Epidermal Growth Factor Receptor Ectodomain in the Urine of Patients with Squamous Cell Carcinoma

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ABSTRACT
In this study we determined the levels of the epidermal growth factor receptor (EGFR) in the urine of patients with squamous cell carcinoma compared to levels in the urine of normal volunteers and patients with nonsquamous cell carcinoma. A 24-h urine specimen was collected from 50 normal volunteers, 50 patients with nonsquamous cell carcinoma, and 42 patients with squamous cell carcinoma. An ELISA using mAbs to the external domain of the EGFR was used to measure levels of the receptor in the urine samples. Measurement of the EGFR ectodomain in the 24-h urine specimens showed detectable levels in 15 (36%) of 42 squamous cell carcinoma patients compared to 3 (6%) of 50 controls and 8 (16%) of 50 nonsquamous patients. It was also observed that 10 (53%) of 19 patients with metastatic squamous cell carcinoma had detectable EGFR ectodomain levels compared to 5 (22%) of 23 squamous cell patients with localized disease. Thus, we concluded that the EGFR ectodomain was detectable in the urine in a significantly higher number of patients with squamous cell carcinoma than normal volunteers or patients with nonsquamous cell carcinoma.

INTRODUCTION
EGF is a mitogenic polypeptide which binds to a cell surface receptor. EGF is an important regulator of differentiation and growth in normal and neoplastic cells in vitro and in vivo (1). The Mr 170,000 EGFR consists of an extracellular binding domain, a transmembrane region, and an intracellular domain possessing tyrosine kinase activity. When EGF or transforming growth factor α bind to the EGFR, the tyrosine kinase is activated, resulting in intracellular responses leading to mitosis (2).

Elevated levels of the EGFR have been reported in a variety of human tumors and cell lines, supporting its role in tumor cell growth (3). Its overexpression has been reported in breast cancer (4, 5), adenocarcinoma and squamous lung cancer (5-9), glioma (10), bladder cancer (11, 12), gynecological carcinomas (13), and prostate cancer (14). Sainsbury et al. (15) found that EGFR levels in combination with estrogen receptor status predicted prognosis in breast cancer.

The EGFR gene has been suggested to be the proto-oncogene of the v-erbB oncogene (16). Amplification of the EGFR gene itself, as well as increased expression of receptor mRNA, has been found in renal cell carcinoma (17), breast cancer (18) bladder tumors (11), and squamous cell carcinomas (19-21).

Amplification of the EGFR gene and increased levels of the protein have been found in a number of cell lines derived from squamous cell carcinomas (9). The A-431 cell line, derived from a human squamous cell carcinoma, is known to express 10-50 times more EGFRs than other cell lines (22). Ishiroya et al. (23) reported that the EGFR gene was overexpressed in 53% of squamous cell carcinomas of the head and neck. When imaging studies were done with a mAb to the EGFR, 94% of primary lesions and metastatic sites of squamous cell carcinoma of the lung were detected (24, 25).

Receptors are frequently released from the cell of origin. Examples of this include the interleukin 2, insulin, tumor necrosis factor, and HER2/neu receptors. Shedding of the extracellular domain of the EGFR may enable its detection in body fluids. This study examined EGFR levels in the urine of patients with squamous cell carcinoma and compared those with levels found in the urine of normal volunteers or patients with nonsquamous cell carcinoma.

MATERIALS AND METHODS
Patient Samples
A 24-h urine sample was collected from 50 normal volunteers, 50 patients with untreated or progressing nonsquamous cell carcinoma, and 42 patients with untreated or progressing squamous cell carcinoma. The urine samples were kept at 4°C for 2 days and then frozen at −70°C until analysis of EGFR levels. Imagery studies were done with a mAb to the EGFR, 94% of primary lesions and metastatic sites of squamous cell carcinoma of the lung were detected (24, 25).

Receptors are frequently released from the cell of origin. Examples of this include the interleukin 2, insulin, tumor necrosis factor, and HER2/neu receptors. Shedding of the extracellular domain of the EGFR may enable its detection in body fluids. This study examined EGFR levels in the urine of patients with squamous cell carcinoma and compared those with levels found in the urine of normal volunteers or patients with nonsquamous cell carcinoma.

