

Evaluation of the Therapeutic Potential of the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor Gefitinib in Preclinical Models of Bladder Cancer

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ABSTRACT

The epidermal growth factor receptor (EGFR) is associated with aggressive phenotypes and is an independent predictor of stage progression and mortality in bladder cancer. Gefitinib ('Iressa,' ZD1839) is an orally active EGFR-tyrosine kinase inhibitor. The objective of this study was to evaluate the *in vitro* and *in vivo* effects of gefitinib in the EGFR-expressing human bladder cancer cell lines 253J B-V, RT-112, and T24. EGFR expression was 3- and 2-fold higher in 253J B-V and RT-112, respectively, compared with T24 cells. Ten μM gefitinib inhibited EGFR, p42/44 extracellular signal-regulated kinase (ERK), and Akt/protein kinase B phosphorylation in all three of the cell lines. Inhibition of ERK by gefitinib was significantly greater in 253J B-V compared with RT-112 and T24 cells (9:2:1 in 253J B-V:RT-112:T24), whereas inhibition of Akt phosphorylation was less in 253J B-V compared with RT-112 and T24 cells (1:9:30 in 253J B-V:RT-112:T24). When cultured in serum-free medium supplemented with epidermal growth factor, 10 μM gefitinib inhibited DNA synthesis in T24 and RT-112 cells, whereas 1 μM gefitinib was sufficient to inhibit DNA synthesis in 253J B-V cells. Similarly, in the presence of serum, 10 μM gefitinib induced a significant reduction in S-phase and viable cell number in T24 and RT-112 cells, whereas 1–10 μM gefitinib caused a dose-dependent effect on these phenotypes in 253J B-V cells. Gefitinib significantly enhanced the ability of ionizing radiation to reduce colony

forming ability in 253J B-V and RT-112 cells. In nude mice, a daily oral dose of 150 mg/kg gefitinib induced regression of tumors produced by 253J B-V cells growing at s.c. sites and suppression of tumors produced by these cells at orthotopic sites but had no effect on tumors produced by RT-112 cells growing at s.c. sites. The data indicates that gefitinib has potential therapeutic value, alone or in combination with ionizing radiation, in a subset of EGFR-expressing bladder cancers. However, there is a differential response to gefitinib in these EGFR-expressing bladder cancer cell lines. Although gefitinib can inhibit phosphorylation of EGFR, ERK, and Akt, and inhibit growth of bladder cancer cells *in vitro*, it does not necessarily inhibit growth of bladder cancer cells *in vivo*. It is likely that optimized therapy approaches will require an accurate "molecular" diagnosis allowing effective, selective, tailored therapeutic strategies to be designed.

INTRODUCTION

A high level of expression of the epidermal growth factor receptor (EGFR), a *trans*-membrane protein tyrosine kinase of the erb family (erb-B1), has been found in many epithelial neoplasias including head and neck, breast, colon, lung, prostate, kidney, ovary, brain, pancreas, and bladder (1–7). EGFR expression in bladder cancer correlates with histological grade, tumor stage, and recurrence (8–10). Its presence has been confirmed in multivariate analysis to be an independent predictor of stage progression and mortality (11–13). Expression of EGFR and one of its ligands, transforming growth factor α , is correlated with tumor recurrence in superficial transitional cell carcinoma (TCC) of the bladder (14), and coexpression of transforming growth factor α protein and EGFR protein has been found to be significantly associated with tumor stage (15). Although transforming growth factor α could be detected in both benign and malignant specimens, levels of transforming growth factor α were significantly higher in the latter, and it has been suggested as the likely ligand for EGFR in bladder cancer (16). EGFR signaling involves *ras*-dependent (*e.g.*, Raf-mitogen-activated protein kinase and phosphatidylinositol 3'-kinase-Akt) and *ras*-independent mechanisms (*e.g.*, STAT 3), resulting in pleiotropic effects on bladder cancer cells including induction of proliferation (17), angiogenesis (18), motility (19), invasion (20, 21), and metastasis (11, 22).

Therapeutic strategies that target EGFR are currently being developed. There are two main approaches to EGFR inhibition: (a) monoclonal antibodies directed to the extracellular domain of the receptor and blocking ligand binding, such as cetuximab (Erbix), h-R3(23), ABX-EGF, and EMD-700 (24–26); and (b) small molecule inhibitors directed against the intracellular domain of the EGFR blocking tyrosine kinase activity. These compounds, known as tyrosine kinase inhibitors (TKIs), include

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Note: J. Kelly and D. Neal are currently at the Department of Oncology, University of Cambridge, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom. 'Iressa' is a trademark of the AstraZeneca group of companies.

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EGFR-specific TKIs such as the reversible gefitinib ('Iressa,' ZD1839) and OSI-774 (Tarceva), the irreversible EKB-569 and dual EGFR-HER2 inhibitors such as the reversible GW572016 and the irreversible CI-1003 (26).

The quinazoline-derived molecule gefitinib is an orally active, reversible, EGFR tyrosine kinase inhibitor already licensed in the United States and Japan for stage-IV non-small cell lung cancer. Single-agent gefitinib has been shown to effectively inhibit EGF-induced EGFR phosphorylation in diverse cell lines and to have antitumor activity in several preclinical models of solid tumors *in vivo* (27, 28). Moreover, gefitinib has been shown to potentiate radiotherapy in human colon, ovarian, non-small cell lung, and breast cancer cell lines (29–31), and potentiate the cytotoxic effects of various anticancer drugs in GEO human colorectal cancer xenografts (32).

Because the EGFR is a potentially important therapeutic target in bladder cancer, we have evaluated the effects of gefitinib on a panel of EGFR-expressing human bladder cancer cell lines. We report that single agent gefitinib is able to induce tumor regression in an orthotopic model of bladder cancer and that this drug has potential therapeutic application in bladder cancer in combination with radiotherapy.

MATERIALS AND METHODS

Tumor Cell Lines. T24 is a human differentiated TCC cell line harboring the *H-ras* oncogene (HTB-4; American Type Culture Collection) derived from recurrent bladder tumor. RT112 is a human differentiated TCC cell line obtained from primary urinary bladder tumor. 253J B-V is a metastatic human TCC cell line (kindly donated by Prof. Colin P. N. Dinney, M. D. Anderson Cancer Center, Houston, TX; Ref. 33). Cell lines were cultured as a monolayer in "routine medium"; this was composed 90% RPMI 1640 supplemented with 10% fetal bovine serum and 50 IU penicillin and 50 μ g/ml streptomycin (for T24 and RT-112 cell lines) and 95% Eagle's MEM supplemented with 5% fetal bovine, vitamins, nonessential amino acids, 50 IU penicillin, and 50 μ g/ml streptomycin (for 253J B-V; Ref. 33). Cell lines were grown in a humidified incubator, supplemented with 5% CO₂, at 37°C.

