Mutations Within the Kinase Domain and Truncations of the Epidermal Growth Factor Receptor Are Rare Events in Bladder Cancer: Implications for Therapy


Abstract

Purpose: It has previously been reported that the patient response to gefitinib depends on the presence of mutations within the kinase domain of epidermal growth factor receptor (EGFR) or the expression of its truncated form, EGFR variant III (EGFRvIII). The focus of this study was to determine if these alterations are present within the tyrosine kinase and ligand-binding domain of EGFR in urothelial carcinoma.

Experimental Design: The kinase domain found within exons 18 to 21 of the EGFR from 11 bladder cancer cell lines and 75 patient tumors were subjected to automated sequencing. EGFRvIII expression was determined by immunohistochemistry using a urothelial carcinoma tissue microarray, and its expression was subsequently verified by reverse transcription PCR, real-time PCR, and Western blot analysis, using an EGFRvIII-transfected glioblastoma cell line and glioblastoma tumors as positive controls.

Results: Our analysis failed to detect mutations within the tyrosine kinase domain of EGFR in the 11 cell lines and 75 patients tested. The initial analysis of EGFRvIII expression by immunohistochemistry revealed that at least 50% of the patient tumors expressed EGFRvIII in a urothelial carcinoma tissue microarray. Conflicting reports exist, however, regarding the extent of EGFRvIII expression in tissues owing to the specificity of the antibodies and the methodologies used. Therefore, we sought to validate this observation by reverse transcription PCR, real-time PCR, and Western blot analysis. In these assays, none of the samples were positive for EGFRvIII except for control transfectants and glioblastomas.

Conclusions: When our results are taken together, we conclude that alterations within the tyrosine kinase domain and expression of EGFRvIII are rare events in bladder cancer. The present study has clinical implications in selecting tyrosine kinase inhibitors for the therapy of urothelial carcinoma.

The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane protein that exhibits intrinsic tyrosine kinase activity following the binding of one of its cognate ligands. Activation of EGFR leads to a wide variety of biological responses such as proliferation, differentiation, migration, modulation of apoptosis, invasion, and metastasis (1, 2).

Overexpression of EGFR has been described in many tumor types, including non–small cell lung cancer (NSCLC), colorectal, gastric, pancreatic, ovarian, breast, and bladder cancers (3). However, it is unclear whether EGFR overexpression would be an accurate prognostic factor in these tumors. In addition to gene amplification, other structural alterations occur, such as mutations in the kinase domain; truncations have been reported in several tumor types such as NSCLC, glioblastoma, and pancreatic cancer (4–8). Based on these observations, EGFR has become an attractive molecular target for therapy in a wide variety of tumor types. The activity of several monoclonal anti-EGFR and small drug inhibitors (i.e., gefitinib and erlotinib) is a subject of intensive investigations and clinical trials (9).

Recently, point mutations or short deletions within the kinase domain of EGFR have been reported in lung, head and neck, and colorectal cancers (4, 5, 10–13). All of these alterations occur in exons 18 to 21 of EGFR. These mutations within the kinase domain of EGFR raised considerable interest because they have been reported to correlate, in some patients with NSCLC, with clinical responses to the tyrosine kinase inhibitor gefitinib (ZD1839, Iressa; refs. 4, 5, 14). Clinical trials
with gefitinib in patients with NSCLC revealed a low response rate in most studies carried out in the U.S. and Europe, whereas a higher response rate was reported in Japan, especially among nonsmoking women with EGFR mutations.

In addition to mutations within the kinase domain of EGFR, the most frequent alteration, the EGFR variant III (EGFRvIII), is characterized by a consistent and tumor-specific in-frame deletion of 801 bp from the extracellular domain. This truncation splits a codon and results in a novel glycate at the fusion junction (15, 16). The resulting protein has a constitutively active tyrosine kinase (17) that greatly enhances the tumorigenicity of these cells (18, 19). The EGFRvIII form is in 40% of glioblastomas, but not in any normal tissues, making it an ideal target for tumor-specific immunotherapy for glioblastomas (15, 20–22). Indeed, the expression of EGFRvIII has been clinically correlated with enhanced tumor cell growth, invasion, and metastasis (23, 24), due to its constitutive kinase activity and its ability to deregulate signal transduction and cell cycle checkpoints (24–27). Furthermore, it has recently been reported that the neoplastic phenotype of EGFRvIII is relatively resistant to gefitinib and that higher doses, repeated dosing, and longer exposures are required to decrease receptor phosphorylation (28).

