Detection of Epidermal Growth Factor Receptor mRNA in Peripheral Blood: A New Marker of Circulating Neoplastic Cells in Bladder Cancer Patients

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ABSTRACT

Despite the large number of studies performed in solid tumors, few attempts at molecular detection of urothelial cells in blood have been made. Specifically, only uroplakin II (UP-II) and cytokeratin 20 (CK-20) have been suggested as tumor markers in the blood of bladder cancer patients. Epidermal growth factor receptor (EGFR) mRNA expression was found in the blood of patients with some types of carcinoma; nevertheless, its expression has been never investigated in the blood of patients with urothelial tumors. We used a EGFR-based reverse transcription-PCR assay for the detection of tumoral cells in the blood of 27 patients with bladder cancer, in 30 healthy donors, and in 9 patients with cystitis. EGFR expression was compared with that of known markers of circulating epithelial cells, CK-19 and CK-20, and to a urothelial-specific marker, UP-II. Analysis by reverse transcription-PCR and Southern blot hybridization showed no evidence of EGFR and UP-II mRNA expression in any of the samples used as controls. Analysis of healthy donors showed mRNA expression for CK-19 and CK-20 in 6 of 30 and in 4 of 30 samples, respectively. All patients with cystitis resulted negative for EGFR expression, whereas 3 of 9, 2 of 9, and 3 of 9 were found expressing CK-19, CK-20, and UP-II, respectively. Among blood samples from tumoral patients, 74% had EGFR mRNA and 41% had positive signals for CK-19, whereas positivity for CK-20 and UP-II was found in 15% and 37% of patients, respectively. These results seem to indicate that EGFR mRNA in the blood may be a useful tumor marker in bladder cancer patients, as well as in other patients with epithelial tumors.

INTRODUCTION

Superficial tumors of the urinary bladder represent 80% of bladder neoplasms and are characteristically confined within bladder mucosa. After TUR-B or radical cystectomy, >50% of patients affected by superficial bladder cancer show no clinical evidence of residual disease on conventional staging; nevertheless, ~20% of them progress to muscle invasion (1). This is the reason why the term “superficial” is considered unsuitable to describe the real prognosis of these patients. Furthermore, 50% of patients with muscle-invasive disease develop metastases in the first 2 years of follow-up despite radical cystectomy. These cases cannot be identified in the initial assessment and are thus assumed to have micrometastases in the presurgical phase. Moreover, systemic chemotherapy for invasive forms has not yet proved to be beneficial in a large series of patients (2). Additionally, despite an increasing number of studies, the role of molecular markers of disease progression in urothelial tumors is still uncertain (3).

The detection of circulating tumor cells has crucial prognostic and therapeutic implications in all cancer patients (4). In recent years, protocols based on RT-PCR allowed the detection of cancer cells in the peripheral blood, bone marrow, and lymph nodes of patients with several cancer types such as breast, colon cancer, melanoma, neuroblastoma, prostate cancer, and lymphoma (5, 6). In the first studies, the detection of epithelium-specific markers such as CK-19 and CK-20 have shown a good correlation with the clinical occurrence of metastases; nevertheless, recent reports concluded that such methods lack a sufficient specificity because of the increasing number of false-positives observed in patients without cancer (7, 8). Both an illegitimate transcription and the presence of a pseudogene have been described to explain the possibility of false-positive results using RT-PCR for CK-19 (9). Furthermore, RT-PCR amplification of CK-20 mRNA, considered a promising candidate method for the detection of circulating epithelial cells (10, 11), seems to lack specificity because its expression is not limited to epithelial cells...
but also occurs in normal granulocytes (12). The diagnostic specificity of the method seems restored just after Ficoll-paque centrifugation of the specimens, with separation of the granulocyte fraction.

More recently, a RT-PCR assay using EGFR primers was, for the first time, evaluated for the detection of micrometastases in the blood of patients with breast cancer, because EGFR mRNA was reported to be present in almost 90% of primary breast cancer, but not in normal human peripheral blood mononuclear cells (13).

A similar EGFR RT-PCR assay was used by De Luca et al. (14), who detected EGFR-expressing cells in the blood of patients affected by lung, breast, and colon cancer, thus suggesting that this assay may be used in patients with different carcinoma types.

