Acquired Resistance to Erlotinib in A-431 Epidermoid Cancer Cells Requires Down-regulation of MMAC1/PTEN and Up-regulation of Phosphorylated Akt

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Introduction

The epidermal growth factor receptor (EGFR) is overexpressed in several types of solid tumors. EGFR protein levels correlated with EGFR gene copy numbers. Activation of EGFR signaling in tumor cells has been linked with increases in proliferation, angiogenesis, and metastasis and with decreases in apoptosis and poor prognosis, collectively making EGFR an attractive target for anticancer therapy. Erlotinib (Tarceva) is a small molecule targeting the ATP-binding site of EGFR, which results in inhibition of tyrosine kinase activity. Erlotinib is the first anti-EGFR agent to extend survival among patients with advanced non–small cell lung cancer. Although EGFR overexpression or amplification has been reported to be a predictive factor for disease response and survival after treatment with EGFR tyrosine kinase inhibitors (TKI; refs. 2–5), other reports showed that EGFR expression level does not predict sensitivity to EGFR TKIs, such as erlotinib or gefitinib (6–10). Thus, identification of predictors of response to such inhibitors is a topic of active investigation. Some groups have reported that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway could be involved in erlotinib sensitivity (3–5, 11–17); others have found that the presence of somatic mutations in the EGFR kinase domain of EGFR in non–small cell lung cancer, or coexpression of EGFRvIII and mutated in multiple advanced cancers 1/phosphatase and tensin homologue (MMAC1/PTEN) in glioblastoma, may reflect sensitivity to EGFR tyrosine kinase inhibition (18–25). Screening in other types of sensitive tumors has not revealed similar mutations, suggesting that other molecular characteristics exist that predict response to EGFR inhibitors.

Another important question of clinical relevance is how disease that initially responds to EGFR TKIs acquires resistance to these drugs. Although the presence of somatic mutations in the tyrosine kinase domain of EGFR in non–small cell lung cancer has been reported to predict initial response, it did not predict prolonged survival and some patients with EGFR mutations had progressive disease (4, 5). Attempts to address this question have included the study of gefitinib in the non–small cell lung cancer cell line PC-9 and the prostate cancer cell line DU-145 (23, 25–28). However, the mechanism by which lung cancer cells escape erlotinib-mediated growth inhibition and acquire resistance to erlotinib after long-term exposure has not been studied in vitro. To evaluate possible mechanisms of acquired resistance of erlotinib, we chose to study A-431 cells, an erlotinib-sensitive, EGFR-overexpressing epidermoid cancer cell line without functional mutations or deletions in the EGFR tyrosine kinase domain. This work provides the first demonstration that acquired resistance to erlotinib depends on activation of phosphorylated Akt (p-Akt) after the down-regulation of MMAC1/PTEN and could be overcome by using PI3K inhibitors.

Materials and Methods

Cell lines, chemicals, and viruses. We used a human epidermoid carcinoma cell line (A-431) obtained from the M. D. Anderson Breast Cancer Translational Research Core Laboratory Cell Line Depository because this...
Figure 1. Establishment and characterization of erlotinib-resistant A-431 cells. A, all experiments were done in quadruplicate and SDs were calculated. Dose-response curves of A-431 parental cells and resistant pools after treatment with erlotinib at final concentrations of 0.03, 0.06, 0.1, 0.2, 0.3, 0.6, 1, 2, 3, 6, 10, or 20 μmol/L for 72 h. Parental cells and erlotinib-resistant pools were seeded in 96-well plates at a density of 200 cells in 90 μL per well, cultured overnight, and treated with erlotinib for 72 h in a humidified incubator at 37°C with 5% CO2. The surviving fractions were determined by MTT assay. Absorbance was measured at 570 nm. Results are the mean percentage of viable cells at each time from six wells. Results are shown as percentage of viable cells, (absorbance of treated cells minus absorbance of cell-free control) / (absorbance of untreated control minus absorbance of cell-free control) × 100, from which IC50 values were calculated. Bars, SD. B, erlotinib and clonogenic inhibition in A-431 parental cells and resistant pools. Numbers of colonies in treated cultures are expressed as a percentage of untreated controls. C, flow cytometry analyses of the DNA contents of cells. Cells were treated with 0 or 10 μmol/L of erlotinib and collected after 72 h. The percentages of apoptotic cells (the sub-G1 peak) are given in each histogram. A-431 parental cell lines showed G1 arrest and increased apoptosis (indicated by an increase in the sub-G1 peak) after treatment with 10 μmol/L erlotinib. However, erlotinib-resistant pools showed no obvious changes in cell cycle distribution. D, flow cytometry analyses of BrdUrd-positive cells. Cells were treated with 0 or 10 μmol/L of erlotinib and collected after 72 h. The percentages of BrdUrd-positive cells are given in each dot blot. A-431 parental cell lines showed a decrease in BrdUrd-positive cells; however, erlotinib-resistant pools showed no obvious changes in BrdUrd-positive cell populations.
cell line is sensitive to EGFR TKIs via suppression of the EGFR signaling pathway (29). Erlotinib was kindly provided by OSI Pharmaceuticals, Inc. Stock solutions of erlotinib (5 mM) were prepared in DMSO and stored in aliquots at −20°C. The maximum dose of erlotinib for the studies reported here was 20 μmol/L because the drug precipitated out of solution at higher concentrations. Akt-specific inhibitor IV, which targets the ATP-binding site of a kinase upstream of Akt but downstream of PI3K (30), was obtained from Calbiochem.

