Gefitinib Inhibits the Growth and Invasion of Urothelial Carcinoma Cell Lines in which Akt and MAPK Activation Is Dependent on Constitutive Epidermal Growth Factor Receptor Activation

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Abstract

Purpose: Abnormally high levels of epidermal growth factor receptor (EGFR) protein are associated with advanced tumor stage/grade. The objective of this study was to evaluate the effects of the specific EGFR tyrosine kinase inhibitor gefitinib on activation of the Akt and mitogen-activated protein kinase (MAPK) pathways in human urothelial cell carcinoma (UCC) cell lines and to identify potential markers of gefitinib responsiveness in biopsy samples of UCC.

Experimental Design: Changes in markers of UCC growth and invasion after exposure to gefitinib were studied in six human UCC cell lines expressing various levels of EGFR. The findings were related to activation of Akt and MAPK. We studied the influence of gefitinib on intraepithelial expansion of the responsive 1207 cell line. EGFR, Akt, and MAPK activation was studied by Western blot analysis of a panel of 57 human UCC.

Results: Gefitinib had a growth-inhibitory and anti-invasive effect in two of six UCC cell lines (i.e., 647V and 1207). Gefitinib was also able to block the expansion of 1207 at the expense of normal urothelial cells. These effects did not depend on the level of expression of EGFR but they were associated with the down-regulation of MAPK and Akt activity; in 1207 cells, gefitinib activity was associated with p27 up-regulation and p21 and matrix metalloproteinase-9 down-regulation. Similarly, the Akt and MAPK pathways were found to be strongly phosphorylated in association with EGFR activation in a subset of human UCC specimens.

Conclusions: Activation of EGFR, Akt, and MAPK defines a subset of UCC which might provide information for the identification of gefitinib responders.

There is considerable experimental evidence to suggest that epidermal growth factor receptor (EGFR) signaling is involved in urothelial cell proliferation (1) and invasion (2). In an organlike model, members of the EGFR family are expressed during the migration and proliferation of normal urothelial cells. Increases in the levels of mRNA for transforming growth factor α (TGFα), heparin-binding EGF-like growth factor, and epiregulin are associated with a 3% to 15% increase in normal urothelial cell proliferation (3). We have shown in this model that regeneration of the urothelium after trauma is inhibited by anti-EGFR antibodies and by AG1478, an EGFR tyrosine kinase–specific inhibitor (4). We also established a human urothelial carcinoma cell line (1207) from an invasive urothelial cell carcinoma (UCC). EGF induces proliferation in this cell line and TGFα increases the ability of this cell line to invade matrigel (5). EGFR is responsible for at least some of the invasive properties of 647V, another UCC cell line (6).

The overexpression of EGFR and/or its ligands is associated with urothelial carcinoma progression in experimentally induced neoplasms (7) and in human tumors. High levels of EGFR protein are associated with advanced tumor stage and grade (8) and cell proliferation (9). These findings strongly suggest that EGFR could be a key therapeutic target in bladder cancer (10). A whole new class of tools has recently become available that may improve the results of treatment for EGFR-dependent tumors in the bladder (11, 12). These tools include antibodies, antisense oligonucleotides, small interfering RNA, and small inhibitory molecules specifically inhibiting the receptor tyrosine kinase. These therapeutic approaches may

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Note: This work is dedicated to Professor Domininique K. Chopin, a great man who is gone too early.

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have a significant effect on the outcome of treatment for bladder cancer. There is thus a strong biological rationale for using EGFR inhibitors for therapeutic purposes: to prevent the recurrence and progression of UCC. Gefitinib ("Iressa," ZD1839) acts as an inhibitor in a wide range of tumor types, including non–small-cell lung cancer, breast cancer (13), and metastatic colorectal cancer (14). However, the mechanisms underlying the antitumor activity of this molecule are unclear. Gefitinib is an orally active, selective EGFR tyrosine kinase inhibitor that causes complete inhibition of EGF-stimulated EGFR autophosphorylation in cell lines at submicromolar concentrations (IC_{50}, 0.02-0.08 μmol/L; ref. 15). It is a synthetic anilinoquinazoline that competes for the Mg-ATP binding site and blocks the signal transduction pathways involved in the proliferation and survival of cancer cells. Based on its promising antitumor activity and favorable toxicity profile in preclinical tests, gefitinib has recently entered clinical trials. A particular challenge in the clinical development of this compound is the exploration of its biological activity against the EGFR and receptor-dependent processes. No clear association between EGFR levels and response to gefitinib has been found. The evaluation of signaling components from inhibition of EGFR by gefitinib provides information about the activation of the EGFR pathway in urothelial carcinogenesis (16). The limited efficacy of gefitinib in preliminary clinical trials extended the search for molecular mechanisms beyond EGFR expression to the identification of EGFR-dependent tumors (17, 18). The initial failure of small-molecule tyrosine kinase inhibitors may also be due to the involvement of multiple pathways. Crosstalk and the redundancy of cell signaling pathways may favor tumor recurrence or progression if a single pathway is inhibited. An understanding of the molecular basis of responsiveness to gefitinib is therefore of great clinical importance to identify the subset of tumors likely to benefit from treatment.

