Effect of an epidermal growth factor receptor tyrosine kinase inhibitor on actin remodeling in an in vitro bladder cancer carcinogenesis model

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Abstract
Alteration of actin remodeling is a marker of malignant-associated field defect and a potential surrogate biomarker for chemoprevention trials. We tested erlotinib, a specific tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR), on actin remodeling in a bladder carcinogenic model consisting of untransformed HUC-PC cells and transformed MC-T11 cells, both derived from the same normal human urothelial clone immortalized by SV40. Erlotinib had a selective growth inhibitory and actin remodeling effect on MC-T11 cells over HUC-PC cells, as examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and immunofluorescence labeling with laser scan cytometer analysis, respectively. The IC50 of untransformed HUC-PC cells was significantly higher than that of transformed MC-T11 cells (P < 0.05, t test). The actin remodeling effect was more prominent at lower dosage levels (1/8-1/4 of IC50), which was accompanied by an increased cell adhesion and decreased motility. At higher dosage levels (1/2 of IC50), erlotinib induced a decreased adhesion and anokisis (detachment-associated apoptosis). The transformed MC-T11, but not HUC-PC, showed a weak constitutive EGFR phosphorylation activity, which was inhibited by erlotinib in a dose-response manner. However, on epidermal growth factor stimulation, both cell lines showed a similar dose-response inhibitory effect on phosphorylated EGFR and mitogen-activated protein kinase (MAPK; P44/P42) activities, and MAPK inhibitor PD98059 showed no specific effect on erlotinib-induced actin remodeling, suggesting that pathways other than MAPK (P44/P42) may be responsible for erlotinib-induced actin remodeling. The findings provide evidence to support erlotinib-based bladder cancer chemoprevention and using actin remodeling as a marker for erlotinib-based intervention trials. [Mol Cancer Ther 2006;5(7):1754–63]

Introduction
Bladder cancer is the fourth most common cancer site, with 60,240 new cases and a projected 12,710 deaths in 2004, representing an important health problem in the United States (1). Approximately 90% of bladder tumors arise from the urothelial lining, and urothelial carcinoma or transitional cell carcinoma, is the most common histologic type in the United States. Between 30% and 70% of patients with superficial tumors will develop new superficial transitional cell carcinoma, often within 12 months of diagnosis, and 10% to 20% progress to infiltrate muscularis propria (2). Because these tumors have a very high incidence of recurrence, the psychological and economic burdens to the health care system of repeated diagnostic evaluations and therapy are substantial.

Although the exact molecular mechanisms for bladder cancer recurrence remains to be fully elucidated, previous studies from this laboratory suggest that molecular “field” defects play an important role (3, 4). Many of such molecular “field” defects may not be detectable by morphologic-based, histologic, or cytologic analysis; however, our study showed that cellular cytoskeletal actin remodeling in the cancer-bearing “normal” urothelium serves as a useful biochemical marker for the “field” defect (3, 4). Cytoskeletal proteins provide the basic infrastructure in maintaining the cell shape and function. Alteration of actin remodeling not only plays an important role in regulating the morphologic and phenotypic events of a malignant cell but also provides a potential target for anticancer drug development. (for review, see ref. 5) The actin remodeling can be measured by a simple double-fluorescence labeling assay (6). In this assay, the relative fluorescence intensity of F-actin and G-actin can be measured, with the ratio of these two measurements reflecting the actin polymerization status. In general, premalignant cells of intraepithelial lesions have a lower F/G-actin ratio than normal cells owing to the loss of cell adhesion. However, the F/G-actin ratio may be increased when tumor cells become more invasive. This simple assay has been used in the past for the analysis of several cancer types, including bladder (7, 8), breast (9), and prostate (10).