Several studies have shown that gefitinib can inhibit bladder cancer cell proliferation and promote apoptosis in vitro (29–32), supporting the idea that a subset of patients might benefit from treatment with this drug. In this study, we sought to examine the status of EGFR mutations/truncations in gefitinib-responding and nonresponding urothelial carcinoma cell lines and in the tumor specimens of patients with bladder cancer. We report that EGFR mutations were very rare within the kinase domain, and thus, must not account for the response of urothelial carcinoma cells to gefitinib. Moreover, the expression of EGFRvIII in bladder cancer was extremely rare, and therefore, does not contribute to the malignant phenotype of this tumor.

Materials and Methods

Cell lines. The 253J-P cell line was purchased from American Type Culture Collection (Manassas, VA). The 253 J-B V metastatic variant was isolated from the 253-J-P cells by orthotopic “recycling” as described previously (33). KU7 cells were provided by Dr. William Benedict (M.D. Anderson Cancer Center, Houston, TX). The UM-UC series of urothelial carcinoma cell lines, UM-UC-3, UM-UC-5, UM-UC-6, UM-UC-9, UM-UC-10, UM-UC-13, UM-UC-14, and UM-UC-16, were maintained and genotyped by the specimen core of the Genitourinary Specialized Programs of Research Excellence in bladder cancer. We have previously reported that the UM-UC-5, UM-UC-6, UM-UC-10, and 253 J-B V cell lines are sensitive to gefitinib in vitro (34). The U87MG glioblastoma cell line was transfected with EGFRVIII using Lipofectamine (Invitrogen, Carlsbad, CA). All of the cells were incubated at 37°C and 5% CO2/95% air.

Human tissue samples. Mutational analysis of EGFR was done on DNA extracted from 75 frozen human bladder tumor samples. The immunohistochemical expression patterns of EGFR were analyzed in a tissue microarray containing 251 samples of bladder tumors. The expression levels of full-length and truncated forms of EGFR were verified by Western blotting, quantitative real-time PCR, and reverse transcription PCR in 24, 12, and 9 samples, respectively.

The cohort of patients selected for the different experiments (i.e., mutational analysis, immunohistochemistry, PCR, real-time PCR, and Western blot) was chosen at random and was based on the availability of DNA, RNA, and protein. Among the 75 cases chosen for sequencing, 21 samples were also included in the tissue microarray slide. These 21 samples were also used for reverse transcription PCR and real-time PCR analysis. Eight of the 24 samples analyzed by Western blot were included in the tissue microarray. There were no patients treated with gefitinib in this cohort.

For DNA extraction, tumor tissue samples from transurethral resections were grossly dissected and the presence of tumor tissue in a sample selected for DNA extraction was confirmed on parallel frozen section. The portion of the tissue containing predominantly tumor cells (~90%) was dissected, cut into small pieces, and transferred to a conical tube containing 5 mL of PBS.

The 12 paired samples of adjacent urothelium and tumor were prepared from cystectomy specimens as previously described (36). In brief, one representative section from the central area of grossly identified tumor was removed, cut into small pieces, and transferred to a conical tube containing 5 mL of PBS. The tube was mechanically agitated for 5 minutes using a vortex mixer to release the tumor cells. The presence of the tumor in the tissue was confirmed by microscopic analysis of a parallel frozen section stained with H&E. Cell suspensions of urothelium adjacent to tumors were prepared by mechanical scraping of the mucosal surface with a razor blade. First, a selected adjacent mucosal area was marked with India ink and its surface urothelium was scraped with a razor blade. Multiple histologic sections of the scraped area were submitted for microscopic examinations to rule out contamination with invasive tumor or with grossly occult microscopic papillary lesions. The samples were then transferred to conical tubes containing PBS, and their purity was determined by cytoligic examinations of cytospin preparations. The presence of cells of interest in all samples was confirmed by microscopic analysis of cytospin preparations. The baseline expression levels of EGFR were tested on cell suspensions of normal urothelial cells prepared from the ureters of nephrectomy specimens done for other medical reasons.

Only those tissue samples that yielded >90% microscopically recognizable intact normal, dysplastic, or tumor cells were used for DNA and RNA extraction. Six transitional cell carcinomas were classified according to the three-tier WHO histologic grading system (grades 1-2, low grade; grade 3, high grade) and growth pattern (papillary versus nonpapillary; ref. 37). The levels of invasion were defined according to the tumor-node-metastasis staging system (38). Stage T1 (lamina propria invasion) was divided into T1a (no muscularis mucosae invasion) and T1b (muscularis mucosae invasion), which have a significantly higher risk of progression. So, as in our previous publications, the tumors were dichotomized into superficial (Ta-T1a) and invasive (T1b and higher) groups (39).

