Prognostic Significance of Urokinase-type Plasminogen Activator Expression in Squamous Cell Carcinomas of the Esophagus

Michael Torzewski, Mario Sarbia,1 Patrick Verreet, Philipp Dutkowski, Hansjörg Heep, Reinhart Willers, and Helmut E. Gabbert

Departments of Pathology [M. T., M. S., H. E. G.] and Surgery [P. V., H. H.], Heinrich Heine University, 40225 Düsseldorf, Germany; Department of Surgery, Johannes Gutenberg University, 55131 Mainz, Germany [P. D.]; and Computer Center, Heinrich Heine University, 40225 Düsseldorf, Germany [R. W.]

ABSTRACT

In the present study, urokinase-type plasminogen activator (uPA) expression in 150 potentially curatively resected SCCs of the esophagus was analyzed immunohistochemically by means of a murine monoclonal antibody (American Diagnostica, Greenwich, CT) and correlated with survival. Altogether, 122 of the 150 tumors (81.3%) expressed different levels of uPA. Among the 122 uPA-positive tumors, 104 (85.2%) showed a weak staining intensity, and 18 (14.8%) showed a strong staining intensity. Among the uPA-positive tumors, 29 (23.8%) tumors showed a uPA immunoreactivity in 6–25% of all tumor cells, 30 (24.6%) showed a uPA immunoreactivity in 26–50% of all tumor cells, 41 (33.6%) showed a uPA immunoreactivity in 51–75% of all tumor cells, and 22 (18.0%) showed a uPA immunoreactivity in 76–100% of all tumor cells. No significant correlation could be shown between the different patterns of uPA expression and various clinicopathological parameters, such as pT category, pN category, tumor size, histological grade, blood vessel invasion, lymphatic vessel invasion, and inflammatory response. Concerning the overall postoperative survival, no significant differences between uPA-positive and uPA-negative tumors could be verified. This also held true when different cut points in the percentage of uPA-positive tumor cells were used. In contrast, the intensity of uPA staining provided significant prognostic information in that patients with strongly uPA-positive tumors had a poorer outcome than patients with weakly uPA-positive or uPA-negative tumors. Moreover, as shown by stepwise multivariate Cox regression analysis, the intensity of uPA expression was an independent prognostic factor.

INTRODUCTION

Dissolution of and migration through basement membranes and extracellular matrices by tumor cells have been shown to be important intermediate steps in the process of cancer invasion and metastasis (1–3). In this connection, basic and clinical research has focused on the role of tumor-associated proteolytic systems (proteases-antiproteases). One of the most extensively studied systems is the urokinase pathway of plasminogen activation (4, 5). The uPA2 is a serine-protease catalyzing the conversion of plasminogen into plasmin, a proteolytic enzyme of broad substrate specificity capable of degrading (directly or through activation of latent collagenases) most proteins of the extracellular matrix. It occurs as either a single chain, an inactive proenzyme, or a two-chain active enzyme form (4–6). The active enzyme form can be blocked by a variety of physiological inhibitors, such as PAI-1 and PAI-2 (7). Binding of uPA to its specific receptor (termed uPAR or CD 87) accelerates the conversion of pro-uPA to active uPA on the cell surface (8–10). Furthermore, the receptor ensures the availability of active uPA on the cell surface by rapid internalization of inactive uPA: PAI-1 or uPA:PAI-2 complexes (11, 12).

In many human tumors, an overexpression of uPA has been found to correlate with a more aggressive biological behavior. More recently, uPA overexpression was identified as a prognostic factor predicting unfavorable outcome of patients with breast (13), gastric (14), colorectal (15), ovarian (16), and cervical cancer (17), as well as malignant melanomas (18) and soft-tissue sarcomas (19).

Concerning SCC of the esophagus, previous studies showed increased uPA levels in carcinomatous tissues compared with those in normal epithelium (20–22). However, to our knowledge, no data are available demonstrating the potential prognostic role of uPA in esophageal cancer. In the present study, we investigated the expression of uPA in 150 SCCs of the esophagus, its relation to clinicopathological parameters, and possible prognostic significance for patient survival.