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It has been well shown that epidermal growth factor (EGF) receptor (EGFR)–mediated signaling mechanisms are fundamental to the tumorigenicity of human bladder cancer and comprise an important pathway regulating cellular division and differentiation; the latter may involve actin-related signaling pathways. The level of EGFR correlates with stage, grade, and progression of human transitional cell carcinoma (11, 12). Dinney et al. (13) reported previously that therapy with either protein tyrosine kinase inhibitors or anti-EGFR monoclonal antibodies inhibits the growth of established human transitional cell carcinoma growing orthotopically in athymic nude mice (14). Although the literature is conflicting, some studies have described higher EGFR expression in tumors that continued to be superficial on relapsing compared with tumors that became infiltrative of the urinary bladder wall (15). EGFR-specific tyrosine kinase inhibitors have been reported in nitrosamine-induced animal models of transitional cell carcinoma to inhibit tumor proliferation and progression (16). Although the effect of the EGF signaling pathway on actin remodeling has been studied previously (17) and seems to be mediated by Ras small GTPase proteins Rac and Rho (18), there has been no direct studies of the effect of EGFR-specific tyrosine kinase inhibitor on actin remodeling.

In this project, we tested directly the effectiveness of erlotinib on actin remodeling in an in vitro bladder cancer carcinogenic model. This model consists of untransformed HUC-PC cells and transformed low-grade MC-T11 cells, both derived from the same normal human urothelial cell immortalized by SV40. Studies have shown that, by exposure to the carcinogen 4-aminobiphenyl (4-ABP), HUC-PC cells can be induced to undergo malignant transformation, whereas MC-T11 cells can be progressed to a high-grade tumor (19). This system provides a fast model to test the efficacy of chemopreventive agents. The results of this study might provide additional evidence to support the use of the anti-EGFR kinase inhibitor for bladder cancer chemoprevention trials and at the same time may test the possibility of using actin remodeling as a potential biological effector or surrogate end point marker to monitor the effect of erlotinib on bladder cancer recurrence.

Materials and Methods

Materials

Erlotinib was kindly provided by OSI Pharmaceuticals, Inc. (Farmingdale, NY). Other chemicals, including carcinogen 4-ABP and culturing reagents, were purchased from Sigma Chemical Corp. (St. Louis, MO).

Cell Culture

Both HUC-PC and MCT-11 cells were grown in 90% F-12 Nutrient Mixture (Ham) medium (Life Technologies, Grand Island, NY) with 1% penicillin and 10 mg/mL streptomycin and either with or without 10% fetal bovine serum. Cultures were maintained at 37°C in 5% CO2 and 95% air, and the medium was changed twice weekly.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The IC50 of erlotinib was determined by a calorimetric tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical) was dissolved in PBS at 5 mg/mL and filtered. Serial triplicate dilutions of the extract at 50 μL in volume were added to 1.5 × 105 cells/mL to 96-well flat-bottomed plates with various concentrations of erlotinib (0.1563, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μmol/L). Plates were incubated for 24 hours at 37°C, pulsed with 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL), and incubated for 4 hours at 37°C. DMSO (100 μL) was then added to all wells and mixed thoroughly for 30 minutes at room temperature. The plates were then read on a microplate reader Bio-Rad 550 (Bio-Rad Laboratories, Hercules, CA). Cell survival was calculated as the percentage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide inhibition using the following formula: percentage survival = (mean experimental absorbance / mean control absorbance) × 100.

Apoptotic Analysis

Apoptosis was detected by two different methods. First, the number of apoptotic cells in various conditions was assessed in triplicate by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling technique using a commercially available kit (Guava terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling kit, Guava Technologies, Hayward, CA). Second, Annexin V-Cy3 labeling method was used to further confirm the observation. During apoptosis, phosphatidylserine was translocated from the cytoplasmic face of the plasma membrane to the cell surface. Annexin V has a strong, Ca2+-dependent affinity for phosphatidylserine and therefore serves as a probe for detecting apoptosis. In this simple method, cells cultured directly on a 1-cm diameter cover glass were fixed with 3.7% paraformaldehyde for 30 minutes followed by a sequential incubation with Annexin V primary antibody (1:100; Abcam, Inc., Cambridge, MA) for 1 hour and Cy3-conjugated AffiniPure goat anti-rabbit IgG (1:200; H+L; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 minutes. Between each incubation step, the cover glass was rinsed with PBS thrice. The stained cover glass was then transferred onto a regular microscopic slide, which was then mounted in 100 mmol/L n-propyl gallate (Sigma Chemical) in spectrapurified glycerol (pH 6.5; Fisher Scientific, Tustin, CA), for fluorescence microscopic examination. Images were generated using a Nikon TE300 microscope equipped with an Imaging Microimagier II digital camera (Nikon, Melville, NY).

