Epidermal Growth Factor Receptor Protein Expression and Gene Amplification in Small Cell Carcinoma of the Urinary Bladder

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Abstract

Purpose: Small cell carcinoma of the urinary bladder is a highly aggressive malignancy with an average life expectancy of only a few months. Epidermal growth factor receptor (EGFR) has been implicated in the pathogenesis and progression of many malignancies. This study was done to investigate EGFR protein expression and gene amplification in a large series of small cell carcinomas of the urinary bladder.

Experimental Design: Fifty-two cases of urinary bladder small cell carcinoma were included in this study. Immunostaining for EGFR was done on paraffin-embedded tissue sections, and gene amplification for EGFR was done by fluorescence *in situ* hybridization. EGFR expression was correlated with clinicopathologic characteristics and clinical outcome.

Results: All 52 patients, except 1, had advanced disease (T_2 or above) at presentation. Immunohistochemically, positive EGFR expression was observed in 14 of 52 (27%) cases. No EGFR gene amplification was observed in any of 52 cases by fluorescence *in situ* hybridization. Forty cases had polysomy and the remaining 12 cases displayed disomy. No correlation between EGFR protein expression and gene amplification was shown. There was no correlation between EGFR expression and clinicopathologic characteristics.

Conclusions: EGFR is expressed in a subset of urinary bladder small cell carcinomas; however, expression of EGFR does not correlate with clinicopathologic variables. At the molecular level, EGFR overexpression in small cell carcinoma of the urinary bladder does not seem to be caused by gene amplification. The expression of EGFR raises the possibility that EGFR may be a potential therapeutic target in the treatment of this malignancy.

Small cell carcinoma of the urinary bladder is a rare and devastating malignancy with a marked propensity to metastasize (1–3). Demographically, most patients are male, and most present with the disease during their 6th to 7th decade of life (1, 4). Presenting clinical complaints include hematuria, dysuria, and urinary obstructive symptoms. Occasionally, patients have paraneoplastic syndromes. Coexistence with other types of carcinoma, such as urothelial carcinoma, is quite common. The mortality is very high despite combined treatment modalities, including surgery, radiation therapy, and chemotherapy (5). The molecular pathogenesis of the disease is not fully elucidated and merits additional investigation.

A small number of cytogenetic and molecular genetic studies have identified several genetic foci that may harbor tumor suppressor genes and oncogenes that may influence the pathogenesis of small cell carcinoma of the urinary bladder. A cytogenetic study by Atkin et al. (6) showed *hypertriploidy and hypertetraploidy*, associated with extensive chromosomal rearrangements involving chromosomes 1 to 3, 5 to 7, 9, 11, and 18. Subsequently, Terracciano et al. (7) observed frequent deletions of 10q, 4q, 5q, and 13 q and gains of 8q, 5q, 6p, and 20q, and Leonard et al. (8) showed monosomy 9, homozygous deletion of p16, and trisomy 7.

Epidermal growth factor receptor (*EGFR*) gene, one of the oncogenes on chromosome 7, has been implicated by several investigators in the pathogenesis and progression of many malignancies (9). Overexpression of *EGFR* in various types of carcinoma has prompted the development of two classes of anti-*EGFR* agents: monoclonal antibodies directed at the extracellular domain of the receptor (cetuximab, panitumumab, etc.) and small-molecule, competitive inhibitors of the tyrosine kinase of the receptor (gefitinib, erlotinib, etc.) (10). Because targeted therapies against *EGFR* have shown considerable success in the treatment of advanced colorectal adenocarcinoma and non–small cell carcinoma of the lung, it seems prudent to investigate the potential utility of these therapies in the management of other neoplasms, such as small cell carcinoma of the urinary bladder.

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Received 8/30/06; revised 11/6/06; accepted 11/14/06.

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doi:10.1158/1078-0432.CCR-06-2167

This investigation was designed to provide further insights into the molecular pathogenesis of small cell carcinoma of the urinary bladder and to help define potential therapies for this rare malignancy. We evaluated *EGFR* protein expression by immunohistochemical staining and gene amplification by fluorescence *in situ* hybridization (FISH) in a series of 52 cases of small cell carcinoma of urinary bladder and sought correlations between *EGFR* protein expression and gene amplification in this tumor. Furthermore, correlations between *EGFR* protein expression and clinicopathologic variables and clinical outcome were investigated.