We investigated these issues in bladder cancer by studying the effect of gefitinib on a panel of human UCC cell lines. We found that the cell growth and invasion were inhibited by gefitinib in 1207 and 647V cells; gefitinib inhibited the cell cycle in 1207 cells and caused cell death in 647V. We found also that gefitinib was able to block the expansion of 1207 at the expense of normal urothelial cells. Other cell lines were unaffected in the pharmacologic range tested. We also showed the absence of a clear relationship between the response to gefitinib and the activation of potential erbB autocrine loops, which was evaluated by assessing receptor and ligand expression. Gefitinib activity was associated with the activation of EGFR and with two downstream signaling pathways: those involving mitogen-activated protein kinase (MAPK) and Akt. Finally, we assessed these potential biological predictors in a representative panel of human UCC tissues.

Materials and Methods

Cell lines and culture medium. The human bladder cancer cell lines T24, J82, RT112, 647V, and SD48 were obtained from originators' laboratories or from the American Type Culture Collection (Manassas, VA) and were grown in RPMI (Life Technologies, Inc., Rockville, MD) with 10% heat-inactivated fetal bovine serum (FBS), antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin), and 2 mmol/L L-glutamine. The 1207 bladder cancer cell line was cultivated as previously described, without insulin (5). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Reagents and antibodies. Gefitinib (Iressa, ZD1839) was provided by AstraZeneca (Macclesfield, United Kingdom). It was dissolved in DMSO to generate a concentrated stock solution, which was then diluted in serum-free RPMI before the experiment and added to cells after overnight incubation for cell adhesion, except for invasion assays. The following antibodies were used: rabbit polyclonal anti-EGFR, anti–phospho-Akt (S473), anti-Akt 1/2, anti–phospho-p44/p42 MAPK (T202, Y204), and anti–p44/p42 MAPK antibodies (Cell Signaling, Beverly, MA), rabbit polyclonal anti–phospho-EGFR (Y1068), mouse monoclonal anti-p21 and anti-p27 antibodies (BioSource International, Inc., Camarillo, CA), and mouse monoclonal anti-α-tubulin antibody (Merck KGaA, Darmstadt, Germany). As secondary horseradish peroxidase–conjugated antibodies, we used goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-mouse antibody (Jackson ImmunoResearch, Baltimore, MD).

Tissue samples. We obtained 57 fresh bladder biopsy samples (T_a, $n = 4$; T_1, $n = 26$; T_2, $n = 9$; T_3, $n = 3$; T_4, $n = 11$) from the Department of Urology (Henri Mondor Hospital, Créteil, France) after the patients had given written information consent during various urological procedures (transurethral resection for superficial tumors or cystectomy for invasive tumors). One fragment was fixed for histologic control and the rest of the sample was collected in a tube, snap-frozen in liquid nitrogen, and stored at −80°C for protein extraction.

Western blotting. Tissue samples from biopsies and cell lines were lysed with radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Lake Placid, NY) supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (25 μmol/L orthovanadate and 50 mmol/L NaF). For cell lines, proteins were extracted after 48 hours of incubation with gefitinib in appropriate medium. The total protein concentration of the soluble extract was determined using the Bicinchoninic Acid Kit (Sigma, St. Louis, MO). Each protein sample (40 μg) was separated with appropriate polyacrylamide-SDS gels, transferred onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA), and incubated with specific antibodies. Immune complexes were visualized by enhanced chemiluminescence detection (ECL plus kit, Amersham Biosciences, Little Chalfont, United Kingdom).

RNA analysis. Total RNA was extracted from the six cell lines with Trizol (Gibco Life Technologies BRL, Eragny, France) and used as the template for first-strand cDNA synthesis by random priming. Real-time PCR (7900 HT, ABI Prism, Applied Biosystems, Foster City, CA) was done with 1 μg of cDNA, 25 μmol/L of primers, and signals were quantified with SYBR Green. The 5′-to-3′ sense and antisense primer pair sequences were as previously described (3). Experimental values were compared with those for the ubiquitous transcription factor TATA-binding protein (19), which was used as an internal control.

Cell viability as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. For growth assays, we incubated cells overnight at a density of 5,000 per well (10,000 per well for 1207) in 96-well plates. The cells were washed in HBSS and then in appropriate medium with various concentration of FBS in the presence or absence of the indicated amounts of gefitinib. After 48 hours, cells were first incubated with MTT (150 μg/well) for 90 minutes and then with acidic isopropanol. Absorbance at 550 nm was then determined with a Labsystems Multiskan RC Microplate Reader (Labsystems, Helsinki, Finland). We carried out two independent experiments, each in triplicate. Data are expressed as percent inhibition with respect to the corresponding untreated controls.

Apoptosis assay. Cells were grown in appropriate medium in the presence or absence of gefitinib for 48 hours. We harvested 5×10^5 cells by treatment with trypsin and detected apoptosis using an Annexin V Detection Kit (Santa Cruz Biotechnology) according to the instructions of the manufacturer. Samples were analyzed using flow cytometer (Becton Dickinson, Bedford, MA).