Antibodies. The primary antisera were as follows: anti-phospho-EGFR (Y1173, mouse monoclonal IgG, clone 9H2; Upstate), anti-EGFR (sheep polyclonal IgG; Upstate), anti-phospho-ERK (E-4: mouse monoclonal IgG, clone sc-7383; Santa Cruz Biotechnology Inc.), pan-ERK antibody (Mouse Monoclonal IgG; BD Transduction Lab.), Phospho-Akt antibody (clone Ser473; rabbit polyclonal IgG; Cell Signaling Tech.), Akt antibody (rabbit IgG polyclonal; Cell Signaling Technology), anti-bromodeoxyuridine (BrdUrd; clone Bu20a; Dako-Cytomation), and anti- α -tubulin (clone DN 1A; mouse monoclonal; Sigma). Appropriate horseradish peroxidase or biotin-conjugated secondary antibodies were purchased from Dako-Cytomation Ltd.

Measurement of Viable Cells. Relative numbers of viable cells were measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (34). Cells were plated out in the middle-60 wells of 96-well plates at a density of 1×10^4 cells/well in 250 μ l of routine medium. Gefitinib (1, 5, or 10 μ M) dissolved in vehicle (0.1% DMSO) or DMSO vehicle alone

was then added. Plates were incubated at 37°C. Each assay condition was carried out in sextuplet. After 72 h and 96 h, the medium was removed, replaced with 100 μ l of medium, and 11 μ l of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added to each well. The plate was then incubated at 37°C for 3 h. To solubilize the purple formazan product, 100 μ l DMSO was added to each well. The plates were read immediately on a Dynex plate reader at 420 nm.

DNA Synthesis Assays. DNA synthesis was measured by incorporation of [³H]thymidine into DNA. Cells were plated in the middle-60 wells of 96-well plates at a density of 5×10^3 cells/well in serum-free medium containing 250 μ g/ml BSA supplemented with 100 ng/ml recombinant human EGF and drug dissolved in vehicle (0.1% DMSO), or 100 ng/ml recombinant human (rh)EGF and vehicle alone. Each assay was carried out in sextuplet. After 24 h incubation with test drug/control vehicle at 37°C, cells were harvested onto glass-fiber filter mats using 12% trichloroacetic acid, and the amount of incorporated [³H]thymidine was measured by β -scintillation counting for 1 min in a scintillation counter (Wallac 1450 Microbeta Plus).

Flow Cytometry Analysis of Cell Cycle. Cells were plated, in triplicate, into 12-well plates (Corning) at a density of 5×10^4 cells/well. After being serum-starved for 24 h, cells were cultured in serum-containing medium, and either gefitinib (1 μ M or 10 μ M) or vehicle control (0.1% DMSO) was added. Medium was changed daily and samples analyzed at 24, 48, and 72 h. For each time point, wells were washed twice with PBS, harvested with trypsin-EDTA, and centrifuged at 1000 rpm for 5 min. Pellets were resuspended into 500 μ l PBS. Propidium iodide (125 μ l; 0.25 mg/ml in 5% Triton-Isoton II) was added together with 50 μ l of 1 mg/ml RNAase type I-A (Sigma). Samples were incubated at 4°C for 1 min and analyzed by flow cytometer (FACScan Becton Dickinson) and CellquestPro Software.

Western Blotting. Relative EGFR expression and the effects of gefitinib on EGF-induced EGFR phosphorylation and components of downstream signal transduction pathways (Akt and mitogen-activated protein kinase) were analyzed by Western blotting. Near-confluent cells were incubated in serum-free medium supplemented with 250 μ g/ml BSA for 24 h. Cells were then stimulated with 100 ng/ml rhEGF for different periods of time before preparation of cell extracts. Cells were either incubated with 10 μ M of gefitinib dissolved in vehicle (0.1% DMSO) or vehicle alone for 10 min before preparation of cell extracts. At various times after stimulation with EGF (5, 10, 30, and 60 min) cells were washed twice with PBS and scraped into protein lysis buffer [4% SDS, 0.125 M Tris-HCl and 10% sucrose (pH 6.8)]. Cell extracts were briefly sonicated (Soniprep 150; MSE), boiled in 2% β mercaptoethanol, and subjected to 6–10% SDS-PAGE before electroblotting onto Hybond-C extra membranes (Amersham). After blocking and sequential incubation with the relevant primary and secondary antisera, signal was visualized using an ECL-Plus revelation kit (Amersham) according to the manufacturer's instructions. Autoradiography signals for each product were scanned as bitmaps and densitometry carried out using Scion Image analysis software (Version β 4.0.2; Scion Corporation).

Combination Therapy with Ionizing Radiation. Effects of gefitinib in combination with ionizing radiation were assessed by colony-forming assays. Cells cultured for 48 h in serum-containing medium in the presence of gefitinib (1 μ M or 10 μ M) or

0.1% DMSO vehicle control were irradiated with 0, 2, 4, 6, and 8 Gy. After irradiation, cells were plated in triplicate at a low concentration (1×10^3 cells/plate) in $100 \text{ mm} \times 20 \text{ mm}$ tissue culture cell plates (Corning Incorporated) and cultured in serum-containing medium for 14 days. At this point, plates were washed twice with PBS, fixed (25% acetic acid in methanol) for 10 min, and stained with methylene blue (Sigma) for 30 min. Plates were then washed in running water and allowed to dry. The number of colonies was counted by two investigators blinded to treatment.

Tumorigenicity Studies. Six-week-old male athymic CD1 nude mice were purchased from Harlan United Kingdom Ltd. and maintained in a laminar air-flow unit under aseptic conditions. Experiments were reviewed and approved by the Local Animal Welfare Committee, performed under a Home Office License, and following the guidelines of the United Kingdom Coordinating Committee on Cancer Research. Mice were fed with a commercial pelleted diet (R & M No. 3; SDS Ltd.) and tap water *ad libitum*. Cells were given fresh medium 24 h before harvesting with trypsin-EDTA. Harvested cells were washed twice in PBS and resuspended in PBS at a concentration of 2×10^7 cells/ml. For s.c. injections, 4×10^6 cells (0.2 ml of suspension) were injected into the flank. Orthotopic implantation of 1×10^6 cells in a volume of $50 \mu\text{l}$ into the bladder wall was carried out essentially as described previously (33).