Mutational analysis. DNA was isolated from tumor specimens using the QIAmp DNA kit, whereas the DNA from the cell lines was isolated by a genomic DNA extraction kit (Qiagen, Valencia, CA). PCR was used to amplify the four exons comprising the kinase domain of the EGFR gene. The primer pairs used were previously described by Lynch et al. (4) and generated by Integrated DNA Technologies, Inc. (Coralville, IA): exon 18, 5′-CAA ATG AGC TGG CAT TGG CGG TGT C-3′ (sense) and 5′-GAT TCC CCC AAA AAC TCA GTG AAA C-5′ (antisense); exon 19, 5′-CTT TAG GTG CGG CTC CAC AGC-3′ (sense) and 5′-CAT TTA GGA TGG GAT GAT GAC C-5′ (antisense); exon 20, 5′-CCA TGA GTA CTT ATG TGG AAA CTC-3′ (sense) and 5′-CAT ATC CCC ATG GCA AAT TCG TCT TCG-5′ (antisense); and exon 21, 5′-CTA CGC TTT ACC AGG CAT AGC-3′ and 5′-GTC GCG ACG TCA CCA AGA ATG TCT GG-5′ (antisense). PCR was done using Taq DNA polymerase (Promega, Madison, WI) with the following cycle conditions for exons 18, 20, and 21: 5 minutes of denaturation at 94°C, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by an additional extension for 5 minutes at 72°C. The PCR reaction for exon 19 was done with Titanium Taq.
DNA polymerase (Clontech, Mountain View, CA) with the following cycle conditions: 3 minutes of denaturation at 95°C, followed by 30 cycles of 95°C for 30 seconds, 70°C for 30 seconds, and 72°C for 1 minute then followed by an additional extension for 5 minutes at 72°C. Nested PCR primers were used for sequencing for exons 18, 20, and 21: exon 18, 5′-CAACGTCGGTCTCCTGCGACCACGCA-3′ (sense) and 5′-CCA AAC ACT CAG TGA AAC AAA GAG-3′ (antisense); exon 20, 5′-GAATCAAATCGAGCCATTATGGC-3′ (sense) and 5′-GCCA AAC TCT TGC TAT CCG AGG AG-3′ (antisense); exon 21, 5′-CAG CCA TAA GTC CTG CAG GCG G-3′ (sense) and 5′-CAT CCT CCG CTG CAT CTG TTA AAC-3′ (antisense). For sequencing, exon 19 primers were the same as amplification primers.

**Reverse transcription PCR analysis.** RNA was extracted from the tumors and cell lines using TRIzol reagent protocol (Invitrogen) and was converted to cDNA using high-capacity cDNA archive kit (Applied Biosystems). Using 150 ng of cDNA in a 25 μl reaction AmpliTaq Gold PCR Mix (Applied Biosystems), PCR was done with the following cycle conditions: 3 minutes of denaturation at 94°C, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes followed by an additional extension for 7 minutes at 72°C. EGFR primers used were as previously described (40) and purchased from Integrated DNA Technologies: the forward primer sequence was 5′-ATG CGA CCC TCC GCG AGG-3′ and the reverse sequence was 5′-GAT GTG TGA AGG ATG-3′. For loading control, the glyceraldehyde-3-phosphate dehydrogenase forward primer was 5′-GAG CCA CAT CGG TCA-3′ and the reverse primer was 5′-CTT CTC ATG GTT CAC ACC C-3′. The amplified fragments were analyzed on 2% agarose gel stained with ethidium bromide and visualized using UV light.

**Western blot.** Whole-cell lysate was prepared using Triton X-100 lysis buffer at 4°C. Lysates were cleared by a 10-minute centrifugation at 14,000 × g, and protein concentration was determined by the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Each sample contained 100 μg, which was boiled for 5 minutes at 100°C in buffer containing SDS, and was separated by 6% and 10% SDS-PAGE. The proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked overnight in blocking solution [5% nonfat dry milk/TBS plus 0.1% Tween 20 (TTBS)], and then incubated with two primary EGFR antibodies (2232 and Ab-15) targeting the cytoplasmic domain at 1:1,000 dilution (Cell Signaling, Inc., Beverly, MA and Neomarker, respectively). The primary antibody was washed in TTBS and then incubated in secondary antibody peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse (Amersham Biosciences) at a 1:2,000 dilution with PBS. Streptavidin-horseradish peroxidase and then 3-amino-9-ethylcarbazole were added. The samples were counterstained with Gill’s no. 3 hematoxylin and then washed with water and mounted with universal mount.