Recombinant adenovirus containing cDNA for myr-Akt as an active Akt (Ad.constitutively active Akt, Ad.CA-Akt) was kindly provided by Dr. Ugra

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**Figure 2.** A, FISH with an EGFR probe. Metaphases were analyzed for A-431 parental cells and resistant pools. Bottom right, gene copy numbers. Each cell line showed high amplification of EGFR. B, mutation analyses of the EGFR tyrosine kinase domain. Silent mutation was observed in both A-431 parental cells and resistant pools. However, no difference in the sequence of EGFR tyrosine kinase domain was detected in either cell line.
Establishment of erlotinib acquired resistant cell lines. To establish erlotinib-resistant cell lines, A-431 cells were continuously exposed to erlotinib for 6 months. Pool 1 was created by exposing cells to 3 μmol/L erlotinib for 1 month followed by 5 μmol/L erlotinib for another month and finally 10 μmol/L for 4 more months; pool 2 was created by exposure to 3 μmol/L erlotinib for 1 month followed by 5 μmol/L for 5 months. Medium was replaced twice weekly and cells were passed at 70% confluence. The resistant pools were maintained in medium without erlotinib for at least 1 week before each experiment.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as described previously (32). These experiments were repeated thrice.

Clonogenic assay. A-431 parental cells and erlotinib-resistant pools were seeded at a density of 100 cells per 100-mm culture dish and incubated overnight. Erlotinib was added to a final concentration of 5 or 10 μmol/L, and the cells were incubated for 12 days in a humidified incubator at 37°C with 5% CO₂. Colonies were counted when they reached 50 cells by staining with 0.1% crystal violet in 20% ethanol for 5 min at room temperature. Numbers of colony-forming units in the treated cultures are expressed as percentages of the untreated controls. These experiments were repeated thrice.

Flow cytometric analysis of cell cycle distribution. A-431 cells and erlotinib-resistant pools were plated in 60-mm dishes, cultured overnight, and then treated or not treated with 10 μmol/L erlotinib in a humidified incubator at 37°C with 5% CO₂. After 72 h, floating and adherent cells were collected by trypsinization, fixed overnight in 70% ethanol, and resuspended in propidium iodide (25 μg/mL) supplemented with 0.1% RNase A and 0.1% Triton X-100. DNA content was measured with a FACScan flow cytometer (BD Biosciences). These experiments were repeated thrice.

Bromodeoxyuridine incorporation assay. A-431 parental cells and the two erlotinib-resistant pools were plated on 60-mm dishes, treated or not treated with 10 μmol/L erlotinib for 72 h, and then incubated with bromodeoxyuridine (BrdUrd, Sigma Chemical Co.) for 30 min at 37°C with 5% CO₂. Floating and adherent cells were collected by trypsinization, washed with PBS containing 1% bovine serum albumin, fixed overnight in 70% ethanol, stained with anti-BrdUrd-FITC (BD Biosciences) for 30 min, and then sorted by a FACScan flow cytometer to determine the percentages of proliferating cells. These experiments were repeated thrice.