Cell Lines
Cell lines A-431 (human epidermoid carcinoma), SW620 (human colon adenocarcinoma), lymph node metastasis, MDA-MB-468 (human breast adenocarcinoma), BT-20 (human breast carcinoma), SK-BR-3 (human breast adenocarcinoma), CaLu 1 (human lung epidermoid carcinoma), and SK-OV-3 (human ovarian adenocarcinoma) were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI...
1640 media supplemented with 10% FCS. The DiFi cell line (human colorectal adenocarcinoma provided by Dr. B. Boman, Creighton University School of Medicine, Omaha, NE) was also grown in RPMI 1640 with 10% FCS. NIH3T3 cells transfected with either c-erbB-2 or EGFR (provided by Dr. S. Aaronson, National Cancer Institute, NIH, Bethesda, MD) were grown in DMEM supplemented with 10% FCS.

**Antibodies**

The specificity of the capture mAb 31G7 (EGFR mAb; Ciba Corning Diagnostics, Alameda, CA), using Western blot analysis has been described previously (26), and the utility of this antibody as an immunohistochemical reagent shown (27). The mAb 225 has been described extensively (25, 28, 29).

The anti-EGFR mAb 4C7 (Ciba Corning Diagnostics), which functions as the label in the EGFR ELISA, was generated by fusing splenocytes from a BALB/c mouse immunized with A-431 cells with the SP2/0 myeloma cell line using standard techniques (30).

The polyclonal antibody EGFRp1 used in immunoprecipitation experiments was produced at Ciba Corning Diagnostics in rabbits immunized with EGFR purified from A-431 cells. Specificity of the antibody was shown with the direct ELISA as described below, as well as by Western blotting.

The anti-EGFR polyclonal antibody RK2 (provided by Dr. J. Schlessinger, New York University Medical Center, New York, NY), also used in the immunodepletion assay, was raised in rabbits against a peptide corresponding to the 984-996 residues of EGFR. This antibody has been extensively used to immunoprecipitate the EGFR protein from human cells (31-33).

**Preparation of Cell Extracts**

Extracts were made by disrupting cell pellets in homogenization buffer (50 mM Tris, pH 7.2, 1 mM EDTA, 150 mM NaCl) using a Brinkman (Westbury, NY) homogenizer. The homogenate was centrifuged in a microfuge (Beckman, Fullerton, CA) for 15 min, and the pellet was resuspended in extraction buffer (homogenization buffer with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride added). The mixture was incubated for 15 min on ice with occasional vortexing. Residue was removed by centrifugation in the microfuge. The protein concentration of the extract was determined using a bicinechonic acid protein assay (Pierce, St. Louis, IL). Conditioned supernatant fluid from A-431 cells at confluence was concentrated 7-fold in a Centriprep concentrator (Amicon, Beverly, MA). A-431 cells were also grown in the presence of tunicamycin (2 μg/ml for 48 h) to inhibit glycosylation of the receptor.

**ELISA Procedures**

**Direct ELISA for Screening Hybridomas.** Supernatants from the hybridomas were selected by ELISA using A-431 and SW620 extracts coated onto 96-well plates. After cloning by limiting dilution, the hybridomas were rescreened against a more extensive panel of antigens. The plates were coated with A-431 extract (250 ng/well), SW620 extract (250 ng/ml), purified EGFR (5 ng/well), and purified ectodomain (5 ng/well). After blocking the plates with 4% nonfat dry milk, 10 μl of each supernatant were added to wells containing 50 μl PBS and allowed to incubate for 2 h. Plates were washed three times with PBS-0.05% Tween 20. Following a 1-h incubation with 100 μl goat anti-mouse horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) and three washes, 100 μl TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were added to each well and allowed to incubate for 15 min. The reaction was stopped with 1 M phosphoric acid (100 μl) and the absorbance read at 450 nm.

