_{50} of each drug which caused 50% inhibition of cell proliferation and the exposure dose was

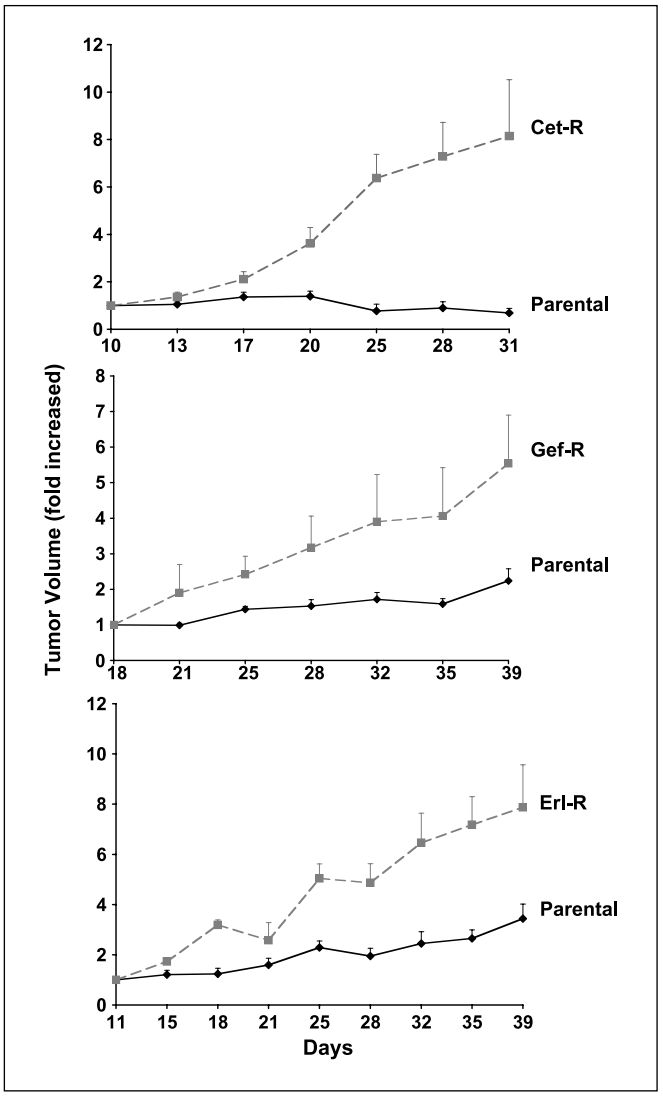

Fig. 2. EGFR inhibitor – resistant cells maintain resistance as tumor xenografts. Parental or EGFR inhibitor – resistant cells (Cet-R, Gef-R, or Erl-R) were injected s.c. into the dorsal flank of athymic mice. Following the establishment of tumor, mice were treated with cetuximab, gefitinib, or erlotinib as described in Materials and Methods. Tumor volume was monitored and the growth of tumor was expressed as the fold increase of tumor volume following initiation of drug treatment ($n = 8$).

progressively doubled every 10 to 14 days until 7 to 8 dose doublings had been achieved. The cetuximab-resistant lines (Cet-R) were treated up to a maximal dose of 640 to 1280 nmol/L of cetuximab, whereas the gefitinib- (Gef-R), and erlotinib-resistant (Erl-R) lines reached a maximal dose of 6.4 μmol/L each. After the establishment of EGFR inhibitor resistant lines, we characterized their resistant phenotype by doing cell proliferation assays when challenged with EGFR inhibitors (Fig. 1). We consistently observed higher proliferative potential and a 10-fold increase or greater in the IC_{50} for all EGFR inhibitor–resistant cell lines as compared with parental cells (ΔIC_{50}). Cell cycle analysis showed that Cet-R, Gef-R, and Erl-R cells did not exhibit a G_1 arrest or marked reduction in S-phase when challenged with cetuximab, gefitinib, or erlotinib as compared with the sensitive parental controls (Supplementary Fig. S1). These results indicate that characteristic cell cycle checkpoints in EGFR inhibitor–resistant lines are no longer affected by EGFR blockade. We then confirmed the establishment of stable EGFR inhibitor–resistant cells in a drug-free culture system. Results showed that EGFR inhibitor–resistant SCC-1 cells still exhibited the resistant phenotype even when cells were cultured in drug-free medium for at least nine months (Supplementary Fig. S2).

