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

Jose L. Dominguez-Escrig, John D. Kelly, David E. Neal, Sonya M. King, and Barry R. Davies
Northern Institute for Cancer Research, School of Surgical and Reproductive Sciences, Faculty of Medical Sciences, University of Newcastle, Newcastle-Upon-Tyne, United Kingdom

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 (Erbitux), 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...
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 Cell Lines. 253J B-V is a metastatic human bladder tumor regression in an orthotopic model of bladder cancer and that this drug has potential therapeutic application in 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 μg/ml streptomycin (for T24 and RT-112 cell lines) and 95% Eagle’s MEM supplemented with 5% fetal bovine, vitamins, nonessential amino acids, 50 μg/ml penicillin, and 50 μg/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: antiphospho-EGFR (Y1173, mouse monoclonal IgG, clone 9H2; Upstate), anti-EGFR (sheep polyclonal IgG; Upstate), antiphospho-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 μl of routine medium. Gefitinib (1, 5, or 10 μM) 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 μl of medium, and 11 μl 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 μl 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 μg/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 × 10^5 cells/well. After being serum-starved for 24 h, cells were cultured in serum-containing medium, and either gefitinib (1 μM or 10 μM) 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 μl PBS. Propidium iodide (125 μl; 0.25 mg/ml in 5% Triton-Isoton II) was added together with 50 μl of 1 mg/ml RNAse 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 μg/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 μM 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 electrophotography to 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 μM or 10 μM) or...
Gefitinib in Preclinical Models of Bladder Cancer

Sections 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 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 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 μl 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 × 5 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 × 10 mm or > 15 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 0.1% 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 mm × 20 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.

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**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 253J B-V cells was 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, ± SD.
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 \( \mu M \) 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 \( \mu M \), 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 \( \mu M \) 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 \( \mu M \) 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 \( \mu M \) 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 \( \mu M \) 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 \( \mu M \) gefitinib caused a significant reduction in DNA synthesis of 68% (\( P < 0.0001 \)) and 61% (\( P < 0.0001 \)) in T24 and RT-112 cells, respectively (Fig. 4, A and B). Concentrations of 1 \( \mu M \) and 10 \( \mu M \) 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 EGFR-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 \( \mu M \) 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 \( \mu M \) gefitinib induced a significant inhibition of EGFR phosphorylation to 20% after 5 min and 30% after 10 min (Fig. 5B). Incubation of 253J B-V cells with 10 \( \mu M \) gefitinib resulted in inhibition of EGFR phosphorylation to 17% after 5 min and 20% after 10 min (Fig. 5C). 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).
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 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 μM 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 < 0.0001, Student’s t test); ***, significant reduction of [3H]thymidine incorporation compared with vehicle control (P = 0.0004, Student’s t test); bars, ±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 ~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).

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the absence of irradiation, 253J B-V cells produced 50% (P = 0.0021) fewer colonies in the presence of 1 μM 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 μM 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 × 10⁶ 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 ~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 ~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 antisera 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
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.

<table>
<thead>
<tr>
<th>Number of palpable tumors</th>
<th>Tumor size ± SD*</th>
<th>Tumor weight ± SD</th>
<th>Mean time until animal culled (days)</th>
<th>Incidence of lung metastasis in animals with bladder tumors</th>
<th>BrdUrd labeling index ± SD</th>
<th>Apoptotic index ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>8/10</td>
<td>1127 ± 363 mm³</td>
<td>0.67 ± 0.1 g</td>
<td>50</td>
<td>2/8</td>
<td>23.5 ± 4.1</td>
</tr>
<tr>
<td>Gefitinib 150 mg/kg</td>
<td>2/10</td>
<td>2.3 ± 1.2 mm³</td>
<td>&lt;0.04 g</td>
<td>All healthy at 63 days</td>
<td>0/2</td>
<td>8.0 ± 2.0</td>
</tr>
</tbody>
</table>

* 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.

** Significantly less than vehicle control group (P < 0.0001, Student’s t test).

*** Significantly more than vehicle control group (P = 0.0008, Welch’s corrected t test).

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 improve 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 μM 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 μM and 10 μM 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-

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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 pleomorphic 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 antiseraum 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 antiseraum to bromodeoxyuridine. E and F, two primary tumor nodules produced by 253J B-V cells after treatment with gefitinib stained with antiseraum 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 nodules 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. 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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