Materials and Methods

Patients. This study included 52 cases of small cell carcinoma of the urinary bladder that were obtained from participating institutions. The medical records were reviewed. Formalin-fixed, paraffin-embedded tissue was available for all cases. H&E-stained sections were examined. A diagnosis of small cell carcinoma was made only when the morphologic criteria established by the 2004 WHO classification system were met (11). Tumor-node-metastasis stage was assigned according to the criteria specified in the AJCC Cancer Staging Manual, 6th edition (12). The Institutional Review Boards approved this research.

Immunohistochemical analysis. Immunohistochemical staining for *EGFR* was done on formalin-fixed and paraffin-embedded tissue sections using the peroxidase-labeled streptavidin-biotin method. Tissue sections (5 μm) from each patient were used for *EGFR* immunohistochemical staining. Sections were deparaffinized in xylene for 5 min and then rehydrated through graded ethanol to distilled water. Antigen retrieval was done by heating sections for 15 min (Target Retrieval, DAKO, Carpinteria, CA). Endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 5 min. Tissue sections were incubated with primary antibodies against *EGFR* (DAKO) for 10 min followed by biotinylated secondary antibody (DAKO) and peroxidase-labeled streptavidin (DAKO). 3,3′-Diaminobenzidine was used as the chromogen. The extent of staining was evaluated by visual examination microscopically. Each section was scanned at low magnification for membranous staining and scored by evaluating the percentage of tumor cells staining positively and assigning each case to one of the following categories: 0%, 0% to 10%, 10% to 25%, 25% to 50%, 50% to 75%, and 75% to 100% staining, respectively. The intensity of staining was defined as negative (0), weak (1+), moderate (2+), or strong (3+). According to the scoring system recommended by Tsao et al. (13), results were recorded as positive expression (≥10% of cells staining) or negative expression (<10% of cells staining).

Tissue preparations and FISH. Sections (4 μm thick) were cut from paraffin blocks from each patient. The slides were deparaffinized with two washes of xylene, 15 min each, and subsequently washed twice with absolute ethanol, 10 min each, and then air dried in the hood. Next, the slides were treated in 0.1 mmol/L citric acid (pH 6.0; Zymed Laboratories, San Francisco, CA) at 95°C for 10 min and rinsed in distilled water for 3 min followed by a wash of 2× SSC for 5 min. Digestion of the tissue was done by applying 0.4 mL pepsin [5 mg/mL in 0.9% NaCl (pH 1.5); Sigma, St. Louis, MO] at 37°C for 40 min. The slides were rinsed with distilled water for 3 min, then washed with 2× SSC for 5 min, and air dried.

FISH was done with LSI *EGFR*/CEP 7 Dual Color Probe containing centromere chromosome 7 (Centromeric Enumeration Probe, CEP7, Spectrum Green) and *EGFR* gene located at 7p12 (*EGFR*, Spectrum Orange; Vysis, Downers Grove, IL). The probe was diluted with tDenHyb2 (Insitus, Albuquerque, NM) in a ratio of 1:50. Diluted probe (5 μL) was applied to each slide in reduced light conditions; the slides were then covered with a coverslip measuring 22 × 22 mm and sealed with rubber cement. Denaturation was achieved by incubating the slides at 80°C for 10 min in a humidified box followed by hybridization at 37°C overnight. The coverslip was removed and the slides were washed extensively with two 45°C prewarmed 0.1× SSC/1.5 mol/L urea, 20 min for each, followed by a wash with 2× SSC for 20 min and a 2× SSC/0.1% NP40 for 10 min at 45°C. The slides were further washed with 2× SSC at room temperature for 5 min and air dried. The slides were counterstained with 10 μL 4′,6-diamidino-2-phenylindole (Insitus), covered with coverslips, and sealed with nail polish.

The slides were examined using a Zeiss Axioplan 2 microscope (Zeiss, Göttingen, Germany) with the following filters from Chroma (Brattleboro, VT): SP-100 DAPI, FITC MF-101 for Spectrum Green (CEP7), and Gold 31003 for Spectrum Orange (*EGFR*). The images were acquired with a CCD camera and analyzed with MetaSystem Isis software (MetaSystem, Belmont, MA). Four sequential focus stacks with 0.4-μm intervals were acquired and then integrated into a single image to reduce thickness-related artifacts.