**Sandwich EGFR ELISA.** Ninety-six-well plates were coated overnight with 100 μl of the anti-EGFR mAb 31G7 at a concentration of 10 μg/ml in PBS. After the plates were blocked for 1 h with 1% BSA in PBS-Tween 20, known concentrations of the EGFR standard (0, 0.625, 2.5, and 5.0 fmol/ml) or the urine test sample (50 μl) were added. A second anti-EGFR mAb, 4C7, conjugated to horseradish peroxidase (50 μl) was added and the sample:antibody:horseradish peroxidase solution was incubated for 2 h at room temperature. Plates were washed three times with the PBS-Tween 20 solution. TMB solution (100 μl) was added for 15 min and then 100 μl phosphoric acid were added to stop the reaction. Absorbance at 450 nm was determined on a Bio-tek (Bio-tek Instruments, Winooski, VT) automated microtiter plate reader. Detectable EGFR levels were those which had absorbance values greater than twice the absorbance seen with the 0 fmol/ml standard.

**Purification of EGFR and EGFR Ectodomain**

Full-length EGFR was purified from A-431 cell extract using affinity chromatography. EGFR extracellular domain was purified similarly from the conditioned media of A-431 cells that had been concentrated 10-fold on a Minimac system (Amicon, Beverly, MA). The affinity column was made by coupling mAb 225 to CNBr-activated Sepharose using the protocol recommended by the supplier (Pharmacia Biotech, Piscataway, NJ). The extract or media was applied to the column and recirculated slowly at 4°C overnight. The column was then washed thoroughly with 20 mM HEPES (pH 7.5)-150 mM NaCl-0.1% Triton X-100-10% glycerol buffer followed by 1 mM NaCl, and then with additional 20 mM HEPES (pH 7.5)-150 mM NaCl-0.1% Triton X-100-10% glycerol buffer. The EGFR, full-length or ectodomain, was eluted with 50 mM triethylamine (pH 10.5). Purity was shown by SDS-PAGE to be greater than 90% for both preparations.

**Western Blotting with the Polyclonal Antibody**

Cell extracts were electrophoresed on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose using the Towbin method (34). After blocking in 4% nonfat dry milk for 1 h, the filter was incubated with the anti-EGFR polyclonal antibody at a 1:300 dilution for 2 h. Three washes were done with 50 mM Tris-Tween 20. A goat anti-rabbit antibody conjugated to alkaline phosphatase (Promega, Madison, WI) was diluted 1:3000 and allowed to incubate with the filter for 1 h. After washing, the immunoreactive bands were stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate. Protein molecular weight markers (Amersham, Arlington Heights, IL) were transferred on each blot and were used to estimate the molecular weight of immunoreactive bands.
Western Blotting after Immunoprecipitation of Urine

Urine samples were concentrated in Centriprep (Amicon) and incubated with the anti-EGFR polyclonal antibody EGFPrp1 at 4°C for 20 h. Protein A-Sepharose was added and an additional 1-h incubation at 4°C was done. The resulting pellet was washed with 10 mM Tris (pH 7.4), 100 mM NaCl, 0.2% NP40 buffer, and electrophoresed on a 7.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, the filter was blocked with 4% nonfat dry milk for 1 h. The filter was then incubated for 2 h with the anti-EGFR mAb 31G7. Three washes were done with PBS-Tween 20. Vectastain reagents (Vector Laboratories, Burlington, MA) were added according to the manufacturer’s guidelines, and the final staining was done with TMB solution. Affinity purified EGFR ectodomain was used as a control.

Immunodepletion

To remove EGFR, the urine was immunoprecipitated for 2 h at 4°C with anti-EGFR mAb 225: rabbit anti-mouse IgG: protein A-Sepharose conjugate or anti-EGFR polyclonal antibody RK2:protein A-Sepharose conjugate (32). After removing Sepharose beads by centrifugation at 8160 \( \times \) g for 5 min, the urine was concentrated and again analyzed by the EGFR ELISA.

Statistical Analysis

Two-way frequency tables were constructed to describe the categorical variables such as detectable EGFR and group status (control, nonsquamous carcinoma, squamous carcinoma). \( \chi^2 \) tests were applied for ascertaining statistically significant differences among the groups. Also, a \( \chi^2 \) test was applied to compare local versus metastatic within the squamous carcinoma group. All calculations were performed using PROC FREQ in Version 6.09 of SAS.

