A Subclass of HER1 Ligands Are Prognostic Markers for Survival in Bladder Cancer Patients

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ABSTRACT

Members of the epidermal growth factor (EGF) family have been suggested as prognostic markers in patients with bladder cancer. Thus far, there has been no consensus on their usefulness. We report an analysis of six ligands and two receptors of which a subset correlate to tumor stage and survival.

Biopsies from bladder cancer tumors were obtained from 73 patients followed for a median of 28 months. The mRNA content for six ligands [EGF, transforming growth factor α (TGF-α), amphiregulin (AR), beta-cellulin (βCL), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPI)] and two receptors [EGF receptor I Human EGF Receptor (HER1) and 2 (HER2)] was examined by a newly developed quantitative reverse transcription-PCR method.

Five ligands and two receptors (HER1 and HER2) were present in median concentrations of (10−15 mol/μg RNA) 0.39 (AR), 11 (βCL), 2.4 (EPI), 40 (HB-EGF), 1.4 (TGF-α), 75 (HER1), and 39,000 (HER2). EGF was barely detectable. A significantly higher expression of EPI (P < 0.001), HB-EGF (P < 0.001), and TGF-α (P < 0.05) was observed in T2-T4 tumors as compared with Ta tumors. Especially the expression of EPI mRNA correlated strongly to survival (P < 0.0005), but increased expression of TGF-α (P < 0.005), AR, and HB-EGF (P < 0.02) was also associated with a reduced life span.

For the first time, mRNA expression of six ligands and two receptors of the EGF family have been examined in bladder cancer tumors. Our data emphasize that members of the EGF family, especially EPI, may be potential bladder tumor markers.

INTRODUCTION

The incidence of human bladder cancer has increased extensively through the last decades. Despite several attempts, it is still difficult to predict tumor progression, optimal therapy, and finally clinical outcome (1, 2). Tumor staging is considered to be one of the best prognostic markers, but several markers including the presence of members of the EGF family have been suggested (3–7).

Originally, the EGF system was considered to include only one receptor (EGF receptor/HER1/ERbB1) and one ligand (EGF). During the last decade, five additional ligands have been identified (TGF-α, AR, βCL, HB-EGF, and EPI; Refs. 8, 9). Binding of any of the six ligands to HER1 induces a specific dimerization between one of the four receptors (HER1, HER2, HER3, or HER4), and HER2 is the potential bladder tumor markers.

MATERIALS AND METHODS

Patients. Seventy-three patients with primary bladder cancer were included. Biopsies were obtained by transurethral tumor resection. Tumor stage and grading were assigned according to the Union Internationale Contre le Cancer Tumor-Node-Metastasis system (14). The relationship between tumor stage, age, and sex distribution is presented in Table 1. Patients were allocated into three groups depending on tumor stage: Ta, superficial tumors; T1, superficial invasive tumors; and T2-T4, muscle-invasive tumors. At the time of inclusion, eight patients had received treatment in the form of radiation, chemotherapy, or intravesical therapy with bacillus Calmette-Guerin. The follow-up period was from the date of biopsy to the day of death or to August 1999. The median follow-up was 28 months (range, 1–64 months). The regional Committee of Scientific Ethics, Aarhus approved the study, and the procedures were in accordance with the Helsinki Declaration.

Preparation of Total RNA. Tumor samples used for mRNA analysis were immediately placed in a denaturing solution [4 mol/liter guanidine thiocyanate, 25 mmol/liter sodium citrates (pH 7), 0.5% sarkosyl, and 0.1 mmol/liter 2-mercaptoethanol] and stored at −80°C. A frozen biopsy (<20 mg) was homogenized by a Heidolph D2o 600 mixer. Total RNA was extracted from tissues according to a slightly modified method of Chomczynski and Sacchi (15). The RNA was resuspended in diethyl pyrocarbonate-treated double distilled water and stored at −150°C. RNA was quantified using a UV spectrophotometer (A260 nm = 1 corresponds to 40 μg/ml RNA).