In vitro *invasion chamber assay*. Cells were used to seed a six-well Matrigel Invasion Chamber (Becton Dickinson Labware) at a density of 25×10^4 per well. We added appropriate medium to the bottom of the insert in the presence or absence of 2 μmol/L of gefitinib. Culture supernatant from T24 cells was placed under the insert as the chemoattractant. After 48 hours, the inserts were scrubbed with a cotton-tipped swab, rinsed in PBS, fixed in methanol, and the cells were stained with H&E. This experiment was done in triplicate. The surface area covered by cell outgrowth was measured under an Olympus microscope coupled to a computer-assisted image analysis system (Measure, ClaraVision, France). Invasion was quantified with Perfect Image Software.

Cell cycle analysis. Cells were cultured in appropriate medium (3% FBS) and 2 μmol/L gefitinib for 72 hours. We harvested 10^6 cells by treatment with trypsin and stained them with propidium iodide using the Con3 DNA Staining Kit (Consults, Rivolta de Torino, Italy). Samples were analyzed by flow cytometry in a Coulter Epics XL Cytometer (Beckman Coulter, Miami, FL). We used Multicycle Software (Phoenix Flow Systems, San Diego, CA) to determine cell cycle phase distribution. This experiment was done in triplicate.

Intraepithelial expansion assay. The cocultivation model to study intraepithelial expansion of 1207 urothelial carcinoma cells was previously described (20). In confluent murine explant cultures, a single standardized circular area was denuded. The urothelium in the injured areas was scraped away from the cyclopore membrane with the tip of a micropipette, the cultures were washed twice with PBS, followed either by seeding of 10^5 1207 UCC cells or no cells in 1.5 mL medium. After allowing the 1207 cells to adhere for 24 hours, the nonadherent cells were washed away by culture medium, and medium containing various concentrations (0, 2, 10, and 20 μmol/L) of gefitinib was added. The cultures were discontinued by fixation in ethanol 70% after 24 hours, 7 days, and 14 days to evaluate the intraepithelial expansion by selective immunostaining of the 1207 cells by anti – keratin 19 antibody RCK 108.

Zymography. Zymography was done in a polyacrylamide gel containing 0.25% gelatin. Supernatants of 1207 and 647V cell cultures were incubated for 48 hours with or without gefitinib. We mixed 30 μL with sample buffer [0.5 mol/L Tris (pH 6.8), 10% SDS, glycerol, bromophenol blue]. Electrophoresis was done in Tris-glycine-SDS buffer. Gel was renatured by incubation in 50 mmol/L Tris (pH 7.5), 2.5% Triton X-100 buffer for 30 minutes, then incubated for 24 hours in development buffer, stained in 0.5% Coomassie brilliant blue G-250 in 10% ethanol, 5% acetic acid for 1 hour, and destained in water for 24 hours. Bands corresponding to proteins displaying matrix metalloproteinase (MMP) activity were quantified with ImageMaster software (Pharmacia Biotech, Uppsala, Sweden).

SNP GeneChip assay. Preparation of target DNA from tumor and control lymphoblastoid cell lines, DNA labeling, hybridization, washing, and staining were done according to the Affymetrix GeneChip Human Mapping 50K protocol (Affymetrix, Inc., Santa Clara, CA; available on the internet). Hybridization to the microarray was detected with an Affymetrix Fluidics FS450 station using the Mapping100 Kv1_450 script and a GCS3000 Scanner. The signal intensity data were analyzed with GeneChip DNA Analysis Software and with the Affymetrix GeneChip Chromosome Copy Number Tool to identify regions of gain or loss for each chromosome.

EGFR mutational analysis. We did PCR to amplify exons 17 to 22. Primer pairs used were exon 17, ggcactgctttccagcat (F) ccccaccagaccatgaga (R); exon 18, agatcactgggcagcatgt (F) cagctgccagacatgagaaa (R); exon 19, cattcatgcgtcttcacctg (F) catatccccatggcaaactc (R); exon 20, agccataagtcctcgacgtg (F) cctggtgtcaggaaaatgct (R); exon 21, gggtcctggggtgatctg (F) aaagaaaatacttgcatgtcagagg (R); and exon 22, gaagcaaattgcccaagact (F) gcctcagctgtttggctaag (R). PCR amplicons were purified using exoSap (Amersham Biosciences Europe, Buc, France) before sequencing. Purified DNA was diluted and cycle sequenced using the ABI BigDye Terminator kit v3.1 (Applied Biosystems, Les Ulis, France). Sequencing reactions were electrophoresed on an ABI9100 genetic analyzer and analyzed in both sense and antisense directions for the presence of mutations using Sequence Navigator software.

Results

Antiproliferative and cell cycle effects of gefitinib in human bladder cancer cell lines. MTT assays showed that 2 μmol/L gefitinib for 2 days did not affect the growth of the T24, J82, SD48, and RT112 cell lines (5 and 10 μmol/L gefitinib also tested had no effect in these cell lines) but had a strong growth-inhibitory effect on 647V and 1207 cell lines. We produced dose-response curves for the growth-inhibitory effects of gefitinib in the six bladder cancer cell lines. Gefitinib inhibited the cell growth of 1207 cell line in a serum-dependent manner. We observed a shift of IC_{50} when the concentration of FBS changed. The maximal growth-inhibitory effect of gefitinib was 80% in 10% FBS (IC_{50}, 0.25 μmol/L), 90% in 3% FBS (IC_{50}, 0.15 μmol/L), and ~100% in serum-free condition (IC_{50}, 0.05 μmol/L; Fig. 1A). These results were not observed with the 647V cell line; the maximal growth-inhibitory effect of gefitinib was 50% for all concentrations of serum. The addition of 50 ng/mL of exogenous EGF did not modify these data; the maximal growth-inhibitory effect of gefitinib was ~90% for 1207 cells and 50% for 647V cells (data not shown).