**Real-time PCR.** RNA (2 μg/50 μL) from urothelial cell lines (UM-UC-1, UM-UC-2, UM-UC-3, UM-UC-6, UM-UC-9, UM-UC-10, UM-UC-12, UM-UC-13, UM-UC-14, UM-UC-15, UM-UC-16, and UM-UC-17) as well as tumor and adjacent specimens were made into cDNA using TaqMan reverse transcriptase reagents (Applied Biosystems). The cDNA was then diluted to a 1:10 concentration for subsequent reverse transcription PCR. RNA was extracted from the globlastoma tumor samples (4A2, 5A2, 47, being positive for EGFVRVIII expression and B1, C2, E1, being negative for EGFVRVIII) using High Pure RNA Paraffin Kit (Roche, Indianapolis, IN). The primer and fluorescence probes were designed according to the Assays-by-Design manufacturer’s instructions, followed by exposure to X-ray film. Equal loading of protein samples was confirmed by incubating membranes with a primary antibody for pan-actin (Ab-5) at 1:1,000 dilution (Neomarker).

**Results**

**Mutational analysis of exons 18 to 21.**

To determine the status of mutations within the kinase domain of EGFR, 75 urothelial carcinoma patient samples, and 11 cell lines, 4 of which have previously been shown to be sensitive to gefitinib, were sequenced for mutations in exons 18 to 21. Representative samples of sequencing analysis are seen in Fig. 1. No mutations were found in the 75 patient samples. In addition, no mutations were found in the 11 cell lines tested: 253JP, 253J B-V, KU17, UM-UC-3, UM-UC-5, UM-UC-6, UM-UC-9, UM-UC-10, UM-UC-13, UM-UC-14, and UM-UC-16. Based on this sequence analysis, the rationale behind the gefitinib sensitivity previously reported (34) in the bladder cancer cell lines, 253J B-V, UM-UC-5, UM-UC-6, and UM-UC-10 cannot be solely attributed to mutations within the kinase domain of EGFR. We concluded that mutations within the kinase domain of EGFR in bladder cancer are rare events.
Expression of EGFRvIII by tissue microarray. We next determined the incidence of EGFRvIII expression in bladder cancer in order to evaluate its potential as a target for tumor-specific cancer therapies. To that end, we determined the expression of EGFRvIII on a bladder cancer tissue microarray. Using a monoclonal antibody for EGFRvIII, initial analysis of the 251 bladder tumor samples revealed that 50% of the tumor samples stained positive for EGFRvIII (Fig. 2A and B). The four normal urothelium on the array also stained positive (Fig. 2C), a contradiction with previous reports that normal tissue did not express EGFRvIII (21). This immunohistochemistry staining raised concerns about the specificity of the antibody used to detect EGFRvIII expression and prompted us to validate its expression by real-time PCR, reverse transcription PCR, and Western blot analysis.

Lack of expression of EGFRvIII in bladder cancer. Due to inconsistencies reported previously by Rae et al. (41) in the detection of the truncated EGFR expression by immunohistochemistry, real-time PCR was used for validation. Real-time PCR was done using primers targeting exons 2 to 3 to amplify the wild-type EGFR, and primers targeting exons 13 to 14 to amplify both wild-type and truncated EGFR. The relative level of expression of exons 2 to 3 was subtracted from exons 13 to 14 to acquire the level of EGFRvIII on 12 bladder tumors and their adjacent specimens, as well as six glioblastomas, of which three were known to express EGFRvIII. The three EGFRvIII-expressing glioblastomas showed positive expression of EGFRvIII, but the three negative glioblastoma tumors, as well as the bladder tumors and adjacent specimens lacked such expression (Fig. 3A). The 12 bladder cancer cell lines showed no expression of truncated EGFR, whereas the U87MG-EGFRvIII glioblastoma cell line transfected with EGFRvIII expressed the truncated variant and served as a positive control (Fig. 3B). Note that wild-type EGFR expression was observed in samples tested with overexpression in ~30% of the samples. To confirm the lack of EGFRvIII expression in the tested samples, reverse transcription PCR of wild-type and truncated EGFR as well as Western blot analysis was done. For reverse transcription PCR, the U87MG-EGFRvIII cell line was again used as a positive control. The 352 bp band corresponded with the expression of EGFRvIII and was only seen in the positive control, U87MG-EGFRvIII (Fig. 4). Wild-type EGFR is detected in the 253J B-V cell line (Fig. 4). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal loading control, as represented by the 420 bp band. Reverse transcription PCR verified the absence of expression of EGFRvIII at the mRNA level (Fig. 4). A total of
21 samples were analyzed by real-time PCR and reverse transcription PCR. Ten of these samples were negative for EGFRvIII expression and 11 were positive for EGFRvIII as determined by tissue microarray staining, indicating that the observed lack of EGFRvIII expression was not due to the a priori selection of samples that were negative for EGFRvIII expression by immunohistochemistry. Western blot analysis of normal urothelium, 24 patient tumors, and 4 cell lines using EGFR antibodies targeting the cytoplasmic domain revealed no EGFRvIII protein expression. Of these patients’ samples, five specimens were EGFRvIII-negative and three were EGFRvIII-positive based on immunohistochemistry. Representative samples are shown in Fig. 5A. Protein expression of EGFRvIII was seen only in the positive control, U87MG-EGFRvIII cell line (Fig. 5A and B). Taken together, our results show that the expression of the truncated variant of EGFR in urothelial carcinoma is a rare event.

