

## Sensitivity to Epidermal Growth Factor Receptor Inhibitor Requires E-Cadherin Expression in Urothelial Carcinoma Cells

Peter C. Black,<sup>1</sup> Gordon A. Brown,<sup>1</sup> Teruo Inamoto,<sup>1</sup> Marissa Shrader,<sup>2</sup> Ameeta Arora,<sup>1</sup> Arlene O. Siefker-Radtke,<sup>3</sup> Liana Adam,<sup>1,2</sup> Dan Theodorescu,<sup>6</sup> Xifeng Wu,<sup>4</sup> Mark F. Munsell,<sup>5</sup> Menashe Bar-Eli,<sup>2</sup> David J. McConkey,<sup>1,2</sup> and Colin P.N. Dinney<sup>1,2</sup>

**Abstract Purpose:** Epidermal growth factor receptor (EGFR) is an attractive target for the treatment of urothelial carcinoma, but a clinical response can be expected in only a small proportion of patients. The aim of this study was to define molecular markers of response to cetuximab therapy in a panel of urothelial carcinoma cell lines.

**Experimental Design:** Eleven cell lines were investigated for antiproliferative response to cetuximab based on [<sup>3</sup>H]thymidine incorporation. A variety of markers, including EGFR expression, phosphorylation, and gene amplification, as well as the expression of other growth factor receptors, their ligands, and markers of epithelial-to-mesenchymal transition were investigated. Cohen's  $\kappa$  statistic was used to estimate the agreement between response and expression of these markers. E-cadherin was silenced by small interfering RNA in two sensitive cell lines, and the effect on the response to cetuximab was measured.

**Results:** We were able to identify a panel of relevant markers pertaining especially to alternate growth factor receptor expression and epithelial-to-mesenchymal transition that predicted response to cetuximab. The data suggested that expression of intact HER-4 ( $\kappa$ , 1.00;  $P = 0.008$ ), E-cadherin ( $\kappa$ , 0.81;  $P = 0.015$ ), and  $\beta$ -catenin ( $\kappa$ , 0.81;  $P = 0.015$ ) and loss of expression of platelet-derived growth factor receptor  $\beta$  ( $\kappa$ , 0.57;  $P = 0.167$ ) were associated with response to cetuximab therapy. Silencing E-cadherin in two sensitive cell lines reduced responsiveness to cetuximab in both ( $P < 0.001$ ).

**Conclusions:** A panel of predictive markers for cetuximab response has been established *in vitro* and is currently being evaluated in a prospective clinical trial of neoadjuvant EGFR-targeted therapy. Most importantly, E-cadherin seems to play a central role in modulation of EGFR response in urothelial carcinoma.

The epidermal growth factor receptor (EGFR) is expressed in a variety of human malignancies, including head and neck, breast, colorectal, lung, prostate, kidney, ovary, brain, pancreas, and bladder cancers (1). EGFR activation and overexpression of its ligands have been associated with advanced disease, development of a metastatic phenotype, and poor prognosis (2). Blockade of EGFR is therefore an attractive strategy for therapy in multiple tumor types, and it has shown clinical uti-

lity in combination with conventional chemotherapy (1, 3–8). Enthusiasm for EGFR targeted therapy has diminished due to the disappointing activity of the EGFR tyrosine kinase antagonist gefitinib (9), but studies continue with the tyrosine kinase inhibitor erlotinib and the inhibitory monoclonal antibody cetuximab (IMC225). Importantly, clinical trials using gefitinib in patients with non-small cell lung cancer have shown that a significant fraction of patients derive substantial benefit from single-agent therapy, and some of the unique molecular features of these tumors that make them responsive to EGFR-directed therapy have subsequently been identified (10). Therefore, it is likely that other solid tumors are heterogeneous with respect to their dependence on the EGFR for growth and survival. Identifying the molecular characteristics associated with EGFR dependency could allow for the prospective identification of patients who are most likely to benefit from cetuximab and other EGFR antagonists.

In urothelial carcinoma cell lines, we have observed a spectrum of sensitivity to EGFR-directed therapy that makes these cells a useful model system to define the molecular markers of response to EGFR targeting. Characterization of the cell lines should reveal markers of response that can be translated into clinical practice by validation in patient specimens within the

**Authors' Affiliations:** Departments of <sup>1</sup>Urology, <sup>2</sup>Cancer Biology, <sup>3</sup>Genitourinary Medical Oncology, <sup>4</sup>Epidemiology, and <sup>5</sup>Biostatistics, The University of Texas M. D. Anderson Cancer Center, Houston, Texas and <sup>6</sup>Department of Urology, University of Virginia Health System, Charlottesville, Virginia

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**Note:** P.C. Black and G.A. Brown contributed equally to this work.

**Requests for reprints:** Colin P.N. Dinney, Department of Urology, The University of Texas M. D. Anderson Cancer Center, Unit 1373, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-792-3250; Fax: 713-794-4784; E-mail: cndinney@mdanderson.org.

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context of clinical trials. E-cadherin may be a particularly relevant marker in bladder cancer because it is involved in EGFR signaling (11), and loss of E-cadherin has proved to be an important predictor of aggressive disease (12, 13). Epithelial-to-mesenchymal transition is associated with disease progression in bladder cancer (12, 14) and other solid tumors (15). The defining feature of epithelial-to-mesenchymal transition is the loss of E-cadherin and gain of mesenchymal markers, such as vimentin and N-cadherin. A recent gene profiling study showed that resistance to erlotinib correlated closely with epithelial-to-mesenchymal transition in non-small cell lung cancer cell lines and patient tumors (16). We have recently reported similar findings with gefitinib in a series of urothelial carcinoma cell lines that we have expanded upon in this report (17).

Here, we characterized the effects of the blocking anti-EGFR antibody cetuximab on DNA synthesis in a panel of 11 human bladder cancer cell lines. We then correlated relative cetuximab responsiveness with expression of several different growth factor receptors and their ligands, invasive potential, tumorigenicity, and epithelial-to-mesenchymal transition status. The results show that markers of epithelial-to-mesenchymal transition and enhanced invasive/tumorigenic potential predict cetuximab resistance in the cells. E-cadherin, especially, seems to play a central role in modulation of EGFR response in urothelial carcinoma. If validated in primary tumors, these data will help guide us toward better patient selection for EGFR-directed therapy of urothelial carcinoma.