We also found that 2 μmol/L gefitinib for 72 hours had no significant effect on the cell cycle of any of the cell lines tested, with the exception of 1207 cells. Analysis of the cell cycle profiles of asynchronous growing cells indicated that gefitinib increased the proportion of 1207 cells in the G_0-G_1 phase from 47.1% to 76.3% and decreased the number in the S phase from 34.4% to 11.9% (data not shown). FITC-conjugated Annexin V was used to detect cells in apoptosis by flow cytometry. Cells (1207 and 647V) cultured in the absence or presence of 2 and 10 μmol/L of gefitinib for 48 hours were then incubated with

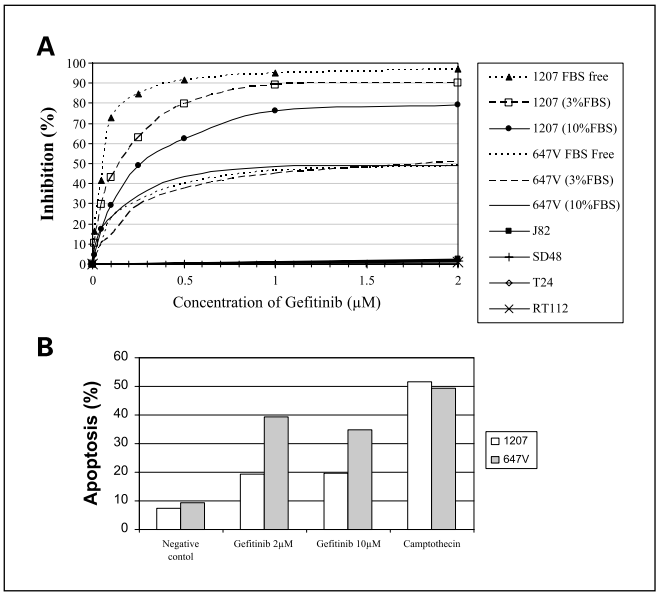

Fig. 1. *A,* growth-inhibitory effect of gefitinib on six bladder cancer cell lines exposed to various concentrations of the drug for 48 hours, as assessed by MTT; cells were exposed to 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2, 5, and 10 μmol/L. Cell growth was inhibited by gefitinib in 1207 and 647V cells, but not in T24, RT112, J82, and SD48 cells. The growth-inhibitory effect of gefitinib was 80% in 10% FBS (IC_{50}, 0.25 μmol/L), 90% in 3% FBS (IC_{50}, 0.15 μmol/L), and ~100% in serum-free condition (IC_{50}, 0.05 μmol/L). *B,* apoptosis assays. Apoptosis was measured using Annexin V kit and flow cytometer. Gefitinib 2 μmol/L induced significantly greater apoptotic cell death in the 647V cell line (36.2%) than in the 1207 cell lines (19.5%) with regard to controls.

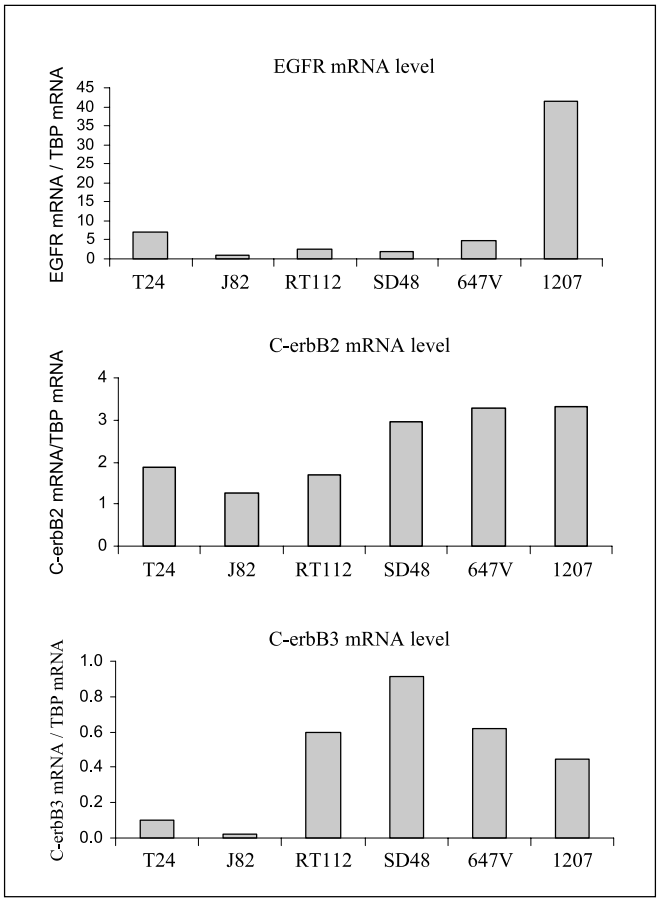

Fig. 2. Quantitative reverse transcription-PCR measurement of growth factors receptor expression at mRNA level. Expression of EGFR, c-erbB2, and c-erbB3 in six bladder cell lines.