RESULTS

Antibody Specificity. mAbs 31G7 and 4C7 were selected on the basis of their positive reactivity in ELISA to A-431 extract and purified EGFR, and their lack of reactivity to SW620 extract. After cloning by limiting dilution, specificity of the antibodies was examined with the ELISA using not only A-431, SW620, and purified EGFR, but also purified EGFR ectodomain. Both monoclonals showed strong absorbance values to the A-431 and to purified EGFR (Table 1). Moderate reactivity was seen to the purified ectodomain. This lesser reactivity may be due simply to a less efficient coating of the antigen onto the plastic plate. A mAb recognizing c-erbB-2 was used as control. It recognized none of the EGFR-containing preparations, yet showed strong reactivity against NIH3T3 cells transfected with c-erbB-2.

<table>
<thead>
<tr>
<th>mAb</th>
<th>A-431 extract</th>
<th>SW620 extract</th>
<th>Purified EGFR</th>
<th>Purified ectodomain</th>
<th>NIH3T3/c-erbB-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4C7</td>
<td>1.388</td>
<td>0.015</td>
<td>1.655</td>
<td>0.606</td>
<td>0.021</td>
</tr>
<tr>
<td>31G7</td>
<td>1.278</td>
<td>0.188</td>
<td>1.368</td>
<td>0.963</td>
<td>0.056</td>
</tr>
<tr>
<td>Anti-c-erbB-2</td>
<td>0.018</td>
<td>0.019</td>
<td>0.064</td>
<td>0.016</td>
<td>2.070</td>
</tr>
</tbody>
</table>

* Values expressed are absorbance at 450 nm.

EGFR Sandwich ELISA Specificity. Assay specificity was demonstrated by testing cell extracts of established cell lines known to either express or not express EGFR. Conditioned media from A-431 confluent cells was also tested in the assay. Extracts were tested at a single dilution (100 \( \mu \)g/ml), and 7-fold concentrated conditioned media was tested undiluted. All of the cell lines known to overexpress EGFR had high absorbance values in the ELISA (Table 2). SK-BR-3, SW620, NIH3T3, and NIH3T3 transfected with c-erbB-2, all lacking in EGFR expression, were negative (Table 2). Conditioned media from A-431 cells reacted positively in the assay. Because the media has been reported to contain the ectodomain of the EGFR (35), this result also suggests that both mAbs recognize epitopes located on this portion of the EGFR. In a separate experiment, cell extracts of NIH3T3 cells transfected with either c-erbB-2 or EGFR or untransfected NIH3T3 control cells were serially diluted in PBS and retested in the assay. Extracts from NIH3T3 cells transfected with EGFR showed a concentration-dependent reactivity as shown in Fig. 1.

<table>
<thead>
<tr>
<th>Assay specificity data with cell extracts</th>
</tr>
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<tbody>
<tr>
<td>Cell extract</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>A431</td>
</tr>
<tr>
<td>SW620</td>
</tr>
<tr>
<td>DiFi</td>
</tr>
<tr>
<td>Calu-1</td>
</tr>
<tr>
<td>MDA-MB-468</td>
</tr>
<tr>
<td>BT-20</td>
</tr>
<tr>
<td>SK-BR-3</td>
</tr>
<tr>
<td>SK-OV-3</td>
</tr>
<tr>
<td>NIH3T3</td>
</tr>
<tr>
<td>NIH3T3 transfected with c-erbB-2</td>
</tr>
<tr>
<td>NIH3T3 transfected with EGFR</td>
</tr>
<tr>
<td>A431 conditioned media</td>
</tr>
</tbody>
</table>