FISH analysis. The method of analysis was as previously described in detail (14, 15). In brief, for each slide, 100 neoplastic nuclei were scored for signals from both CEP7 and *EGFR* probes under the fluorescence microscope with ×1,000 magnification. Nuclei of non-neoplastic urothelial cells from the same patient were used as control. Signals from nonoverlapping nuclei were included in the counts. Two signals of the same size in close proximity, not connected by a link, were counted as two signals. A diffuse signal was regarded as a signal if it was contiguous and within an acceptable boundary. Two small signals connected by a visible link were counted as one signal. Overlapping nuclei and nuclei with uncertain signal were not included in the counts. In accordance with the criteria of Alvarez et al. (15), *EGFR* amplification was considered to be present if >10% of the nuclei contained multiple *EGFR* signals and the EGFR/CEP7 ratio was >2. Polysomy for an individual tumor was defined as follows: >10% of the nuclei contained greater than two signals of CEP7 or EGFR and the EGFR/CEP7 ratio was <2. Individual tumors were considered to show disomy if the percentage of disomic cells (with two copies of EGFR and CEP7) fell into the range of summarized average ± three SD of the normal control cells and did not meet the criteria for amplification or polysomy. For each FISH preparation, known positive and negative cells were used as controls. Preparations were considered valid if >90% of the cells showed bright signals.

Statistical analysis. Data were analyzed using Statistical Analysis System version 9.1 (SAS Institute, Cary, NC). Correlations between EGFR protein expression and gene amplification and between *EGFR* protein expression and various clinicopathologic variables were evaluated by using Cochran-Mantel-Haenszel tests for correlated ordered categorical outcomes. A P value of <0.05 was considered significant, and all P values were two sided.

Results

Immunohistochemical study of EGFR gene expression and its correlation with clinicopathologic variables. Fifty-two patients were included in the study. There were 41 males and 11 females. The patients' ages ranged from 36 to 85 years (mean, 67 years). The average follow-up time was 20.6 months and median follow-up time was 11 months. The average survival time was 14.1 months after treatment. All patients, except one, had advanced disease (T_2 or above) at the time of initial clinical presentation. Pathologic stage in these cases was as follows: T_1 (1 patient), T_2 (26 patients), T_3 (22 patients), and T_4 (3 patients). Positive immunohistochemical expression of EGFR was observed in tissue sections from 14 of 52 (27%) patients: 7 with 10% to 25% staining, 4 with 25% to 50% staining, 1 with 50% to 75% staining, and 2 with 75% to 100% staining (Fig. 1). In contrast, immunohistochemical expression of EGFR was absent