Annexin V and propidium iodide and subjected to flow cytometric analysis. The results showed that 2 μmol/L gefitinib induced significantly greater apoptotic cell death in the 647V cell line (36.2%) than in the 1207 cell lines (19.5%); results were compared with their respective negative control (9.3% and 7.3% respectively; $P < 0.001$; Fig. 1B). These data might explain that gefitinib did not modify cell cycle of 647V cells whereas it had a growth-inhibitory effect.

Protein and mRNA levels for erbB receptors in various cell lines. Quantitative reverse transcription-PCR showed that the six bladder cancer cell lines tested in this study produced various levels of *c-erbB1*, *c-erbB2*, and *c-erbB3* mRNA. All the cell lines contained all three receptors. Levels of receptor mRNA and protein (Western blotting) were found to be correlated (Fig. 2). We also compared levels of mRNA for EGF, TGFα, amphiregulin, heparin-binding EGF-like growth factor, and epiregulin in the same cell lines to identify a potential autocrine loop. The T24 cell line showed the highest expression of amphiregulin, epiregulin, heparin-binding EGF-like growth factor, and TGFα genes whereas the J82 cell line showed the lowest expression of these genes; the RT112 cell line showed a high expression of amphiregulin, HB-EGF, and TGFα genes; the 647V cell line mainly expressed TGFα; the SD48 cell line mainly expressed amphiregulin and HB-EGF; finally, the 1207 cell line mainly expressed amphiregulin and TGFα (data not shown). No relationship was found between the response to gefitinib and the activation of potential erbB autocrine loops, as assessed by receptor and ligand expressions.

EGFR signaling pathways and effect of gefitinib. Using an antibody specific for the Tyr1068-phosphorylated form of the EGFR, we showed that the receptor was phosphorylated at this residue in all cell lines, although the level of phosphorylation differed between cell lines. The 1207 gefitinib-responsive cell line had the highest proportion of phosphorylated receptors and both gefitinib-responsive cell lines (1207 and 647V) displayed a decrease in phosphorylation following gefitinib treatment for 48 hours (in 10% FBS condition). This decrease in receptor phosphorylation was associated with a strong decrease in phosphorylation of the MAPKp44/42 (T202, Y204) and Akt (S473) downstream effectors (Fig. 3A). In T24, J82, RT112, and SD48 cells, 2 μmol/L gefitinib had little or no effect on EGFR phosphorylation and downstream signaling pathways. The effect of gefitinib on EGFR phosphorylation seemed to be dose dependent in 1207 cells and was accompanied by a parallel increase in p27 expression (Fig. 3B), which was not the case for 647V cells. There was a switch from EGFR phosphorylation to p27 expression at a concentration of 0.25 μmol/L gefitinib, which corresponds to the IC_{50} for 1207 cell growth. These results suggest that the inhibitory effects of gefitinib on cell proliferation and progression through the cell cycle are mediated through an increase in the amount of p27 in the 1207 cell line. We also found that the effects of gefitinib on EGFR phosphorylation were accompanied by a parallel decrease in p21 expression in 1207 and 647V cell lines (Fig. 3B).

In the two gefitinib-responsive cell lines, we looked for genetic alterations underlying EGFR overexpression and activation using the 50K SNP GeneChip. The *c-erbB1* gene locus on chromosome 7 is highly amplified in the 1207 cell line (no other locus is amplified on this chromosome); this

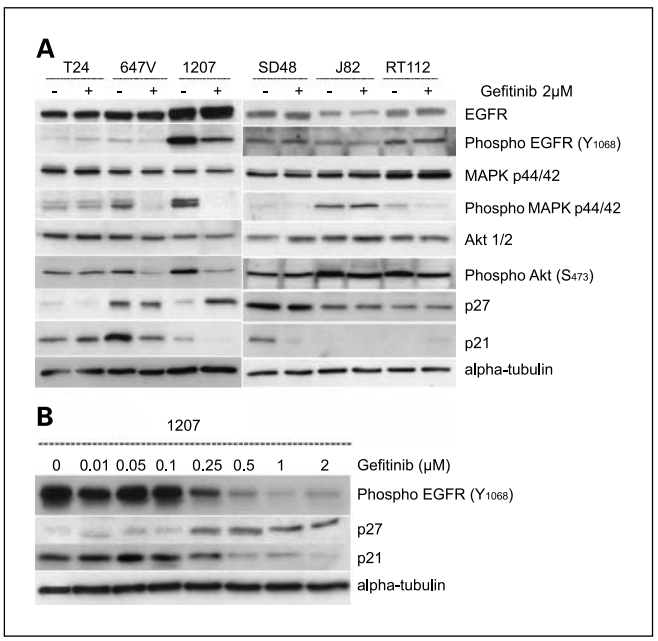

Fig. 3. *A,* EGFR signaling pathways and MAPK and Akt phosphorylation in UCC cell lines treated and untreated with 2 μmol/L of gefitinib for 48 hours. The drug decreased EGFR, MAPK, and Akt activities in 1207 and 647V cells. *B,* effect of various concentrations of gefitinib on phospho-EGFR, p27, and p21 levels in 1207 cells. The drug clearly up-regulated p27 and down-regulated p21 expression.

amplification was also present in the tumor from which this cell line was produced. In contrast, the 647V cell line displayed no detectable amplification of this locus (data not shown). We isolated DNA from 647V and 1207 cell lines and carried out a PCR to amplify exons 17 to 22 overlapping the ATP binding pocket of the *c-erbB1* gene. No mutation was detected in subsequent sequence analysis.