## Materials and Methods

**Cell lines and culture conditions.** The metastatic human urothelial cell line 253J B-V was generated in our laboratory from the 253J P cell line ("P" indicates "parental" lineage; ref. 18). The UM-UC series of urothelial carcinomas was isolated and genotyped by the specimen core of the M.D. Anderson Genitourinary Specialized Program of Research Excellence in bladder cancer. The UM-UC6 cell line is a variant cell line developed from prolonged *in vitro* cell culture that has some characteristic differences to low-passage UM-UC6. KU7 was supplied by William Benedict at MD Anderson. The cell lines were maintained at 37°C in modified Eagle's MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, penicillin, streptomycin, and nonessential amino acids.

**Drugs and antibodies.** ImClone Systems, Inc. generously provided cetuximab, a selective antihuman EGFR antagonist monoclonal antibody in a stock concentration of 2 mg/mL. The following antibodies were purchased from the manufacturers listed and were used for Western blot analysis: EGFR, HER2, HER3, and HER4 (Lab Vision); E-cadherin (Zyomed); Zonula Adherens Sampler Kit (BD Transduction Laboratory); vimentin, actin, and vinculin (Sigma-Aldrich); c-MET, HER4, platelet-derived growth factor receptor  $\beta$  (PDGFR- $\beta$ ), and insulin-like growth factor-1 receptor (IGF-1R; Santa Cruz Biotechnology, Inc); PDGFR- $\alpha$  and pEGFR (Tyr<sup>1068</sup>; Cell Signaling Technology); matrix metalloproteinase 2 (MMP2) and MMP9 (Calbiochem); PDGFC and HB-EGF (R&D Systems, Inc.); and tissue inhibitor of metalloproteinase 2 (TIMP2) and TIMP3 (Chemicon).

**Cell proliferation assay.** Cells ( $5 \times 10^3$ ) were plated in 96-well plates for 24 hours, serum-starved for 24 hours, and then treated with or without cetuximab at increasing concentrations in epidermal growth factor-stimulated and nonstimulated environments for 48 hours. We measured the effect of cetuximab on DNA synthesis by pulse-labeling cells with [<sup>3</sup>H]thymidine (MP Biomedicals) for 2 hours followed by lysis in 100  $\mu$ L of 0.1 mol/L KOH. Cells were harvested onto fiberglass filters and incorporated tritium was quantified by  $\beta$ -counter.

**Western blot analysis.** Cells were harvested at 70% to 80% confluency and disrupted on ice in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% IGEPAL, 0.5 mmol, L-phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL leupeptin, 5  $\mu$ g/mL pepstatin A, 2.1  $\mu$ g/mL aprotinin]. The protein concentration was assayed using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). About 30 to 50  $\mu$ g of each protein sample was boiled in sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 10% (w/v) glycerol, 100 mmol/L DTT, 2.3% SDS, 0.002% bromophenol blue] for 5 minutes and separated on a 10% SDS-polyacrylamide gel at 120 V in electrophoresis buffer [25 mmol/L Tris-HCl (pH 8.3), 192 mmol/L glycine, 0.1% SDS]. Proteins in the gels were electrophoretically transferred onto polyvinylidene difluoride membranes in transfer buffer (25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol) at 30 mV overnight at 4°C. The membranes were washed in TBS-T [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20], blocked in 5% nonfat dry milk for 2 hours at room temperature with shaking and then rinsed once briefly with TBS-T. The membranes were incubated with 1:200 to 1:1,000 dilutions of primary antibodies overnight, followed, after further washing, by incubation for 1 hour at room temperature in horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology) diluted in milk (antirabbit 1:3,000, antimouse 1:5,000). The probed proteins were detected using the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

**Fluorescent *in situ* hybridization.** EGFR gene copy number was determined by fluorescence *in situ* hybridization in urothelial cell lines fixed with methanol and acetic acid using the dual probe LSI EGFR Spectrum Orange/CEP7 Spectrum Green Probe (Vysis) according to the manufacturer's instructions and as previously described (19). For each cell line, 50 individual nuclei were scored for copy number of EGFR (red) and the centromere of chromosome 7 (green).

**ELISA.** Cell-free culture supernatants were assayed for transforming growth factor- $\alpha$ , insulin-like growth factor-I, PDGF-AA, PDGF-BB, and PDGF-AB by ELISA (R&D Systems). The resultant concentrations (pg/mL) were corrected to the total protein amount in the cells from which the supernatant was acquired.

**Gene expression profiling.** Gene expression analysis was done on a panel of 41 urothelial carcinoma cell lines using the HG-U133A GeneChip from Affymetrix, as previously reported (20, 21). For each cell line, mRNA was generated from a single log-phase culture. This data array was not collected specifically for this study, but rather, we queried according to cell lines and genes of interest to our study the results of profiling done by D. Theodorescu for other studies. Genes representative of epithelial and mesenchymal differentiation were selected for analysis, and data were processed by established techniques (22). We selected from the panel of 41 cell lines, those for which we have sensitivity data. The median expression across all 41 cell lines was calculated, and the expression of the selected genes in each of the cell lines of interest was classified as increased or decreased compared with this median.

**FGFR3 mutation analysis.** DNA was isolated from the urothelial carcinoma cells using a genomic DNA extraction kit (Qiagen). PCR was done to amplify exons 7 and 10 using AmpliTaq Gold DNA polymerase (Applied Biosystems) and the primers 5'-CGGCAGTGGCGGTGGTGTG-3' (sense) and 5'-AGCACCGCCGTCTGGTT GGC-3' (antisense) for exon 7 (23) and 5'-CCTCAACGCCATGTCTTT-3' (sense) and 5'-AGGCAGCTCAGAACCTGGTA-3' (antisense) for exon 10 (purchased from Sigma Genosys). Five picomoles of each primer were added to the 20- $\mu$ L reaction volume, and 1  $\mu$ L DMSO was added for exon 7. The following cycling variables were used: 95°C for 10 minutes, then 35 cycles of 95°C for 30 s, 65°C (exon 7) or 58°C (exon 10) for 30 s, and 72°C for 30 s, followed by a final incubation at 72°C for 10 minutes (23). Unincorporated primers and deoxynucleotides were removed using shrimp alkaline phosphatase and exonuclease I (U.S. Biochemical). Direct sequencing was done by Big Dye Terminator Cycle Sequencing (Applied Biosystems), and the data were analyzed with Sequencing Analysis 3.0 software (Applied Biosystems). Visual

inspection of the electropherograms was carried out using Sequence Scanner Software v1.0 (Applied Biosystems). Mutation analysis was done independently in our laboratory and by Dr. François Radvanyi, Ph.D. (Institut Curie).