Building upon these results, we used a mouse xenograft model to determine if the resistance to EGFR inhibitors developed *in vitro* would retain the resistance phenotype *in vivo*. To carry out these experiments, we inoculated the EGFR inhibitor sensitive lines (Parental) and the EGFR–inhibitor resistant lines into the dorsal flank of athymic nude mice. After two weeks, systemic administration of either vehicle or the corresponding EGFR inhibitor was delivered to mice. These *in vivo* results, presented in Fig. 2, indicate that EGFR inhibitor–resistant cells established in culture maintain their resistant phenotype in the *in vivo* xenograft model system. Taken together, these results indicate that we have developed SCC-1 cell lines resistant to cetuximab, erlotinib, and gefitinib. In addition, these cells can grow in the absence of drug for long periods of time and maintain their resistant phenotype as well as a resistant phenotype *in vivo*.

EGFR signaling of EGFR inhibitor–resistant cells. To determine if the EGFR signaling cascade was altered in EGFR inhibitor–resistant lines, we investigated the active EGFR, AKT, MAPK, and STAT3. Using immunoblotting analysis, we found that treatment with EGFR inhibitors efficiently blocked EGF-stimulated activation of EGFR, AKT, MAPK, and STAT3 in a dose-dependent manner in parental cells but not in Cet-R, Gef-R, and Erl-R cells (Fig. 3). These results not only confirm the resistant characteristic of resistant cells to EGFR inhibitor but also imply that alternative signaling pathways related to MAPK, AKT, or STAT3 may play an important role in the development of resistance to EGFR targeting agents.

Apoptotic response of EGFR inhibitor–resistant cells. To determine if EGFR inhibitor–resistant lines had decreased apoptotic potential, we measured apoptosis using Annexin V/propidium iodide staining in parental and resistant SCC-1 cells. As shown in Fig. 4, treatment with cetuximab, gefitinib, or erlotinib resulted in the significant induction of apoptosis in a dose-dependent manner in parental cells. However, there was no significant change of early apoptotic cell populations in Cet-R, Gef-R, or Erl-R cells following EGFR inhibitor exposure. These results indicate that EGFR inhibitor–resistant lines may