Lower levels of cancer cell invasion following EGFR inhibition: effect on MMP-9 activity. *In vitro* invasion assays using modified Boyden chambers coated with Matrigel showed that 647V, 1207, T24, and J82 cells were able to invade Matrigel chambers but that 647V and T24 cells were much more invasive than 1207 and J82 cells. Gefitinib (2 μmol/L) inhibited the invasion of 647V and 1207 cells by 94% and 83%, respectively, but did not inhibit the invasion of T24 cells (Fig. 4A). We also evaluated MMP-9 activity by zymography in the 1207 and 647V cell lines treated with gefitinib for 48 hours. Gefitinib (2 μmol/L) decreased MMP-9 activity in both 1207 (by 55%) and 647V (by 33%) cells whereas this effect was not observed in the T24 cell line (Fig. 4B). Thus, gefitinib may block the invasion of these two cell lines by down-regulating MMP-9.

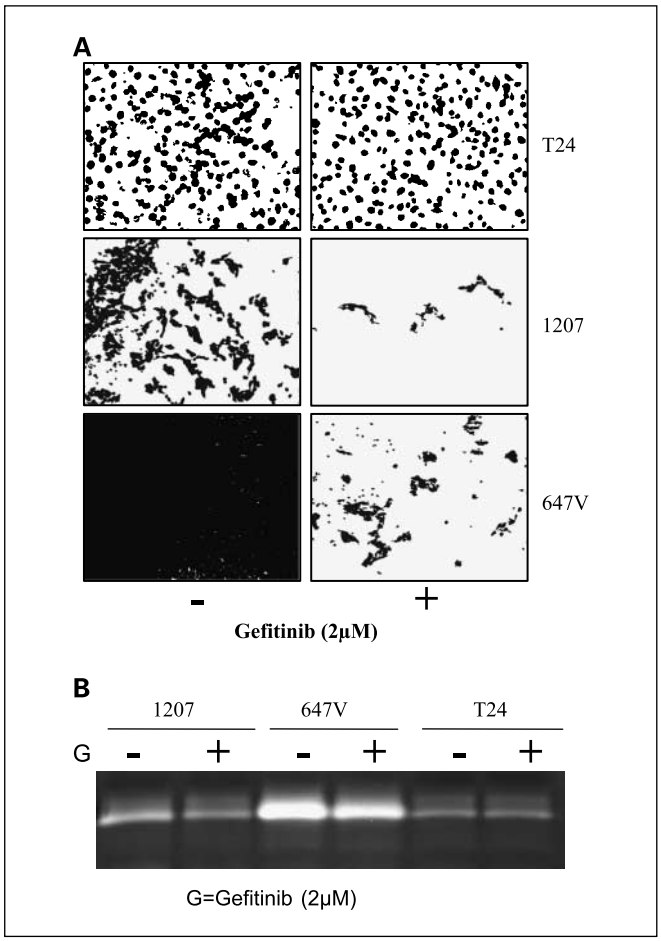

Fig. 4. *A,* invasion assays. 1207, 647V, and T24 cell lines were incubated in the presence or absence of gefitinib (2 μmol/L) for 48 hours on Matrigel. We used T24 supernatant as the chemoattractant at the bottom of the Matrigel. Gefitinib inhibited invasion in the 1207 (by 83%) and 647V (by 94%) UCC cell lines but did not inhibit T24 invasion. *B,* zymogram of conditioned medium of 1207, 647V, and T24 cells treated with gefitinib (2 μmol/L) for 48 hours. Gefitinib decreased MMP-9 levels in 1207 (by 55%) and 647V (by 33%) cell lines.

Influence of gefitinib on intraepithelial expansion of 1207. UCC often expand in the bladder mucosa by replacing normal urothelium by carcinoma cells. *In vitro* intraepithelial expansion assays were done to show the potential of gefitinib to block the expansion of 1207 at the expense of normal urothelial cells. At the dose of 20 μmol/L, the normal wound healing process (in the absence of 1207 cells) was delayed, resulting in a closure of the wounds at 48 hours instead of 24 hours (data not shown), but at a dose of 2 and 10 μmol/L, this delay was not observed. A dose-dependent decrease in the surface covered by 1207 cells was observed (Fig. 5), although even at the concentration of 20 μmol/L, residual 1207 cells remained at the center of the wounded area. These residual 1207 cells continued to incorporate bromodeoxyuridine (data not shown) but they showed multinucleation.