Discussion

NSCLC and bladder cancer share a few similarities. Like NSCLC, bladder cancer is more prevalent in men than in women and is associated with tobacco smoking. Based on these similarities, and recent reports suggesting that EGFR mutations within the kinase domain are associated with selected NSCLC patient groups and confer a greater response rate to EGFR inhibitors such as gefitinib, we determined the prevalence of mutations in exons 18 to 21 of EGFR in 11 cell lines and 75 tumor specimens of urothelial carcinoma.

Interestingly enough, four of the cell lines tested were found to be sensitive to gefitinib in vitro, whereas the other seven cell lines were resistant to the cytotoxic effects of the drug (34). Resistance to gefitinib was associated with uncoupling between the EGFR and mitogen-activated protein kinase, and could be predicted by analyzing the activation of Gsk-3β and cyclin D1. In certain cell lines, this uncoupling was due to a dependence...
Fig. 5. Western blot analysis for the expression of EGFR and EGFRvIII in six tumor specimens (A) and four bladder cancer cell lines (B). The EGFRvIII-transfected U87MG cells and normal urothelium served as positive and negative controls, respectively. Actin was used as an indication for equal loading. EGFRvIII expression was not observed in any of the tumor specimens or cell lines tested but only in the positive control. Wild-type EGFR was detected in the cell lines as well as in one tumor sample.

on platelet-derived growth factor-β signaling for cell cycle progression (34). Our data indicate that the response to gefitinib by bladder cancer cells cannot be attributed solely to the status of EGFR mutations within the kinase domain and that other variables and cell properties must affect the response to gefitinib.

Indeed, recent work, based on the retrospective analysis of a group of patients with NSCLC treated with gefitinib, suggests that increased EGFR gene copy number and EGFR protein overexpression may be associated with improved survival (42).

In bladder cancer, it was shown that EGFR is overexpressed in 31% to 48% of tumors, mainly in association with muscle invasion (43–45). Thus, it will be important to correlate EGFR gene amplification and protein expression with the response to gefitinib or other tyrosine kinase inhibitor drugs targeting EGFR in the ongoing clinical trials in patients with bladder cancer (46).

In this study, we were also unable to detect measurable levels of EGFRvIII expression in any tumor cell line or patient specimens. Although our initial immunostaining for EGFRvIII in urothelial carcinoma tissue microarrays detected EGFRvIII expression in 50% of the samples, subsequent analysis by real-time PCR, reverse transcription PCR, and Western blot failed to detect its expression in the tumor samples. These conflicting results between immunohistochemistry staining and reverse transcription PCR and real-time PCR for the expression of EGFRvIII were also documented for breast cancer (41). We now stress the point of validating any data that emerge from using antibody and tissue microarrays to measure the expression of EGFRvIII, which may produce false-positive results.

Although we could not detect EGFRvIII mRNA or protein expression in any of the investigated bladder cancer cell lines, or in the large number of tumor specimens, in contrast, many of these samples do express wild-type EGFR at various levels. Given that there was expression of wild-type EGFR, that it has several potential ligands, and that it can be activated in several ways, it is doubtful that EGFRvIII is a major drive in the EGFR pathway in bladder cancer. Moreover, our study shows that EGFRvIII does not play a role in bladder cancer and therefore does not represent a viable target for bladder cancer therapy.

These results have clinical implications in the sense that when considering treatment with tyrosine kinase inhibitors for bladder cancer, there is no need to consider EGFRvIII-preferential inhibitors such as AG1478 (47). However, the challenge of predicting which patients will respond to gefitinib and other tyrosine kinase inhibitors remains to be elucidated.

References


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