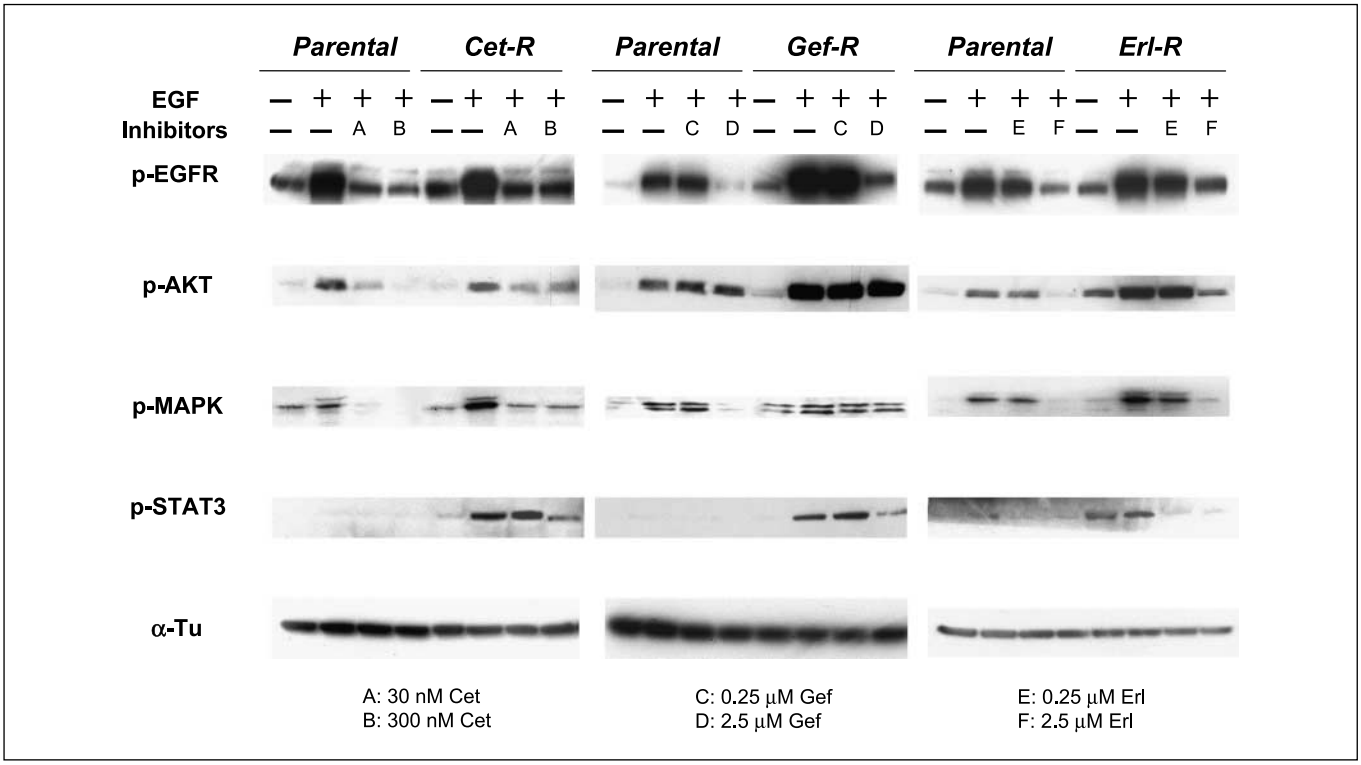

Fig. 3. EGFR signaling profile of EGFR inhibitor – resistant cells. Parental and EGFR inhibitor – resistant cells (Cet-R, Gef-R, or Erl-R) were exposed to different concentrations of corresponding inhibitors for 2 h followed by 45 min of EGF (10 μg/mL) stimulation. Following harvesting, cells were lysed and processed for immunoblotting using antibodies directed against p-EGFR, p-AKT, p-MAPK, and p-STAT3 as described in Materials and Methods. The α-tubulin (α-Tu) serves as a loading control.