**Gelatin zymography.** Each urothelial carcinoma cell line was incubated in serum-free media at 37°C in 10-cm plates. The culture medium was collected after 24 hours and centrifuged at 1,500 rpm for 5 minutes at 4°C to remove cell debris. The supernatant was mixed with 6× nonreducing sample buffer (5:1, v/v), and 20 µg were separated by 10% SDS-PAGE with the gel containing 1% gelatin as the protease substrate. The procedure was followed by incubation in zymogram renaturation buffer (2.5% Triton X-100) for 1 hour at room temperature to remove SDS. The buffer was replaced with zymogram development buffer (50 mmol/L Tris, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L CaCl<sub>2</sub>, and 0.2% Brij 35) and incubated overnight on a rotary shaker. The gels were stained in 30% methanol, 10% acetic acid, and 0.5% (w/v) Coomassie brilliant blue for 1 hour followed by destaining. Gelatinolytic activity was manifested as horizontal white bands.

**In vitro growth curve.** Cell numbers were determined in triplicate after growth in full culture media in a six-well plate format. Counts were determined daily by a Coulter counter (Coulter Electronics, Inc.).

**Invasion assay.** Invasion chambers containing Matrigel-coated polyethylene terephthalate membranes with 8-µm pores in a 24-well plate format were purchased from BD Biosciences. Cells ( $5 \times 10^3$ ) were released from their tissue culture flask by EDTA (1 mmol/L), centrifuged, resuspended in DMEM, and placed in the upper compartment of the invasion chamber. Each cell line was plated in duplicate. NIH-3T3-conditioned medium was placed in the lower compartment as a chemoattractant. After incubation for 36 hours at 37°C, the membranes were removed and stained in propidium iodide (Sigma-Aldrich) without removing cells from the upper surface of the membrane. The filters were mounted on glass slides and inspected under the fluorescent microscope at 400× magnification. By adjusting the plane of focus, the noninvaded cells could be distinguished from the invaded cells. The number of each was counted in 10 separate fields per membrane. The ratio of invaded to noninvaded cells was calculated.

**Orthotopic tumor inoculation.** *In vivo* xenograft growth was evaluated in 6-week-old male athymic mice (obtained from NIH). All animals were cared for, and all experiments were done according to institutional guidelines established by the Animal Core Facility at M. D. Anderson Cancer Center. Cultured cells grown to 70% confluence were harvested with 0.1% EDTA/0.25% trypsin, washed, and resuspended in HBSS (Invitrogen) for injection. The mice were anesthetized with i.p. pentobarbital. After exposure of the bladder through a lower abdominal incision,  $1 \times 10^6$  tumor cells in 50 µL were injected intramurally into the bladder dome ( $2 \times 10^6$  for UM-UC5 and 253J P). Tumorigenicity was assessed grossly by necropsy at 4 weeks. Variable numbers of mice were injected for each cell line, as some of the mice were used concomitantly for other experiments, and mice that died within 7 days of injection were excluded: 10 253J P, 10 UM-UC5, 9 UM-UC9, 20 UM-UC10, 13 UM-UC13, 115 253J B-V, 129 KU7, and 158 UM-UC3.

**Silencing of E-cadherin with small interfering RNA.** 253J B-V and UM-UC14 were transfected with small interfering RNA (siRNA) targeting E-cadherin using oligofectamine (Invitrogen Corp.). Targeted oligonucleotides (sense 5'-CGAAUGUGGUACCUUUUUGAtt-3', antisense 5'-UCAAAAGGUACCACA UUCGtc-3') and a nontargeting negative control were purchased from Ambion, Inc. Total protein lysate was collected 60 hours after transfection and analyzed by Western blotting. In a parallel experiment, siRNA transfected cells were trypsinized at 60 hours and plated in 96 wells for a [<sup>3</sup>H]thymidine incorporation assay as described above.

**Statistical analysis.** We used Cohen's  $\kappa$  statistic (24) to estimate the agreement between sensitivity and expression.  $\kappa$  values of >0.75 were considered to represent excellent agreement, 0.40 to 0.75 for good agreement, and <0.40 for poor agreement. Dose responses of UMUC14 and 253J B-V to cetuximab were analyzed by the methods of Cuzick (25), and a linear regression model was fit to the log<sub>10</sub> transformed data

for each cell line with and without silencing of E-cadherin to assess the difference in response to cetuximab induced by the silencing of E-cadherin. All statistical analyses were done using SAS 9.1 for Windows (Copyright 2002-2003 by SAS Institute, Inc.) and Stata/SE 9.0 for Windows (Copyright 1985-2005 by StataCorp LP).