Quantitative RT-PCR Analysis. The mRNA expression of the EGF system was quantified according to our procedure published previously (13). Briefly, 0.1 μg of RNA was reverse transcribed with a fixed amount of RNA standards for each component of the EGF system analyzed, followed by PCR with a specific primer set for each of the investigated mRNAs. Coamplification

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of standard and target RNA was possible because the two RNA molecules contained identical primer-binding sequences. RT-PCR was performed on a set of RNA calibrators (RNA purified from a HCV29 cell line) with known amounts of the specific mRNA species from the EGF system, which made it possible to generate standard curves (13, 16). After RT-PCR, the samples were loaded on a 1.5% agarose gel (Fig. 1A). Computer scanning (GelDoc 1000; Bio-Rad) was used to determine the intensity of target and standard band for each sample. The ratio of the target and standard band was determined for the samples and the calibrators, and the mRNA concentration in the unknown samples was determined from the calibration curve (Fig. 1B). The mRNA concentration of the calibrators was determined by traditional competitive RT-PCR, where a fixed amount of calibrator RNA was coamplified with a series of RNA standard concentrations (16). The RNA standard concentrations, where equal amounts of target and standard bands are generated, correspond to the mRNA concentration in the calibrator.

### Immunohistochemical Analysis

To compare the localization of the ligands in the tumors, representative samples of tissues were examined by immunohistochemistry. Tissues were fixed in 4% buffered paraformaldehyde for 24 h, embedded in paraffin, and cut into 10-μm sections using a microtome. After incubation for 30 min in 10% normal rabbit serum (code number X0902; Dako, Copenhagen, Denmark), sections were incubated 18 h at room temperature with the primary antisera, AR (Ab 1 from Neomarkers, polyclonal), TGF-α (Oncogene, ab 1, monoclonal), βCL (AB-261-NA from RD, polyclonal), and HER1 (E30 from Oncogene, monoclonal). The primary antisera were diluted 1/100 and 1/400. The immunoreaction was visualized by means of biotinylated rabbit antirabbit immunoglobulins (monoclonal antibodies; code number 0354; Dako) or biotinylated swine antirabbit immunoglobulins (polyclonal antibodies) diluted 1/40 for 1 h as the second layer, followed by StreptABComplex/horseradish peroxidase (code number K0377) diluted 1/100 as the third layer, and finally staining by means of 3,3-diaminobenzidine for 30 min. Sections were counterstained with hematoxylin. For controls, the sections were incubated with nonimmune serum, and similar sections with immunoreagents to the various ligands were compared to exclude nonspecific reactions.

### Statistical Analysis

Nonparametric tests were used throughout this study. Two-sided P values less than 0.05 were considered to be significant. The Mann-Whitney U test and Kruskal-Wallis test were used to compare the expression of the EGF family members in Ta, T1, and T2-T4 bladder tumors. Correlations were examined using Spearman rank correlation test. Kaplan-Meier survival curves were used to estimate the survival of the patients. Log rank test for trend was used to compare the survival. The software Graph Pad Prism (version 2.00, 1995) was used for statistical analyses.

### RESULTS

#### The mRNA Expression of the EGF Family

The mRNA expression of six ligands and two receptors was quantified in biopsies from 73 bladder cancer patients. All of the six ligands and two receptors were identified in varying amounts. βCL and HB-EGF were the most abundant ligands, whereas AR, EPI, and TGF-α occurred in concentrations approximately 10-100-fold lower (Table 2). The concentration of EGF was low and could not be quantified. The median concentration of EPI, TGF-α, βCL, AR, and HB-EGF increased in muscle-invasive bladder tumors. The mRNA content of TGF-α, EPI, and HB-EGF were 6-10 times higher in T2-T4 biopsies as compared with Ta tumors. The differences were highly significant for EPI (P < 0.001), HB-EGF (P < 0.001), and TGF-α (P < 0.05). Only the mRNA expression of EPI was significantly altered in biopsies classified as T1 in comparison with T2-T4 (P < 0.05). The median concentrations of the receptors were not statistically significantly altered.