Mice were monitored on a daily basis for adverse effects and signs of tumor development. s.c. tumor nodules were measured twice weekly with calipers, and daily treatment p.o. with gefitinib (75 mg/kg/day or 150 mg/kg/day dissolved in 1% Tween 80) or vehicle (1% Tween 80) was commenced when the nodules measured at least $5 \times 5 \text{ mm}$. Orthotopic bladder tumors were monitored by the presence of hematuria and palpation. Hematuria was evident in the cages after 7 days when treatment with gefitinib or vehicle was commenced. Tumorigenicity studies in mice with s.c. tumors were terminated when the tumor nodules reached $10 \times 10 \text{ mm}$ or $> 15 \text{ mm}$ in one dimension. Mice with orthotopic tumors were culled when they displayed at least one of the following adverse effects associated with tumor development: rapid loss of body weight of 10% maintained for at least 72 h, immobile/lethargic behavior, lack of response to gentle stimuli, tented skin tone, labored respiration, or unconsciousness. Two h before culling, the animals were given a single i.p. injection of 160 mg/kg BrdUrd (Sigma). At postmortem, tumor dimensions and bladder weight were measured, and mice were examined carefully for the presence of metastases. Samples of primary tumors/bladders, lungs, and any other tissues of abnormal appearance were fixed in formalin, processed for histology, and embedded in paraffin wax. Sections were stained with H&E and examined by light microscopy.

Immunocytochemistry for BrdUrd. Sections were rehydrated through a series of graded alcohols and incubated with hydrogen peroxide in methanol to remove endogenous peroxidase activity. Sections were subsequently treated with 1 mg/ml trypsin (Dako-Cytomation) in 0.1% calcium chloride for 30 min at 37°C and then incubated in 95% formamide in 0.15 M trisodium citrate for 45 min at 70°C . After extensive washing in PBS, sections were blocked with 10% normal rabbit serum dissolved in PBS/1% BSA for 15 min. The sections were then incubated with primary anti-serum to BrdUrd (Dako Clone Bu20a; 1:20 dilution) dissolved in PBS/1% BSA overnight. Bound antibody was detected after se-

quential incubation with biotin-conjugated rabbit antimouse immunoglobulins, the streptABC kit (Dako-Cytomation), and Sigma fast diaminobenzidine tablets (Sigma). Sections were counterstained with Meyer's hematoxylin, mounted, and examined by light microscopy. Three fields containing the highest density of positively staining cells were counted at $\times 40$ magnification to obtain the Labeling Index (LI), where $\text{LI} = \text{no. of BrdUrd-positive cells}/\text{total no. of cells} \times 100$.

Cell Death Assay. Apoptotic cells in formalin-fixed, paraffin-embedded sections were identified using the "In Situ Cell Death Detection Kit POD" (Roche), according to the manufacturer's instructions. Three fields containing the highest density of positively staining, apoptotic cells were counted at $\times 40$ magnification to obtain the Apoptotic Index (AI), where $\text{AI} = \text{no. of apoptotic cells}/\text{total no. of cells} \times 100$.

RESULTS

Relative Expression of EGFR and Growth Rates of Human Bladder Cancer Cell Lines. The relative expression of the EGFR protein in three human bladder cancer cell lines was assessed by Western blotting. In three different experiments, EGFR protein was found to be ~ 2 -fold and 3-fold higher in RT-112 and 253J B-V cells compared with T24 cells (Fig. 1A). Whereas the difference in expression between RT-112 and

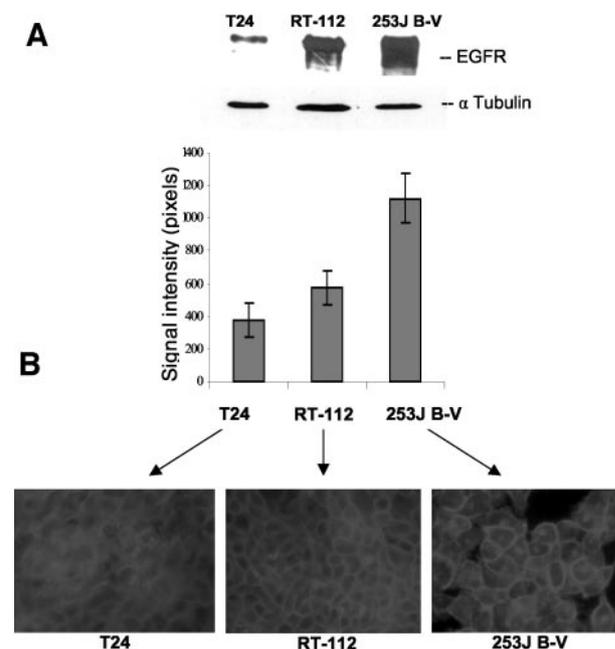


Fig. 1 Relative expression of epidermal growth factor receptor (EGFR) in human bladder cancer cell lines T24, RT-112, and 253J B-V. **A**, Western blot analysis of cell extracts obtained from serum starved cells without recombinant human epidermal growth factor stimulation and hybridized with anti-EGFR receptor antibody. Signal intensity was quantified by Scion Software and results expressed as area of signal in pixels. Relative expression of EGFR was ~ 2 -fold and > 3 -fold higher in RT-112 and 253J B-V cell lines when compared with T24 cells. Expression of EGFR was significantly higher in 253J B-V ($P = 0.0159$, $t = 4.021$, Student's t test). $P = 0.0133$ ANOVA. **B**, immunofluorescence microscopy showing cellular location of expressed EGFR protein in T24, RT-112, and 253J B-V cells; bars, \pm SD.

T24 was not statistically significant, expression of EGFR was significantly higher in 253J B-V ($P < 0.05$; Student's *t* test). In all three of the cell lines EGFR was confirmed by immunofluorescence microscopy to be predominantly located on the plasma membrane (Fig. 1B). When cultured in serum-containing medium, the doubling times of the cell lines were T24 = 17.0 h, RT-112 = 26.4 h, and 253J B-V = 29.5 h.

Gefitinib Reduces Viable Bladder Cancer Cell Number *in Vitro*. After 72 h, gefitinib induced a dose-dependent, cell type-dependent reduction in the number of viable cells. A concentration of 10 μM gefitinib inhibited cell viability in all three of the cell lines. The magnitude of the reduction was greater in 253J B-V cells (48%; $P < 0.0001$) compared with T24 (35%; $P = 0.0001$) and RT-112 (17%; $P = 0.0079$) cells. At a concentration of 1 μM , gefitinib inhibited the number of viable cells in the 253J B-V cell line by 28% ($P = 0.0085$), but there was no significant reduction in T24 (5%; $P = 0.9$) or RT-112 (11%; $P = 0.08$) cells, compared with vehicle control alone (Fig. 2).

Effects of Gefitinib on Cell Cycle. Flow cytometry analysis showed that 10 μM gefitinib caused a significant reduction in the number of cells in S phase in all three of the bladder cancer cell lines. In T24 and RT-112 cells, there were, respectively, 20% ($P = 0.0004$) and 5% ($P = 0.0008$) fewer cells in S phase after 48 h incubation with 10 μM gefitinib (Fig. 3, A and B). In 253J B-V cells, gefitinib caused a dose-dependent significant decrease in the number of cells in S phase, along with a concomitant increase in the number of cells in G₁ (Fig. 3C). A dose of 1 μM gefitinib induced a reduction in the S-phase fraction of the order of 25% after 24 h and 20% ($P < 0.0001$) after 48 h, whereas a dose of 10 μM gefitinib induced reductions of 27% and 33% ($P < 0.0001$) after 24 and 48 h, respectively.