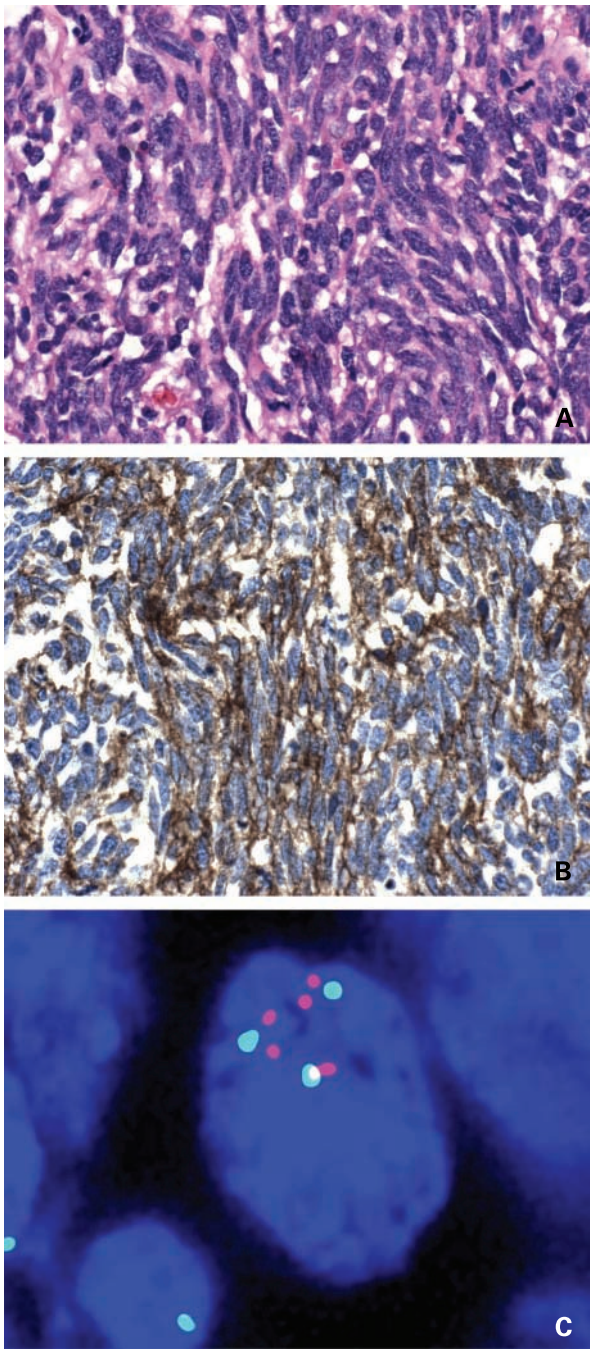

Fig. 1. Analysis of EGFR expression. *A,* small cell carcinoma of the urinary bladder is composed of small round or spindle-shaped cells in compact sheets. *B,* immunohistochemistry showed EGFR expression in a mixed membrane and cytoplasmic distribution. *C,* FISH reveals a polysomy pattern. Green, centromere 7 signals; red, *EGFR* signals.

or very limited in 38 of 52 (73%) cases: 34 with no staining and 4 with 1% to 10% staining. There was no correlation between EGFR expression and several pertinent clinicopathologic characteristics, including age (P = 0.64), gender (P = 0.26), history of smoking (P = 0.67), clinical stage (P = 0.13), pathologic T stage (P = 0.53), presence or absence of lymph node metastasis (P = 0.27), and presence or absence of distant metastasis (P = 0.34). No significant difference was shown between the survival of patients whose tissues showed positive versus negative EGFR expression (P = 0.91).

FISH study for EGFR gene amplification. No *EGFR* gene amplification was observed in any of the 52 cases. However, polysomy (more than two copies of CEP7 and *EGFR*/CEP7 ratio <2) of chromosome 7 was observed in 40 cases (Fig. 1), and the remaining 12 cases displayed disomy. No significant difference was detected between the survival of patients whose tissues showed disomic versus polysomic FISH patterns (P = 0.39).

In addition, normal bladder urothelium from the same patient was also evaluated. At least 50 normal urothelial cells were counted for the signals of CEP7 and *EGFR*. None showed polysomy or amplification of EGFR. EGFR/CEP7 ratio in the normal control ranged from 0.9 to 1.1, with two copies of EGFR and CEP7 in 67% to 79% of cells. In addition, these normal controls showed no gain of EGFR or CEP7 in >10% of cells.

Correlation between EGFR protein expression and gene amplification. We compared EGFR gene amplification data with EGFR protein expression data in all 52 cases of small cell carcinoma of the urinary bladder (Table 1). Positive EGFR protein expression was present in 14 of 52 cases (12 cases had polysomy and 2 cases had disomy). EGFR protein expression was absent or very limited in 38 of 52 cases (28 patients had polysomy and 10 cases had disomy). In contrast, no EGFR gene amplification was observed in any of these 52 cases. Forty of 52 cases had polysomy (12 patients had positive EGFR protein expression and 28 cases had no protein expression). The remaining 12 cases had disomy (2 cases had EGFR protein expression and 10 cases had no EGFR protein expression). No correlation between EGFR protein expression and gene amplification was shown (P = 0.33).

Discussion

It is well known that activation of proto-oncogenes and inactivation of tumor suppressor genes are involved in tumorigenesis. Proto-oncogenes influence normal cell growth pathways and cell cycle regulation. When proto-oncogenes undergo mutations, rearrangements, insertions, or amplification, this can result in constitutive activation of genes (oncogenes) and uncontrolled cellular proliferation (16). Various classes of oncogene proteins have been characterized at the molecular level, including growth factors, growth factor receptors, transcriptional factors, etc.

Table 1. EGFR immunostaining and FISH of small cell carcinoma of the urinary bladder

EGFR immunostaining		EGFR FISH	
%	No. cases	Polysomy	Disomy
0	34	24	10
1-10	4	4	0
10-25	7	6	1
25-50	4	4	0
50-75	1	1	0
75-100	2	1	1
Total	52	40	12

The *EGFR* superfamily is composed of several transmembrane growth factor tyrosine kinases that share similarities in structure and function. They include four distinct receptors (EGFR/erB-1, *HER2/erB-2*, *HER3/erB-3*, and *HER4/erB-4*) that play important roles in cell survival and proliferation (17). EGFR (HER-1 and c-erbB-1) is expressed in many human epithelial malignancies, including carcinomas of the head and neck, breast, lung, ovary, uterine cervix, prostate, kidney, and colon (17–21). Overexpression of EGFR in these carcinomas correlates with a poor prognosis and decreased survival (22, 23).