Fluorescence Analysis

For fluorescence analysis, cells cultured directly on 1-cm diameter cover glass were fixed with 3.7% paraformaldehyde for 30 minutes and subjected for triple labeling of either F-actin/G-actin/DNA or F-actin/paxillin/DNA. For the former, cells were incubated sequentially with Alexa Fluor 647–conjugated DNase I (1:150; Molecular Probes,
Inc., Eugene, OR) for 30 minutes, Bodipy-conjugated phallolidin (100 μL; 1:200 v/v; Molecular Probes) for another 30 minutes, and 4,6-diamidino-2-phenylindole (1:1,000; 10 mg/mL; Molecular Probes) for 5 minutes. For the latter, cells were incubated with monoclonal anti-paxillin (1:100; clone 5H11, Upstate Biotechnology, Lake Placid NY) for 1 hour, Cy3-conjugated AffiniPure goat antimouse IgG (1:150; H+L; Jackson ImmunoResearch Laboratories) for 30 minutes, Bodipy-phallacidin (1:40; for F-actin; Molecular Probes) for 30 minutes, and 4,6-diamidino-2-phenylindole (1:1,000) for 5 minutes. Between each incubation step, the cover glass was rinsed with PBS thrice. The stained cover glass was then transferred onto a regular microscopic slide, which was then mounted in 100 mmol/L n-propyl gallate in spectranalyzed glycerol (pH 6.5), for fluorescence microscopic examination. Images were generated using a Nikon TE300 microscope equipped with an Imaging Microimager II digital camera. A laser scanning cytometer (Compucyte Corp., Cambridge, MA) equipped with a 20-mW argon ion, air-cooled laser, and a 30-mW violet diode laser was used to measure F-actin (green) and G-actin (red) fluorescence. The green immunofluorescence was excited using the 488-nm argon laser (20 mW), and the red fluorescence was excited using 633-nm helium-neon laser (20 mW). The 4,6-diamidino-2-phenylindole–stained nuclei excited with 395-nm UV diode laser (10 mW) were used to identify single cells for measurement. About 1,000 to 2,000 cells were analyzed on each slide, and the mean fluorescence intensity of F-actin and G-actin was used to calculate the F/G-actin ratio.

**Cell Adhesion and Migration Assays**

For adhesion analysis, 96-well tissue culture plates coated with 50 μg/mL fibronectin for 1 hour at 37°C were used. After blocking with 1% bovine serum albumin for 30 minutes, plates were seeded at a concentration of 5 × 10^4 cells per well with untreated cells and cells treated with erlotinib at different time points (1, 2, and 3 hours). After washing off the nonattached cells with 1× PBS and shaking, the remaining attached cells were fixed in 1% glutaraldehyde for 15 minutes and stained with 0.1% (w/v) crystal violet for 20 minutes. Cells were lysed in 1% SDS. The intensity of the stain, in direct proportion to the number of adherent cells, was quantitated by absorbance at 540 nm using a microplate reader. Each cell line was tested in three separate wells over three independent experiments. For migration assay, cells exposed to 4-ABP for 24 hours were further incubated with erlotinib for an additional 24 hours. A uniform cell-free area was created by scratching confluent monolayers immediately before the incubation of erlotinib (time 0) with a plastic pipette tip, and the wound area was inspected at different time intervals (0, 6, and 24 hours) to determine the distance migrated by the cells. The closer the gap, the faster the cell migrates. At each time point, four photographs were taken, and the number of cells/mm^2 that migrated into the area of wound was counted. For both assays, results were expressed at mean ± SD of three independent experiments.