Gefitinib Inhibits DNA Synthesis in Bladder Cancer Cells *in Vitro*. When bladder cancer cells were incubated with serum-free medium supplemented with 100 ng/ml rhEGF, a concentration of 10 μM gefitinib caused a significant reduction in DNA synthesis of 68% ($P < 0.0001$) and 61% ($P < 0.0001$)

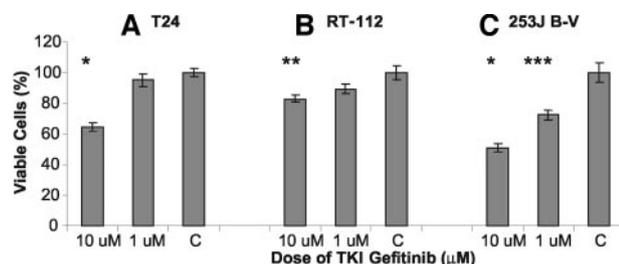


Fig. 2 Effects of the tyrosine kinase inhibitor (TKI) gefitinib on viable cell number in human bladder cancer cell lines T24, RT-112, and 253J B-V. Human bladder cell lines were incubated in serum-containing medium in the presence of 1–10 μM gefitinib or 0.1% DMSO vehicle control alone. The reduction of the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, corresponding to the number of viable cells, was determined after 72 h in culture. Results are expressed as percentage of viable cells relative to DMSO vehicle control. *, significantly fewer viable cells than vehicle control ($P < 0.0001$, Student's *t* test); **, significantly fewer viable cells than vehicle control ($P = 0.008$, Student's *t* test); ***, significantly fewer viable cells than vehicle control ($P = 0.0086$, Student's *t* test); bars, \pm SD.

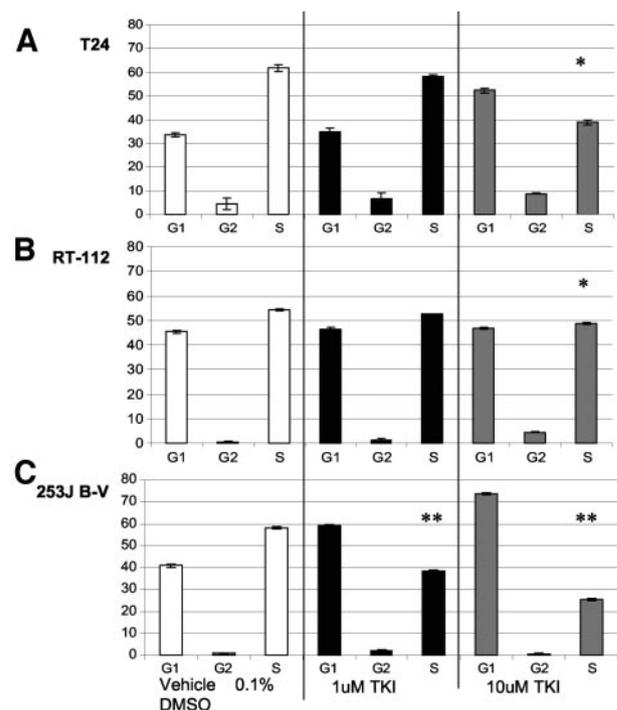


Fig. 3 Effects of tyrosine kinase inhibitor (TKI) gefitinib on the cell cycle in human bladder cancer cell lines T24, RT-112, and 253J B-V. T24, RT-112, and 253J B-V cells were cultured for 48 h in serum-containing medium in the presence of 10–1 μM gefitinib or 0.1% DMSO vehicle control alone. After staining with propidium iodide, the G₁ and S-phase fractions were measured by flow cytometry. *, significant reduction of S-phase fraction ($P < 0.001$, Student's *t* test); **, significant reduction of S-phase fraction ($P < 0.0001$, Student's *t* test); bars, \pm SD.

in T24 and RT-112 cells, respectively (Fig. 4, A and B). Concentrations of 1 μM and 10 μM gefitinib were able to significantly reduce DNA synthesis in 253J B-V cells by 43% ($P = 0.02$) and 45% ($P = 0.01$), respectively (Fig. 4C). Interestingly, when bladder cancer cells were maintained in serum-containing medium, gefitinib either failed to inhibit DNA synthesis or even slightly induced DNA synthesis in all three of the bladder cancer cell lines (data not shown).

Gefitinib Inhibits Phosphorylation of EGFR, ERK, and Akt in Bladder Cancer Cell Lines.

Gefitinib inhibited EGF-induced phosphorylation of EGFR in T24, RT-112, and 253J B-V bladder cancer cells within 5 min of stimulation with rhEGF (Fig. 5). In T24 cells, incubation with 10 μM gefitinib resulted in a reduction of EGFR phosphorylation to only 25% after 5 min and 17% after 10 min, compared with control levels of EGFR phosphorylation in the presence of vehicle alone (Fig. 5A). Similarly, in the RT-112 cell line, incubation with 10 μM gefitinib induced a significant inhibition of EGFR phosphorylation to 20% after 5 min and 30% after 10 min (Fig. 5A). Incubation of 253J B-V cells with 10 μM gefitinib resulted in inhibition of EGFR phosphorylation to 17% after 5 min and 20% after 10 min (Fig. 5A). Inhibition of EGFR phosphorylation by gefitinib persisted for at least 30 min in RT-112 and 253J B-V cells but did not differ from controls in T24 cells at 30 min (data not shown).

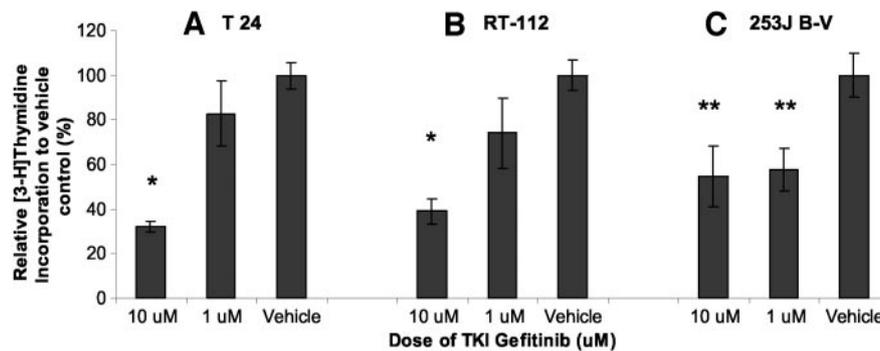


Fig. 4 Effects of the tyrosine kinase inhibitor (TKI) gefitinib on DNA synthesis in human bladder cancer cell lines T24, RT-112, and 253J B-V. Human bladder cancer cell lines were incubated in serum-free medium supplemented with 100 ng/ml recombinant human epidermal growth factor, in the presence of the TKI Gefitinib 10–1 μ M or 0.1% DMSO vehicle control alone. After 24 h, [3 H]thymidine was added, and incorporation of [3 H]thymidine was determined after an additional 24 h by scintillation counting. Results are expressed as percentage of [3 H]thymidine incorporation relative to DMSO vehicle control. *, significant reduction of [3 H]thymidine incorporation compared with vehicle control ($P < 0.0001$, Student's t test); **, significant reduction of [3 H]thymidine incorporation compared with vehicle control ($P < 0.05$, Student's t test); bars, \pm SD.