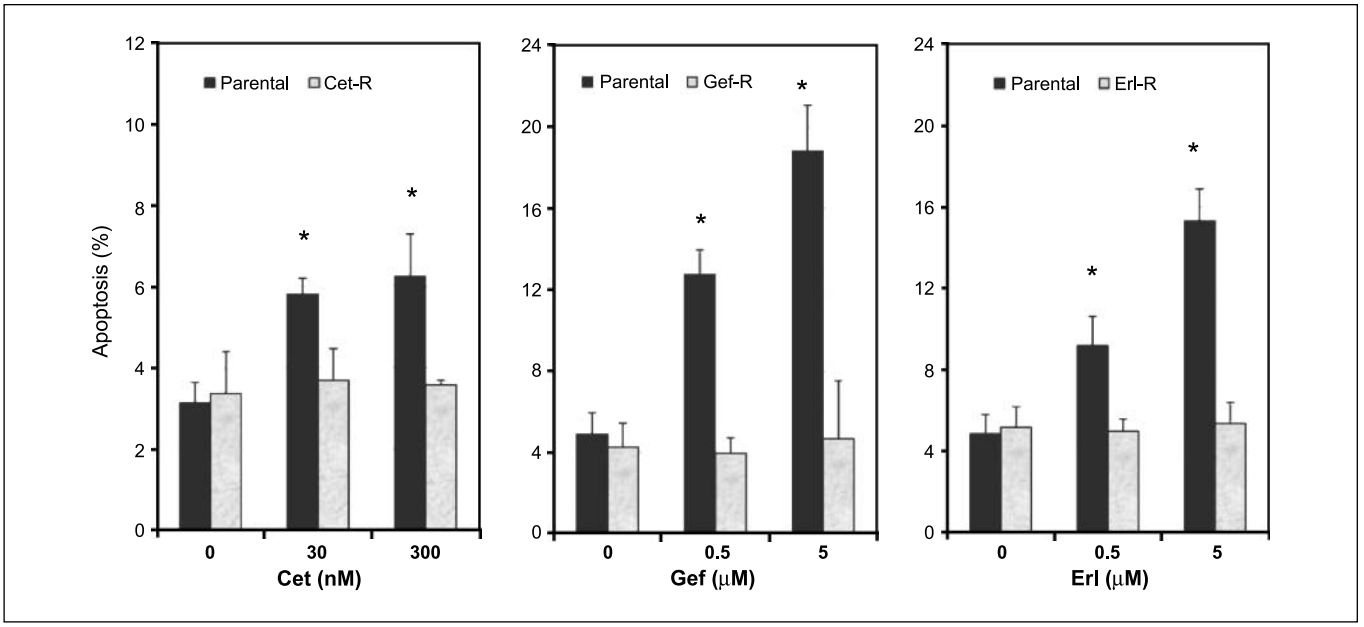

Fig. 4. Apoptosis response of EGFR inhibitor – resistant cells following EGFR inhibitor treatment. Apoptosis was examined by flow cytometry using Annexin V and propidium iodide staining as described in Materials and Methods. Parental and EGFR inhibitor – resistant cells (Cet-R, Gef-R, or Erl-R) were exposed to different concentrations of cetuximab, gefitinib, or erlotinib for 72 h. The percentage of cells in early apoptotic population was analyzed by Cell Quest software (Becton Dickinson). *, $P < 0.05$. Similar results were obtained in replicate experiments.

be able to escape the characteristic apoptotic response induced by EGFR inhibitors.

Angiogenesis potential of EGFR inhibitor–resistant cells. Alteration of EGFR signaling in EGFR inhibitor–resistant cells may cause changes in angiogenic potential. To test this hypothesis, we investigated tumor vascularization by s.c. implanting tumor cell-imbedded Matrigel plugs into nude mice as described previously (13). In this assay, parental and EGFR inhibitor–resistant SCC-1 cells were introduced into separate Matrigel plugs in the same mouse. Following implantation with tumor cells, blood capillaries were observed growing from the edge of the Matrigel plugs toward the implanted tumor as visualized by fluorescent microscopy. As shown in Fig. 5, plugs with parental SCC-1 cells were visibly clear, with limited identifiable blood vessels migrating into the Matrigel. In contrast, plugs containing Cet-R, Gef-R, or Erl-R cells showed extensive vascularization and growth of vessels towards the tumor core that was confirmed by the enhanced intensity of fluorescence (*bottom panel*). These results suggest that the development of acquired resistance to EGFR inhibitors *in vitro* can enhance mechanisms involved in angiogenesis.

Radiation response of EGFR inhibitor–resistant cells. To determine if EGFR inhibitor–resistant cells have increased resistance to radiation treatment, we evaluated EGFR inhibitor–resistant lines using clonogenic survival assays (14). Figure 6 depicts radiation-survival curves for Cet-R, Gef-R, Erl-R, and the corresponding parental SCC-1 cells. The results indicated that EGFR inhibitor–resistant cells had a higher survival rate when treated with 3, 6, or 9 Gy of radiation as compared with parental cells. The reduced cell death in resistant cells was further confirmed by evaluating the apoptosis profile of tumor cells following radiation treatment using Annexin V/propidium iodide flow cytometric analysis as described above. As shown in Fig. 7, treatment with radiation resulted in the induction of apoptosis in a dose-dependent manner in the parental cells. However, there was no significant change in the apoptotic cell populations when Cet-R, Gef-R, or Erl-R cells were treated with radiation. Taken together, these results indicate that tumor cells with acquired resistance to EGFR targeting inhibitors manifest radioresistance possibly by altered abilities to effectively induce apoptosis.