#### Survival of Bladder Cancer Patients as a Function of mRNA Expression

We examined the correlation between survival and the expression of the EGF family. The median concentration of the bladder tumors was selected as the first cutoff point for each of the family members. The second cutoff point was arbitrarily chosen (Table 3). The patients were categorized as low (<first cutoff point), medium (first cutoff point-second cutoff point), and high (>second cutoff point). The distribution is shown in Fig. 2, A–G.

Kaplan-Meier survival curves were used to evaluate the impact of ligand and receptor expression in context to survival of bladder cancer patients (Fig. 3, A–G). EPI mRNA showed a highly significant correlation to survival (P < 0.0005). Moreover, expression of AR, HB-EGF, and TGF-α correlated to survival. βCL, HER1, and HER 2 did not reflect the survival of bladder cancer patients (Table 3).

#### Immunohistochemistry of Bladder Biopsies

To identify the cells expressing the ligands of the EGF family, we analyzed a subset of the biopsies by immunohistochemistry (Fig. 4, A–F). TGF-α and AR showed a distinct staining of the luminal epithelium of Ta biopsies and of tumor cells in T2-T4 biopsies (Fig. 4, A–D). βCL was primarily

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**Table 1 Clinical data**

<table>
<thead>
<tr>
<th>Tumor stage</th>
<th>Ta</th>
<th>T1</th>
<th>T2-T4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient (no.)</td>
<td>19</td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>13</td>
<td>31</td>
</tr>
<tr>
<td>Female</td>
<td>3</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Age</td>
<td>64</td>
<td>68.5</td>
<td>68</td>
</tr>
<tr>
<td>Range</td>
<td>45–83</td>
<td>56–82</td>
<td>44–84</td>
</tr>
</tbody>
</table>

Fig. 1. Quantitation of EPI mRNA. A, a standard curve was generated by combining a fixed concentration of internal standard RNA with a dilution series of mRNA from the HCV29 cell line, followed by RT-PCR amplification as described in “Materials and Methods.” The RT-PCR products were analyzed by gel electrophoresis. Calibrators containing 1.4 × 10^{-12}, 2.1 × 10^{-12}, 2.8 × 10^{-12}, 7.1 × 10^{-12}, 1.4 × 10^{-11}, 2.1 × 10^{-11}, and 2.8 × 10^{-11} mol EPI mRNA were used in Lanes 1–7, respectively. The top and bottom bands indicate the RT-PCR product of the internal RNA standard (418 bp) and EPI mRNA (357 bp), respectively. Lane 8 shows a DNA size marker (4X174 DNA/HaeIII digest) corresponding to 1353 bp, 1078 bp, 872 bp, 603 bp, 310 bp, 281 bp, and 271 bp (running as a single band). B, the intensities of the bands in each lane of the gel were determined, and the ratio of the band originating from EPI mRNA and the RNA standard was determined. On the basis of this, a standard curve was generated by plotting the EPI/standard ratio against the amount of calibrator EPI mRNA.

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expressed by the endothelium of small vessels (Fig. 4E). EGF was negative in all of the biopsies. We could not examine EPI and HB-EGF because no antibodies suitable for immunohistochemistry were available to us. HER1 was localized to the surface membrane of cancer cells rather than from the surrounding tissues. EPI has a potential important marker in patients with bladder cancer. EPI is one of the newest members of the EGF family (21). However, Toyoda et al. (21) have shown previously that most normal human tissues express very low amounts of EPI mRNA. In contrast, a high level of EPI mRNA was observed in the T24 cell line (an invasive transitional cell carcinoma, grade 3). This observation strongly suggests that the high expression of EPI observed in bladder cancer biopsies originates from the cancer cells rather than from the surrounding tissues. EPI has been shown also to be a potent mitogen in vascular smooth muscle cells, and EPI may contribute to angiogenesis through an autocrine loop (22).