	T24				RT-112				253J B-V			
RhEGF	+5min		+10min		+5min		+10min		+5min		+10min	
TKI Gefitinib 10 μ M	+	-	+	-	+	-	+	-	+	-	+	-
p-EGFR												
EGFR												
α Tubulin												
(5a) % Reduction	1 : 4	1 : 6	1 : 5	1 : 3	1 : 6	1 : 5	80%	66%	83%	80%		
p-ERK												
ERK												
α Tubulin												
(5b) % Reduction	1 : 2	1 : 1	1 : 1.3	1 : 3	1 : 8	1 : 9	50%	25%	66%	87%	90%	
p-Akt												
Akt												
α Tubulin												
(5c) % Reduction	1 : 11	1 : 40	1 : 2	1 : 13	1 : 1.5	1 : 1.5	92%	97.5%	50%	93%	33%	33%

Fig. 5 Effects of tyrosine kinase inhibitor (TKI) gefitinib on epidermal growth factor-induced phosphorylation of epidermal growth factor receptor (EGFR) and downstream signaling proteins p42/44 extracellular signal-regulated kinase (ERK) and Akt in human bladder cancer cell lines T24, RT-112, and 253J B-V. Serum-starved human bladder cancer cells were treated with either 10 μ M Gefitinib or 0.1% DMSO vehicle control alone, and then restimulated with 100 ng/ml recombinant human epidermal growth factor (rhEGF) for 5 and 10 min. Proteins were separated by SDS-PAGE (6–10%), transferred onto Hybond-C Protein membranes, and probed with phospho- or nonphosphospecific antisera. Signal intensity, relative to α -tubulin, was quantified by Scion Software. The ratio of signal intensity with the phosphospecific antisera is stated, along with the percentage reduction in signal intensity in the presence of gefitinib; bars, \pm SD.

Gefitinib inhibited p42/44 ERK phosphorylation after stimulation with rhEGF in all three of the human bladder cancer cell lines studied (Fig. 5B). Only a modest reduction to 50% of control levels was seen in T24 cells after 5 min. In the RT-112 cell line, gefitinib induced a reduction in ERK activity to \sim 75% and 33% of control levels after 5 and 10 min. A greater magnitude of inhibition of p42/44 ERK phosphorylation was seen in 253J B-V cells, where phosphorylation was reduced to 13% after 5 min and 11% after 10 min (Fig. 5B).

Gefitinib also inhibited Akt phosphorylation after stimulation with rhEGF in all three of the human bladder cancer cell lines (Fig. 5C). In contrast to p42/44 ERK phosphorylation, the magnitude of gefitinib-induced inhibition of Akt phosphorylation was greatest in T24 cells and least in 253J B-V cells. In T24 cells, a reduction of Akt phosphorylation to 9% was observed compared with controls after 5 min and 2% after 10 min. In RT-112 cells, gefitinib inhibited Akt phosphorylation to 50% after 5 min and 8% after 10 min. In 253J B-V cells, gefitinib inhibited Akt phosphorylation to an extent but not by $>50\%$ compared with controls (Fig. 5C).

Gefitinib Enhances the Cytotoxic Effect of Irradiation in Bladder Cancer Cells *in vitro*. The effect of gefitinib in combination with irradiation on 253J B-V and RT-112 cells was assessed by colony-forming assays. One μ M gefitinib caused a significant enhancement of the cytotoxic effect of 2 Gy irradiation on 253J B-V cells (doubling time = 29.5 h), with 40% fewer colonies being produced after 2 Gy irradiation in the presence of gefitinib compared with vehicle alone relative to the number of colonies produced by nonirradiated 253J B-V cells in the presence of gefitinib or vehicle ($P < 0.0004$, Student's t test). No colonies were formed by 253J B-V cells after doses of irradiation of 4 Gy or above in the presence of 1 μ M gefitinib, whereas some colonies were present after 4 Gy and 6 Gy without gefitinib (Fig. 6A). In RT-112 cells (doubling time = 26.4 h), 1 μ M gefitinib did not significantly enhance the cytotoxic effect of 2 Gy irradiation, but a significant reduction in the number of colonies was observed after 4 Gy (14%; $P = 0.05$) and 6 Gy ($P = 0.0008$) in the presence of 1 μ M gefitinib compared with vehicle alone (Fig. 6B). It should be noted that in

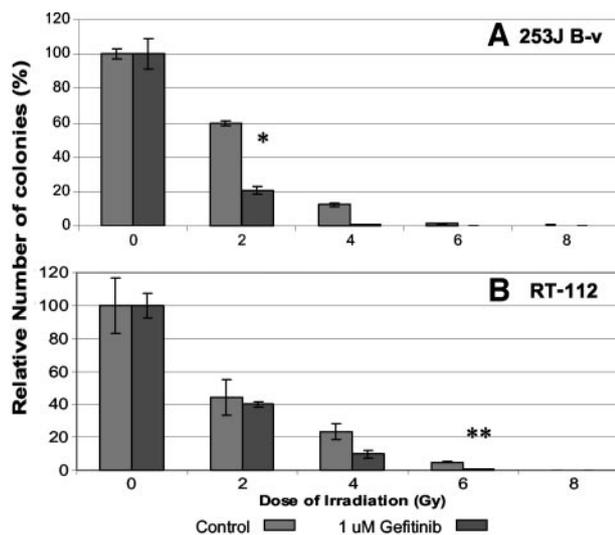


Fig. 6 Effects of tyrosine kinase inhibitor (TKI) gefitinib in combination with ionizing radiation on colony-forming ability in bladder cancer cell lines RT-112 and 253J B-V. Human bladder cancer cells were cultured for 24 h in serum-containing medium in the presence of 1 μ M Gefitinib or 0.1% DMSO vehicle control alone, before they were irradiated with 0, 2, 4, 6, or 8 Gy. After irradiation, cells were seeded at a concentration of 1×10^3 cells/plate in 79-cm² cell culture dishes and cultured for 14 days in serum-containing medium in the presence of either drug or DMSO control. Results are expressed as the percentage of colonies relative to nonirradiated cells in the presence or absence of drug. *, significant reduction in the number of colonies relative to vehicle control ($P = 0.0004$, Student's t test); **, significant reduction in the number of colonies relative to vehicle control ($P = 0.0008$, Student's t test); bars, \pm SD.

the absence of irradiation, 253J B-V cells produced 50% ($P = 0.0021$) fewer colonies in the presence of 1 μ M gefitinib compared with vehicle, but the number of colonies produced by RT-112 cells in the absence of irradiation was not affected ($P > 0.7$) by the presence of 1 μ M gefitinib (data not shown).

