Molecular correlates of gefitinib responsiveness in human bladder cancer cells

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Abstract

We characterized the effects of the small molecule epidermal growth factor receptor (EGFR) inhibitor gefitinib (ZD1839, Iressa) on cell proliferation in a panel of 17 human bladder cancer cell lines. Gefitinib inhibited DNA synthesis in a concentration-dependent fashion in 6 of 17 lines. Growth inhibition was associated with $p27^{Kip1}$ accumulation and decreased cyclin-dependent kinase 2 activity. Gefitinib also inhibited baseline EGFR, AKT, and extracellular signal-regulated kinase (ERK) phosphorylation in the EGFR-dependent cells maintained in serum-free medium, whereas it had no effect on baseline EGFR or ERK phosphorylation in the EGFR-independent cells. Analyses of candidate markers of EGFR dependency revealed that the gefitinib-sensitive cells expressed higher surface EGFR levels than the gefitinib-resistant lines. Gefitinib-sensitive cells generally expressed higher levels of E-cadherin and lower levels of vimentin than the gefitinib-resistant cells, but these correlations were not perfect, suggesting that these markers of epithelial-mesenchymal transition cannot be used by themselves to prospectively predict EGFR-dependent growth. Together, our results show that bladder cancer cells are markedly heterogeneous with respect to their sensitivity to EGFR antagonists. Although surface EGFR levels and epithelial-mesenchymal transition status seem to roughly correlate with responsiveness, they cannot be used by themselves to identify bladder tumors that will be sensitive to EGFR-directed therapy. However, comparing levels of $p27^{Kip1}$ or DNA synthesis before and after gefitinib exposure does identify the drug-sensitive cells. [Mol Cancer Ther 2007;6(1):277–85]

Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa receptor tyrosine kinase that has been implicated in tumor progression and metastasis (1–3). It remains one of the most important targets of investigational anticancer therapies, and a large number of different small-molecule and antibody-based EGFR antagonists have been developed and/or evaluated in late-stage clinical trials. Gefitinib (also known as ZD1839 or Iressa) is an orally active, selective EGFR tyrosine kinase antagonist that inhibits tumor cell growth at nanomolar concentrations in responsive cell lines *in vitro* (4). It displayed very promising activity in preclinical models (5–7) but did less well in patients with non–small cell lung cancer (NSCLC) and other types of cancer (8–12), which has significantly diminished overall clinical enthusiasm for the drug and its target. Importantly, however, molecular analyses showed that many of the NSCLC tumors that did respond to single-agent gefitinib possessed activating mutations within the EGFR tyrosine kinase domain (exons 18–21; refs. 13–21), strongly suggesting that a better understanding of the biological underpinnings of EGFR-dependent tumor growth could allow investigators to prospectively identify those patients who would most benefit from EGFR-directed therapy in NSCLC and other tumors.

Previous work has shown that the EGFR and its ligands are overexpressed in human bladder tumors (22–25), and transgene-driven overexpression of the EGFR within the bladder enhances tumor progression in mice (26), providing direct support for its importance in the biology of this disease. Studies with clinically relevant EGFR antagonists confirmed that they are potent inhibitors of tumor growth in xenograft models where they not only inhibit tumor cell proliferation but also angiogenesis (5–7, 27–30). Thus, the design and implementation of EGFR-directed clinical trials is a high priority in the field of translational bladder cancer research. However, before these trials are initiated, it seems critical that a better understanding of the heterogeneity in tumor responsiveness that is likely to be encountered and of the molecular markers that identify EGFR-dependent tumors be obtained.

With these issues in mind, we initiated the present study to characterize the heterogeneity of gefitinib responsiveness in a fairly large panel of human bladder cancer cell lines and to attempt to identify biological characteristics of EGFR-dependent proliferation that could be used to prospectively or retrospectively identify drug-sensitive tumors. Our data confirm that human bladder cancer cells

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display marked heterogeneity in gefitinib responsiveness *in vitro*, but that high surface EGFR expression and E-cadherin/vimentin levels loosely correlate with drug responsiveness *in vitro*. On the other hand, comparing levels of $p27^{Kip1}$ or proliferation before and after drug exposure can be used to identify all of the drug-responsive cells.

Materials and Methods

Cell Lines

The 253J B-V cells were derived from the 253J parental line by orthotopic ''recycling'' through the mouse bladder as described previously (31). The KU-7 cells were provided by Dr. William Benedict (Department of GU Medical Oncology, University of Texas M.D. Anderson Cancer Center). All other cells lines (UM-UC1–7, UM-UC9–15, and UM-UC17) were provided by H. Barton Grossman (Department of Urology, University of Texas M. D. Anderson Cancer Center). All cells were maintained in MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Life Technologies), 1% MEM vitamin solution (Life Technologies), sodium pyruvate (Bio Whittaker, Rockland, ME), L-glutamine (Bio Whittaker), nonessential amino acids (Life Technologies), and penicillin/streptomycin (Bio Whittaker) under 5% CO_2 in an incubator.