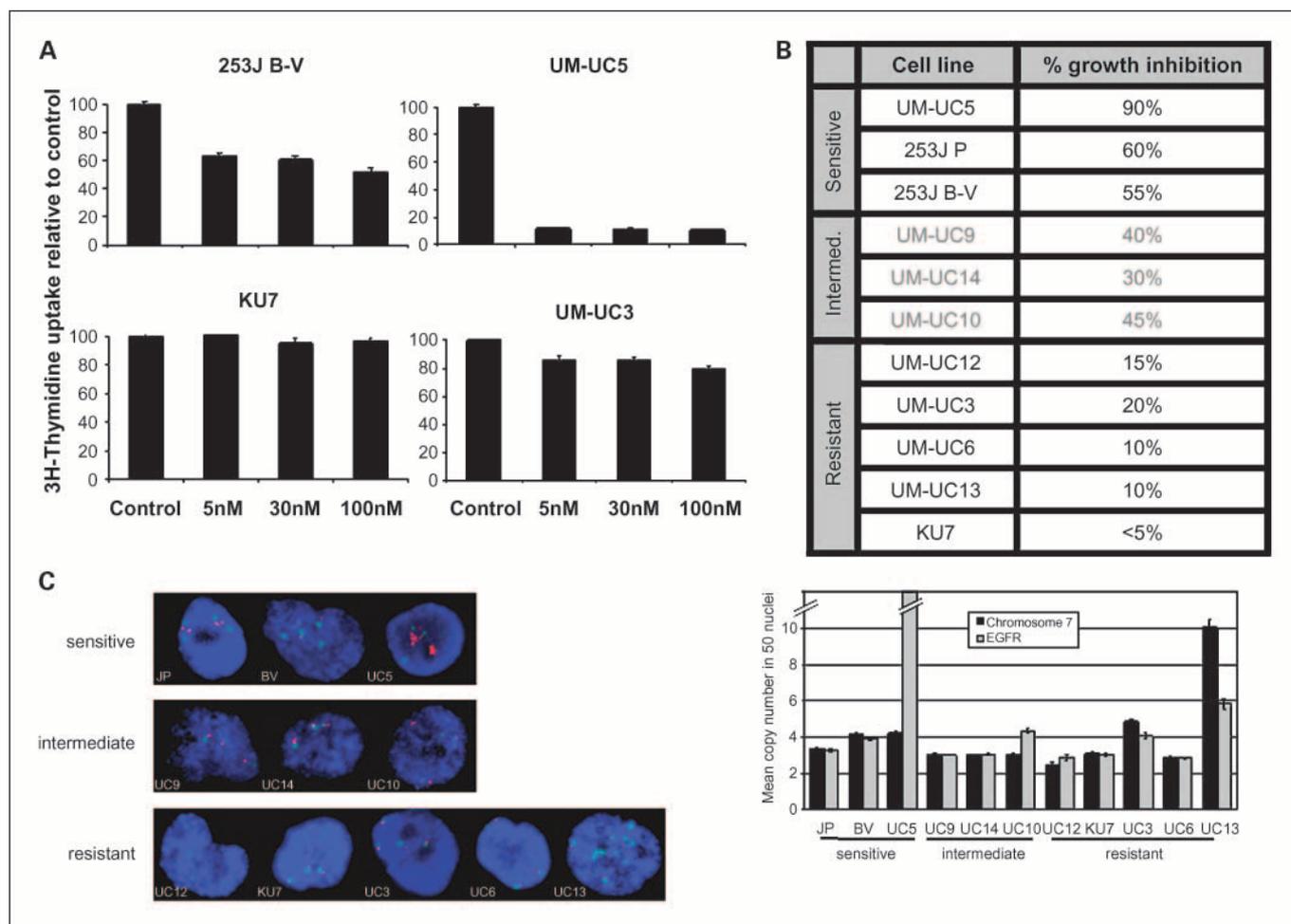
## Results

**In vitro response to EGFR inhibition by cetuximab.** We tested the antiproliferative effect of cetuximab on a panel of urothelial carcinoma cell lines by [<sup>3</sup>H]thymidine incorporation (Fig. 1). Cells were incubated with increasing concentrations of the antibody (5-100 nmol/L). Examples of [<sup>3</sup>H]thymidine incorporation for two sensitive and two resistant cell lines are shown in Fig. 1A. Sensitivity to cetuximab was defined as >50% inhibition of cell growth at a concentration of 100 nmol/L, and resistance to cetuximab was defined as <25% inhibition of cell growth at a concentration of 100 nmol/L. Cell lines with 25% to 50% inhibition were designated intermediate in response. Of the 11 cell lines tested (Fig. 1B), three were sensitive (253J P, 253J B-V, and UM-UC5), three were intermediate (UM-UC9, UM-UC10, and UM-UC14), and five were resistant (UM-UC-3, UM-UC-6, UM-UC12, UM-UC13, and KU-7). We have previously reported similar responsiveness of some of these cell lines to gefitinib *in vitro* and *in vivo* (2).

**EGFR status and ligand secretion in a panel of urothelial carcinoma cell lines.** The EGFR expression profile of our cell lines has previously been published (2) and is reproduced in Table 1. The expression level of EGFR alone did not predict the response of the urothelial carcinoma cell lines to growth inhibition induced by cetuximab. All cell lines expressed EGFR. Although two of the most sensitive cell lines had high expression (UM-UC5 and 253J B-V), so too did two of the highly resistant cell lines (UM-UC3 and UM-UC6). Constitutive phosphorylation of EGFR was similarly seen most prominently in one sensitive (UM-UC5) and one resistant (UM-UC6) cell line and, therefore, did not appear useful for prediction of response (Table 1).

EGFR gene amplification has been described as a marker of sensitivity to EGFR tyrosine kinase inhibition. We analyzed the panel of 11 cell lines for EGFR copy number by fluorescence *in situ* hybridization (Fig. 1C). UM-UC5, the cell line that was most sensitive to cetuximab, was found to have true amplification of EGFR. The remaining cell lines showed balanced polysomy, except for UM-UC13, which showed an excess of the centromere of chromosome 7 compared with the EGFR gene. EGFR gene amplification in urothelial carcinoma, therefore, may not be frequent enough to be used efficiently as a predictive marker, but it does warrant further study in a cohort of bladder cancer patients to define its true prevalence.

Beyond receptor status, ligand secretion could predict responsiveness to EGFR inhibition: cells that produce large quantities of EGFR ligand could be dependent on autocrine stimulation. The expression of ligands for growth factor receptors other than EGFR could implicate these cells in similar autocrine stimulation. We determined transforming growth factor- $\alpha$ , IGF-1, PDGF-AA, PDGF-AB, and PDGF-BB by ELISA and PDGF-CC and HB-EGF by Western blot analysis (data not shown). There was no apparent correlation between any of the ligands and the antiproliferative responsiveness of the cell lines to cetuximab. Some of the resistant cell lines, however, secreted



**Fig. 1.** Sensitivity of urothelial carcinoma cell lines to cetuximab and its correlation with EGFR expression. **A**, the antiproliferative effect of cetuximab for two sensitive cell lines (UM-UC5 and 253J B-V) and two resistant cell lines (KU7 and UM-UC3) as measured by [<sup>3</sup>H]thymidine incorporation. The cells were serum starved for 24 h (2% fetal bovine serum) before treatment with increasing doses of cetuximab for an additional 24 h, a summary of the percentage of cetuximab-induced (100 nmol/L) growth inhibition of a panel of 11 urothelial carcinoma cell lines as measured by [<sup>3</sup>H]thymidine uptake. The cell lines are stratified by response to cetuximab into sensitive (>50% growth inhibition), intermediate (25-50% inhibition), and resistant (<25% growth inhibition). **C**, one sample nucleus for each cell line is shown after fluorescent *in situ* hybridization with a dual probe for EGFR (red) and the centromere of chromosome 7 (green). EGFR amplification was observed in UM-UC5 (indicated by clustering of red fluorescence). The other cell lines had balanced polysomy, except UM-UC13, which had excessive copies of the centromere compared with the EGFR gene.

one or more ligands at a higher concentration than most of the other cell lines. KU7 expressed high levels of PDGF-AA, PDGF-AB, and PDGF-BB, UM-UC12 expressed high levels of HB-EGF, and UM-UC13 expressed high levels of TGF- $\alpha$  and PDGF-CC.