Chromosome preparation and fluorescence in situ hybridization with an EGFR probe. Exponentially growing cultures of A-431 parental cell line and resistant pools were fed 24 h before being harvested for chromosome preparation. Cytologic preparations were made according to standard procedures. Briefly, cells were exposed to demecolcine (colcemid; 0.04 μg/mL) for 1 h, subjected to hypotonic treatment (0.075 mol/L KCl for 20–25 min at room temperature), and fixed in a mixture of methanol and acetic acid (3:1 by volume). Air-dried slides were made and fluorescence in situ hybridization (FISH) was done with a locus-specific identifier EGFR probe from Vysis, Inc. (Abbott Laboratories) according to the manufacturer’s protocol. Briefly, the slides were denatured in 70% formamide/2× sodium chloride-sodium citrate buffer at 73°C for 5 min and 10 μL of the probe, denatured at 72°C for 5 min, were applied on the slide and hybridized overnight at 37°C. The slides were washed in 2× sodium chloride-sodium citrate buffer/0.3% NP40 at 72°C for 2 min and counterstained with 4,6-diamidino-2-phenylindole. Analysis was done on a Nikon Eclipse 800 photomicroscope equipped with a cooled charged-coupled device camera. At least 25 metaphases were analyzed and 10 metaphases were captured from each cell line. Gene copy numbers in each cell line were counted by a pathologist (S.K.). These experiments were repeated thrice.

Mutation analyses of EGFR tyrosine kinase domain. Genomic DNA was extracted from each cell line by using a DNeasy kit (Qiagen), and exons 18 to 21 (the area of the EGFR gene coding for the tyrosine kinase domain) were amplified separately. PCR and sequencing were done with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and 3730XL DNA Analyzer (Applied Biosystems) in the M. D. Anderson DNA Analysis Core Facility. Sense and antisense sequences were obtained from the products of the same amplification reactions. The following primer pairs (sense and antisense) were used to generate EGFR DNA fragments: exon 18, TTGTCCTCTCCTCAATAGGCTTG and TGCCAGGACTCTGGGCTCC; exon 19, ATACACTGGGACAGATGTTG and GGGCAGTGGTCTTCTAAGGG; exon 20, TGCGTCTTCACCTGGAAAG and TCCCCATGGCAACTCTTG; and exon 21, ATAGACCCTGAATTCGAGTG and CCAAAAGCAGCTCGTGCAC. All sequences were confirmed at least twice from independent PCR isolates.

Intracellular erlotinib concentration. Intracellular erlotinib content after a 24-h exposure was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Samples were prepared as follows: Cells (3 × 10⁶ in 30 mL) were plated on 150-mm dishes and incubated for 36 h, after which medium was changed to that containing 0 or 10 μmol/L of erlotinib. Plates were then incubated for 24 h in a humidified incubator at 37°C with 5% CO₂. Cells were then washed twice in PBS, trypsinized, and neutralized with fresh medium. After centrifugation at 1,000 × g for 5 min, cells were resuspended in fresh medium and then counted. The cell pellets were then washed thrice followed by centrifugation at 2,000 × g for 2 min each time and stored at −80°C until analysis by LC-MS/MS. The results were confirmed twice in independent experiments.

Immunoprecipitation of EGFR. To confirm that EGFR signaling was being reduced by erlotinib, cells were plated, incubated overnight, washed twice with serum-free medium, and then incubated in serum-free medium for 24 h. The medium was then replaced with medium containing 0.05% fetal bovine serum, and the cells were treated with 0 or 10 μmol/L of
erlotinib for 3 h and then stimulated with 100 nmol/L EGF (Upstate Cell Signaling Solutions) for 15 min. Cells were then washed twice with PBS, collected by scraping, and lysed for 30 min at 4°C in lysis buffer [20 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 2 mmol/L MgCl₂, 0.5% NP40, 1 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 μL/mL protease inhibitor cocktail]. Total protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions (Pierce Biotechnology). Protein extracts (200 μg per sample) were precleared to reduce nonspecific adsorption to immunoprecipitates by incubating them for 1 h with 15 μL of a protein A-agarose suspension (Calbiochem). The precleared lysates were then subjected to immunoprecipitation with 30 μL of protein A-agarose beads bound to 2 μg of anti-EGFR antibody Ab-13. Precipitates were separated by electrophoresis on 8% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed with anti-phosphotyrosine antibody 4G10 (Upstate Cell Signaling Solutions). The results were confirmed twice in independent experiments.