EGFR Ectodomain Levels in Urine. Twenty-four-h urine collections were obtained from 50 normal volunteers, 42 patients with squamous cell carcinoma, and 50 patients with nonsquamous cell carcinoma. All patients had newly diagnosed or progressive cancer at the time of urine collection. The distribution of diseases in each category is presented in Table 3. Because normal human serum often contains detectable EGFR ectodomain levels, urine tests for hemoglobin were performed on all urine specimens. Several patients in both the squamous and nonsquamous groups had trace amounts of blood in their urine. These patients were found in both the group with detectable and those with nondetectable levels of EGFR ectodomain. We, therefore, concluded that there was no correlation between trace amounts of blood in the urine and urine EGFR ectodomain levels.
The EGFR sandwich ELISA that uses mAbs to the external domain of EGFR was used to measure levels of the receptor in urine samples. Detectable EGFR ectodomain levels were those which had absorbance values greater than a doubling of the absorbance seen with the 0 fmol/ml standard. The external domain of the EGFR was detected in the urine of 3 (6%) of 50 normal volunteers (1.6-4.1 fmol/mM creatinine), 8 (16%) of 50 nonsquamous cell carcinoma patients (0.6-29.0 fmol/mM creatinine), and 15 (36%) of 42 squamous cell carcinoma patients (1.3-45.7 fmol/mM; Fig. 2). There was a significant difference between the number of patients with squamous cell carcinoma with detectable urine EGFR ectodomain when compared with the control and nonsquamous cell carcinoma groups ($P < 0.001$, $P = 0.029$, respectively). There was no statistically significant difference between the control individuals and the nonsquamous cell carcinoma patients ($P = 0.104$). Given that there were only 50 subjects in each of these two groups, the statistical power (1, probability of a type II error) is very small. In fact, for a 0.05 significance level test for detecting a difference of 10% in rates, the statistical power is 0.13. A sample size of 150 per group is necessary for this statistical power to reach an acceptable level (power = 0.80).

Of the 42 squamous cell carcinoma patients, 23 had locoregional disease while 19 had metastatic disease to distant sites. Ten (53%) of the 19 patients with metastatic disease had a positive urine ELISA test for the external domain of the EGFR while only 5 (22%) of the 23 patients with locoregional disease had detectable EGFR in the urine ($P = 0.036$; Fig. 3). Comparing those patients with locoregional disease to those with nonsquamous carcinoma, we found no significant difference in

Table 3  Patients with detectable EGFR ectodomain levels in their urine

<table>
<thead>
<tr>
<th>Tumour Type</th>
<th>Normal Volunteers (n=50)</th>
<th>Nonsquamous Cell Ca (n=50)</th>
<th>Squamous Cell Ca (n=42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophageal cancer</td>
<td>0/2</td>
<td>8/26</td>
<td>0/8</td>
</tr>
<tr>
<td>Head/neck cancer</td>
<td>6/10</td>
<td>0/8</td>
<td>1/4</td>
</tr>
<tr>
<td>Lung cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-squamous cell carcinoma (8/50)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>0/8</td>
<td>0/8</td>
<td>0/3</td>
</tr>
<tr>
<td>Gastrointestinal cancer</td>
<td></td>
<td>1/8</td>
<td>0/3</td>
</tr>
<tr>
<td>Head/neck cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung cancer</td>
<td>3/8</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Melanoma</td>
<td>0/5</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Renal cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1  Specificity of sandwich ELISA for EGFR shown by detection of transfected EGFR in NIH3T3 cell extract. ■, 3T3-tr EGFR; ●, 3T3-tr c-erbB-2; ▲, 3T3-control.

Fig. 2  Levels of EGFR ectodomain in 24-h urine specimens from normal volunteers (n = 50), patients with nonsquamous cell carcinoma (n = 50), and patients with squamous cell carcinoma (n = 42). Comparison among the three groups was performed using the $\chi^2$ test.

Fig. 3  Levels of EGFR ectodomain in 24-h urine specimens from squamous cell carcinoma patients with localized disease (n = 23) and distant metastatic sites (n = 19). Comparison between the two groups was performed using the $\chi^2$ test.
detectable urine EGFR ectodomain levels ($P = 0.580$). There was, however, a significant difference between the number of detectable levels in the locoregional group compared to those in the control group ($P = 0.050$).

**Western Blotting of Immunoprecipitated Urine Samples.** In order to verify that what was detected in the ELISA was indeed the external domain of the EGFR and not merely a cross-reacting substance, two methods were used. In the first method, concentrated urine samples (both reacting positively and negatively in the ELISA) were immunoprecipitated with an anti-EGFR polyclonal antibody, EGFRp1, that had been produced in rabbits. The specificity of this antibody was first determined using Western blotting of cell extracts. Fig. 4 shows clear bands at 170,000 for cell lines known to overexpress EGFR (A-431, MDA-MB-468, BT-20, DiFi); no band was seen at this location for SW620, a cell line lacking in EGFR expression. EGFRp1 recognized epitopes on both glycosylated (170,000) and unglycosylated (130,000) forms of the receptor present in the tunicamycin-treated A-431 cell extract (Fig. 4, Lane 7).