Gefitinib Inhibits Growth of 253J B-V Cells But Not RT-112 Cells at s.c. Sites. The effect of single-agent gefitinib on tumor growth *in vivo* was assessed after s.c. injection of 4×10^6 cells into the flank of groups of six CD1 nude mice. RT-112 cells produced rapidly growing tumors, the growth of which was unaffected by the administration of 150 mg/kg/day gefitinib (Fig. 7A). Tumors produced by 253J B-V cells in animals treated with vehicle alone began to grow rapidly at \sim 14 days after commencing the treatment. After 26 days, all of the animals in this group had to be culled due to excessive tumor burden. In contrast, the mean volume of 253J B-V tumors treated with 75 mg/kg/day gefitinib decreased by \sim 50% during the course of the treatment. Tumor regression was even greater in the group of mice treated with 150 mg/kg/day gefitinib (Fig. 7B). T24 cells failed to produce tumors at s.c. sites even after 3 months.

Gefitinib Inhibits Orthotopic Growth of 253J B-V Cells. The effects of gefitinib on tumor growth and metastasis were studied by orthotopic implantation of 253J B-V cells into the bladder wall of CD-1 nude mice. Results are summarized in Table 1. Seven days after orthotopic injection, hematuria was

evident in the cages, and treatment with gefitinib (150 mg/kg/day) or vehicle control (1% Tween 80) was commenced. Animals treated with vehicle alone developed large palpable tumors in 8 of 10 (80%) subjects and were maintained for a mean period of 50 days before it was necessary to cull them due to excessive tumor burden. After 63 days, all of the tumor-bearing animals in this group had been culled due to tumor burden. The mean weight of the bladders in the control group at postmortem was 0.67 ± 0.10 g, and the mean tumor volume was 1127 ± 363.2 mm³. Examination of sections of the bladder stained with H&E revealed invasive, poorly differentiated tumors growing beneath the lamina propria in the muscular wall of the bladder (Fig. 8A). Two animals from the control group contained micrometastases in the lungs (Fig. 8B), but no other metastases were observed. Immunostaining with antiserum to BrdUrd showed that the tumor cells in these micrometastatic lesions were proliferating (Fig. 8C).

In the gefitinib-treated group, very small palpable tumors were only evident in 2 of 10 animals. After 63 days, all of the

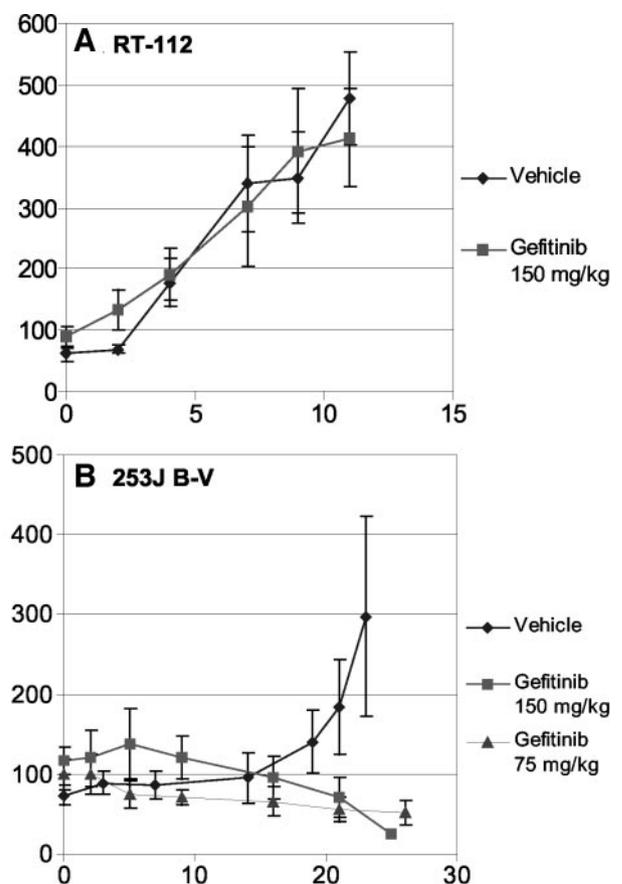


Fig. 7 Effects of tyrosine kinase inhibitor gefitinib on growth of s.c. tumors produced by human bladder cancer cell lines (A) RT-112 and (B) 253J B-V. RT-112 and 253J B-V cells were injected s/c into the flank of 12 CD1 nude mice (4×10^6 cells/animal). Six mice were treated with Gefitinib (75 or 150 mg/kg/day), and 6 mice were treated with vehicle control [1% (v/v) Tween 80]. Treatment was commenced when the tumor size reached 5×5 mm. Tumor size was measured with calipers and the volume determined using the formula $\pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2$; bars, \pm SD.