**Western blotting.** Cells were washed twice with PBS, trypsinized, and washed twice with ice-cold PBS. Cell pellets were then lysed in a buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, 25 mmol/L sodium fluoride, 200 μmol/L sodium orthovanadate, 1 μL/mL protease inhibitor cocktail (Sigma Chemical)] for 30 min on ice. Cell lysates were clarified by centrifugation for 30 min at 14,000 × g, and the total protein concentration in the resultant supernatants was determined with a BCA protein assay kit (Pierce Biotechnology). Equal amounts (50 μg) of protein were heated in SDS sample buffer with DTT (final concentration, 10 mmol/L) at 98°C, fractionated by size on 10% SDS-polyacrylamide gels, and transferred onto PVDF membranes (Bio-Rad Laboratories). Membranes were blocked by

![Figure 4](https://www.aacrjournals.org/)

**Figure 4.** A, screening for basal expression of molecules possibly involved in sensitivity to EGFR TKIs detected by Western blotting. Signals were detected with an Odyssey IR imaging system and the strength of bands was quantified. Relative densitometric units were calculated in comparison with parental cell expression levels. B, protein expression levels of some molecules, including PI3K/Akt pathway, were analyzed by Western blotting with or without treatment of 10 μmol/L erlotinib. Signals were detected by using an Odyssey IR imaging system.
incubation for 1 h with TBS-T [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk and then incubated with the following primary antibodies at 4°C overnight in TBS-T containing 5% nonfat dry milk: anti-EGFR antibody Ab-12 (Lab Vision); anti-c-ErbB2/c-Neu antibody Ab-3, and anti-HER3 antibody (Calbiochem); anti-phosphorylated HER3 (p-HER3), anti-insulin-like growth factor-I receptor (IGF-IR) β, anti-phosphorylated IGF-IR (p-IGF-IR), anti-Akt, anti-p-Akt (Ser473), anti-phosphorylated extracellular signal-regulated kinase.

Figure 5. A, the parental cells and resistant pools were treated with the Akt-specific inhibitor Akt inhibitor IV (Calbiochem) and assayed by MTT for viability. For the immunoblotting experiment, cells were treated for 72 h with 0 or 100 nmol/L of Akt inhibitor IV. B, the resistant pools were transfected Ad.MMAC1/PTEN or Ad.mock before treatment with 10 μmol/L erlotinib and assayed by MTT for viability. For the immunoblotting experiment, cells were transfected for 72 h with Ad.MMAC1/PTEN or Ad.mock. C, the A-431 parental cells were transfected Ad.CA-Akt or Ad.mock 24 h before 5 or 10 μmol/L of erlotinib and assayed by MTT for viability after 72 h of erlotinib treatment. For the cell cycle analyses, parental cells were transfected with Ad.CA-Akt or Ad.mock 24 h before treatment with 5 or 10 μmol/L of erlotinib. After 72 h of erlotinib treatment, cells were harvested and cell cycle analyses were done as described in Materials and Methods. The percentage of apoptotic cells determined by sub-G1 peak is given in each histogram. For the immunoblotting experiment, cells were transfected for 72 h with Ad.CA-Akt or Ad.mock. D, for the immunoblotting experiment, EGFR-overexpressing MDA-MB-468 and BT-549 cells were harvested after 72 h of treatment with 0 or 10 μmol/L of erlotinib. For viability, the MDA-MB-468 and BT-549 cells were treated by erlotinib combined with or without the Akt inhibitor IV and assayed by MTT after 72 h.
(p-ERK), anti-phosphorylated signal transducers and activators of transcription 3 (p-STAT3; Tyr705), and anti-p-STAT3 (Ser272) antibodies (all from Cell Signaling Technology); anti-E-cadherin (BD Biosciences); anti-HER4, anti-Pi3K p85α, anti-phosphorylated Pi3K (p-Pi3K) p85α (Tyr458), anti-PTEN, anti-ERK, anti-p27, anti-poly(ADP-ribose) polymerase (PARP), and anti-STAT3 antibodies (all from Santa Cruz Biotechnology); and anti-p-actin antibody A-5441 (Sigma Chemical). Signals were detected by using an Odyssey IR imaging system (LI-COR Biosciences). Each result was confirmed at least thrice in independent experiments.

Results

Establishment of cell lines with acquired erlotinib-resistant cell lines. To elucidate the mechanism of acquired resistance against erlotinib, we established erlotinib-resistant A-431 cell lines through continuous exposure to this drug for 6 months. Morphologically, the two pools were similar to the parental cells.