Discussion

Molecular inhibition of EGFR signaling is under active investigation as a promising cancer treatment strategy. Despite broad enthusiasm regarding the potential value of EGFR target modulation in cancer therapy, acquired resistance to EGFR inhibitors has been widely observed in preclinical model systems and in cancer patients who initially respond well to treatment (11, 15–18). Similar to the development of acquired resistance to other molecular targeted agents, such as Gleevec (19) and Herceptin (20), acquired resistance to EGFR inhibitors may limit therapy options, as EGFR inhibitor–resistant tumors may also become cross-resistant to other drug or treatment modalities with different mechanisms of action (21). Efforts to better understand the underlying mechanisms of acquired resistance to EGFR inhibitors, and potential strategies to overcome resistance, are greatly needed.

In the current study we present the development of a head and neck squamous cell carcinoma tumor cell line (SCC-1) resistant to cetuximab, gefitinib, and erlotinib to explore mechanisms of resistance to EGFR blockade (Fig. 1). To determine if acquired resistance in this *in vitro* model was stable, we cultured resistant cells in the presence or absence of cetuximab, gefitinib, or erlotinib for nine months. After nine months in drug-free culture, cells maintained their resistance to each individual EGFR inhibitor, indicating that the molecular

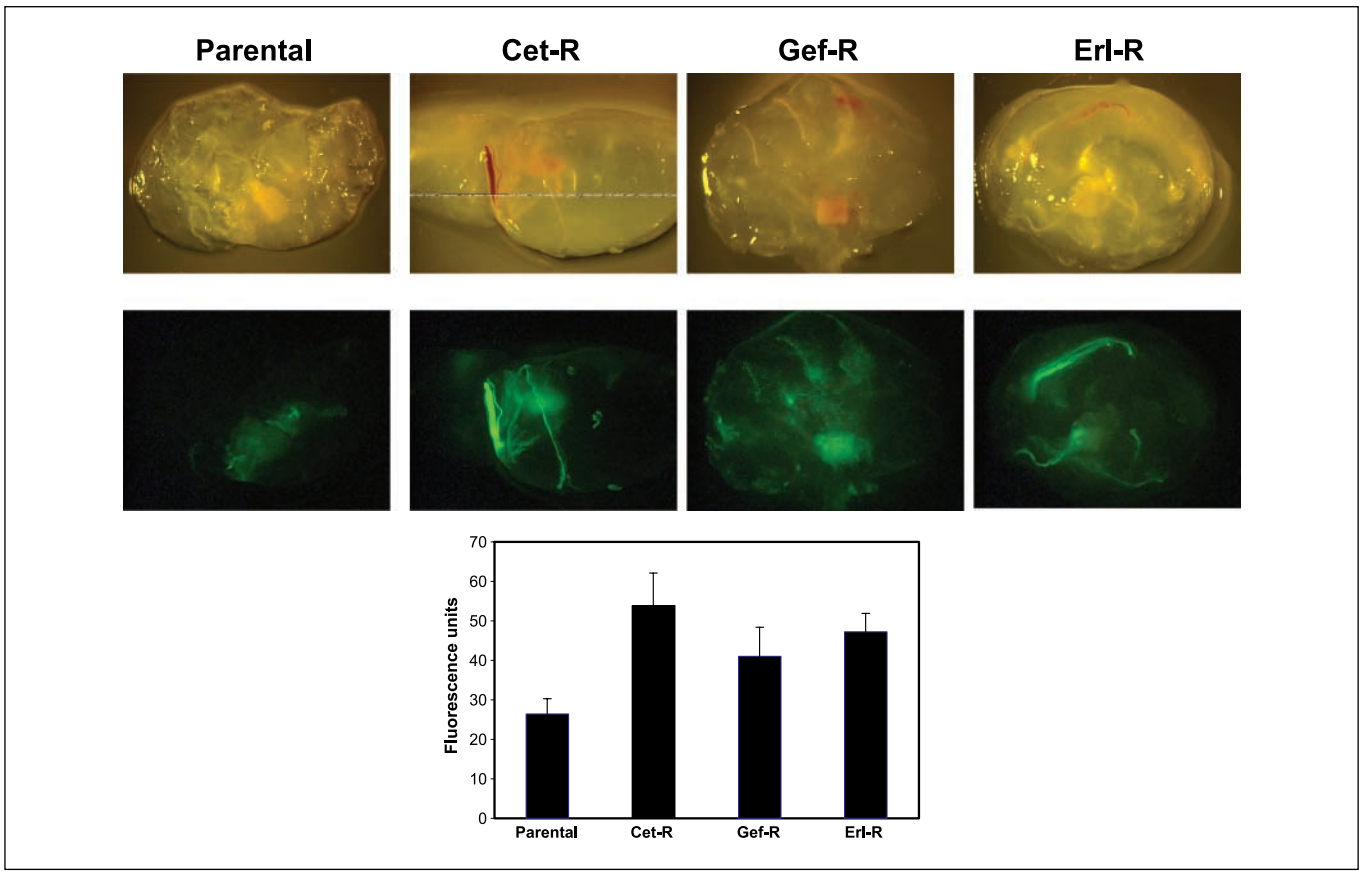

Fig. 5. Angiogenesis potential of EGFR inhibitor – resistant cells. Parental or EGFR inhibitor – resistant (Cet-R, Gef-R, or Erl-R) cells were implanted into dorsal Matrigel plugs (*upper panel*) prepared in athymic mice as described in Materials and Methods. Following 10 d after implantation the Matrigel plugs were removed and examined by fluorescence microscope. Middle panel, green fluorescent blood vessels in Matrigel plug; bottom panel, intensity of fluorescence further quantified. Results were obtained from four mice in two independent experiments. Bars, SD.