Antibodies and Reagents

The antibodies used for immunoblotting and immune complex assays were from the following sources: mouse anti-human $p27^{Kip1}$ (clone 57, BD PharMingen, San Diego, CA), rabbit anti-human EGFR (Upstate Biotechnology, Inc., Lake Placid, NY), rabbit anti-human phosphorylated EGFR (p-EGFR; Y^{1173}, Biosource, Inc., Camarillo, CA), rabbit anti-human cyclin-dependent kinase 2 (cdk2; clone D12, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human actin (Sigma, St. Louis, MO), rabbit anti-human p-AKT, rabbit anti-human AKT, rabbit anti-human phosphorylated extracellular signal-regulated kinase (p-ERK), rabbit anti-human ERK, rabbit anti-human E-cadherin, rabbit anti-human vimentin (all from Cell Signaling Technology, Danvers, MA), and secondary antibodies (horseradish peroxidase–conjugated sheep antimouse or donkey anti-rabbit; Amersham Biosciences, Piscataway, NJ). Gefitinib was obtained from the University of Texas M. D. Anderson Cancer Center pharmacy and was dissolved in DMSO before use (10 mmol/L stock). C225 (cetuximab, Erbitux) was generously provided by ImClone Systems, Inc. (New York, NY).

Quantification of DNA Synthesis

Cells (5,000) were plated into each well of a 96-well plate. The next day, the cells were treated with increasing concentrations of gefitinib in serum-free medium. After 24 h, the medium was removed, and 50 μL of complete medium containing 10 μCi/mL [^{3}H]thymidine was added for 1 h. Medium was again removed, and 100 μL of 1.0 N KOH was added to each well to lyse the cells. Cell lysates were transferred to fibroglass filters (Perkin-Elmer, Boston, MA), and filters were dried in a microwave and then wetted with scintillation fluid (Beta Plate Scint, Perkin-Elmer). Scintillation counts were measured using the Micro Beta Plate Reader from Perkin-Elmer.

Immunoblot Analyses

To detect $p27^{Kip1}$, cdk2, AKT, ERK, E-cadherin, and vimentin, cells were lysed by incubation for 1 h at 4°C in 100 μL of Triton lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 25 μmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and one complete mini protease inhibitor cocktail tablet]. For detection of p-EGFR/EGFR, cells were washed once in sterile PBS and incubated overnight in serum-free MEM. The next day, the cells were pretreated for 2 h in serum-free MEM with gefitinib, and then some cells were exposed to 20 ng/mL EGF (Invitrogen, Carlsbad, CA) for 15 min before lysis. The cells were washed twice in cold wash buffer [1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, and one complete mini protease inhibitor cocktail tablet (Roche, Indianapolis, IN)] and 80μL cold Triton lysis buffer added for 10 min at 4°C. Cells were then scraped and transferred to a microfuge tube where lysis was continued for 30 min on ice. Lysates were centrifuged for 10 min at 12,000 × *g* (4°C), and 20 μg of the postnuclear supernatants were mixed with equal volumes of 2× SDS-PAGE sample buffer (50 mmol/L Tris-HCl, 2% SDS, 0.1% bromophenol blue, 10% glycerol, and 5% β-mercaptoethanol). Samples were then boiled for 5 min at 100°C and resolved by SDS-PAGE. Polypeptides were transferred to nitrocellulose membranes in a transfer buffer containing 39 mmol/L glycine, 48 mmol/l Tris, and 20% methanol. Membranes were blocked for 1 h in either 5% milk diluted in TBS containing 0.1% Tween 20 (TBS-T; p27, cdk2, p-Akt/Akt, p-Erk/Erk, E-cadherin, and vimentin) or 5% bovine serum albumin in TBS-T (p-EGFR/EGFR). Membranes were incubated overnight at 4°C with primary antibodies. Blots were washed 3 × 5 min in TBS-T before incubation with secondary antibodies for 2 h at 4°C. Blots were washed 3 × 10 min in TBS-T and developed by enhanced chemiluminescence (Renaissance; Perkin-Elmer).

Analysis of EGFR Surface Expression

Cells grown under basal conditions were harvested with trypsin and washed twice in fluorescence-activated cell sorting (FACS) buffer (2% fetal bovine serum, 0.1% sodium azide in PBS). Cells were incubated with C225 (1:10) in FACS buffer for 1 h on ice. Cells were washed once in FACS buffer and incubated with goat anti-human Alexa Fluor 488 antibody (1:50) in FACS buffer for 30 min on ice. Cells were washed once in FACS buffer and resuspended in fresh buffer before analysis by FACS.

Immune Complex cdk2 Kinase Assays

Cells were cultured to 60% confluency in 10-cm dishes and treated with various concentrations of gefitinib for 24 h. Cells were then harvested with trypsin and lysed by rotating them for 1 h at 4°C in 1 mL of the Triton X-100 lysis buffer described above. Lysates were cleared by centrifugation for 10 min at 12,000 × *g* (4°C). Supernatants

containing 200 μg of protein were then incubated with an anti-cdk2 antibody overnight followed by incubation with 50 μL protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h at 4°C. The beads were then washed twice with lysis buffer and once more with kinase buffer [25 mmol/L Tris (pH 7.2) and 10 mmol/L $MgCl_2$]. Immunoprecipitates were incubated with 1 μg histone H1, 150 μmol/L ATP, and 20 μCi [γ-^{32}P]ATP in 50 μL of kinase buffer for 15 min at 30°C. SDS sample buffer was used to terminate the reaction, and the mixture was boiled for 5 min at 100°C. Finally, the mixture was loaded onto 12% SDS-PAGE gels and resolved at 100 V for 90 min. The gels were stained with Coomassie blue, destained, dried, and analyzed by autoradiography.