**Expression profile of additional growth factor receptors.** One hypothesis to explain cetuximab resistance is that resistant cell lines are dependent on a dominant growth factor receptor other than EGFR for growth stimulation. We characterized the expression profiles of EGFR, HER2, HER3, HER4, IGF-1R, PDGFR- $\alpha$ , PDGFR- $\beta$ , c-MET, and FGFR3 in our panel of urothelial carcinoma cells by Western blot analysis and tabulated the results in Table 1. There was a clear trend of increased PDGFR- $\beta$  expression in the intermediate and resistant cell lines. There was loss of full-length HER4 (p180) and increased expression of cleaved HER4 (p80) in the resistant cell lines. FGFR3 expression varied little throughout the cell lines, but mutational analysis of exons 7 and 10 revealed mutations in UM-UC9 and UM-UC14 (both exon 7, S249C). The

sensitivity of both of these cell lines to cetuximab can be categorized as intermediate. A statistical analysis of expression variables was done using Cohen's  $\kappa$  statistic to estimate the agreement between sensitivity and expression. The data suggested that expression of intact HER-4 ( $\kappa$ , 1.00;  $P$  = 0.008) and loss of PDGFR- $\beta$  ( $\kappa$ , 0.57;  $P$  = 0.167) was associated with sensitivity to cetuximab therapy. We concluded that these two growth factor receptors (PDGFR- $\beta$  and HER4) are markers of response to cetuximab therapy and concurrently represent potential alternate therapeutic targets.

**Loss of E-cadherin correlates with resistance to cetuximab.** We previously determined that E-cadherin expression is important in bladder cancer aggressiveness and in patient outcome (13). Here, we screened the panel of urothelial cell lines for expression of E-cadherin and markers of mesenchymal differentiation by Western blot analysis (Fig. 2A) and gene expression analysis (Fig. 2B). By Western blot, all of the sensitive and intermediate cell lines expressed E-cadherin, whereas four of five resistant lines did not. An analysis using Cohen's  $\kappa$  statistic

**Table 1.** Expression of alternate growth factor receptors by Western blot analysis

	EGFR	pEGFR	HER2	HER3	HER4 p180	HER4 p80	IGF-1R	PDGFR- $\alpha$	PDGFR- $\beta$	c-MET	FGFR3
JP	+	+	+	+	+	-	+	++	-	+	NT
BV	++	+	++	+	+	-	+	++	-	+	++
UC5	+++	+++	++	-	+	-	++	++	-	++	++
UC9	+	+	++	-	+	-	NT	++	+	++	+*
UC14	+	+	+	-	+	-	+	++	++	++	+*
KU7	+	+	+++	++	+	+	++	+++	+++	+	++
UC3	++	+	++	++	-	+	+	++	+++	+	++
UC6	++	++	++	+	-	+	++	++	++	++	++
UC13	+	+	+	+	-	+	+	++	++	+++	++

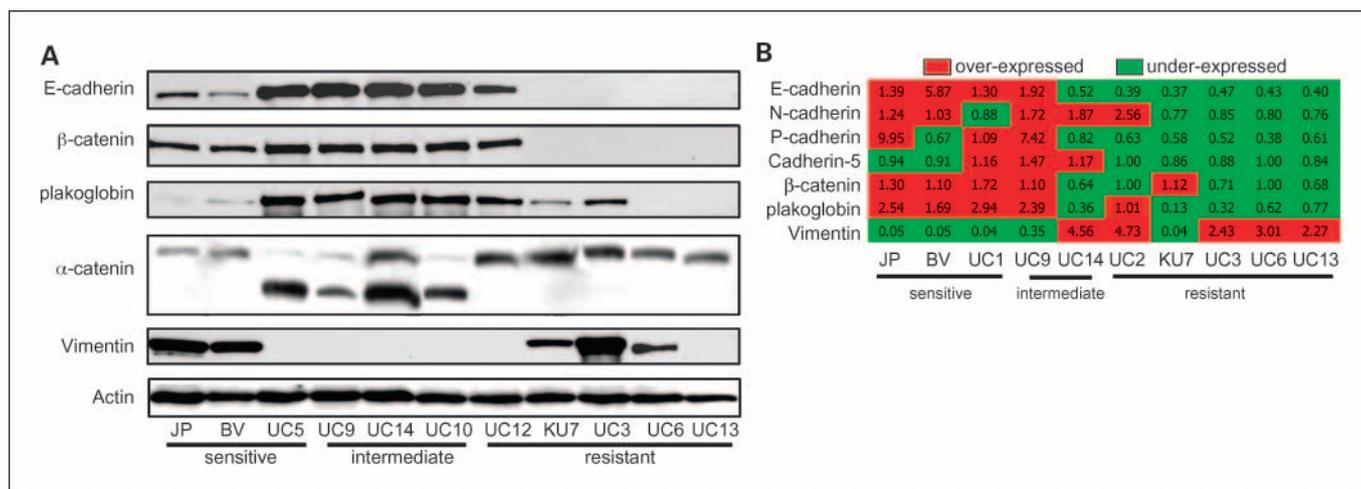
NOTE: Protein expression is rated as follows: -, none; +, weak; ++, moderate; +++, strong. Abbreviation: NT, not tested; HER4 p180, intact receptor with band at 180 kDa; HER4 p80, cleaved receptor with band at 80 kDa.

\*FGFR3 mutations present (S249C).

(24) to estimate the agreement between sensitivity and the loss of E-cadherin showed a significant association ( $\kappa$ , 0.81;  $P = 0.015$ ). The cell lines lacking E-cadherin also lacked  $\beta$ -catenin expression in the total cell lysates. Plakoglobin ( $\gamma$ -catenin) was expressed weakly in two sensitive lines and was absent in two highly resistant lines ( $\kappa$ , 0.42;  $P = 0.182$ ; Fig. 2A). The resistant cell lines and both 253J P and 253J B-V lacked a second lower band (90 kDa) on Western analysis for  $\alpha$ -catenin, in addition to the dominant band of 102 kDa. Three of five resistant cell lines expressed vimentin, but the sensitive lines 253J P and 253J B-V were also strongly vimentin-positive. There was no pattern of correlation between cetuximab response and expression of other adhesion molecules, including M-cadherin, R-cadherin, P-cadherin, cadherin-5, and desmoglein (data not shown). These markers were present in most or all cell lines. N-cadherin, often a marker of mesenchymal differentiation, was seen only in 253J P, UM-UC5, UM-UC12, and UM-UC14.

Gene expression analysis (Fig. 2B) confirmed the finding that epithelial markers are decreased and vimentin is increased in cell lines resistant to cetuximab. In this analysis, N-cadherin again did not seem to act as a marker of mesenchymal differentiation. There were some clear discrepancies between these data and the Western blot data depicted in Fig. 2A. We attributed these to the difference between mRNA and protein expression, as well as to the phenotypic drift that is seen in these cells with prolonged culture and at different centers. The pattern, however, was the same for both sets of data. These cumulative data indicate that E-cadherin expression is an important predictor of cetuximab response.