PATIENTS AND METHODS

Patients. The present study is based on 150 patients who underwent potentially curative resection for SCCs of the esophagus from January 1978 to December 1992. Potentially curative resection was defined as the absence of distant metastases, the removal of all gross tumor, and the histologically confirmed absence of tumor tissue at the surgical margins. No preoperative radio- or chemotherapy was performed. One hundred twenty-one patients were male, and 29 were female. The median age

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1 To whom requests for reprints should be addressed, at Department of Pathology, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany. Phone: 49-2118118487; Fax: 49-2118118353.
was 58 years (range, 35–82 years). The follow-up ranged from 24 to 192 months after surgery (or to the date of death). Two patients were lost to follow-up. Eighteen patients died of postoperative complications (i.e., within 30 days), leaving 130 patients for the survival analyses.

**Tissue Samples.** The surgical specimens from the primary tumors were fixed in 4% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. The pT classification and the pN classification were determined according to the criteria proposed by the Union Internationale Contre le Cancer (23). Accordingly, 25 tumors were categorized as pT1 (16.7%), 26 as pT2 (17.3%), 94 as pT3 (62.7%), and 5 as pT4 (3.3%). Seventytwo cases were categorized as pN0 (48.0%) and 78 as pN1 (52.0%). The grade of tumor differentiation was determined according to the criteria proposed by the WHO (24). Eighteen tumors were graded as G1 (12.0%), 64 as G2 (42.7%), 60 as G3 (40.0%), and 8 as G4 (5.3%). Tumor size was defined as the largest diameter of the tumor. Ninety-nine tumors had a maximum diameter of 5 cm or less (66.0%), and 51 tumors were larger than 5 cm (34.0%).

Additionally, the histological review included the parameters LVI, BVI, and inflammatory response. BVI was assumed when tumor cells and RBCs were noted together in an endothelium-lined vascular space or when tumor cells were found in an endothelium-lined vascular space with a definite smooth-muscle layer. LVI was assumed when tumor cells were detected in a thin-walled endothelium-lined space containing no RBCs but occasionally containing some lymphocytes or faintly stained lymph fluid (25). Evidence of BVI was found in 37 tumors (24.7%), and LVI was found in 54 tumors (36.0%). The inflammatory response was scored as 1, indicating weak lymphomononuclear infiltrates, or 2, indicating strong lymphomononuclear infiltrates (26). Accordingly, weak inflammatory response was found in 82 tumors (54.7%), and strong inflammatory response was found in 68 tumors (45.3%).

**Antibody.** The murine monoclonal antibody [product 3689, IgG1, American Diagnostica, Greenwich, CT (distributed by LOXO, Dossenheim, Germany)], which is a common antibody for evaluating uPA expression by immunohistochemistry (16, 27, 28) was used. The antibody is directed against a β-chain epitope of human urokinase near the catalytic site and recognizes the free and receptor bound, single and two chain urokinase and the β-chain fragment.

**Immunohistochemistry.** For immunohistochemistry, 5-μm-thick slices from one representative paraffin block per tumor were used. After blocking of endogenous peroxidase activity and blocking of nonspecific conjugation with 5% normal horse serum, the slices were incubated with the primary antibody at room temperature for 1 h at a dilution of 1:50. After a second incubation with a biotin-conjugated antimouse antibody, the slices were incubated with an avidin-biotin-peroxidase reagent (26). The reaction products were visualized by immersing the slices in diaminobenzidine-tetrachloride. Finally, the slices were counterstained with hematoxylin.

Paraffin sections of normal kidney served as positive controls for uPA immunoreactivity. Negative controls were performed by replacement of the primary antibody by an irrelevant monoclonal mouse antibody.