Small Interfering RNA – Mediated Silencing of p27^{Kip1}

Cells were grown to 60% confluency in six-well plates and transfected with specific or nonspecific small interfering RNA (siRNA) constructs for 48 h according to the manufacturer's protocols. The constructs used were the siRNA SMARTpool cdk-N-1B (p27^{Kip1}; Upstate Cell Signaling Solutions, Lake Placid, NY) or the siRNA Nonspecific Control IV (Dharmacon RNA Technologies, Lafayette, CO), all at 200 nmol/L. Liposome-mediated transfection was accomplished with Oligofectamine reagent (Invitrogen/Life Technologies, Carlsbad, CA) diluted 1:100 in serum-free MEM. Following silencing, cells were treated with gefitinib (10–1,000 nmol/L) for 24 h, and DNA synthesis was quantified by [^{3}H]thymidine uptake. The efficiency of p27^{Kip1} silencing was verified in each experiment by immunoblotting.

Quantification of Vascular Endothelial Growth Factor Production by ELISA

Cells were plated in equal numbers in 24-well plates in the presence of 10% MEM. The medium was removed after 24 h; cells were washed with 1 mL of PBS and then incubated with 1 mL of fresh medium containing the indicated concentrations of gefitinib for 24 h. Human vascular endothelial growth factor (VEGF) levels in cell-free culture supernatants were determined using the commercial Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN) and following the manufacturer's instructions. The protein concentrations of VEGF were determined by comparison of the absorbance with the standard curve.

Statistics

All results represent the average of at least three separate experiments and are expressed as mean ± SD unless otherwise indicated. Statistics were compared using *t* test, repeated-measures ANOVA, Mann-Whitney, Kruskal-Wallis, and Tukey-Kramer. GraphPad InStat v3.05 software (GraphPad Software, Inc., San Diego, CA) was used. Statistical significance was set at $P < 0.05$.

Results

Gefitinib Inhibits DNA Synthesis in a Subset of Human Bladder Cancer Cell Lines

Previous studies concluded that EGFR antagonists exert largely cytostatic effects on tumor cell proliferation *in vitro*. In an effort to characterize potential heterogeneity in gefitinib responsiveness, we characterized the effects of gefitinib on DNA synthesis in cells maintained in serum-free medium using [^{3}H]thymidine incorporation assays. Gefitinib inhibited DNA synthesis by at least 50% at a biologically and clinically relevant concentration (1 μmol/L) in six of the cell lines (Fig. 1, *gray lines*), whereas the other 11 cell lines were less responsive (Fig. 1, *black lines*).

As cdk inhibitors have been implicated in growth arrest, we next examined the effects of gefitinib on p21$^{cip1/waf1}$ and p27^{Kip1} expression to determine whether or not the proteins served as a marker for drug responsiveness. We incubated three gefitinib-sensitive and three gefitinib-resistant cell lines with 1 μmol/L gefitinib for 24 h in serum-free medium and quantified protein levels by immunoblotting. Our data confirmed that gefitinib increased p27^{Kip1} expression in the sensitive but not the resistant cell lines ($P < 0.05$, sensitive versus resistant; Fig. 2A). We observed no change in p21$^{cip1/waf1}$ protein expression in any of the lines in response to gefitinib exposure for 24 h (data not shown).

p27^{Kip1} accumulation was associated with significant inhibition of cdk2 activity in the two cell lines that displayed the greatest sensitivity to gefitinib-induced growth arrest (UM-UC-5 and 253J B-V), but gefitinib had no effect on cdk2 activity in the resistant cells ($P < 0.009$, sensitive versus resistant; Fig. 2B). Nonetheless, although siRNA-mediated knockdown of p27^{Kip1} expression restored DNA synthesis to near control levels in gefitinib-exposed 253J B-V cells, it had no effect on DNA synthesis in the other gefitinib-sensitive cell line tested (UM-UC-5; Fig. 2C), suggesting that compensatory mechanisms substituted for p27^{Kip1} in the latter. Silencing p27^{Kip1} expression had no effect on DNA synthesis in the gefitinib-resistant cells (Fig. 2C).