**Metalloproteases and their endogenous inhibitors.** In the context of loss of E-cadherin, we previously reported increased predictive power by calculating the ratio of MMP9 to E-cadherin expression on immunohistochemistry (13). To investigate this relationship in the panel of cell lines, we analyzed MMP2 and MMP9 expression in the whole-cell lysates by Western blot

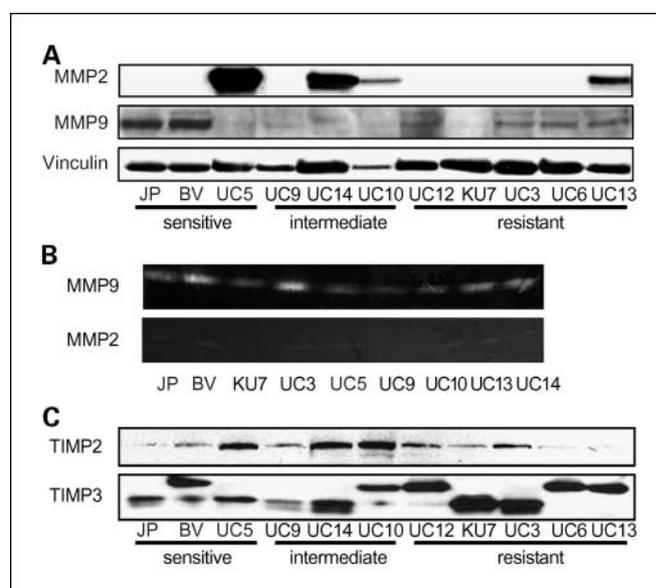


**Fig. 2.** Expression of epithelial and mesenchymal markers in urothelial carcinoma cell lines. *A*, expression of E-cadherin, various catenins, and vimentin. Whole-cell lysates from 11 cell lines growing at 70% to 80% were resolved by 10% SDS-PAGE and analyzed by Western blot analysis using a specific antibody for each marker and visualized by enhanced chemiluminescence. The cell lines are grouped by antiproliferative response to cetuximab. *B*, gene expression analysis by Affymetrix HG-U133A GeneChip of similar markers in 10 urothelial cell lines. UM-UC1 and UM-UC2 are included here, but not elsewhere. UM-UC1 expressed E-cadherin, but not vimentin, by Western blot analysis and was sensitive to cetuximab inhibition, whereas UM-UC2 expressed vimentin, but not E-cadherin, and was resistant to cetuximab (data not shown). Expression is arbitrarily dichotomized in overexpression and underexpression based on the relative value compared with the median of a panel of 41 urothelial carcinoma cell lines.

analysis (Fig. 3A). MMP2 expression was detected in only four cell lines with variable cetuximab sensitivity. As with vimentin, there was increased MMP9 expression in the 253J P and 253J B-V cell lines, but also most prominently in four resistant cell lines (UM-UC3, UM-UC6, UM-UC12, and UM-UC13). This difference was not apparent on zymography (Fig. 3B), where all the cell lines, except KU7 and UM-UC9, showed MMP2 activity and all showed similar levels of MMP9 activity. A MMP9/E-cadherin ratio could not be calculated because there was no detectable E-cadherin expression in the resistant cell lines. To test for a possible interaction with endogenous inhibitors of these metalloproteases, we determined the expression levels of TIMP2 and TIMP3 in total cell lysates by Western blot (Fig. 3C). The majority of sensitive cell lines lacked the glycosylated TIMP3 (upper band on Western blot), but otherwise, no clear correlation to cetuximab response was observed.

**Phenotypic correlates of epithelial markers, mesenchymal markers, and cetuximab sensitivity.** We were interested in establishing phenotypic correlates of the epithelial and mesenchymal marker expression in the panel of urothelial carcinoma cell lines and relating this to cetuximab sensitivity (Fig. 4). Cell morphology (Fig. 4A) correlated to the extent that the E-cadherin-expressing cell lines grew in well-organized and cohesive islands that coalesced as the cells became more confluent. Cell lines lacking E-cadherin grew as single spindle-shaped cells. One cell line in each group did not conform to this pattern; 253J B-V was spindle-shaped, and KU7 formed cohesive epithelial units. The sensitive and intermediately responsive cell lines, therefore, maintained a more epithelial morphology, whereas the resistant cell lines were mesenchymal in appearance. This morphology was congruent with the invasive potential of the cell lines in a Boyden chamber invasion assay (Fig. 4C). The cell lines with mesenchymal morphology were the most invasive cell lines (UM-UC3, UM-UC6, and UM-UC13), and these cell lines were resistant to the antiproliferative effect of cetuximab. These cell lines were also the most tumorigenic in the intramural orthotopic bladder cancer model (Fig. 4D). The cetuximab-sensitive cell line 253J B-V was highly tumorigenic, which is consistent with its selection for tumorigenicity by 5× recycling through the murine orthotopic model. The parental line 253J P was poorly tumorigenic, as was the most sensitive cell line (UM-UC5) and one of the intermediately responsive cell lines (UM-UC10). UM-UC6, UM-UC12, and UM-UC14 were not tested for tumorigenicity. In summary, these data show a clear correlation between transition-to-mesenchymal phenotype, loss of E-cadherin expression, and resistance to cetuximab treatment.

**Silencing of E-cadherin imparts resistance to cetuximab inhibition.** To investigate whether the association between E-cadherin expression and cetuximab response in the cell lines was causally related, we silenced E-cadherin expression with transient siRNA and analyzed the effect of cetuximab on [<sup>3</sup>H]thymidine incorporation (Fig. 5). Both 253J B-V ( $P < 0.001$ ) and UM-UC14 ( $P < 0.001$ ) showed a dose-response to cetuximab inhibition when transfected with nontargeting siRNA (statistical analysis according to methods of Cuzick; ref. 25). With silencing of E-cadherin the dose-response was lost in UM-UC14 ( $P = 0.372$ ), but not 253J B-V ( $P = 0.003$ ). However, a linear regression model revealed that both cell lines were significantly less responsive to cetuximab with silencing of



**Fig. 3.** Expression of metalloproteases and their inhibitors. *A*, the expression of MMP2 and MMP9 in the total cell lysate from the panel of urothelial cell lines was determined by Western blot analysis. *B*, the enzymatic activity of MMP2 and MMP9 was measured by zymography. *C*, the expression of endogenous inhibitors TIMP2 and TIMP3 in the total cell lysates was analyzed by Western blot. The cell lines are grouped in *A* and *C* by response to cetuximab.

E-cadherin ( $P < 0.001$  for each) than without. Successful silencing of E-cadherin was shown by Western blot analysis. The expression level of EGFR remained unchanged (data not shown). These data establish a functional link between E-cadherin expression and the antiproliferative effect of cetuximab.