changes that occur in acquired resistance to EGFR blockade are stable (Supplementary Fig. S2). Furthermore, cells with acquired resistance to cetuximab, gefitinib, or erlotinib could form xenografts in athymic nude mice and maintain their resistant phenotype when challenged with EGFR inhibitors *in vivo* (Fig. 2). Results from cell cycle analysis (Supplementary Fig. S1) provide a potential clue to the resistant phenotype that suggests that cells with acquired resistance to EGFR inhibitors have a large population of cells in S-phase of the cell cycle relative to parental controls. Furthermore, cells with acquired resistance to EGFR inhibitors maintain this population in S-phase after challenge with EGFR inhibitors, whereas the parental cells arrest primarily in G_1. These findings suggest that cells with acquired resistance to EGFR inhibitors receive proliferative signals independent of signaling from the EGFR, and maintain cells in S-phase even when challenged with EGFR inhibitors.

Although EGFR inhibitors are known to inhibit the PI3K/AKT pathway, the consistent observation of elevated level of p-AKT in our resistant cells (Fig. 3) indicates constitutive activation of AKT as an important mediator of EGFR resistance. Our findings are consistent with the findings of Yamasaki et al. in which they observed acquired resistance to erlotinib following constitutively active Akt transfection in A431 cells (15). Janmaat et al. also reported that persistent activity of the PI3K/AKT and/or MAPK pathway associated with gefitinib-resistance of non – small cell lung cancer cell lines and that simultaneous inhibition of both pathways reduces tumor survival more effectively than inhibition of each pathway alone (22). These data are consistent with other reports showing that loss of phosphotase and tensin homolog (PTEN) results in PI3K and AKT hyperactivity and resistance to gefitinib in MDA-468 breast cancer cells (23, 24). Reconstitution of PTEN in these cells reestablished EGFR-driven AKT signaling and thereby restored gefitinib sensitivity (25). More interestingly, recent studies from our group and the Engelman group show that sensitive cancers may adapt to activate the PI3K-AKT pathway as they become resistant via activation of alternative receptor tyrosine kinases, such as ErbB3, c-Met, and insulin-like growth factor receptor (26 – 28). Taken together, these data suggest that AKT-mediated survival-signaling pathways play a key role in resistance to anti-EGFR therapy and that the use of inhibitors that target the AKT pathway in resistant tumors may be beneficial for clinical response.