A somewhat weaker but still significant correlation between ligand expression and survival was observed for HB-EGF and AR. The impact of HB-EGF and AR expression in bladder cancer development is unclear. Ruck and Paulie (23) have shown that HB-EGF and AR are expressed in human bladder carcinoma cell lines. Moreover, like EPI, these two factors are likely to be involved in vessel formation (24, 25). Therefore, it is possible that an increased expression of EPI, HB-EGF, and AR escalates cellular growth and vascular remodeling or angiogenesis, ultimately leading to tumor progression and poor survival in patients with bladder cancer.

**DISCUSSION**

In the present paper, we report on a prospective study of bladder cancer patients followed for a median of 28 months. Using a newly developed quantitative mRNA method, we have analyzed the mRNA expression of six ligands and two receptors belonging to the EGF family in a single biopsy (13). Most importantly, we show a strong correlation between the survival of bladder cancer patients and the mRNA expression of EPI, HB-EGF, AR, and TGF-α.

Increased attention has been paid to growth factors with respect to cancer development and progression. Presently, most studies on bladder cancer and the EGF family have focused on protein expression, and only a few members of this expanding super family have been analyzed. Conflicting reports on this topic have been published, some of which have emphasized the receptors, EGF, and TGF-α to be potential markers for tumor progression and clinical outcome, whereas others have not been able to relate the ligand and receptor expression to reduced life span among these patients (5, 7, 17–19). Because of the variability of methods used in these studies, it is difficult to evaluate or conclude if any of these EGF family members may prove useful as prognostic markers in bladder cancer. Our study is unique because it allows analysis of six ligands and the corresponding receptors in the same sample using a mRNA method with a precision of approximately 25%.

In muscle-invasive bladder tumors (T2-T4), as compared with Ta tumors, we observed an increased expression of HB-EGF, TGF-α, and EPI. Moreover, the survival of bladder cancer patients was significantly impaired with increased expression of EPI, AR, TGF-α, and HB-EGF. Recently (20), we have shown an elevated expression of this subclass of HER1 receptor ligands in androgen-independent prostate cancer cell lines as compared with normal prostate epithelial cells and androgen-sensitive prostate cancer cells. The results suggest a correlation between up-regulation of these four ligands and malignant growth.

We are the first to report on the EPI mRNA expression in patients with bladder cancer. Our data show a strong correlation between the expression of EPI and survival. Unfortunately, we could not expand our results to include the expression of EPI protein because no antibody is currently available. Nevertheless, our data suggest EPI as a potential important marker in patients with bladder cancer. EPI is one of the newest members of the EGF family (21). However, Toyoda et al. (21) have shown previously that most normal human tissues express very low amounts of EPI mRNA. In contrast, a high level of EPI mRNA was observed in the T24 cell line (an invasive transitional cell carcinoma, grade 3). This observation strongly suggests that the high expression of EPI observed in bladder cancer biopsies originates from the cancer cells rather than from the surrounding tissues. EPI has been shown also to be a potent mitogen in vascular smooth muscle cells, and EPI may contribute to angiogenesis through an auto/paracrine loop (22).

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**Table 2** The mRNA expression of selected EGF family members in human bladder tumors

<table>
<thead>
<tr>
<th>EGF</th>
<th>Ta median (range)</th>
<th>T1 median (range)</th>
<th>T2–T4 median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPI (10^−21 mol/μg RNA)</td>
<td>0.9 (0.0–20.9)</td>
<td>1.6 (0.0–18.4)</td>
<td>6.5^ (0.0–144.7)</td>
</tr>
<tr>
<td>HB-EGF (10^−21 mol/μg RNA)</td>
<td>8.5 (0.0–229.1)</td>
<td>34.4 (1.6–80.4)</td>
<td>50.0^ (0.0–2001)</td>
</tr>
<tr>
<td>TGF-α (10^−21 mol/μg RNA)</td>
<td>0.4 (0.0–6.8)</td>
<td>1.0 (0.0–24.7)</td>
<td>4.9^ (0.0–1206)</td>
</tr>
<tr>
<td>HER1 (10^−19 mol/μg RNA)</td>
<td>0.17 (0.0–24.4)</td>
<td>1.01 (0.0–85.1)</td>
<td>0.77 (0.0–76.4)</td>
</tr>
<tr>
<td>HER2 (10^−19 mol/μg RNA)</td>
<td>325 (0.0–4570)</td>
<td>1530 (0.0–1610)</td>
<td>293 (0.0–4149)</td>
</tr>
</tbody>
</table>

^ a P < 0.001,   ^ b P < 0.05.