## Discussion

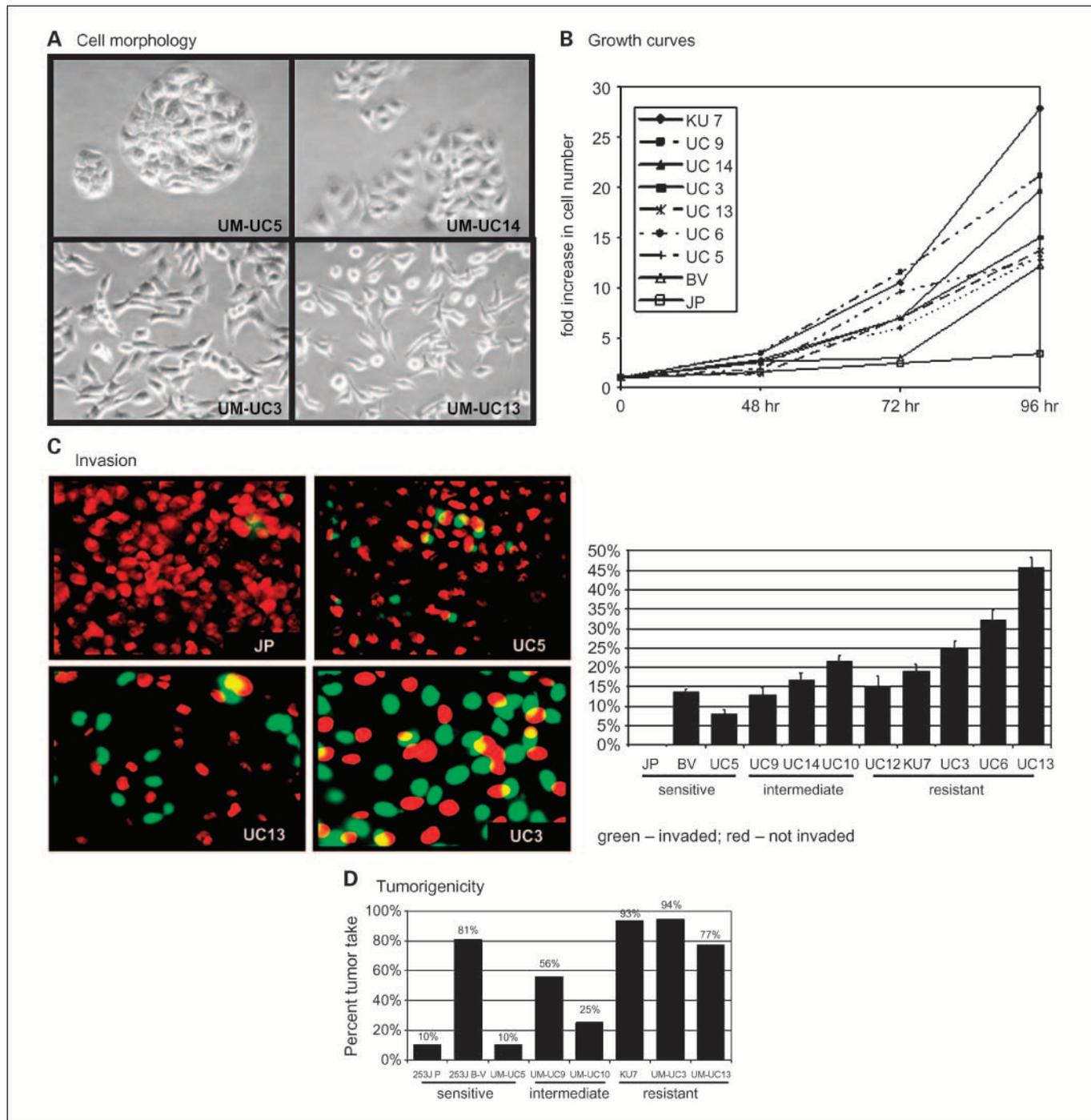
We have shown in a panel of urothelial carcinoma cell lines that loss of E-cadherin expression is a marker of poor response to the antiproliferative effect of EGFR targeting by the monoclonal antibody cetuximab. Loss of other epithelial markers and gain of the mesenchymal marker vimentin showed a similar but looser association. We previously showed a similar relationship with gefitinib (17), but here, we were able to establish a causal association by silencing E-cadherin expression in two sensitive urothelial carcinoma cells and thereby imparting resistance to cetuximab.

Expression of E-cadherin seems to be prerequisite for the antiproliferative response of cetuximab in urothelial carcinoma. This is consistent with reports of a functional link between E-cadherin and EGFR in non-small cell lung cancer (11, 26). E-cadherin is not only an important adhesion molecule, but also a regulator of intracellular signaling cascades, including the Akt and p42/44MAPK pathways (27, 28). Incorporation of E-cadherin into adhesion complexes has been shown to activate EGFR and affect the ability of EGFR to respond to ligand (11). E-cadherin and EGFR are found together in complexes, and their association is dependent on the extracellular domain of E-cadherin (11) and potentially also  $\beta$ -catenin (29). We can hypothesize based on the findings in urothelial carcinoma cell lines that the presence of these complexes and the activation of EGFR by E-cadherin is a prerequisite to the antiproliferative effect of EGFR inhibition. We have seen the

same correlation with both the antibody inhibitor (cetuximab) and the small molecule inhibitor (gefitinib), so that the effect of E-cadherin is not likely related to drug-receptor binding. The implication is that cells lacking E-cadherin have adapted to