Despite the large number of studies performed in most solid tumors, few attempts at the molecular detection of urothelial cells in blood have been made. Specifically, only uroplakin II and CK-20 have been suggested as candidate tumor markers in the blood of bladder cancer patients (15–17).

A nested RT-PCR assay that amplifies CK-20 transcripts was used to detect cancer cells in the blood of urothelial cancer patients for the first time by Fuji et al. (15), and then by Gudemann (11), although a PCR-based assay for detecting UP-II-positive cells in the blood of patients with bladder cancer was firstly developed by Li et al. (16) and more recently by Lu et al. (17), who suggested that this assay is highly specific and might be used as a tumor marker for the molecular staging of urothelial cancers, although the sensitivity does not seem to be optimal.

In the present article, we describe the high sensitivity and specificity of EGFR-based RT-PCR assay as a method for the detection of tumoral circulating cells in blood samples from patients with a diagnosis of primary bladder cancer. In fact, EGFR, which is expressed in all cell types except hemopoietic cells, is found to be overexpressed in bladder tumors and in bladder cancer metastases (18–20).

To our knowledge, EGFR mRNA expression in the blood of patients affected by urothelial tumors has never been investigated.

The specificity of the assay was then compared with that of known markers of circulating epithelial cells, CK-19 and CK-20, and to a urothelial-specific marker, UP-II.

**MATERIALS AND METHODS**

**Patients and Blood Samples.** A total of 27 patients with diagnoses of TCC of the bladder were included in this study. Among those, 6 had superficial (Tis-T1-N0M0), 10 had muscle-invasive (T2–4N0M0), 3 had lymph-node metastases (pN1–2 M0), and 8 had metastatic disease (M1). Patients were graded by histopathological examination and staged according to the Tumor-Node-Metastasis classification.

To avoid contamination as a result of surgery, all blood samples were collected before TUR-B. Thus, at the time of blood drawing, all patients with superficial or locally advanced tumors were completely untreated. Among patients with metastatic disease, 10 of 8 was receiving adjuvant therapy with gemcitabine, 3 of 8 had received the last chemotherapy cycle with M-VAC or M-VEC 6–10 months before blood collection, and 4 of 8 were untreated.

All patients with diagnoses of nonmetastatic disease who were untreated at the moment of blood collection were then subjected to transurethral resection or radical cystectomy.

To test the specificity of the RT-PCR assays, a total of 30 healthy subjects without cancer were included as negative controls. Inclusion criteria were as follows: (a) 19–40 years of age; (b) the absence of any acute or chronic inflammatory disease; and (c) the absence of any nontumorous abnormal condition of the bladder.

A total of nine subjects with nontumorous disease of the bladder were also included, all with clinical diagnosis of acute cystitis. Clinical and pathological features of all patients are summarized in Tables 1 and 2.

**Blood Collection.** Two ml of peripheral blood was collected by forearm venipuncture using EDTA tubes. RNA extraction was performed using a modified Trizol LS-based procedure, as described previously (21). Briefly, blood samples were mixed into a succinyl-linked gelatin (1:1), and the red cells were allowed to settle by gravity to separate all nucleated cells. Then, the supernatant was centrifuged and the pellet underwent subsequent RNA extraction steps. The quantity and quality of RNA preparations were determined by absorbance at 260 and 280 nm, respectively.

**Tumor Specimens.** Tumor tissue samples of 16 TCCs were frozen in liquid nitrogen immediately after surgery and stored at −80°C until use. Total RNAs were extracted from all samples with guanidium isothiocyanate (22) and subjected to EGFR-specific RT-PCR.

Patients in which EGFR expression was investigated in both blood and frozen tissues are shown in Table 1.

**Ethical Issues.** Written informed consent was obtained from all patients for both blood and tissue sampling, from patients with cystitis, and from all healthy donors.

**RT-PCR.** Total RNA (1 µg) from blood samples and frozen tissues was reverse transcribed in a final volume of 20 µl with 100 pmol of random examer and 50 units of MuLV reverse transcriptase (Perkin-Elmer Corporation, Norwalk, CT), according to the manufacturer’s guidelines.

Aliquots from blood samples corresponding to 100 ng of RNA were then amplified in PCR buffer containing 25 pmol each primer and 1.25 units of Taq polymerase in a final volume of 50 µl.