**Table 3** The ErbB family mRNA expression, cut-off points, and comparison of survival using log-rank test for trend

<table>
<thead>
<tr>
<th>EGF</th>
<th>Bladder tumors median (range)</th>
<th>Cut-off point First Second</th>
<th>Survival comparison (log rank test for trend)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR (10^−21 mol/μg RNA)</td>
<td>0.39 (0.0–25.50)</td>
<td>0.39</td>
<td>0.0</td>
</tr>
<tr>
<td>HB-EGF (10^−21 mol/μg RNA)</td>
<td>11.4 (0–1627)</td>
<td>11.4</td>
<td>30.0</td>
</tr>
<tr>
<td>TGF-α (10^−21 mol/μg RNA)</td>
<td>2.4 (0–144)</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td>HER1 (10^−19 mol/μg RNA)</td>
<td>40.0 (0–2000)</td>
<td>40.0</td>
<td>70.0</td>
</tr>
<tr>
<td>HER2 (10^−18 mol/μg RNA)</td>
<td>1.4 (0–121)</td>
<td>1.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>0.75 (0–85.1)</td>
<td>0.75</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>390 (0–4570)</td>
<td>390</td>
<td>1850</td>
</tr>
</tbody>
</table>

^ a NS, not significant.
Several studies have examined the expression of TGF-α in bladder cancer cell lines and biopsies. Malignant cells transfected with TGF-α develop a matrix-degrading potential (26). Moreover, TGF-α stimulation has been shown to induce invasion of human transitional carcinoma cells (27). Recently (3), increased expression of TGF-α was observed in bladder tumors as compared with normal bladders, but TGF-α could not be related to tumor stages. We show an increased expression of TGF-α mRNA in T2-T4 bladder tumors.

Fig. 2. The distribution of AR (A), TGF-α (B), βCL (C), HER1 (D), EPI (E), HER2 (F), and HB-EGF (G) in 73 human bladder tumors. The arrows indicate cutoff points.
patients as compared with Ta patients, but, as reported previously (18), no significant increase was observed between T1 and T2-T4. Like Ravery et al. (5), we show that TGF-α correlates strongly to survival of bladder cancer patients.

βCL did not show any alteration in expression as a function of tumor stage and survival. βCL was first isolated from a β-cell tumor (28). The expression of this ligand has now been observed in a number of tissues (29). Little is known about the cells synthesizing βCL, but

Fig. 3. Kaplan-Meier survival curves for 73 bladder cancer patients plotted for AR (A), βCL (B), EPI (C), HB-EGF (D), TGF-α (E), HER1 (F), and HER2 (G). In all of the groups, the patients were categorized into low, medium, and high.
our results suggest βCL to be produced in the endothelial cells of the small vessels. Hereby, βCL may play a central role in regeneration or remodeling of the vascular bed (28).

The HER1 protein expression in bladder cancer has been studied extensively. Our study confirms that there is no correlation between high abundance of HER1 or HER2 in bladder biopsies and the survival of the patient (5, 18, 30).

In conclusion, we report that the mRNA expression of HB-EGF, EPI, and TGF-α are increased in muscle-invasive tumors as compared with superficial bladder tumors. Moreover, we report that increased expression of EPI, TGF-α, HB-EGF, and AR correlates to poor survival of patients with bladder cancer. Our results strongly support that analysis of especially EPI mRNA may prove to be a useful tumor marker in bladder cancer.

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