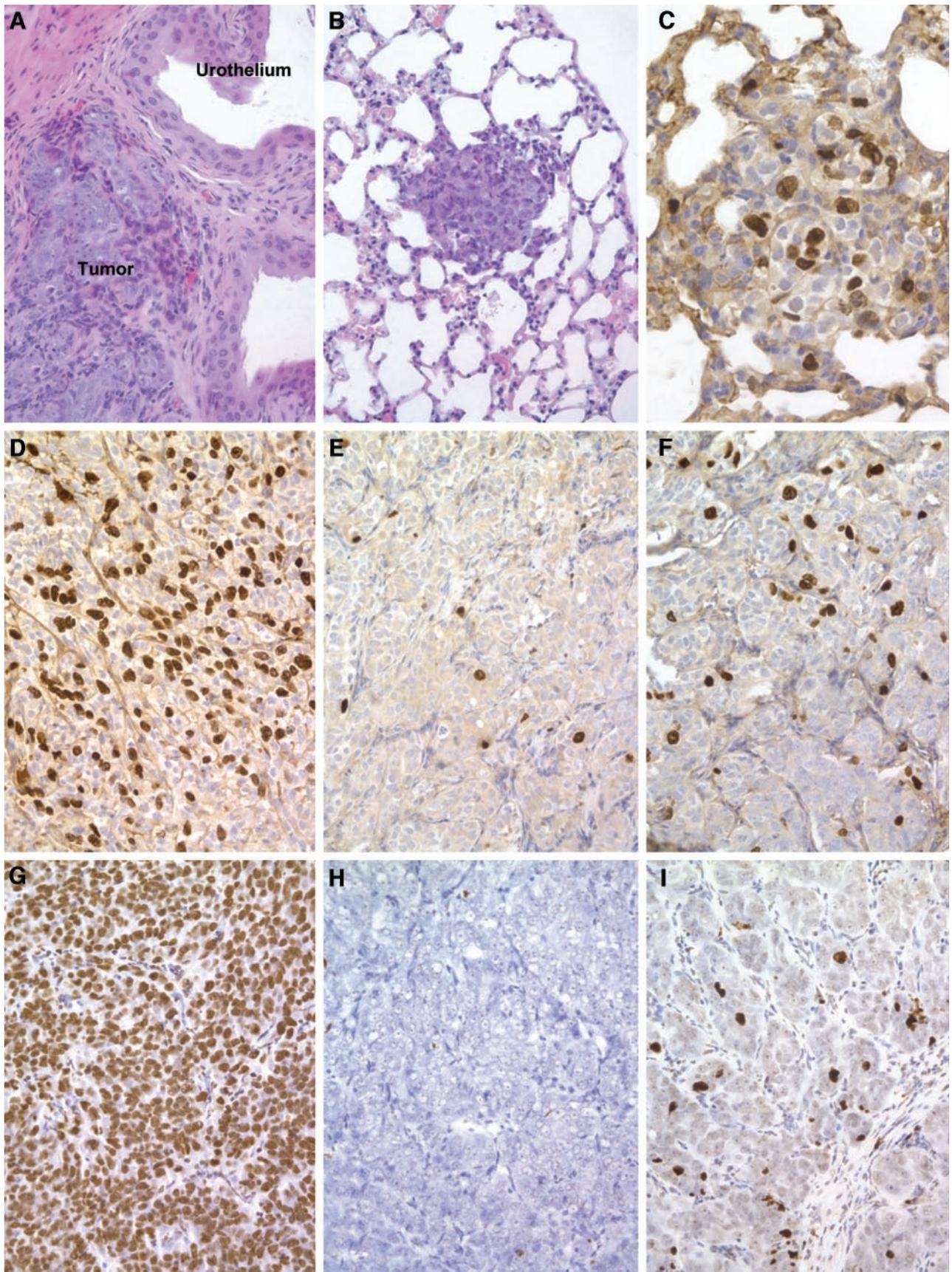


Table 1 Incidence and pathology of tumors produced by 253J B-V cells injected into the bladders of nude mice after treatment with gefitinib or vehicle

	Number of palpable tumors	Tumor size \pm SD ^a	Tumor weight \pm SD	Mean time until animal culled (days)	Incidence of lung metastasis in animals with bladder tumors	BrdUrd ^b labelling index \pm SD	Apoptotic index \pm SD
Vehicle control	8/10	1127 \pm 363 mm ³	0.67 \pm 0.1g	50	2/8	23.5 \pm 4.1	1.1 \pm 0.3
Gefitinib 150 mg/kg	2/10	2.3 \pm 1.2 mm ²	<0.04 g	All healthy at 63 days	0/2	8.0 \pm 2.0 ^c	3.2 \pm 1.6 ^d

^a Average tumor size for the control group has been expressed as volume (mm³) of tumor as measured on autopsy. Average tumor size for the Gefitinib-treated group has been expressed as the average surface area (mm²) of stained tumor cell remnants (three fields) by microscopy.

^b BrdUrd, bromodeoxyuridine.

^c Significantly less than vehicle control group ($P < 0.0001$, Student's t test).

^d Significantly more than vehicle control group ($P = 0.0008$, Welch's corrected t test).

animals in this group appeared healthy, but at this point they were culled to compare the pathology of these animals with the vehicle-treated control group. Macroscopic tumor nodules were only evident in 2 mice. The bladders of the remaining animals appeared to be tumor-free, and the mean weight of the bladders in the gefitinib-treated group was < 0.04 g, which did not differ significantly from nontumor-bearing mice of the same age. However, upon examining serial sections of the bladders of the gefitinib-treated animals, small microscopic foci of tumor cells were observed in 9 of 10 mice. The mean area of these tumor foci was 2.3 \pm 1.2 mm². No metastases were observed in any of the gefitinib-treated animals. The S-phase fraction and apoptotic index of the tumor deposits in the gefitinib-treated mice was compared with tumors in the vehicle-treated mice. Tumors in the vehicle group contained a significantly greater number of BrdUrd-staining cells (Table 1; Fig. 8, D--F) and consequently had a higher S-phase fraction (23.45%; SD = 4.09) than the tumor foci in the gefitinib-treated group (8.03%; SD = 2.01). This difference was found to be statistically significant ($P < 0.0001$, Student's t test). In contrast, the number of apoptotic cells in tumors produced by the vehicle-treated mice was significantly less (1.1% \pm 0.3) than in the tumor foci present in the gefitinib-treated mice (3.2% \pm 1.6); this difference was statistically significant ($P = 0.0008$, Welch's corrected test; Table 1; Fig. 8, G--I).

DISCUSSION

EGFR expression at high levels has been shown to be an independent prognostic indicator of stage progression and poor survival in patients with bladder cancer (11–13). Muscle-invasive TCC carries a poor prognosis, and despite radical cystectomy or radiotherapy the overall 5-year survival is only in the order of 50%. Therefore, new targeted therapeutic strategies are needed to im-

prove the outlook for this group of patients. In this study, we attempted to evaluate the therapeutic potential of a small molecule inhibitor of EGFR, gefitinib ('Iressa,' ZD1839) in preclinical models of human bladder cancer that express EGFR. Although 10 μ M gefitinib was able to inhibit DNA synthesis and reduce viable cell number and the S-phase fraction in all three of the EGFR-expressing human bladder cancer cell lines used in this study, a dose-dependent reduction of these parameters by 1 μ M and 10 μ M gefitinib was only observed in 253J B-V cells. It would appear, therefore, that 253J B-V cells are more sensitive to growth inhibition by gefitinib than T24 and RT-112 cells. This is consistent with the observation that 253J B-V cells express a higher level of EGFR protein than T24 and RT-112 cells. However, this may be coincidental, because studies in other tumor cell systems with both gefitinib and the EGFR-specific blocking antibody C-225 have demonstrated responses in human tumor xenografts and cell lines expressing many EGFR levels, from very low to very high (35), suggesting that responses to tyrosine kinase inhibitors do not necessarily correlate with levels of EGFR protein. Moreover, gefitinib may also inactivate signaling from EGFR/erbB2 and EGFR/erbB3 heterodimers (36).