Aliquots of the same cDNA were amplified with β-actin, EGFR, CK-19, CK-20, and UP-II primers. Amplification for β-actin and EGFR was performed for 35 cycles; a cycle profile consisted of denaturation at 94°C for 1 min 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. Amplification for CK-19 was performed for 40 cycles; each cycle profile consisted of denaturation at 94°C for 1 min 30 s, annealing at 68°C for 30 s, and extension at 72°C for 2 min. A final extension step at 72°C for 7 min completed the reaction. Amplifications for CK-20 and UP-II were also performed for 35 cycles, with annealing temperatures of 55°C and 60°C, respectively.

A sample without RNA was included in each experiment of RT-PCR as a negative control; RNAs extracted from HeLa and MCF-7 cell lines were used as positive controls for the expression of EGFR and CK-19, respectively. RNA from SKOV cell
line was used as a positive control for CK-20, whereas RNA from a patient with metastatic bladder cancer was used as a negative control for UP-II amplification.

In the course of treatment at the moment of blood collection, patients who never received chemotherapy.

At the moment of surgery. Last chemotherapeutic cycle was performed 6–10 mo before blood collection.

Follow-up. From January 1999 to August 2000, all patients with bladder tumors were subjected to periodical follow-up.

Primers for CK-19 were chosen taking care to retain mismatches with the known CK-19 pseudogene at the 3' end, as described (9).

An aliquot of the amplification products was then size-fractionated by agarose gel electrophoresis, transferred to Hybond-N membrane, and hybridized with 1 × 10^6 cpm of 32P-end labeled internal probes for 3 h at 37°C in 6× SSC, 0.5% SDS. After hybridization, filters were washed in 1× SSC/0.5% SDS at 60°C and autoradiographed on Kodak film XAR at −80°C.

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low-up studies. All patients with bladder carcinoma in situ, diagnosed endoscopically with positive cytology and more than one positive biopsy on apparently healthy mucosa, were treated by TUR-B and then by mitomycin C or Bacillus Calmette-Guerin (BCG). These patients were followed every 3 months with urinary cytology and pelvic ultrasonography. Patients with locally advanced or metastatic tumors were followed, after radical surgery, with chest roentgenogram, abdominal-pelvic computerized axial tomography, and bone scintigraphy every 6 months.

**Statistical Analysis.** Statistical analysis of data has been performed using the $z$-test (two sides). A $P < 0.05$ was considered indicative of a statistically significant difference.

**RESULTS**

**EGFR, CK-19, CK-20, and UP-II mRNA Expression in Blood Samples.** The present study evaluated the suitability of EGFR RT-PCR assay for detection of circulating cells in the blood of bladder cancer patients. We compared this method with known methods of detection of circulating urothelial cells, such as CK-19-, CK-20-, and UP-II-RT-PCR amplifications.

To verify the specificity of the amplification products, we used Southern blot hybridization with specific oligonucleotide probes. RNA integrity was confirmed in all samples by RT-PCR using human β-actin primers.

The first part of this study was performed in blood samples from 30 healthy donors with no evidence of cancer or bladder disease. Analysis by RT-PCR and Southern blot hybridization showed no evidence of EGFR and UP-II mRNA expression in any of the samples used as controls. Analysis of healthy donors showed mRNA expression for CK-19 in 6 of 30 and for CK-20 in 4 of 30 samples ($P < 0.05$ for CK-19 and $P = 0.129$ for CK-20 versus EGFR positivity). This data confirms the lack of specificity of CK-19, as demonstrated previously (Fig. 1A).

To determine further the specificity of the target mRNA marker to detect cancer cells in blood, nine samples from patients with cystitis were analyzed for EGFR, CK-19, CK-20, and UP-II. The results of all these samples were negative for EGFR expression, whereas 3 of 9, 2 of 9, and 3 of 9 were found expressing CK-19, CK-20, and UP-II, respectively (Fig. 1B).

In our hands, 20 of 27 (74%) blood samples from tumoral patients showed the presence of EGFR mRNA and 11 of 27...
(41%) had positive signals for CK-19, whereas positivity for CK-20 and UP-II was found in 4 of 27 (15%) and in 10 of 27 (37%) patients, respectively (Fig. 1, **CK-20** and **UP-II** was found in 4 of 27 (15%) and in 10 of 27 (41%) had positive signals for CK-19, whereas positivity for CK-19, CK-20, and UP-II mRNA expression in the blood was found in 8 of 16, 3 of 16, and 4 of 16 patients, respectively (Table 1). The difference between EGFR and the positivity of other markers was found statistically significant (**CK-19, P = 0.004; CK-20, P < 0.0001; UP-II, P < 0.0001**).