**Immunoblot Analysis**

Cells that were washed twice in cold PBS were scraped from culture dishes in lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EGTA, 2 mmol/L MgCl_2, 1% (v/v) Triton X-100, 10% glycerol, 10 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 μg/mL pepstatin A, 50 mmol/L NaF, 1 mmol/L NaVO_4]. Lysates were centrifuged at 12,000 × g and 4°C for 10 minutes. Protein concentrations of lysates were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories). For Immunoblot analyses, the same amount of proteins (30 or 50 μg) was subjected to 8% SDS-PAGE and electrotransferred to nitrocellulose membranes using electroblot buffers. Membranes were blocked in PBS containing 5% nonfat dry milk for 30 minutes. Reactions with the primary antibodies in TBS buffer containing 3% dry milk were carried out at 4°C overnight. After extensive washing, membranes were placed on a shaker with biotinylated secondary IgG for 1 hour. On further washing, membranes were reacted with streptavidin-horseradish peroxidase for 45 minutes and enhanced chemiluminescence detection reagents immediately before autoradiography.

**Inhibition of Mitogen-Activated Protein Kinase Activity**

MC-T11 Cell were seeded in 24-well culture plate with a 1-cm slide at a density of 5 × 10^4 cells per well in medium containing 10% FBS for 8 hours. Cells were then serum starved for 16 hours before erlotinib (387 nmol/L; 1/8 of ID_50) was added for an additional 24 hours. Cells were then treated either with or without mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase 1/2 inhibitor PD98059 (Cell Signaling, Inc., Danvers, MA) at 10 μmol/L for 2 hours. After washing off the inhibitor with serum-free medium, cells were stimulated with EGF at 100 ng/mL for 2 minutes (Cell Signaling).

**Statistical Analysis**

Descriptive statistics, such as the mean and SE, were used to summarize the results. The Student’s t test and ANOVA test were used for the univariate analysis. Statistical significance was defined by a two-tailed P of 0.05.

**Results**

**Effect of Erlotinib on Actin Remodeling on MC-T11 and HUC-PC Cells**

Before analyzing the effect of erlotinib on actin remodeling on these cell lines, we first determined the IC_50 of erlotinib using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. This was done for the purpose of finding optimal concentrations of the drug that could be used to induce actin remodeling without causing significant toxicity. Cells were grown in culture medium containing 10% fetal bovine serum and various concentrations of erlotinib (0, 0.1563, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20 μmol/L) for 24 hours. After three experiments, the for MC-T11 (3.2 ± 0.2 μmol/L) was found to be significantly lower than the IC_50 for HUC-PC (9.5 ± 0.5 μmol/L);
The effect on untransformed HUC-PC cells was much less dramatic, which had abundant F-actin fibers in the untreated control (Fig. 1A). The observation was confirmed by a quantitative analysis of F-actin and G-actin using the laser scan cytometer, as shown in Fig. 1B, which presented the changes of the F/G-actin ratio in response to erlotinib treatment in MC-T11 cells. We also tested the effect of EGF stimulation on actin remodeling because it has been reported that EGF itself stimulates actin polymerization (18). In this experiment, cells were cultured in serum-free medium for 24 hours with various concentrations of erlotinib before incubating with EGF at a final concentration of 100 ng/mL for 2 minutes. Without erlotinib, there was a slightly higher F/G-actin ratio in EGF-treated cells over untreated cells; however, the difference did not reach statistical significance (0.27 versus 0.33; $P > 0.05$, Student’s $t$ test; Fig. 1B). Consistent with the morphologic findings, erlotinib induced actin polymerization in both EGF-treated and untreated MC-T11 cells as indicated by the increase of the F/G-actin ratio; however, the effect was more prominent in cells that were not stimulated with EGF ($P < 0.05$, ANOVA test for both; Fig. 1B). For cells without EGF treatment, an increase of F/G-actin ratio was observed with the increase of concentrations of erlotinib (from 0 to 1/8 to 1/4 of IC$_{50}$), but at a dose level of 1/2 of IC$_{50}$, the F/G-actin ratio slightly decreased but was still higher than the untreated control. On the contrary, no significant change of the F/G-actin ratio was observed for erlotinib-treated untransformed HUC-PC cells (data not shown). These findings suggest that erlotinib has a more pronounced effect on actin polymerization in transformed cells compared with untransformed cells.