The *EGFR* gene is located on chromosome *7p12-p22* and encodes a 170-kDa glycoprotein. Structurally, EGFR is composed of an NH_2-terminal extracellular EGF ligand-binding domain, a transmembrane lipophilic segment, and a COOH-terminal intracellular region containing a tyrosine kinase domain. Two ligands can activate EGFR: EGF and transforming growth factor. After the ligand binds EGFR, the receptors homodimerize or heterodimerize. Subsequently, tyrosine kinase of the intracytoplasmic domain of the receptor is activated and signal pathways are triggered that may lead to cellular growth, differentiation and proliferation, invasion, and increased metastatic potential of the tumor cells (24, 25).

Overexpression of the EGFR has been shown in 40% to 80% of cases of non–small cell carcinomas of the lung and has been associated with a poor prognosis (26). Anti-EGFR therapeutic agents, such as tyrosine kinase inhibitors, have been studied extensively in non–small cell carcinomas of the lung; the results thus far are inconclusive. Increased response rate after treatment of tyrosine kinase inhibitors has been shown in patients with positive EGFR immunostaining by some investigators, but not others. However, some, but not all studies, have revealed that positive EGFR gene amplification by FISH is associated with significantly better survival after treatment with tyrosine kinase inhibitors. Furthermore, new evidence suggests that patients with positive EGFR by both immunohistochemistry and FISH have better response to gefitinib treatment, although more studies are still being conducted to more clearly define variables for patient selection (27). Recent studies have shown that mutations in the EGFR gene tyrosine kinase domain (e.g., in-frame deletion in exon 19 and missense point mutation in exon 21) have been associated with better responsiveness of non–small cell lung carcinomas to treatment with tyrosine kinase inhibitors (13).

Overexpression of EGFR has been regarded as limited or nonexistent in small cell carcinoma of the lung. Despite this, Araki et al. (28) reported the first case of metastatic small cell lung cancer that was successfully treated with gefitinib. *In vitro* studies by Tanno et al. (29) showed that two of five cell lines of lung small cell carcinoma expressed functional EGFR by Western blot and, in addition, showed that gefitinib inhibited phosphorylation of extracellular signal-regulated kinase 1/2 by EGF in cell lines with detectable and undetectable EGFR expression. The above findings imply that the biological behavior of small cell carcinoma may not necessarily correlate well with the degree of observable EGFR expression.

Currently, data about EGFR expression in small cell carcinomas of the urinary bladder are limited. Abrahams et al. (2) did immunostaining for EGFR in 11 cases of small cell carcinoma of the urinary bladder and found positive staining in 4 of 11 (36%) cases. In our study, we showed immunohistochemical evidence of EGFR gene expression in 14 of 52 (27%) cases of small cell carcinoma of the urinary bladder. No correlation between EGFR gene expression and clinicopathologic variables was found in our study. We used a cutoff of 10% of tumor cells, showing EGFR staining as the lower limit of positive EGFR expression in this study because this 10% cutoff value had been adopted by other investigators (13), although a 1% lower limit of positive tumor cell staining has been used as a cutoff value by others (30). Consequently, it should be kept in mind that the percentage of cases expressing EGFR in our study would have been higher if we had used a 1% cutoff as our criteria for EGFR positivity. In addition, Cappuzzo et al. (31) used a semiquantitative approach to evaluate for EGFR immunostaining by Zymed antibody (Zymed Laboratories) based on the fraction of positive cells (0-100%) times staining intensity (0, negative; 1, trace; 2, weak; 3, moderate; 4, intense), generating overall scores ranging from 0 to 400. Specimens with scores 0 to 200, 201 to 300, and 301 to 400 were respectively classified as having negative or low, intermediate, and high levels of expression. However, this scoring system is complicated and possibly subject to variation in reproducibility due to the subjectivity involved in assigning staining intensity, and therefore, we elected not to adopt this method in our study.

No prior study has investigated *EGFR* gene at the molecular level in small cell carcinoma of the urinary bladder. In contrast to our observation that *EGFR* protein expression was evident in 27% of patients, we found no EGFR gene amplification in any of 52 cases. However, we did show frequent polysomy, possibly representative of "low-level" gene amplification, in 40 of 52 cases.