Interestingly, the only patient with superficial disease and positivity to EGFR both in the blood and in frozen tissue (patient 6) had a local relapse 4 months after the primary surgery.

**DISCUSSION**

In this study, circulating EGFR-positive cells were detected in 70% of patients affected by TCC of the bladder, specifically in 90% of patients with invasive tumors (T2–4N0M0), in all patients with regional lymph-node metastases, and in 87% of those with metastatic disease. This result is intriguing in that all patients without apparent distant metastases who had progression of disease in 20 months showed EGFR-expressing cells in the blood. Among patients with superficial disease, only patient 6 showed the presence of EGFR in blood; this patient had a local relapse after 4 months, whereas all of the others with primary diagnosis of superficial tumors, who showed negative results for EGFR expression in blood, are still relapse-free.

To verify the correlation between EGFR expression in peripheral blood and in primary bladder tumors, we analyzed by RT-PCR the expression of EGFR in tumoral tissues and in peripheral blood from 16 bladder cancer patients. According to our expectations, we found that all patients who expressed EGFR mRNA in peripheral blood were also expressing EGFR in primary tumors. We also found a correlation between EGFR positivity in blood and in tumors and the prognosis of patients; in fact, all patients with an absence of EGFR-expressing cells in blood and EGFR-negative tumors did not relapse in the first 20 months of follow-up.

Previous reports have shown EGFR expression in 30–50% of bladder tumors, with a general association with high tumor stage, tumor growth pattern, and clinical outcome of patients (18). Our data for the first time seems to indicate a good correlation between EGFR expression levels in blood samples and in tumors.

We also compared the EGFR molecular marker for detection of circulating urothelial cells with other markers used previously, specifically CK-19, CK-20, and UP-II. EGFR RT-PCR assay seems highly specific and sensitive compared with the other markers analyzed in this study.

Using a single round of PCR Li et al. (16) found circulating UP-II-expressing cells in 30% of metastatic patients and in none of the patients with nonmetastatic disease. We also preferred a single round of PCR with subsequent Southern blot hybridization because of the risk of false-positive results described in the nested PCR assays (23). In our hands, the percentage of UP-II-positive samples was 12.5% in metastatic patients and 47% in nonmetastatic patients, with no significant correlation between UP-II expression and follow-up data. In fact, UP-II-positive cells were found in five patients with superficial disease, with no evidence of relapse after 20 months of follow-up. Furthermore, UP-II expression was found in patients with benign cystitis, a group of patients that Li et al. did not analyze.

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**Table 3** Analysis of EGFR, CK-19, CK-20, and UP-II expression in peripheral blood samples from patients with metastatic bladder cancer and from healthy donors

<table>
<thead>
<tr>
<th>Marker</th>
<th>Stage of Tumors</th>
<th>Positive Samples (%)</th>
<th>P vs. Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>IV</td>
<td>7/8 (87%)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CK-19</td>
<td>IV</td>
<td>6/8 (75%)</td>
<td>0.011</td>
</tr>
<tr>
<td>CK-20</td>
<td>IV</td>
<td>1/8 (12.5%)</td>
<td>0.598</td>
</tr>
<tr>
<td>UP-II</td>
<td>IV</td>
<td>1/8 (12.5%)</td>
<td>0.472</td>
</tr>
</tbody>
</table>

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**Note:**
- Follow-up data after 20 months revealed progression of disease or death in 16 patients; among those, all had EGFR-expressing cells in the blood, whereas CK-19, CK-20, and UP-II mRNA expression in the blood was found in 8 of 16, 3 of 16, and 4 of 16 patients, respectively (Table 1). The difference between EGFR and the positivity of other markers was found statistically significant (**CK-19, P = 0.004; CK-20, P < 0.0001; UP-II, P < 0.0001**).
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EGFR in Blood of Bladder Cancer Patients

Data from Lu et al. (17) shows that they found circulating UP-II mRNA-positive cells in 28.6% of patients with invasive tumors and in 40% of patients with regional lymph-node metastases. Our percentage of UP-II-positive samples among patients with invasive tumors is higher (40%), although we failed to find any positive samples among patients with N1–2 disease. This is probably attributable to the small number of patients with lymph-node metastases accrued in our study. In superficial tumors, all characterized by single, low-grade lesions, we found a high rate of positivity for UP-II (83%). This finding, together with the presence of UP-II-positive circulating cells found in patients with cystitis, seems to suggest that detection of UP-II-positive cells in the blood is not necessarily related to the occult existence of metastasis.