**Effects of Erlotinib on Cell Adhesion, Apoptosis, and Motility**

We further examined the effect of erlotinib-induced actin remodeling on cell adhesion and motility on MC-T11 and HUC-PC cells. For adhesion analysis, cells were grown in fibronectin-coated culture plates. Data from both 1 and 3 hours of incubation are shown in Fig. 2A. At both 1 and 3 hours of incubation, a dose-response increase in cell adhesion was seen in erlotinib-treated MC-T11 cells at dosage levels from 1/8 to 1/4 of IC$_{50}$, with a slight decrease at dosage level of 1/2. This effect was not seen, however, in HUC-PC cells. Figure 2B shows representative images of F-actin and adhesion complex (marked by paclitaxel) in MC-T11 cells. Erlotinib-induced actin polymerization as reflected by increased F-actin fibers was accompanied by an increase in cell adhesion complex formation (Fig. 2B, compare b and c to a, arrowhead indicating adhesion complex) at lower concentrations (1/8-1/4 of IC$_{50}$). However, at a higher concentration of erlotinib (1/2 of IC$_{50}$, Fig. 2B, d), the adhesion complex formation decreased instead. Again, no significant changes were seen in HUC-PC cells; thus, the data is not shown. We hypothesize that the loss of cell adhesion may be associated with detachment-associated apoptosis or anoikis. Figure 2C shows the results of the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling analysis in erlotinib-treated MC-T11 cells. Indeed, there was a significantly
increased number of apoptotic cells at the concentration of 1/2 IC₅₀ level compared with control and low dosage levels (1/8 or 1/4 of IC₅₀). The finding was further confirmed using the Annexin V labeling assay as shown in Fig. 2D.

To further explore the biological effect of erlotinib on actin remodeling, we studied the antagonizing effect of erlotinib on carcinogen 4-ABP-stimulated motility in MC-T11 and HUC-PC cells using the wound-scratch assay. Previously, we found that when both HUC-PC and MC-T11 cells were exposed to 4-ABP, cell motility was increased (20). For these experiments, cells were exposed to 4-ABP for 24 hours until confluence were further incubated with erlotinib.
erlotinib for an additional 24 hours. A wound was created by a micropipette tip at 0 hour, and phase-contrast images were taken at 0, 6, and 24 hours. Figure 3A shows representative images of MC-T11 and HUC-PC cells after the incubation with erlotinib at 1/4 of IC_{50} for three time points, and Fig. 3B shows the mean of the three experiments of the actual count of cells that migrated in the scratched area. In MC-T11 cells, those treated with erlotinib had significantly fewer cells that migrated into the wound area relative to the control at both 6 and 24 hours time points (P < 0.05; Fig. 3B). For HUC-PC cells, a significant difference between erlotinib-treated cells and the control was seen only at the 24-hour time point (Fig. 3B).

**Effects of Erlotinib on Constitutive or EGF-Stimulated EGFR Activity in MC-T11 and HUC-PC Cells**

To determine if the observed effect of erlotinib on MC-T11 and HUC-PC cells correlates with the anti-EGFR activity of erlotinib, the Western blot analysis for phosphorylated EGFR (p-EGFR) and MAPK (P44/P42) in MC-T11 and HUC-PC cells either with or without EGF stimulation was done, and the results are shown in Fig. 4. Cells were cultured in serum-free medium for 24 hours in the presence of various concentrations of erlotinib (0, 1/8, 1/4, and 1/2 of ID_{50}). Cells were then further incubated with EGF for 2 minutes. Without EGF stimulation, only the transformed MC-T11 cells had slightly detectable p-EGFR activity in 1 and 6 hours, which was inhibited by erlotinib in a dose-responsive manner. Such activity was undetectable even in the control cells at 24 hours. HUC-PC cells also had very weak constitutive p-EGFR activity. However, in EGF-treated cells, a dose-response anti-EGFR activity of erlotinib was detectable in both cell types. Such an effect was detectable at 1 hour after incubation, which was maximized at 6 hours and sustained until 24 hours. To be noted, the concentrations that were tested in two cell lines were different, in that much higher concentrations were used in HUC-PC cells. For both cell lines, however, there was a decreased total EGFR in EGF-stimulated cells that were not treated with erlotinib. This may be explained by a partial loss of reactivity of anti-total EGFR antibodies for the P-EGFR because the immunoblot was done on the same membrane, whereas anti-P-EGFR was done first followed by anti-total EGFR.