Activation of signaling pathways in human UCC of the bladder. A rationale for the use of gefitinib must be defined if we are to avoid the problem of treatment failure. *In vitro*, we have shown that gefitinib had an activity when Akt and MAPK activation was dependent on constitutive EGFR activation. Thus, we studied EGFR phosphorylation and MAPK and Akt activation in a panel of 57 human UCC tumors to make it possible to compare our *in vitro* findings with the situation *in vivo* in humans. We did this analysis by Western blot, loading equal amount of protein on each well. We observed no EGFR phosphorylation in 19 (31.6%) tumors, moderate phosphorylation in 27 (52.6%) tumors, and strong activation in 11 (15.8%) tumors (Fig. 6). No significant association was observed between pathologic stage of tumors and EGFR phosphorylation levels (data not shown). We identified nine tumors in which EGFR (Y1068) was strongly phosphorylated in association with MAPKp44/42 and Akt activation. This similar situation was observed in 1207 cell line responding to gefitinib.

Discussion

In this work, we found that the response to gefitinib (Iressa, ZD1839) was not related to the expression of erbB receptors and ligands. The six bladder cancer cell lines tested all contained the c-erbB1, c-erbB2, and c-erbB3 receptors and at least one erbB ligand transcript. These data are consistent with clinical studies in which EGFR levels were found not to be correlated with clinical response (11, 21). We assessed the expression of c-erbB2 and c-erbB3 because erbB receptors signal through heterodimerization (22) and because it has been suggested that studying the interaction between the various forms of this receptor may make it possible to predict whether a cell line will respond to gefitinib (23). Similar findings have been obtained with lung cancer cell lines (24).

We found that the growth and invasion of two UCC cell lines (647V and 1207) was inhibited by gefitinib. Gefitinib inhibited the cell growth of 1207 cell line in a serum-dependent manner; we obtained a better growth inhibition in serum-free condition than in serum conditions. These results are in accordance with Nutt et al. (25) who observed that in bladder tumor cell lines, cells were more sensitive to growth inhibition in the serum-free medium. These data suggest that other pathways are probably activated in the presence of serum which contained a lot of various growth factors. With the 647V cell line, these results were not observed; we found a partial growth inhibition even in absence of serum.

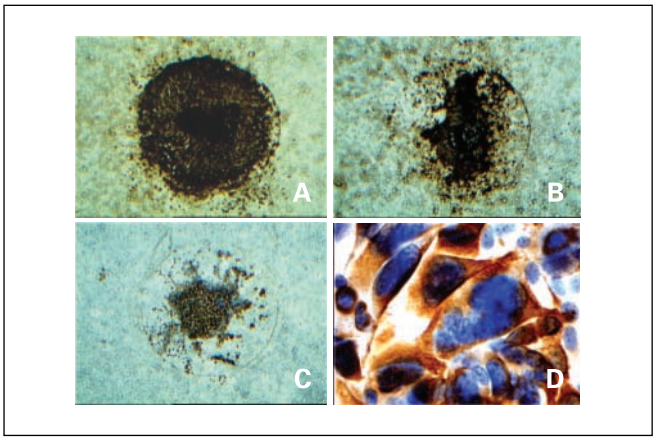

Fig. 5. Intraepithelial expansion assay testing the inhibitory effect of gefitinib on 1207 cells cocultivated with normal murine urothelium. Cocultivation for 14 days in absence (*A*) and at a dose of 2 μmol/L (*B*) or 20 μmol/L (*C*) gefitinib showing a dose-dependent decrease of cytokeratin 19–stained 1207 cells. *D,* higher magnification of residual 1207 cells in coculture after 14 days at a dose of 20 μmol/L.

In the responsive cell lines, c-erbB1 was constitutively phosphorylated, and the downstream MAPK and phosphatidylinositol 3-kinase/Akt pathways were also activated; these two pathways were inhibited by gefitinib. We also found that EGFR phosphorylation was inhibited in a dose-dependent manner, consistent with the growth inhibitory effects. The activation of MAPK by MAPK/extracellular signal–regulated kinase kinase 1 and 2 is the best-characterized response to EGFR signaling for cell survival and proliferation. This pathway has been shown to be involved in cancer cell invasion (26). It has been suggested that MAPK phosphorylation is an important biological marker of EGFR activation (27) and that the phosphatidylinositol 3-kinase/Akt pathway is the other main signaling pathway involved in EGFR activation and targeted by gefitinib (28). Abnormal activation of this pathway affects cell survival and plays a crucial role in the resistance to apoptosis induced by growth factor deprivation and chemotherapy.

Gefitinib induces G_1 arrest in malignant cell lines of several origins (29). The G_1-S transition and cell cycle exit are governed by the coordination of cyclin-dependent kinases and cyclin-dependent kinase inhibitors. We have shown here that gefitinib inhibits the growth of 1207 cells by up-regulating p27 in a dose-dependent manner, but it is not the case in 647V cells. Increases in p27 levels were also observed in oral carcinoma cell lines treated with gefitinib (29). Exit from the cell cycle mediated by p27 may limit the efficacy of therapies based on DNA damage and requires proliferating cells. It has been suggested that control of entry into and exit from the cell cycle by the selective administration of cytotoxic agents may make it possible to combine various innovative therapies with conventional chemotherapy (30). The expression of p27 could also be considered a surrogate marker for the effect of gefitinib.