After immunoprecipitation of the concentrated urine samples with EGFRp1, Western blotting was done on the samples using mAb 31G7. The samples were from three squamous cell carcinoma patients with elevated levels in the ELISA and from one normal patient without detectable EGFR ectodomain. A $M_r$ 95,000 band was detected only in the samples that had shown detectable EGFR ectodomain in the ELISA (Fig. 5). This band was not present in the urine from the normal volunteer. EGFR ectodomain was precipitated from A-431-conditioned supernatant and run on the Western blot as a control. The A-431 ectodomain band had a slightly larger $M_r$, 105,000, consistent with that reported in Weber et al. (35). The bands are broad due to the relatively high amount of glycosylation on the ectodomain. A rabbit polyclonal, rather than another monoclonal, was used for the immunoprecipitation step in an effort to minimize the reactivity of the antimouse secondary antibody. Unfortunately, due to the large amount of rabbit immunoglobulin present, cross-reactivity of the antimouse secondary resulted in clearly visible broad bands at approximately $M_r$ 50,000 and 25,000.

The immunoprecipitation experiment was repeated three times and none of the Western blots gave immunoreactive bands that stained significantly darker. The faintness of the bands is due to the fact that the levels of EGFR ectodomain in the urine specimens were initially very low, and the ability to concentrate the urine was limited by the increasing viscosity of the concentrate. In all three experiments, the $M_r$ of the immunoreactive bands was approximately 95,000, as determined by comparison to protein molecular weight markers, as well as to the ectodomain purified from A-431 cells.

**Immunodepletion of Urine Samples.** The second method used to verify that the species detected in the ELISA was EGFR ectodomain was an immunodepletion of urine from four squamous cell carcinoma and one nonsquamous cell carcinoma patients. Urine specimens were incubated with either a mAb to the EGFR ectodomain (225) or the polyclonal antibody to the carboxyl terminus of EGFR (RK2). EGFR levels in the concentrated urine were determined before and after immunodepletion using the ELISA. After treatment with 225, greater than 90% of the activity was removed from the urine of the squamous cell carcinoma patients and 70% of the EGFR activity from the urine of the nonsquamous carcinoma patient (Table 4). In contrast, treatment with the polyclonal antibody RK2 removed less than 15% of the EGFR activity from the urine of the squamous cell carcinoma patients and less than 25% of the activity from the urine of the nonsquamous carcinoma patient. Thus, the activity detected by the ELISA method appears to be predominantly the ectodomain of the EGFR.
DISCUSSION

The EGFR gene and its protein product are overexpressed in many human cancers. This is especially true for squamous cell carcinomas of the head and neck and also squamous cell lung cancer where overexpression of the EGFR gene occurs in 53 and 94% of patients (23-25).

We have used an ELISA using mAbs recognizing epitopes of the external domain of the EGFR. EGFR ectodomain activity was detected in the urine of 15 (35%) of 42 squamous cell carcinoma patients compared to 8 (16%) of 50 nonsquamous cell carcinoma patients and 3 (6%) of 50 control individuals. Furthermore, elevated urine EGFR ectodomain levels were more frequently seen in patients with metastatic than with locoregional squamous cell carcinoma (53% versus 22%). Admittedly, the sample size is small in this study, yet not only are significant differences seen between the groups for the number of patients with detectable EGFR ectodomain, but also much lower EGFR ectodomain values (1.6-4.1 fmol/mM creatinine) are seen in the normal patient group as compared to the nonsquamous (0.6-29.0 fmol/mM creatinine) and squamous cell carcinoma groups (1.3-45.7 fmol/mM creatinine).

The EGFR consists of an ectodomain as well as a cytoplasmic region that contains tyrosine kinase activity. The structure is similar to that of other peptide growth factor receptors. Growth factor receptors are found in the circulation in other patient populations. These include the insulin receptor, interleukin 2 receptor, the c-erbB-2 (HER2/neu) receptor, and the tumor necrosis factor receptor. Two possible mechanisms exist for release of the receptor from the cell. The receptor may be enzymatically clipped and released from the cell surface, or it may be a product of alternate splicing, production, and secretion of only the external domain of the receptor.