We first confirmed resistance to erlotinib in the pools by MTT assay; IC50 values were calculated from the percentage of viable cells after exposure to erlotinib at various concentrations (Fig. 1A). The IC50 value for the parental cells was 1.67 μmol/L compared with 17.6 μmol/L for resistant pool 1 and 13.2 μmol/L for resistant pool 2. Erlotinib resistance was also confirmed by clonogenic assay (Fig. 1B). Under continuous exposure to 5 μmol/L erlotinib, parental cells formed very few colonies, whereas resistant pools formed colonies after exposure to erlotinib concentrations as high as 10 μmol/L (pool 1, 91.7 ± 13.0% of untreated control; pool 2, 74.7 ± 3.46% of untreated control). Cell cycle distribution studies also showed that 72 h of erlotinib exposure increased the proportion of parental cells in sub-G1 (0.09% at 0 μmol/L, 26.6% at 10 μmol/L) and G1 arrest (39.5% at 0 μmol/L, 81.1% at 10 μmol/L) and reduced the proportion of parental cells in S phase (60.5% at 0 μmol/L, 16.0% at 10 μmol/L). In contrast, 72 h of erlotinib exposure had no effect on cell cycle distribution in the resistant pools (Fig. 1C). Notably, the S-phase fractions remained high after exposure to 10 μmol/L erlotinib in both resistant pools (57.1% in pool 1 and 54.6% in pool 2). DNA synthesis rates, assessed by BrdUrd staining, were substantially reduced from 38.5% to 4.84% in parental cells exposed to erlotinib but were unchanged in the resistant pools (Fig. 1D). Treatment with 10 μmol/L erlotinib increased the amount of cleaved 85-kDa PARP in parental cells but not in the resistant pools, indicating that apoptosis had been induced only in the parental cells (data not shown).

Extent of EGFR gene amplification is not involved in erlotinib acquired resistance. To clarify the possible molecular mechanisms underlying resistance of the A-431 pools to erlotinib, we examined the amount of EGFR gene amplification by FISH. All three cell lines showed high levels of amplification, with more than 20 gene copies (Fig. 2A). This result suggests that EGFR gene amplification was not involved in the acquired resistance of our A-431–resistant pools.

Mutation of the EGFR tyrosine kinase domain is not involved in erlotinib acquired resistance. We next examined the sequence of exons 18 to 21 of EGFR (the area of the gene that codes for the tyrosine kinase domain). We identified a silent mutation at exon 20 in both the parental cells and the resistant pools (Fig. 2B), but we found no differences between the parental cells and the resistant pools. This result confirmed that mutation of the EGFR tyrosine kinase domain was not involved in the acquired resistance of our A-431–resistant pools.

Erlotinib crossed the cell membranes in the resistant cells and reduced EGFR phosphorylation. Subsequently, we investigated whether the cellular uptake of erlotinib was altered in resistant versus parental cells. Intracellular erlotinib content was measured by LC-MS/MS after a 24-h drug exposure as follows: parental cells, 0.71 ± 0.04 ng/107 cells; pool 1, 1.01 ± 0.29 ng/107 cells; and pool 2, 0.79 ± 0.08 ng/107 cells (Fig. 3A). Thus, intracellular drug uptake and retention were similar in the parental and resistant cells, suggesting that the differential sensitivity to erlotinib observed was not related to differences in cellular drug uptake.

To establish whether erlotinib-mediated inhibition of EGFR was altered in the resistant cells, we examined EGFR phosphorylation after stimulation with EGF with or without erlotinib (Fig. 3B). Immunoprecipitation with EGFR antibody followed by detection with a phosphotyrosine antibody showed that the extent of EGFR autophosphorylation resulting from EGF stimulation was reduced by erlotinib both in resistant pools (density ratios at 10 μmol/L/0 μmol/L erlotinib were 0.307 for pool 1 and 0.478 for pool 2) as well as in the parental cells (density ratio, 0.431). We concluded from these results that erlotinib effectively reduced EGFR kinase activity in both the resistant and parental cells.

Basal MMAC1/PTEN protein expression was down-regulated in both resistant pools. Next, we examined molecules reported to be involved in sensitivity to EGFR TKIs by Western blotting. Expression of EGFR, HER2, Akt, ERK1/ERK2, p-ERK1/ERK2, p27, STAT3, p-STAT3 (Tyr705), and p-STAT3 (Ser272) levels was similar in the parental cells and the resistant pools (Fig. 4A). Expression of HER3, p-HER3, IGF-IR, p-IGF-IR, and E-cadherin proteins was down-regulated in both resistant pools relative to that in the parental cells, with particularly marked decreases in HER3, p-HER3, IGF-IR, and p-IGF-IR (Fig. 4A). HER4 was not detected in any of the three cell lines, and expression of the Pi3K subunit p85α and that of p-Pi3K was no different in the parental cells than in the resistant cells. Notably, expression of MMAC1/PTEN was down-regulated in resistant pools (density ratios versus parental cell expression were 0.338 for pool 1 and 0.473 for pool 2). Further, expression of p-Akt (Ser473) was modestly up-regulated in resistant cells (density ratios versus parental cell expression were 2.06 for pool 1 and 1.30 for pool 2).