Beyond targeting AKT, an alternative strategy to overcome acquired resistance to EGFR inhibitors is to combine EGFR agents with angiogenesis-targeting agents. This approach is highly appealing in view of observations that the angiogenic process is involved in the development of resistance to anti-EGFR therapy. By using the Matrigel plug neovascularization assay, we show that plugs with acquired resistance to cetuximab, gefitinib, or erlotinib exhibit a higher vessel density than parental cells (Fig. 5). Consistent with these findings, Viloria-Petit et al. found that vascular endothelial growth factor

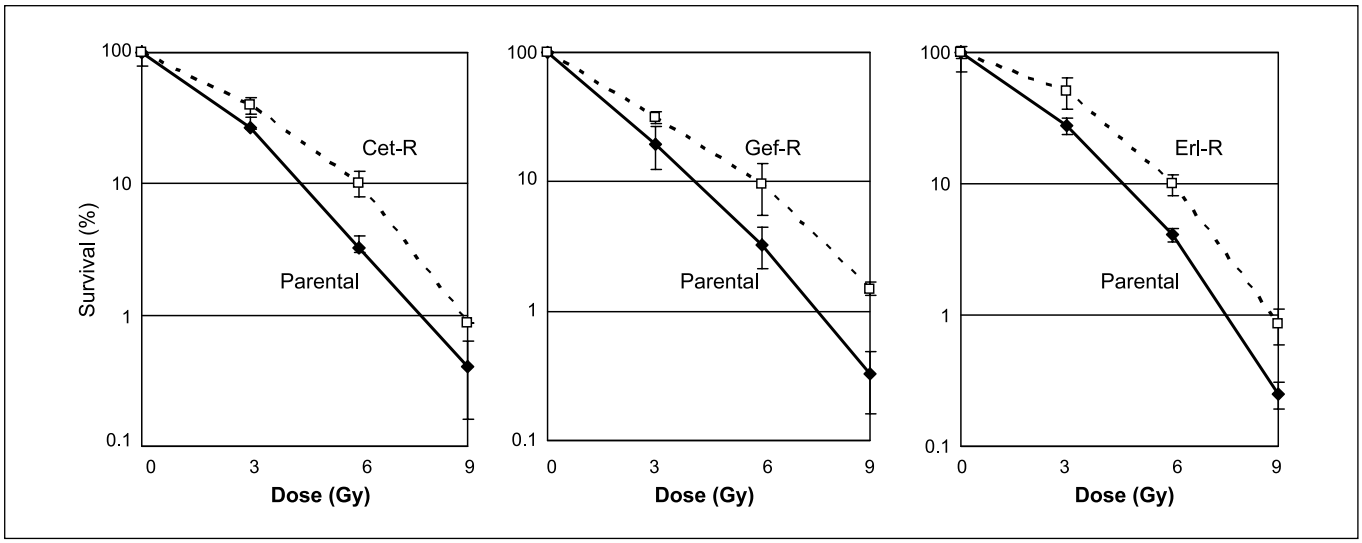

Fig. 6. Radiation response of EGFR inhibitor – resistant cells. Radiosensitivity of EGFR inhibitor – resistant (Cet-R, Gef-R, or Erl-R) and parental tumor cells were examined by clonogenic survival analysis following 3, 6, or 9 Gy of radiation as described in Materials and Methods. Results were expressed as the percentage of colony formation relative to controls. Data points, means of duplicates with five cultures per replicate per point; bars, SD.

expression was elevated in cetuximab-resistant A431 cells developed *in vivo* (18). In addition, Ciardiello et al. determined that vascular endothelial growth factor expression was elevated in gefitinib-resistant cells when screening a panel of colon cancer cell lines (29). Thus, up-regulation of vascular endothelial growth factor may contribute to increased angiogenesis and to resistance to EGFR inhibitors. Combining antiangiogenic agents with EGFR inhibitors may confer the resistance to anti-EGFR therapy. To test this possibility, combined treatment of EGFR targeting agent and vascular endothelial growth factor receptor targeting agent ZD6474 has been evaluated in gefitinib- and cetuximab-resistant tumor cells (30, 31). The results showed that the combined treatment achieved significantly greater tumor growth inhibition in both sensitive and resistant tumor cells. Furthermore, ZD6474 inhibited tumor growth in cells that were refractory to anti-EGFR therapy. These results provide a clinical rationale for further investigation of antiangiogenenic agents as a potential treatment option for EGFR inhibitor-resistant tumors.