Substances that cross-react with the antibodies to the EGFR could result in a false positive in the EGFR ELISA. Reactivity of the individual antibodies was shown with a direct ELISA using purified EGFR, ectodomain, and cell extracts both positive and negative for EGFR. The antibodies used in the EGFR ELISA were shown to recognize epitopes present on the EGFR ectodomain. Assay specificity was also evaluated using cell extracts. The assay was shown to have no cross-reactivity with c-erbB-2 (highly homologous to EGFR) and specific reactivity to EGFR after transfection into NIH3T3 cells.

Verification that what was being measured in the urine ELISA was EGFR ectodomain was performed by two methods. In the first method, Western blotting using the 31G7 mAb after immunoprecipitation with an anti-EGFR polyclonal antibody revealed a Mr 95,000 band. Although not identical in molecular weight to A-431 derived ectodomain, the similarity in size and apparent degree of glycosylation indicated by the band's broadness are suggestive that what was immunoprecipitated was EGFR ectodomain. In the second method, anti-EGFR antibodies bound to Sepharose beads were incubated with urine. Greater than 90% of the urine EGFR activity was removed from the urine of four squamous cell carcinoma patients and 70% of the activity was removed from the urine of one nonsquamous cell carcinoma patient using mAb 225 which recognizes the EGFR ectodomain. In comparison, less than 15% of the activity was removed from the urine of the squamous cell carcinoma patients and less than 25% of the activity was removed from the urine of the nonsquamous carcinoma patient after treatment with the antibody to the carboxyl terminus of the EGFR. It would, therefore, appear that the activity being measured in the urine is predominantly the ectodomain.

Urinary levels of EGFR ectodomain were more frequently elevated in patients with metastatic squamous cell carcinoma than in patients with locoregional squamous cell carcinoma (53% versus 22%). It would seem unlikely that a urine assay for the external domain of the EGFR would be useful as a screening test for squamous cell carcinoma. Further investigation would seem to be indicated for using EGFR ectodomain levels to monitor results of treatment in patients with metastatic squamous cell carcinoma.

In this study, 6 (60%) of 10 patients with squamous cell carcinoma of the lung had detectable EGFR ectodomain in their urine. This may be due to the fact that 9 of the 10 squamous cell lung cancer patients had metastatic disease. However, the small sample size of 10 patients warrants further investigation with a larger number of patients to determine if measurement of urine EGFR ectodomain levels is a useful diagnostic method for squamous cell lung cancer.

ACKNOWLEDGMENTS

We thank Michelle F. Moore for her excellent help in the preparation of this manuscript. We also thank Drs. John Mendelsohn and Hideo Masui (Memorial Sloan-Kettering Cancer Center, New York, NY) for providing the anti-EGFR mAb 225 used in the immunodepletion assays.

Table 4  Immunodepletion of EGFR ectodomain from 24-h urine specimensa

<table>
<thead>
<tr>
<th>Patients</th>
<th>% EGFR removed with mAb 225 to the ectodomain</th>
<th>% EGFR removed with antibody RK2 to the COOH terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-1</td>
<td>&gt;90</td>
<td>10</td>
</tr>
<tr>
<td>S-2</td>
<td>&gt;90</td>
<td>13</td>
</tr>
<tr>
<td>S-3</td>
<td>&gt;96</td>
<td>15</td>
</tr>
<tr>
<td>S-4</td>
<td>&gt;98</td>
<td>0</td>
</tr>
<tr>
<td>Nonsquamous cell carcinoma</td>
<td>70</td>
<td>&lt;25</td>
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</tbody>
</table>

a The urine specimens from four squamous cell carcinoma patients and one nonsquamous cell patient were analyzed for EGFR levels by ELISA before and after immunodepletion with either mAb 225 or antibody RK2 as described in "Materials and Methods."
REFERENCES


Epidermal growth factor receptor ectodomain in the urine of patients with squamous cell carcinoma.

L M Witters, E M Curley, R Kumar, et al.


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