Erlotinib suppressed p-Akt (Ser473) in parental cells but not in the resistant cells. At this point, we chose to focus on p-Akt because it was the only molecule that was up-regulated in both erlotinib-resistant pools and was a potential candidate for involvement in the mechanism of acquired resistance. The protein expression levels of some molecules in the EGFR-Akt signaling pathway were evaluated after erlotinib treatment by Western blotting (Fig. 4B). Notably, p-Akt (Ser473) was completely suppressed in parental cells treated with 10 μmol/L erlotinib but not in the resistant pools. In addition, MMAC1/PTEN expression was increased in parental cells treated with 10 μmol/L erlotinib but not in the resistant pools. Expression of other molecules was not affected by erlotinib treatment in the parental or the resistant cells.

Treatment with Akt inhibitor IV blocked the expression of p-Akt (Ser473) and restored erlotinib sensitivity in both resistant pools. To determine whether Akt activity is critical for erlotinib sensitivity, we treated parental and resistant A-431 cells with the Akt-specific inhibitor IV and erlotinib. The Akt inhibitor IV (100 nmol/L) effectively blocked the expression of p-Akt (Ser473; Fig. 5A) and restored erlotinib sensitivity in both resistant pools (Fig. 5A).

Transfection with MMAC1/PTEN blocked the expression of p-Akt (Ser473) and restored erlotinib sensitivity in both resistant pools. We then transfected the erlotinib-resistant pools.
with Ad.MMAC1/PTEN to assess the role of MMAC1/PTEN in acquired resistance. Overexpression of MMAC1/PTEN in these cells led to down-regulation of p-Akt (Fig. 5B) and restored erlotinib sensitivity in both resistant pools (Fig. 5B).

**Transfection of active Akt in A-431 parental cells caused resistant to erlotinib.** To investigate whether p-Akt activation could cause resistant to erlotinib in erlotinib-sensitive cells, we transfected A-431 parental cells with Ad.CA-Akt or Ad.mock, treated them with 5 or 10 μmol/L of erlotinib, and tested cell viability by MTT assay. Transfection with Ad.CA-Akt caused activation of p-Akt (Ser\(^{473}\)) and resistance to erlotinib (Fig. 5C). Transfection with Ad.CA-Akt also resulted in decreases in the percentage of apoptotic cells (the sub-G\(_1\) peak; Fig. 5C).

**Expression of p-Akt (Ser\(^{473}\)) protein in intrinsically erlotinib-resistant breast cancer cell lines was not down-regulated by erlotinib.** Finally, to examine the mechanistic difference between acquired and intrinsic resistance to erlotinib, we examined p-Akt (Ser\(^{473}\)) expression level in two intrinsically erlotinib-resistant breast cancer cell lines that also overexpress EGFR-MDA-MB-468 and BT-549 cells. The IC\(_{50}\) value of erlotinib for A-431 cells was 1.67 μmol/L; by comparison, the IC\(_{50}\) values for MDA-MB-468 and BT-549 cells were >20 μmol/L (data not shown). Treatment with 10 μmol/L of erlotinib did not down-regulate p-Akt (Ser\(^{473}\)) expression in MDA-MB-468 and BT-549 cells (Fig. 5D). Finally, the combination of 10 μmol/L of erlotinib plus the Akt inhibitor IV partially increased sensitivity to erlotinib in both the MDA-MB-468 and BT-549 cells (Fig. 5D).

**Discussion**

Our results show that acquired resistance to erlotinib can be caused by down-regulation of MMAC1/PTEN and subsequent activation of p-Akt. We further found that the acquired resistance could be overcome by overexpression of MMAC1/PTEN or inhibition of Akt activity. The findings of MMAC1/PTEN down-regulation in erlotinib-resistant cells and the inability of erlotinib to induce MMAC1/PTEN expression are novel and have not been reported previously as possible mechanisms for acquired resistance to EGFR TKIs. Unlike previous studies, we used A-431 epidermoid cancer cells, which do not have mutations in EGFR and were used in the development of the EGFR TKIs erlotinib and gefitinib.

A previous study of gefitinib-resistant PC-9 lung adenocarcinoma cells with an EGFR mutation suggested that changes in adaptor protein-mediated signal transduction from EGFR to Akt may represent a possible mechanism by which cells become resistant to gefitinib (26). The same group showed in a subsequent study that, after long-term exposure to gefitinib, PC-9 cells stop overexpressing EGFR and acquire gefitinib resistance (27). Others have reported that persistent activity of signaling pathways from EGFR to p-Akt was associated with sensitivity to EGFR TKIs (4), and p-Akt activation, especially EGFR-independent p-Akt activation, has been recognized as an intrinsic resistance factor to EGFR TKIs (11, 12).