Currently, methods for scoring EGFR FISH signals have been variable among different studies. In this study, we adopted the criteria of Alvarez et al. (15). Polysomy was defined by the presence of at least 10% of nuclei with greater than two signals of CEP7 and EGFR and the EGFR/CEP7 ratio was <2. Amplification for EGFR was defined by the presence of >10% of nuclei containing multiple EGFR signals and by an EGFR/CEP7 ratio that was >2 (15). In cases of non–small cell carcinoma of the lung, Cappuzzo et al. (31) classified EGFR FISH into six categories according to the frequency of tumor cells with specific copy numbers of the *EGFR* gene and the chromosome 7 centromere: (*a*) disomy (≤2 copies in >90% of cells), (*b*) low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10-40% of the cells, ≥4 copies in <10% of cells), (*c*) high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells, ≥4 copies in <10% of cells), (*d*) low polysomy (≥4 copies in 10-40% of cells), (*e*) high polysomy (≥4 copies in 40% of cells), and (*f*) gene amplification (defined by the presence of tight EGFR gene clusters and a ratio of *EGFR* gene to chromosome of ≥2 or ≥15 copies of EGFR per cell in ≥10% of analyzed cells). For further analysis, they divided the patients into two groups: (*a*) $FISH^+$ groups, high gene copy numbers (gene amplification or high polysomy), and (*b*) $FISH^-$ groups, all other categories (disomy, low trisomy, high trisomy, and low polysomy).

Disomy in our study is equivalent to category 1 of the system proposed by Cappuzzo et al. (31). Polysomy in our study corresponds to all tumors assigned to categories b, c, d, e in the aforementioned system. Criteria for gene amplification as defined in our study were virtually the same as those used in the system of Cappuzzo et al. (31). In short, our scoring system varies from, but also overlaps with, Cappuzzo's scoring system.

Cappuzzo et al. considered tumors with high polysomy and those with amplification to be FISH$^+$ cases; in contrast, we did not observe amplification in our cases of small cell carcinoma of the urinary bladder. However, for reasons stated previously, we elected not to use the complex scoring system proposed by Cappuzzo et al.

Several mechanisms have been proposed to explain overexpression of EGFR, including gene amplification, activating mutations, increasing EGFR transcription or translation causing increased mRNA and protein production, decreased protein destruction, overexpression of receptor ligands, etc. (10, 32). The mechanisms of EGFR protein overexpression are not well understood and seem to differ markedly among various tumor types. In glioblastoma, amplification is a major cause for increased expression of EGFR (32). However, this pathway is not common in carcinoma of the breast, ovary, bladder, prostate, or head and neck (18, 20, 33).

Based on our results, we conclude that EGFR gene amplification does not seem to induce protein overexpression. Expression of the EGFR protein may be influenced by other regulatory pathways as stated previously. Further studies are needed to evaluate these pathways. Significantly, frequent expression of EGFR suggests that therapy targeted against EGFR (such as cetuximab or gefitinib) may be beneficial in a subset of cases of small cell carcinoma of the urinary bladder; this hypothesis is worthy of further investigation. Currently, no information is available about EGFR mutations in small cell carcinoma of the urinary bladder. Future sequencing studies of the EGFR gene in this tumor may help unravel its molecular pathogenesis and may provide additional information about the potential role of anti-EGFR therapy in the management of small cell carcinoma of the urinary bladder.

To our knowledge, this study is not only the largest series that has addressed the incidence of EGFR expression in small cell carcinoma of the urinary bladder but also the first study that has shown absence of EGFR gene amplification at the molecular level, absence of correlation between EGFR overexpression and gene amplification, and absence of correlation between EGFR overexpression and clinicopathologic variables.

In summary, we found in our series of 52 patients with small cell carcinoma of the urinary bladder that EGFR was expressed in 14 (27%) cases. However, we did not find any correlation between EGFR expression and clinicopathologic characteristics, such as age, gender, history of smoking, pathologic T stage, clinical stage, lymph node status, the presence or absence of distant metastases, or survival. No EGFR gene amplification was identified in any of the cases. No correlation between protein expression by immunohistochemical studies and gene amplification by FISH was shown. Our observation of EGFR expression in 27% of cases of urinary bladder small cell carcinoma suggests the possibility that anti-EGFR therapy may be beneficial for a subset of patients with this aggressive and devastating cancer.

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