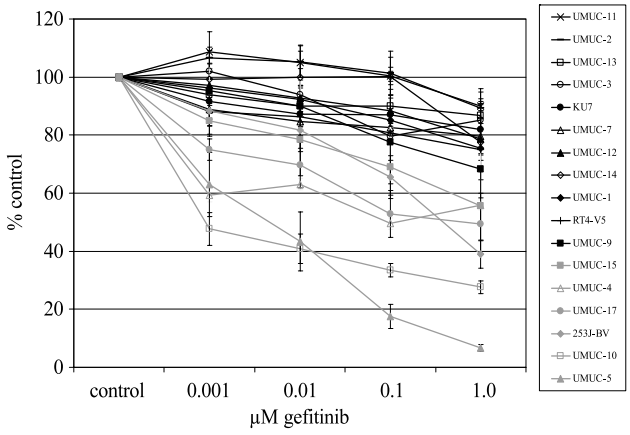

Figure 1. Concentration-dependent effects of gefitinib on DNA synthesis. [^{3}H]thymidine incorporation was used to evaluate the effects of increasing concentrations of gefitinib (1 nmol/L to 1 μmol/L). Percentage inhibition of [^{3}H]thymidine incorporation relative to untreated cells. Gefitinib-sensitive cells (*gray*) and gefitinib-resistant cells (*black*). Representative results of at least three separate experiments. *Points,* mean; *bars,* SE.

Aside from their direct effects on tumor cell proliferation, EGFR antagonists also down-regulate angiogenesis by blocking tumor cell production of proangiogenic factors. We therefore characterized the effects of gefitinib on VEGF production in a subset of our cell lines. Consistent with the [^{3}H]thymidine incorporation data, the drug significantly inhibited VEGF secretion in the UM-UC-5 and 253J B-V cells but had no effect on cytokine secretion in the KU-7 or UM-UC-3 cells (Fig. 3).