We also studied p21 expression and found that 1207 and 647V cell lines expressed p21 and that this expression was decreased by gefitinib. Initially, p21 was characterized as a negative cell cycle regulator binding cyclin-dependent kinase 4/cyclin D and cyclin-dependent kinase 6/cyclin D, and regulates the activity of these molecules in early G_1. In fact, this protein has both a positive and a negative regulatory effect on the cell cycle (31). The function of this protein in normal cells ensures appropriate cyclin-dependent kinase inhibition during cell cycle progression. However, in some tumors, p21 may also promote cell survival via an Akt-dependent mechanism (32). Gefitinib probably regulates p21 negatively through Akt inhibition.

Understanding the deregulation and overproduction of protein kinases has constituted a major breakthrough in the clinical development of new drugs in oncology. The success of Gleevec (imatinib mesylate), a platelet growth factor receptor inhibitor, lies in the genetic alterations underlying chronic lymphocytic leukemia and gastrointestinal stromal tumors (BCR-ABL and c-kit). Other tyrosine kinase inhibitors, such as gefitinib and erlotinib, were found to be much less effective in large phase III studies in non–small-cell lung cancer (28). These studies suggest that the definition of validated biomarkers is urgently required and that patient populations should be characterized carefully before treatment. The importance of unraveling the mechanisms of action of potential therapeutic targets has been shown by in-depth studies in which the constitutive activation of EGFR due to mutations was found to be associated with a response to gefitinib (33). The overexpression of EGFR is well documented and has been linked to proliferation (9). In our model, the 1207 cell line and the original tumor harbored *EGFR* gene amplifications but the 647V cell line did not. This suggests that the growth of 1207 cell line is completely under the dependence of the EGFR pathway and might explain a strong activity of EGFR in these cells. By contrast, in the 647V cell line, it seems that other mechanism(s) could be involved in the proliferation.

We detected Akt and MAPK phosphorylation in all the cell lines tested and in most of the human UCC specimens. Gefitinib had biological and biochemical effects in only two cell lines (647V and 1207). These observations and our results for a subset of 57 human UCC (EGFR, Akt and MAPK phosphorylation) indicate that in a subgroup of human UCC, Akt and MAPK activation is not dependent solely on constitutive EGFR activation. This situation of alternative signaling activation may account for insensitivity to gefitinib. To confirm our results, testing the antiproliferative effects of gefitinib in the primary patient tumor samples *ex vivo* is needed.

The results of the intraepithelial expansion assay, which is considered to represent an *in vitro* parallel of carcinoma *in situ* in the urinary bladder mucosa (20), indicate that gefitinib selectively affects 1207 cells, reversing the balance of growth and expansion from the 1207 cells in favor of that of the normal urothelial cells. Our finding that MMP-9 is down-regulated by

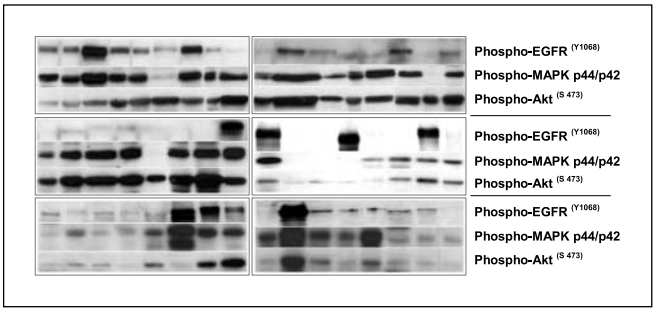

Fig. 6. EGFR, MAPK, and Akt activation of human bladder UCC as assessed by Western blotting. The phosphorylation of Akt (S473) and MAPK (T202, Y204) is dependent on EGFR (Y1068) phosphorylation in a subset of tumors. The figures shows 49 of 57 tumors samples studied.

gefitinib in the 1207 and 647V cell lines may partly account for the inhibition of invasion observed with this compound. EGF induces MMP-9, but not MMP-2, in bladder cancer cells, and both total gelatinase activity and MMP-9 activity in urine are associated with high-stage bladder tumors (34). Because gefinitib is able to reduce both intraepithelial expansion as well as invasiveness of a subset of UCC cell lines, this tyrosine kinase inhibitor may have the potential to be of benefit for a subset of patients with carcinoma *in situ* and invasive UCC. Our data suggest that activation of EGFR pathway may be the marker by which this subset of patients could be identified.

Conclusions

We show here that gefitinib inhibits growth and invasion in some bladder cancer cell lines and may be effective in a subset of UCC similar to the 1207 cell line, those in which Akt and MAPK are predominantly activated by EGFR. The effects of gefitinib on cell proliferation and invasion are mediated by the differential regulation of p21, p27, and MMP-9. *EGFR* gene amplification is one of the mechanisms underlying constitutive EGFR activation. The search for other mechanisms involved in EGFR activation or the alternative activation of Akt and MAPK pathways might provide essential information for the identification of gefitinib responders and for the design of alternative or combined targeted therapies in bladder cancer.

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