Gefitinib was shown to inhibit phosphorylation of EGFR and at least two downstream molecules that are involved in signal transduction from EGFR, namely p42/44 ERK and Akt/protein kinase B (PKB), in all three of the bladder cancer cell lines studied. The magnitude of ERK inhibition by gefitinib was greatest in 253J B-V cells, and the magnitude of inhibition of Akt by gefitinib was least in 253J B-V cells. Interestingly, in the 253J B-V cell line, levels of phosphorylated ERK were high even in the absence of EGF stimulation, suggesting the presence of an autocrine mechanism in this cell line, whereas levels were low in the T24 and RT-112 and only raised after stimulation with rhEGF. Thus, it would appear that *in vitro* growth inhibi-

Fig. 8 Histology and immunocytochemistry of tumors produced by orthotopic injection of 253J B-V tumors into the bladder wall. A, H&E staining of a primary tumor nodule produced by 253J B-V cells in the bladder wall after 63 days of treatment with gefitinib, showing the host urothelium and pleiomorphic tumor cells residing in the bladder wall. B, H&E staining of a lung micrometastasis produced by 253J B-V cells after treatment with vehicle control. C, the lung micrometastasis in B stained with antiserum to bromodeoxyuridine shows that the tumor cells are proliferating. D, a primary tumor produced by 253J B-V cells, after treatment with vehicle control stained with antiserum to bromodeoxyuridine. E and F, two primary tumor nodules produced by 253J B-V cells after treatment with gefitinib stained with antiserum to bromodeoxyuridine, showing fewer positively stained cells than the tumor in D. G, positive control of a primary tumor produced by 253J B-V cells pretreated with DNase and stained with the *in situ* cell death detection kit, showing that DNA strand breaks stain positively. H, primary tumor produced by 253J B-V cells treated with vehicle control stained with the *in situ* cell death detection kit, showing no positively staining cells. I, primary tumor nodule produced by 253J B-V cells treated with gefitinib treated with the *in situ* cell death detection kit, showing scattered positively staining cells.

tion by gefitinib correlates with high constitutive activation of ERK and the ability of the drug to inhibit ERK rather than Akt. The p42/44 ERK pathway is known to be important in cell proliferation, whereas the Akt/PKB pathway has been more strongly implicated in cell survival. Inhibition of the Akt/PKB pathway is known to be important in terms of enhancing the sensitivity of tumor cells to radiotherapy and chemotherapeutic drugs (37). Indeed, 1 μM gefitinib was sufficient to increase the cytotoxic effect of doses of irradiation > 4 Gy in RT-112 bladder cancer cells. This having been said, gefitinib was also able to enhance the cytotoxic effect of irradiation in 253J B-V after only 2 Gy of irradiation. A possible confounding variable in the current studies is the relative growth rate of the three cell lines; 253J-BV, which is most sensitive to growth inhibition by gefitinib, was the slowest growing cell line. However, although RT-112 cells grew slightly faster than 253J B-V cells, they were more radioresistant than 253J B-V cells.

Single-agent gefitinib was sufficient to cause regression of tumors produced by 253J B-V cells and significantly suppress growth of primary tumors and metastases produced by 253J B-V cells after orthotopic injection into the bladder wall. However, gefitinib did not completely eradicate tumors produced by 253J B-V cells in the bladder wall during the 63-day experimental period in the current study, as evidenced by the persistence of foci of tumor cells in the bladder wall following histological examination. These microscopic foci of tumor cells that persist in the gefitinib-treated mice are characterized by a significantly reduced S-phase fraction and the presence of a significantly greater number of apoptotic cells than observed in orthotopic tumors that grow in the vehicle-treated animals. Persistence of viable tumor cells after prolonged treatment with gefitinib occurs in preclinical models of other solid tumor types, as evidenced by the rapid regrowth of tumors after withdrawal of gefitinib treatment (28).

It has been reported previously that orthotopic tumor burden produced by 253J B-V cells can be partially inhibited by the EGFR inhibitors 4.5-dianilinophthalimide (38) and monoclonal antibody cetuximab (39). In the latter study, inhibition of the angiogenic factors VEGF, interleukin 8, and FGF-2, both at mRNA (Northern blot) and protein level (ELISA) and a concomitant reduction in microvessel density was reported in tumors that arose in the cetuximab-treated group, suggesting that inhibition of EGFR signaling in 253J B-V cells *in vivo* can inhibit angiogenesis. It is possible that in the present study, gefitinib is able to induce regression of tumors produced by 253J B-V cells to a "preangiogenic" phase. Inhibition of angiogenesis by gefitinib has also been reported in squamous cell carcinomas (30).

Our data present compelling evidence that the EGFR-TKI gefitinib may have therapeutic potential, alone or in combination with ionizing radiation, in a subset of EGFR-expressing human bladder cancers. However, expression of EGFR in homogeneous bladder cancer cell lines is not necessarily predictive of a therapeutic response to gefitinib *in vivo*. Although inhibition of EGFR phosphorylation by gefitinib in bladder cancer cell lines results in some degree of inhibition of phosphorylation of p42/44 ERK and Akt/PKB, a short-term inhibition of DNA synthesis in cells maintained in serum-free medium supplemented with rhEGF, and an inhibition of viable cell number *in vitro*, it does not necessarily result in the inhibition of tumor

growth *in vivo*, at least using a dose of 150 mg/kg/day. In nude mice, a dose of 75 mg/kg gefitinib results in plasma concentrations of 10.7 μM after 1 h, decreasing to 4.8 μM after 8 h, and 0.4 μM after 24 h.¹ We would not expect the plasma concentrations achieved by dosing with 150 mg/kg to exceed twice these values. Given that 253J B-V cells respond to 10 μM and 1 μM *in vitro*, whereas RT-112 cells respond to 10 μM gefitinib but not 1 μM *in vitro*, it may be that a dose of 150 mg/kg/day is not sufficient to see responses in the RT-112 cell line *in vivo*. Although it is difficult to draw general conclusions from only three cell lines, particularly when the responsive 253J B-V cell line may or may not reflect the biology of typically responsive bladder cancers, our data suggest that response to single-agent gefitinib *in vivo* may correlate with high EGFR expression, slow growth rate *in vitro*, constitutively high ERK activation, or strong inhibition of ERK rather than Akt by gefitinib *in vitro*.

The present studies suggest that not all of the EGFR-expressing bladder cancer cell lines are dependent on EGFR signaling alone for growth and survival *in vivo*. It is likely that this reflects the redundancy of receptor tyrosine kinase-mediated signaling in solid carcinomas. Phase I studies of gefitinib have shown an acceptable tolerability profile and promising antitumor activity (40–43). In two large, double-blind, Phase II monotherapy trials, IDEAL ('Iressa,' Dose Evaluation in Advanced Lung Cancer) 1 and 2, objective tumor response rates of 18% (IDEAL 1) and 12% (IDEAL 2) were observed at the recommended dose of 250 mg/day, in previously treated patients with advanced stage III and IV non-small cell lung cancer (44, 45) Phase III clinical trials of gefitinib in combination with chemotherapy (gemcitabine + cisplatin or carboplatin + paclitaxel) have confirmed the safety of gefitinib in a placebo-controlled setting (46–48). However, there is still a requirement for the rational use of TKIs and other small molecules to optimize therapeutic responses. An accurate "molecular" diagnosis will allow for a targeted, selective tailored therapeutic approach to each particular tumor, possibly by combination with radio-, chemotherapy, and possibly other small molecules. An important future challenge will be to identify what molecular characteristics predict response or lack of response to a given TKI. Accurate molecular diagnoses may allow effective, selective tailored therapeutic strategies to be designed.

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