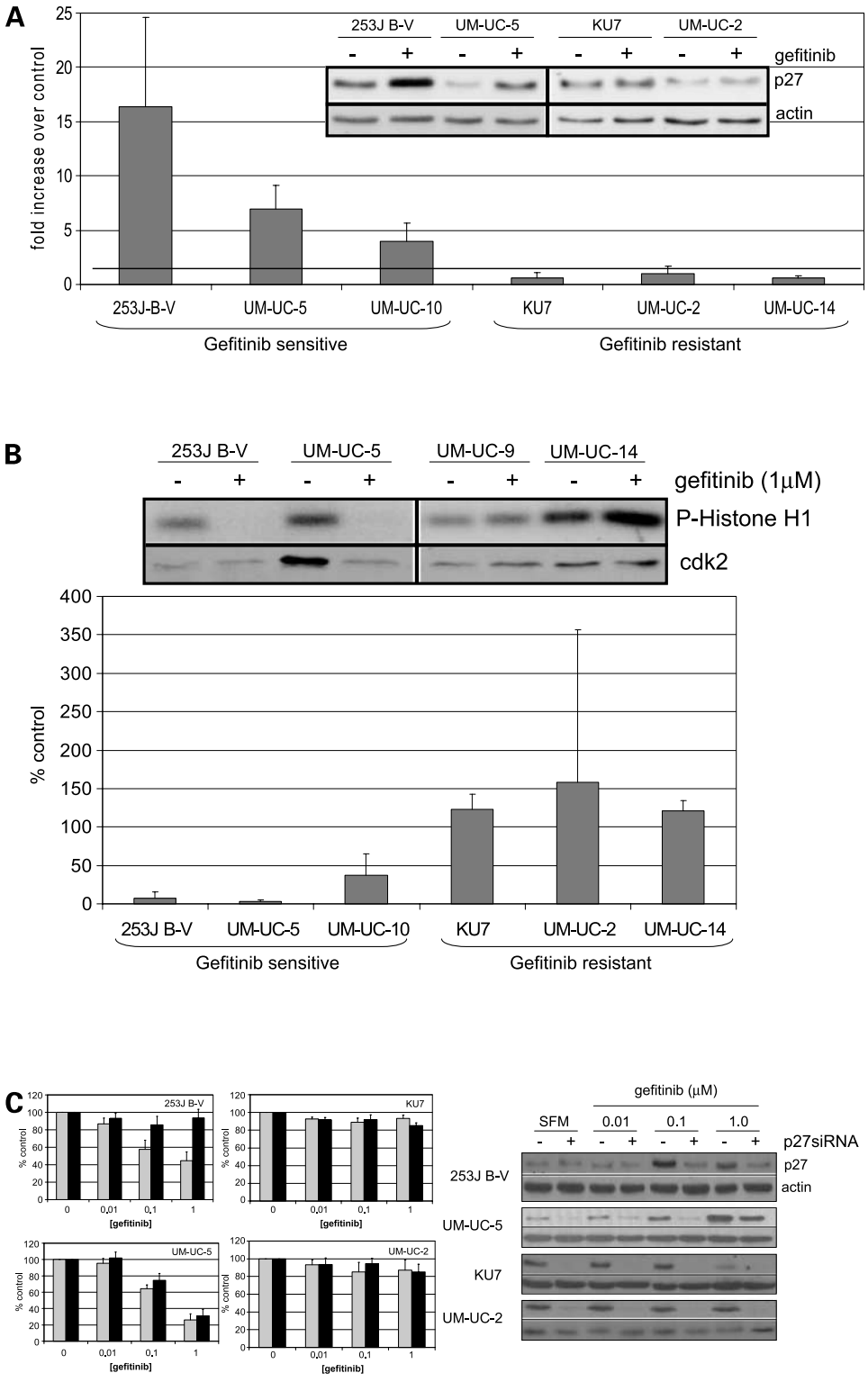

Figure 2. Effects of gefitinib on regulators of G_1-S progression. **A,** effects on $p27^{Kip1}$ protein expression. Cells were incubated in the absence or presence of 1 μmol/L gefitinib for 24 h in serum-free medium. $p27^{Kip1}$ expression was measured in whole-cell lysates by immunoblotting (*top*). Expression of actin was measured in parallel as loading control. Quantitative comparisons of relative protein expression (*bottom*). **B,** effects cdk2 kinase activity. Cells were incubated with 1 μmol/L gefitinib for 24 h in serum-free medium, and cdk2 activity was measured in immunoprecipitates as described in Materials and Methods (*top*). Expression of cdk2 protein was quantified in parallel by immunoblotting as a loading control (*bottom*). Representative of results obtained in three separate experiments. **C,** effects of silencing $p27^{Kip1}$ on gefitinib-mediated inhibition of DNA synthesis. Cells were transfected with a p27-specific construct or an off-target control, and the effects of gefitinib on [^{3}H]thymidine incorporation were measured as described in Materials and Methods.

Molecular Correlates of Gefitinib Sensitivity

We next compared the concentration-dependent effects of gefitinib on EGFR phosphorylation in gefitinib-sensitive and gefitinib-resistant cell lines. Gefitinib was equally effective at blocking EGF-induced EGFR phosphorylation in gefitinib-sensitive and gefitinib-resistant cell lines (IC_{50} = 10 nmol/L across the panel, Fig. 4A; data not shown), indicating that the receptors expressed by all of the cell lines were sensitive to drug-mediated inhibition. To directly test this possibility, we sequenced the EGFR tyrosine kinase domain in our bladder cancer cell lines and in >100 primary tumors. The results confirmed that none of the cells or tumors contained activating EGFR mutations (32). We then compared the effects of gefitinib on the baseline EGFR phosphorylation observed in serum-starved cells. The two cell lines that consistently displayed the greatest gefitinib sensitivity (253J B-V and UM-UC-5) expressed high levels of constitutively phosphorylated EGFR, and this phosphorylation was inhibited in a concentration-dependent fashion by gefitinib (Fig. 4B). Interestingly, two of the gefitinib-resistant cell lines (KU-7 and UM-UC-3) also displayed constitutive EGFR phosphorylation, but this baseline phosphorylation was not affected by concentrations of gefitinib up to 10 μmol/L (Fig. 4B). Analysis of downstream signal transduction pathways confirmed that constitutive EGFR phosphorylation was associated with constitutive phosphorylation of the ERKs and AKT, and gefitinib inhibited ERK and AKT phosphorylation in parallel with its effects on EGFR phosphorylation in the 253J B-V and UM-UC-5 cells (Fig. 4B). In contrast, baseline AKT phosphorylation was lower in the KU-7 and UM-UC-3 cells, and gefitinib had no further effect on AKT phosphorylation (Fig. 4B). Furthermore, although the ERKs were constitutively phosphorylated in the KU-7 and UM-UC-3 cells, gefitinib had no effect on ERK phosphorylation (Fig. 4B; ref. 33). Together, these results show that AKT and ERK phosphorylation are directly controlled by the EGFR in the gefitinib-sensitive but not in the gefitinib-resistant cells.

We then examined whether or not gefitinib sensitivity correlated with surface EGFR expression. We included six of the gefitinib-sensitive lines and six randomly selected gefitinib-resistant lines in these experiments. The gefitinib-sensitive lines expressed significantly higher levels of surface EGFR compared with the drug-resistant cells ($P < 0.02$; Fig. 4C). In spite of this, EGFR expression did not directly correlate in a linear fashion with gefitinib sensitivity (r^2 = 0.466). We did parallel studies to determine whether or not ligand (transforming growth factor-α) expression also correlated with sensitivity to gefitinib-mediated growth arrest using ELISA. We examined three of the gefitinib-sensitive lines and three randomly selected gefitinib-resistant lines in these experiments and found no difference in transforming growth factor-α expression between the gefitinib-sensitive and gefitinib-resistant cell lines (data not shown).

Epithelial-Mesenchymal Transition and Gefitinib Resistance

Recent studies have reported a correlation between resistance to EGFR inhibition and expression of markers of epithelial-mesenchymal transition (EMT; refs. 34–37). We therefore analyzed the baseline expression levels of the two most familiar markers of EMT (E-cadherin and vimentin) in our cell lines to examine the likelihood of a correlation between gefitinib sensitivity and EMT. Immunoblotting was done on baseline lysates from four gefitinib-sensitive and four gefitinib-resistant cell lines as described in Materials and Methods. The results show that all four of the gefitinib-sensitive cell lines and one of the gefitinib-resistant lines expressed the epithelial marker E-cadherin, whereas all four gefitinib-resistant cell lines and one gefitinib-sensitive cell line expressed the mesenchymal marker vimentin (Fig. 5). Therefore, it seems that expression of E-cadherin and vimentin do not precisely cosegregate with gefitinib sensitivity and resistance, respectively.