With regard to intrinsic resistance to EGFR TKIs, our findings agree with those of a previous study that knockdown of MMAC1/PTEN reduced sensitivity to erlotinib and that expression of MMAC1/PTEN in a MMAC1/PTEN-null cell line increased sensitivity to erlotinib (20). Dysfunction or loss of MMAC1/PTEN, which blocks PI3K/Akt signaling, is thought to be involved in intrinsic resistance to EGFR TKIs (11, 13, 17, 20). Indeed, the MDA-MB-468 and BT-549 cell lines used in the present study are known to lack MMAC1/PTEN function through deletion or mutation of the MMAC1/PTEN gene (33). Interestingly, the extent of MMAC1/PTEN down-regulation was nearly equivalent in both resistant pools but p-Akt activation was more prominent in pool 1 (also the most resistant to erlotinib). These results could imply that down-regulation of MMAC1/PTEN may represent an early step in acquired resistance, and the subsequent up-regulation of p-Akt by other mechanisms could increase the extent of resistance. Future studies are necessary to determine the precise mechanisms of MMAC1/PTEN down-regulation and p-Akt activation in this process. In particular, MMAC1/PTEN could be regulated by a variety of mechanisms, including deletion/mutation, phosphorylation, oxidation, subcellular localization, lipid ligands, and protein-binding partners (34). Research is under way to clarify the molecular mechanisms of MMAC1/PTEN down-regulation.

Our findings confirmed that the mechanisms of acquired and intrinsic resistance have some pathways in common (e.g., p-Akt and MMAC1/PTEN). However, the effect of the combination of the Akt inhibitor IV plus erlotinib on cell viability was more modest in the intrinsically resistant (breast cancer) cell lines than in the acquired resistant A-431 cells. The specificity of Akt inhibitor IV is unclear, but it contains planar heterocycles that may target the ATP-binding sites of kinases (30). It inhibits phosphorylation of Akt by targeting its immediate upstream kinase, PDK1, but it does not directly affect p-Akt or its upstream target PI3K (30) nor does it affect EGFR-induced ERK1/ERK2 (35). However, we found that Akt inhibitor IV down-regulated MMAC1/PTEN in both resistant cell lines (data not shown). Therefore, one can speculate that Akt inhibitor IV down-regulated p-Akt independently of MMAC1/PTEN. Hence, the modest effect of Akt inhibitor IV could be explained by the existence of different mechanisms for intrinsic and acquired resistance, or by the MMAC1/PTEN-null nature of MDA-MB-468 and BT-549 cells (33), or by the possibility that p-Akt is deregulated from the upstream molecular effect of Akt inhibitor IV. An Akt inhibitor that directly affects Akt activity but not that of upstream or downstream molecules (e.g., Akt inhibitor II) might be able to increase cellular sensitivity to erlotinib (35, 36). Future studies of the PI3K/Akt pathway are necessary to distinguish the mechanisms underlying acquired versus intrinsic resistance.

Transfection of A-431 parental cells with Ad.CA-Akt caused high expression of p-Akt, but the erlotinib resistant in those cells (Fig. 5C) was modest compared with that of the resistant pools (Fig. 1A). These results imply that acquired resistance to erlotinib involves not only activation of p-Akt but also some other mechanisms. Our findings indicate that the resistant pools had lost the expression of MMAC1/PTEN, HER3, IGF1R, and E-cadherin, suggesting that these molecules may be associated with loss of sensitivity to erlotinib in A-431 cells. These results also suggest that acquired resistance may occur by mechanisms similar to those operating in intrinsic resistance.

EGFR is involved in many signaling pathways, including Ras/Raf/mitogen-activated protein kinase (MAPK)/ERK kinase/MAPK, Janus-activated kinase/STAT, protein kinase C, phospholipase C\(_{\gamma}\), and other cytosolic pathways (13). Our investigation of STAT3 and ERK1/ERK2 indicated that these pathways were not involved in the acquired resistance mechanism, at least in this model. Interestingly, erlotinib down-regulated the ERK1/ERK2 pathway in both parental and resistant cell lines. In A-431 parental cells, p-Akt was down-regulated by 12 to 24 h after erlotinib treatment followed by a decrease in cell viability at 48 to 72 h (data not shown). However,
erlotinib did not down-regulate p-Akt in the resistant pools. These results imply that EGFR could be an attractive target if erlotinib were combined with an agent targeting other downstream molecules (e.g., p-Akt or MMAC1/PTEN).