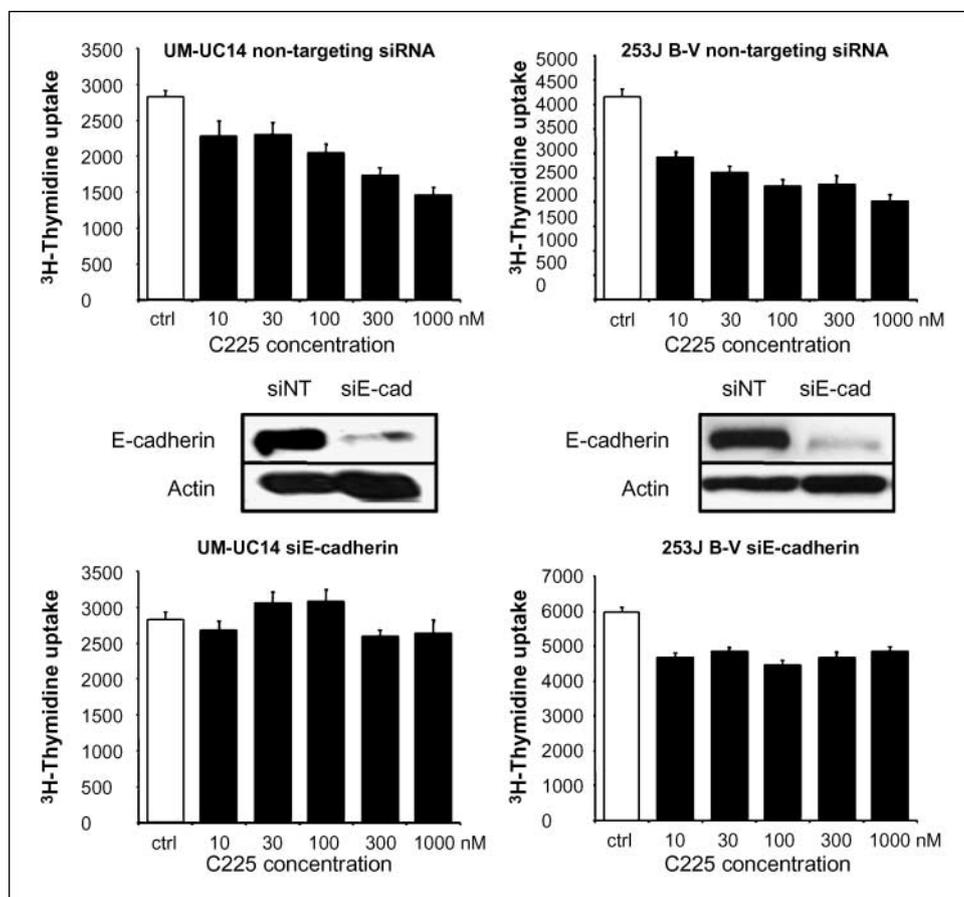
develop other activating mechanisms that bypass EGFR signaling, thereby making them resistant to EGFR inhibition.

A further relevant correlate was observed between HER4 and E-cadherin expression; resistant cell lines lacking E-cadherin



**Fig. 4.** Phenotypic correlates of resistance to cetuximab and loss of epithelial marker expression. *A*, sample images of two sensitive (UM-UC5 and UM-UC14) and two resistant (UM-UC3 and UM-UC13) cell lines. The cells were grown in the usual culture conditions on 100-mm plates, and the images were acquired at 200 $\times$  magnification using phase contrast. *B*, relative growth rates of each cell line. The cells were plated in triplicate at 25,000 cells per well in a six-well plate and counted after release with 0.25% trypsin 48, 72, and 96 h later. The number of cells is represented as the fold increase over the number plated. *C*, more invasive cell lines are resistant to cetuximab. In a Boyden chamber assay, invaded and noninvaded cells were stained with propidium iodide and analyzed by fluorescent microscopy at 400 $\times$  magnification. Images were acquired in different planes of focus corresponding to the top and bottom of the Matrigel insert. Cell nuclei were arbitrarily labeled red in the plane of noninvaded cells and green in the plane of invaded cells. The images were overlaid, as shown in these sample images, and the ratio of invaded to total (invaded plus noninvaded) cells was calculated. These ratios are plotted in the bar diagram. *D*, tumorigenicity correlates loosely and inversely with cetuximab sensitivity. For each cell line, mice underwent orthotopic intramural injection of  $1 \times 10^6$  cells in the bladder ( $2 \times 10^6$  for UM-UC5 and 253J P). Gross tumor take was assessed by necropsy at 4 wk and expressed as a percentage of total mice injected.

**Fig. 5.** Silencing of E-cadherin by siRNA induces resistance to cetuximab. One sensitive (253J B-V) and one intermediate (UM-UC14) cell line with baseline E-cadherin expression were transfected transiently with siRNA targeted to E-cadherin or with nontargeting control siRNA. A high degree of E-cadherin silencing was shown by Western blot at 60 h posttransfection. The silenced cells were assayed for response to cetuximab inhibition by [<sup>3</sup>H]thymidine incorporation.



expression were noted to express cleaved HER4, whereas sensitive cell lines maintaining E-cadherin expression were found to express intact HER4, but not cleaved HER4. This implicates HER4 cleavage as potentially being involved in E-cadherin expression and response to EGFR inhibition. These mechanisms remain to be elucidated.

Expression of PDGFR- $\beta$  was also identified as a marker of resistance to cetuximab therapy. We previously showed that in response to gefitinib, downstream signaling through GSK- $\beta$  predicts response to treatment (2), and in unpublished studies, we have shown the importance of heterodimerization between EGFR and PDGFR- $\beta$ . These findings are particularly relevant because they underline the heterogeneity of growth factor receptor expression between tumors and suggest that PDGFR- $\beta$  may be suitable as an alternative target for treatment. FGFR3 also seems to deserve investigation as an additional target of directed therapy (30–32).

The most important predictor of response to EGFR inhibition in non-small cell lung cancer has been the presence of activating mutations in the tyrosine kinase domain of the receptor (33, 34). Such mutations, however, have not been identified in bladder cancer (35). In this study, we also showed that other potential markers related to EGFR, including expression levels, constitutive phosphorylation, and ligand expression, were not correlated to response. EGFR amplification was identified in one cell line that was highly sensitive to cetuximab (UM-UC5), which suggests that patient specimens should be studied to assess the frequency of amplification in bladder tumors (10, 36–39).

We have identified several markers of sensitivity to EGFR-targeted therapy in a preclinical model. The translational application of these findings will be to validate the markers in patient tumors. We are conducting a neoadjuvant trial, in which an EGFR-targeting agent is given to patients undergoing radical cystectomy for invasive urothelial carcinoma. We will correlate marker findings at initial biopsy with biological markers of response, including Ki-67 expression, p27 up-regulation, GSK- $\beta$  phosphorylation, and cyclin D1 degradation. We expect, in particular, that patients with cleaved HER4 and loss of E-cadherin expression will respond poorly to treatment.

## Conclusions

We believe that the low response rate of EGFR-targeted therapy does not render this treatment strategy obsolete, but rather that individual patient profiling is required to ensure that an appropriate targeted agent or combination of inhibitors is given in each case. Profiling a panel of bladder cancer cell lines has yielded several potential markers of response to EGFR-targeted therapy, including expression levels of HER4, PDGFR- $\beta$ ,  $\beta$ -catenin, and E-cadherin, and we intend to validate these markers in a neoadjuvant trial with EGFR-targeted therapy. E-cadherin expression seems to be particularly important in modulation of EGFR response. Exploring mechanistic relationships between the markers and EGFR function will improve our understanding of resistance to EGFR-targeted therapy and lead us to additional potential targets to evaluate for use as novel treatment strategies.

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Peter C. Black, Gordon A. Brown, Teruo Inamoto, et al.

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