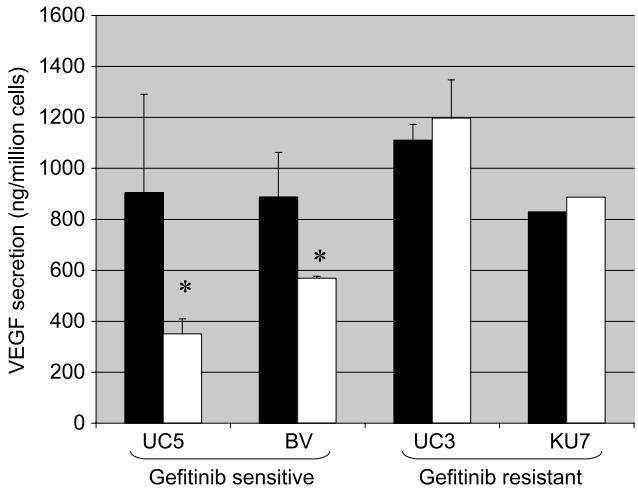

Figure 3. Effects of gefitinib on VEGF secretion. Cells were exposed to 1 or 10 μmol/L gefitinib for 24 h, and VEGF levels in the conditioned medium were quantified by ELISA as described in Materials and Methods. *Columns,* mean; *bars,* SE.

Discussion

The primary goals of the present study were to define the extent of the heterogeneity in gefitinib responsiveness in human bladder cancer cells and to identify biological characteristics of EGFR dependency that might be used to prospectively identify tumor cells that are most sensitive to EGFR-directed therapy. To these ends, we examined the effects of gefitinib on DNA synthesis in a panel of 17 human bladder cancer cell lines. Other methods could have been used to characterize gefitinib responsiveness (e.g., colony formation in soft agar or invasion/migration), and it is possible that the use of these methods could identify additional cell lines that are dependent upon the EGFR for some aspect of their malignant behavior. However, our studies of the effects of gefitinib or C225 on VEGF production strongly suggest that gefitinib inhibits angiogenic factor secretion in the same subset of cells that is responsive to the inhibitor in the DNA synthesis assays. Our data confirmed the existence of marked heterogeneity