On the other hand, our results using CK-20 and CK-19 seem to indicate a very low specificity, being expressed both in samples from healthy donors and in those from patients with benign cystitis. Although our results with CK-19 are in agreement with data from literature (24, 25), those with CK-20 are in contrast with Gudemann et al. (11) and Fujii et al. (15). Even so, this discrepancy may be because Fujii worked on a mononuclear cell fraction, thus eliminating CK-20 expression attributable to the presence of granulocytes. Thus, the positivity for CK-20 that we observed in samples from healthy donors and those with benign cystitis may be attributed to the presence of the granulocyte fraction in the blood samples. Nevertheless, in our hands, the percentage of CK-20-expressing cells in blood samples from patients with locally advanced bladder cancer is similar to that observed by Fujii (14%), but does not correlate to either the stage of tumor nor the propensity to relapse. Follow-up data seem to indicate a role of EGFR, but not of CK-19, CK-20, and UP-II, in predicting the progression of disease.

In this view, patient 6 is interesting for having a superficial tumor which relapsed early. In this patient, EGFR mRNA expression was found both in tissue and in blood. Furthermore, 50% of patients who showed negative results for CK-19 mRNA expression underwent progression to higher stage of disease. Moreover, UP-II and CK-20 expression was found in 4 and 3, respectively, of the group of 16 patients who relapsed.

Our results using EGFR-based RT-PCR assay on the blood samples of patients with bladder cancer are similar to those obtained by Leitzel et al. (13) on breast cancer, and by De Luca et al. (14) on colon and lung carcinomas. Leitzel identified EGFR mRNA expression in metastatic breast cancer patients, but not in healthy donors nor in patients treated with adjuvant therapy, thus concluding that such a marker may be useful in the identification of circulating micrometastases in a proportion of breast cancer patients. More recently De Luca et al. (14) found EGFR mRNA expression in blood samples from 59% of patients with metastatic carcinoma and in 10% of healthy donors, whereas we failed to find any positive signal in samples from healthy donors. This discrepancy may be attributable to the difference in sensitivity between the single-round and the nested RT-PCR.

In the last decade, EGFR expression has been proposed as a prognostic marker in bladder tumors, being associated with rapid proliferation of cell lines; various reports indicate a bad prognosis in EGFR-positive bladder tumors, suggesting that EGFR expression is a stronger predictor of tumor proliferation than stage or grade (26).

Previous studies have confirmed that systemic administration of anti-EGFR monoclonal antibody is able to inhibit growth of TCC in nude mice; such effect is mediated also by a block of angiogenesis attributable to normalization of such angiogenesis-related markers as basic fibroblast growth factor and vascular...
endothelial growth factor (27). Thus, the identification of bladder cancer patients with circulating EGFR-expressing cells may be useful in selecting patients who may benefit from EGFR-targeted therapies.

In bladder cancer, the frequency of lymph-nodal disease observed at the time of surgery, together with the high rate of metastatic disease after radical cystectomy, suggests the inefficiency of available staging modalities for detection of micrometastatic disease. Thus, the risk of understaging patients with bladder cancer is still very high. In this view, an EGFR-based RT-PCR assay could be useful in the presurgical staging of the tumor through identification of patients with higher risk to relapse, as well as in the clinical management of patients included in follow-up studies. Such a conclusion is supported by two main considerations: EGFR mRNA is detected in the peripheral blood of the majority of metastatic patients; and furthermore its expression in the blood of nonmetastatic patients correlates well with the emergence of relapse, independent of the clinical stage of tumors at the moment of surgery.

We thus suggest that detection of this marker in peripheral blood also may be useful in anticipating the diagnosis of metastatic disease in patients affected by bladder cancer, as well as in all patients with tumors of epithelial origin. Longer clinical follow-up of patients with superficial and locally advanced disease is required to select patients with a high risk of relapse.

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