For MAPK (P44/P42) activity, there was no discernable difference of activity seen in response to erlotinib cells in either cell lines without EGF stimulation (Fig. 5). However, in EGF-treated cells, a dose-response inhibition of MAPK (P44/P42) similar to p-EGFR was seen in both cell lines after 24 hours of erlotinib incubation, although the overall level of activity seems to be lower in HUC-PC than in MC-T11 cells. MAPK-specific inhibitor PD98059 did not have any effect on erlotinib-induced actin remodeling in EGF-stimulated cells as shown in Fig. 6. Similar findings were seen in non-EGF-treated cells (data not shown). Together, the findings suggest that, although the differential effects of erlotinib on these cell lines for actin remodeling correlate in general with the constitutive activity of p-EGFR, pathways other than MAPK (P44/P42) may be responsible for erlotinib-induced actin remodeling.

**Discussion**

Our study shows the effect of the EGFR tyrosine kinase inhibitor erlotinib on actin remodeling in urothelial cells in vitro. Erlotinib is a quinazolin derivative that is orally active and a specific inhibitor of the tyrosine kinase moiety of EGFR. Erlotinib acts through direct and reversible...
inhibition of the EGFR tyrosine kinase and its use in epithelial-derived, EGFR-expressing, and overexpressing tumors is grounded in strong biological rationale. There has been tremendous interest and effort in developing erlotinib as an antitumor agent, and the safety and efficacy of this agent has been extensively shown in a large series of human trials. Whereas most of these studies are focused on the therapeutic aspect of erlotinib, there have been relatively few studies testing the efficacy of this agent for cancer prevention. Because alterations of actin remodeling are “field” disease markers associated with bladder cancer recurrence (3, 4), our work showing that erlotinib has a direct effect on actin remodeling in both transformed and untransformed urothelial cells in vitro provides the first, albeit indirect, evidence to suggest that erlotinib may also have potential beneficial effects in preventing bladder cancer recurrence. This is important because the cost per bladder cancer patient from diagnosis to death is the highest among all cancers (e.g., $96,000–$187,000 per patient) in the United States mainly because of the high risk for recurrence of these tumors (21).

Our data showed selective effects of erlotinib on transformed MC-T11 cells compared with untransformed HUC-PC cells for both antigrowth and proliferation and actin remodeling. In MC-T11 cells, low doses of erlotinib (1/8 of IC50 or 387.13 nmol/L) induce cell differentiation as indicated by increased actin polymerization. However, at higher dosage levels (1/2 of IC50 or 1548.5 nmol/L), the adhesion complex formation slightly decreased instead (but was still higher than the untreated control), which may be related to anoikis (or detachment-associated apoptosis). Actin was first identified in nonmuscle cells only decades ago, and at the same time, it was found that actin filaments were disrupted in the malignant transformed cells (22). Actin is a ubiquitous protein present in all eukaryotic cells and is one of the major structural and functional proteins involved in maintaining cell morphology, cell adhesion, cell movement (motility), exocytosis and endocytosis, as well as cell division (23–25). There is overwhelming evidence showing the importance of actin remodeling in regulating the morphologic and phenotypic events of a malignant cell (26–29). Extensive studies have