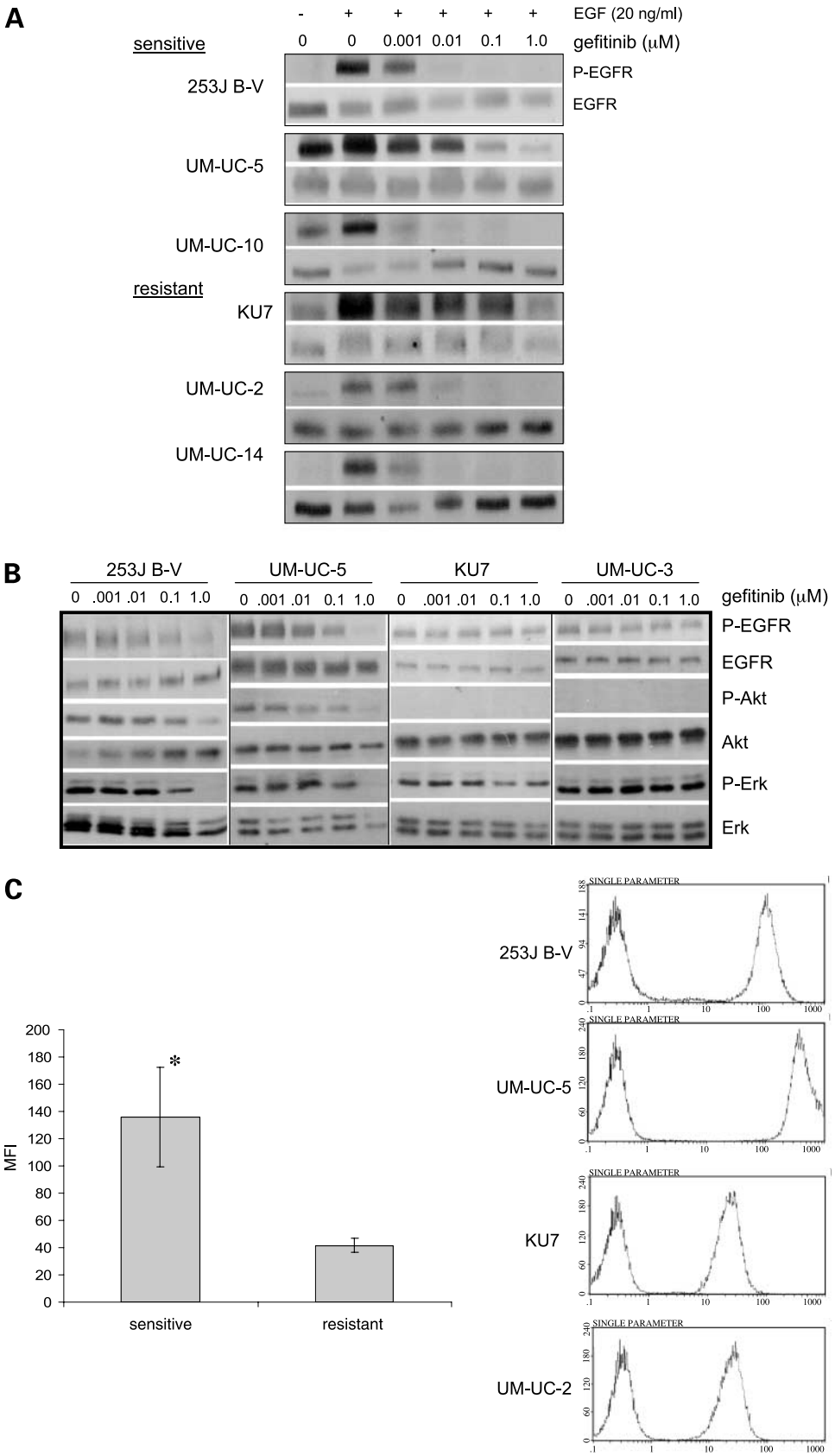

Figure 4. Expression of potential correlates of EGFR dependency. **A,** effects of gefitinib on EGF-induced phosphorylation of EGFR. Whole-cell lysates from cells that had been serum starved overnight and preincubated with the indicate doses of gefitinib for 2 h were prepared 15 min after stimulation with 20 ng/mL EGF. Total EGFR was also detected by immunoblotting as a control for protein loading. Note that gefitinib inhibits EGF-mediated EGFR phosphorylation at similar concentrations in all of the cell lines. **B,** effects of Iressa on baseline EGFR phosphorylation. Whole-cell lysates from cells that had been incubated for 24 h with increasing concentrations of Iressa in serum-free medium were prepared and immunoblotted as described in Materials and Methods. Note that only the gefitinib-sensitive cell lines display high basal EGFR, AKT, and ERK phosphorylation that can be partially inhibited by the drug. **C,** baseline EGFR surface expression. Cells were stained with an antibody that recognizes the extracellular domain of the EGFR (C225), and receptor levels were quantified by FACS. Representative overlays are provided showing background fluorescence (*blue traces*) and EGFR-specific fluorescence (*red traces*) for four of the cell lines. *Columns,* mean EGFR expression in the sensitive and resistant cells; *bars,* SE. MFI, mean fluorescent intensity. Representative results of at least three separate experiments. *, $P < 0.05$.

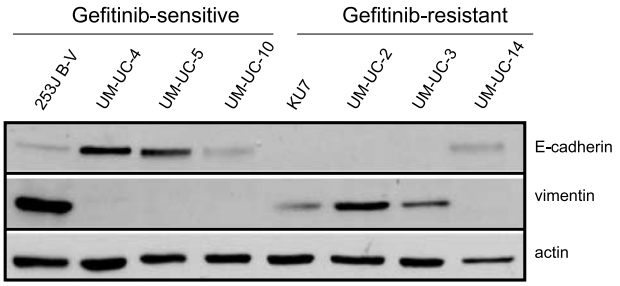

Figure 5. Markers of EMT correlate with gefitinib sensitivity. E-cadherin and vimentin expression was measured in whole-cell lysates by immunoblotting. Expression of actin was measured in parallel as loading control.

in gefitinib responsiveness, and less than half of the lines (6 of 17) were significantly growth inhibited by clinically relevant concentrations of the drug (≤1 μmol/L). Other groups have found similar heterogeneity in their studies of bladder cancer cell lines (34, 35). In the cell lines displaying higher gefitinib responsiveness, there was a correlation among inhibition of cell proliferation, EGFR surface expression, and inducible $p27^{Kip1}$ protein expression. This correlation was especially obvious in two of the most sensitive cell lines (253J B-V and UM-UC-5). These cell lines displayed the highest EGFR surface expression compared with the other sensitive cells and also had the highest inducible $p27^{Kip1}$ protein expression correlating with decreased cdk2 kinase activity. However, only the 253J B-V cells seemed to be dependent on $p27^{Kip1}$ for gefitinib-mediated growth arrest as silencing $p27^{Kip1}$ restored cell proliferation in these cells but not in the UM-UC-5 cells. In the case of the UM-UC-5 cells, another mechanism must have compensated for loss of $p27^{Kip1}$. A decrease in cyclin D1 expression may be responsible for the lack of effect of p27 siRNA in the UM-UC-5 cells. In fact, another study has shown that gefitinib decreases cyclin D1 expression in several bladder cancer cell lines, including UM-UC-5 (33).

Despite the strong correlation among gefitinib responsiveness, EGFR surface expression, and $p27^{Kip1}$ protein expression in the most responsive lines, gefitinib responsiveness was not as tightly linked to surface EGFR expression within the panel of cell lines as a whole. This was unexpected given our experience with human pancreatic cancer cell lines, where cellular transforming growth factor-α expression correlated directly with gefitinib sensitivity, as did baseline EGFR phosphorylation, which is driven by autocrine stimulation of the receptor (36). However, we observed no link between gefitinib responsiveness and baseline EGFR phosphorylation as well as other features associated with the EGFR signaling pathway, such as ERK and AKT activation. Furthermore, none of our cell lines contained the activating exon 18 to 21 EGFR mutations implicated in gefitinib sensitivity in NSCLC (32).