Two recent studies described potential mechanisms of acquired resistance to EGFR TKIs in lung cancer involving acquired or selection of mutations in EGFR (23, 25). In those studies, an additional mutation involving substitution of methionine for threonine at position 790 within the EGFR kinase domain in erlotinib-sensitive cells led to the development of resistance in lung adenocarcinoma (23, 25). In contrast to our findings, those studies showed that EGF phosphorylation was not blocked by EGFR TKIs in cells with the acquired mutation. The previously noted PC-9 lung cancer cell line, which has a mutation in EGFR tyrosine kinase domain, did not acquire additional mutations in the EGFR tyrosine kinase domain in resistant clones established either by 6 weeks of exposure (26) or by 1 year of exposure (27). Our finding that our resistant pools did not show mutations in the EGFR tyrosine kinase domain agrees with these reports (26, 27).

EGFR amplification has been proposed to correlate with disease response and/or prolonged survival after treatment with EGFR TKIs (3–5). However, in our study, neither EGFR amplification nor increases in EGFR expression were involved in the acquired resistance mechanisms in A-431 cells. The presence of efflux pumps and metabolizing enzymes in tumors could be important in regulating the intracellular concentrations of EGFR TKIs (37, 38). Therefore, we assessed the intracellular content of erlotinib in resistant cells to determine whether these mechanisms were involved in the acquisition of resistance. However, drug levels were similar in the parental and acquired resistance cells, indicating that activation of drug efflux pumps or drug-metabolizing systems was not involved in the mechanism of acquired resistance to erlotinib in A-431 cells. During our screening of various other molecules potentially involved in the acquisition of resistance, we noted down-regulation of HER3, IGF, and E-cadherin. HER3 (ErbB3) also activates PI3K, upstream of p-Akt, and EGFR-dependent activation of PI3K activity in A-431 cells is accompanied by the binding of p85 to HER3 (39). Gefitinib reportedly prevents heterodimerization of EGFR to HER2 and HER3 and blocks heregulin signaling (40). Although overexpression of HER3 protein could modulate sensitivity to erlotinib (41), HER3 gene gain was not a predictor of response to EGFR TKIs in a clinical setting (42). Some evidence exists to suggest that HER3 is overexpressed only in gefitinib-sensitive cell lines and acts to couple EGFR with the PI3K/Akt pathway in those cells (41, 43), whereas gefitinib-resistant cells do not activate the PI3K pathway through HER3 (41). Our finding that our resistant cells did not overexpress HER3 agrees with these reports (41, 43).

IGF-IR activates both the Akt and ERK1/ERK2 pathways as well as participates in cross-talk between IGF-1 and the EGFR pathway (44). This IGF signaling is subject to positive regulation by EGF, which in turn suppresses insulin-like growth factor binding protein-3 at the mRNA and protein levels by activating MAPK in an EGFR tyrosine kinase–dependent manner, thereby restoring cellular response to IGF-1 (45). IGF-IR is also considered an attractive therapy target; indeed, some reports have shown that adding an anti-IGF-IR agent to an EGFR TKI may be more effective than the single agent alone (46–48). However, we found both IGF-IR and p-IGF-IR expression to be down-regulated in the resistant pools. These findings stand in contrast to those describing IGF-IR and acquired resistance to gefitinib (28).

Non–small cell lung cancer cells that express the epithelial cell junction protein E-cadherin have shown greater sensitivity to EGFR inhibition in vitro (49) and in vivo (50). In contrast, cells that have undergone the epithelial-to-mesenchymal transition (and thus express the mesenchymal markers vimentin or fibronectin rather than E-cadherin) were insensitive to inhibition by erlotinib in vitro and in xenografts (49). In our study, E-cadherin was suppressed in both resistant pools, with the extent of the suppression being greater in the “high-dose” pool 1 than in the “low-dose” pool 2.

In summary, down-regulation of MMAC1/PTEN and constitutively activated p-Akt seem to be critical for acquired resistance to erlotinib in this model. We propose that erlotinib resistance is associated with down-regulation of MMAC1/PTEN and increased p-Akt levels and that resistant cells could be susceptible to treatments that inhibit p-Akt activity. The precise roles of HER3, E-cadherin, and MMAC1/PTEN in this resistance, and the clinical relevance of these findings, require further study.

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References


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