Figure 4. Dose- and time-response effect of erlotinib on constitutive and EGF-stimulated EGFR activity (p-EGFR) in MC-T11 and HUC-PC cells. Cells were treated with various concentrations of erlotinib [0, 1/8, 1/4, 1/2 of IC50] for indicated times (1, 6, and 24 h) and further treated with or without EGF (100 ng/mL) for 2 min before they were washed with cold PBS thrice. Total EGFR (T-EGFR) and P-EGFR were detected by the Western blot. X axis, actual intensity of P-EGFR measured by densitometry.
shown that actin filaments are regulated by actin signaling proteins that are components of important oncogenic signal transduction pathways, the most notable one being the small GTPase of Ras superfamily proteins Rac, Rho, and Cdc42 (30, 31). On the other hand, hundreds of actin-binding proteins have been cloned, and some of these proteins showed distinctive expression patterns in cancer carcinogenic and progressive processes (32–36). Numerous studies have shown the direct relationship between actin polymerization and EGFR signaling, which is mediated by Ras small GTPase proteins Rac and Rho (18). Furthermore, the actin polymerization is obligatory for negative feedback regulation of the EGFR tyrosine kinase through the C-kinase pathway (37). On the other hand, actin polymerization is a major effector for EGF and EGFR signal cascade leading to proliferation and motility control (38), and alteration of the actin-binding domain of the EGFR has direct effect for EGF-stimulated invasion (39). Thus, it is entirely consistent with observations from the current study that the anti-EGFR erlotinib has a direct effect on actin remodeling in the urothelial cells in vitro. Our ultimate goal is to determine if modulating the change of actin polymerization, for example by measuring the cellular G-actin content, provides a marker to monitor the effect of anti-EGFR therapy and/or prevention.

One of the major focuses of anti-EGFR research today is to determine the mechanisms of therapeutic resistance, either antibody based or with small tyrosine kinase inhibitors, such as erlotinib. Previously, two landmark studies by Lynch et al. (40) and Paez et al. (41) reported that specific mutations in the kinase domain of EGFR in some lung carcinomas are associated with markedly improved response rates to an EGFR tyrosine kinase inhibitor. Mutations in the EGFR receptor seem to play a significant role in determining the sensitivity of tumor cells to EGFR inhibitor therapy by altering the conformation and activity

**Figure 5.** Dose- and time-response effect of erlotinib on MAPK (P44/42) activity. Cells were treated with various concentrations of erlotinib with or without EGF stimulation afterward as described in Fig. 4. Total and phosphorylated MAPK (T-44/42 and P-44/42, respectively) were detected by the Western blot. X axis, actual intensity of P-44/42 measured by densitometry.
of the receptor. However, recently, it has become clear that other mechanisms may have to be involved because the mutations found in patients with lung carcinoma were not found in many other tumor systems, including colon carcinoma, head and neck cancer, pancreatic cancer, etc. Furthermore, whether such mutations play a role in the resistance of a nonantibody-based, tyrosine kinase inhibitor, such as erlotinib, remains a question. Recently, several potential mechanisms have been proposed, which include the presence of redundant tyrosine kinase receptors (42), epithelial to mesenchymal transition (43), and the dynamics of EGFR (44). It should be noted that our study did not directly examine the association of drug sensitivity with actin remodeling; however, it is possible that actin remodeling may also play a role in drug sensitivity because cells with more polymerized actin (represented with higher F-actin staining) are associated with less sensitivity for erlotinib-induced growth inhibition and actin remodeling. More extensive studies will be needed to address this issue.

Recently, there has been a great deal of attention focused on how epithelial to mesenchymal transition is involved in tumor progression, especially from a noninvasive to invasive status (45). Given the fact that actin remodeling is regulated by pathways associated with epithelial to mesenchymal transition (e.g., Ras signaling and E-cadherin), it is possible that the effects observed on actin remodeling by erlotinib is the downstream overall effect of the drug on epithelial to mesenchymal transition. Although the current study observed the direct effect of anti-EGFR erlotinib on actin remodeling, further studies are also needed to dissect signaling pathways leading to actin remodeling by erlotinib focusing on the Rho pathway. The findings of the current study are limited to in vitro observations, in vivo animal model experiments, as well as human studies are needed. These findings will clarify whether and how actin remodeling is related to the actual anticaner or chemopreventive effect of erlotinib.

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References


Figure 6. Effect of MAPK inhibitor on erlotinib-induced actin remodeling. EGF-stimulated MC-T11 cells treated with or without erlotinib (1/8 of IC50, 387 nmol/L) and MAPK inhibitor (PD98059) were stained with Bodipy-phallacidin (green for F-actin), Texas-Red-DNase I (red for G-actin), and 4',6-diamidino-2-phenylindole (blue for DNA). Images were taken using a Nikon Eclipse E400 microscope at 100× oil immersion object. Note erlotinib increased the F-actin staining intensity and formation of F-actin fibers, whereas no discernable difference of actin remodeling was seen in PD98059 treated versus untreated cells.


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