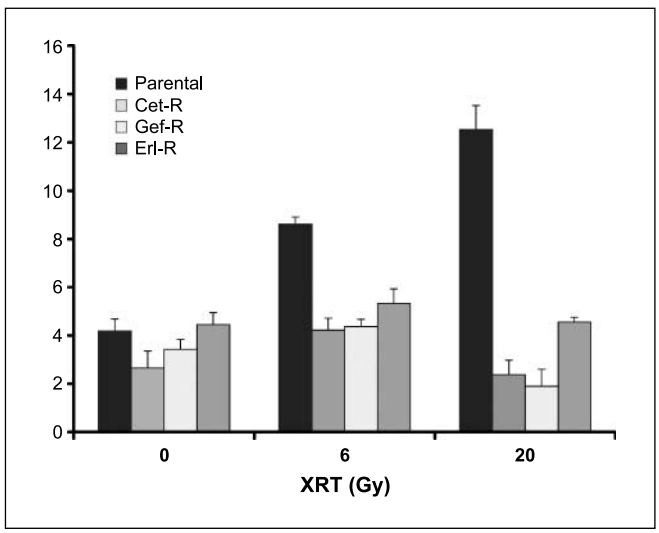

Fig. 7. Radiation-induced apoptosis profile of EGFR inhibitor – resistant cells. Parental and EGFR inhibitor – resistant cells (Cet-R, Gef-R, or Erl-R) were exposed to 0, 6, or 20 Gy of radiation. Twenty-four hours later, cells were harvested and processed for Annexin V/propidium iodide staining followed by flow cytometry analysis as described in Materials and Methods. The percentage of cells in early apoptotic population was analyzed by Cell Quest software (Becton Dickinson). Similar results were obtained in replicate experiments.

Acquired resistance to EGFR inhibitors presents a clinical problem not only due to the development of resistance to EGFR blocking agents, but also due to potential manifestation of resistance to other drug or treatment modalities with distinct mechanisms of action. Data from the present study show that following chronic exposure to EGFR-targeting agents, tumor cells with acquired resistance to EGFR inhibitors developed resistance to ionizing radiation (Figs. 6 and 7). Consistent with our findings, previous reports indicate that dose and schedule of drug administration in combination with radiation seemed to be a critical element to obtain radiosensitization of tumor cells (32, 33). Stea et al. suggested that the optimal combination of gefitinib and irradiation occurred only with a short preincubation period of 30 minutes followed by 8 hours of continuous exposure to gefitinib postradiation. On the other hand, a prolonged preincubation interval of 24 hours not only reduced radiosensitivity, but actually conferred radioprotection in tumor cells (32). These results suggest that the optimal dose of drug for radiosensitivity modulation and sequencing of modalities should be considered with caution. Furthermore, these results warrant consideration in the design of future clinical strategies for EGFR-radiation combinations. Specifically, it may prove most advantageous to deliver EGFR inhibitors during or after radiation as opposed to before radiation. Recent data from Milas et. al. similarly suggest benefit from continuing EGFR inhibitor therapy following radiation treatment (34).

In summary, the work presented herein describes the establishment of resistant tumor cell lines to cetuximab, gefitinib, or erlotinib derived from head and neck SCC-1 cells. Our results show that following long-term exposure to anti-EGFR agents, tumor cells acquire resistance not only to anti-EGFR

therapy but also to radiation therapy. Several lines of data point to potential resistance mechanisms involving the activation of alternative survival pathways, such as the PI3K/AKT, proapoptotic, and angiogenic cascades. Proteomic- and genomic-based approaches are currently underway to further examine molecular and cellular profiles of resistant cell lines to better understand molecular mechanisms that enable bypass of anti-EGFR effects. Utilizing cetuximab-, gefitinib-, and erlotinib-resistant cells, this model system provides a valuable resource to further define molecules involved in EGFR targeting, as well as potential methods to overcome treatment resistance.

Disclosure of Potential Conflicts of Interest

P.M. Harari has served as a consultant for Amgen, AstraZeneca, Genentech, and ImClone.

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