We also investigated whether or not there was a link between EMT status and gefitinib responsiveness as has been observed in NSCLC (37–39). All of the four evaluated gefitinib-responsive cell lines expressed E-cadherin, indicating that these cells are phenotypically epithelial. Conversely, three of the four gefitinib-resistant cell lines examined expressed vimentin, indicating that these cells had undergone an EMT. In one study using 42 NSCLC cell lines, the authors found that baseline expression of E-cadherin mRNA correlated with erlotinib responsiveness (39). In addition, similarly to the mRNA analyses, expression of E-cadherin protein was high with the erlotinib-sensitive cells, whereas vimentin expression was mainly detected in the insensitive cell lines. In another study of 17 NSCLC cell lines, the authors also found that E-cadherin protein expression correlated with erlotinib sensitivity, and vimentin protein expression correlated with erlotinib insensitivity (37). Finally, Witta et al. found a similar correlation between loss of E-cadherin and erlotinib resistance and went on to show that exposure to histone deacetylase inhibitors or transfection with E-cadherin restored EGFR inhibitor sensitivity (38). Thus, prospective analysis of EMT markers may allow for an identification of a subset of tumors that are more likely to respond to EGFR-directed therapy.

Nonetheless, the predictive value of these two EMT markers in bladder cancer cells is not perfect. For example, the 253J B-V cells were one of the two most gefitinib-responsive lines in our panel, and they did express E-cadherin and also expressed high levels of the mesenchymal marker vimentin. The 253J B-V cells were selected by orthotopic ''recycling'' for aggressive local growth and metastasis *in vivo*, and we suspect that as a result of this selection, they are actually in transition between an epithelial and a mesenchymal phenotype. We found another example in the UM-UC-14 cells, which expressed high levels of E-cadherin and low levels of vimentin yet did not respond well to gefitinib. Recent studies indicate that activating mutations within the tyrosine kinase domain of the fibroblast growth factor receptor-3 (FGFR3) accumulate in a substantial subset of superficial bladder tumors (40–42), and it is possible that these mutant receptors function to drive autocrine proliferation in cells that express them, inasmuch as mutant active EGFRs can drive proliferation and survival in a subset of NSCLCs. Indeed, another EGFR-independent cell line within our panel (UM-UC-9) also expresses mutant FGFR3 and high levels of E-cadherin.[4] Thus, mutant FGFR3 may functionally substitute for the EGFR in bladder cancer cells that express E-cadherin but do not respond to gefitinib. We are currently testing this hypothesis in ongoing studies.

It will also be important to identify strategies to inhibit autocrine growth in the ''mesenchymal'' subset of bladder cancer cells. Our results show that downstream signaling pathways are uncoupled from the EGFR in these cells (Fig. 3; ref. 33), and it is possible that oncogene activation

[4] C. Dinney and P. Black, unpublished observations.

and/or loss of tumor suppressors (p16 and Rb) might bypass any requirement for growth factor receptor signaling. Indeed, recent studies suggest that expression of constitutively active forms of K-ras identify gefitinib-resistant NSCLCs (43), and retention of phosphatase and tensin homologue deleted on chromosome 10 and expression of EGFRvIII seem to correlate with gefitinib responsiveness in brain tumors (44). However, there does not seem to be an obvious correlation between the mutational status of any of these cancer-associated pathways and EGFR/FGFR3–independent proliferation in our panel of bladder cancer cells, suggesting that other mechanisms may be involved. One attractive alternative hypothesis is that other growth factor receptors may function in the mesenchymal cells in a manner that is similar to the role of the EGFR and FGFR3 in the E-cadherin–positive cells. Supporting this idea, we have found that UM-UC-3 and KU-7 cells express high levels of platelet-derived growth factor receptor-β and platelet-derived growth factor and undergo p27-dependent growth inhibition when they are exposed to chemical inhibitors of the platelet-derived growth factor receptor.[5] This potential redundancy could have important implications for the emergence of EGFR-resistant tumor growth because we would expect that exposure to EGFR inhibitors would select for cells that rely on a different growth factor receptor for their proliferation. Identifying the molecular mechanisms that drive reliance upon different growth factor receptors will be a high priority for future research. The observation that developmental transcription factors, such as Twist, can drive EMT and metastatic progression (45) strongly suggests that they may play central roles in these processes.

Finally, we would argue that clinical trials with growth factor receptor antagonists should be designed so that their effects on their targets and downstream biological responses can be confirmed in a quantitative fashion. Specifically, it should be possible to confirm that bladder cancer cells are responding to EGFR, FGFR3, or platelet-derived growth factor receptor inhibitors by comparing levels of proliferation markers ($p27^{Kip1}$ and proliferating cell nuclear antigen) in tumor tissues collected before and after therapy, as we have done in previous studies with angiogenesis inhibitors (46–48). In the coming year, we will conduct a neoadjuvant clinical trial with the EGFR antagonist erlotinib (Tarceva) in patients with bladder cancer. The primary purposes of the trial are to comprehensively assess EMT status using Affymetrix microarrays as a means of prospectively identifying EGFR-responsive tumors and to refine our current methods for monitoring response to growth factor receptor inhibitor-based therapy using tissue biopsies. End points in the latter will include levels of phosphorylated EGFR, $p27^{Kip1}$, and Ki-67/proliferating cell nuclear antigen.

[5] G. Brown et al., in preparation.

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