All immunostained slides were examined independently by light microscopy by two observers (M. T. and M. S.), and the following two parameters were assessed. First, the proportion of positive tumor cells was estimated by assessing the whole tumor section and assigned to one of five scores: 0, <5% positive tumor cells; 1, 6–25% positive tumor cells; 2, 26–50% positive tumor cells; 3, 51–75% positive tumor cells; or 4, 76–100% positive tumor cells. Second, the intensity of uPA staining was scored as 1, indicating weak staining intensity, or 2, indicating strong staining intensity. The staining intensity was judged relative to the esophageal mucosa adjacent to the tumor tissue, where a weak cytoplasmic staining of uPA was found throughout all layers of the normal squamous epithelium. When different interpretations existed, a consensus was achieved after reexamination over a double-headed microscope.

**Statistical Analysis.** The correlation between the uPA immunoreactivity and other prognostic parameters was statistically analyzed by means of the Fisher’s exact test. Survival rates were calculated by the Kaplan-Meier method for analysis of censored data. The statistical significance of differences in survival was analyzed by means of the log-rank test, and the prognostic significance of parameters in multiparametric analyses by means of a stepwise forward Cox regression analysis. The parameters that were not dichotomous were dichotomized for Fisher’s exact test and the multivariate analysis as follows: pT category (pT1/pT2 versus pT3/pT4), age (≤55 years versus >55 years), tumor size (≤5 cm versus >5 cm), and grading (G1/G2 versus G3/G4). P values lower than 0.05 were considered statistically significant.

**RESULTS**

In esophageal mucosa adjacent to the tumor tissue, a weak cytoplasmic staining of uPA was found throughout all layers of the normal squamous epithelium.

In SCCs, an immunoreactivity for uPA was found in 122 of the 150 tumors (81.3%). Among the 122 uPA-positive tumors, 104 (85.2%) showed weak (Fig. 1A) and 18 (14.8%) strong staining intensity (Fig. 1B). Among the uPA-positive tumors, the percentage of tumor cells showing uPA expression varied greatly between 6 and 100%.

Compared with the normal squamous epithelium, a weak cytoplasmic staining of uPA was found throughout all layers of the normal squamous epithelium.

No differences could be found between uPA-positive tumors and uPA-negative tumors in relation to various clinicopathological parameters, such as pT category, pN category, tumor size, histological grade, BVI, LVI, and inflammatory response (Table 2). Likewise, no significant correlation between these parameters and uPA expression was found when the tumors were stratified with regard to the percentage of uPA-positive tumor cells and the intensity of uPA staining, respectively (data not shown).

Comparing the overall postoperative survival, no significant difference between uPA-negative tumors and uPA-positive tumors could be observed in the univariate survival analysis (Table 3; for the corresponding Kaplan-Meier plot, see Fig. 2a).
Likewise, no significant differences in survival could be verified, either, when different levels of the percentage of uPA-positive tumor cells were used as cut points (Table 3; corresponding Kaplan-Meier plot not shown). In contrast, the intensity of uPA staining had an unfavorable influence on postoperative survival in that the log-rank test showed that patients with intense uPA staining of the tumors had a significantly poorer outcome than patients with weak or absent uPA staining of the tumors \((P = 0.0271; \text{ Table 3; for the corresponding Kaplan-Meier plot, see Fig. 2b).}\)

In a forward multivariate Cox regression analysis, including the parameters pT category, pN category, tumor grade, tumor size, LVI, and BVI, as well as uPA expression (stratification: strong uPA expression versus weak or absent uPA expression), the latter could be verified as an independent prognostic variable \((P = 0.0001), \text{ pT category } (P = 0.0034), \text{ and pN category } (P = 0.0428; \text{ Table 4}).\)

**DISCUSSION**

In the present study, uPA expression was shown to be an important prognostic parameter associated with a more unfavor-
Table 3  Survival rates (%) of 130 patients with SCCs of the esophagus in relation to the uPA expression (log rank test)

<table>
<thead>
<tr>
<th>uPA immunoreactivity in general</th>
<th>Patients (n)</th>
<th>2-year survival (SE)</th>
<th>5-year survival (SE)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>24</td>
<td>33.3 (±9.6)</td>
<td>25.0 (±8.8)</td>
<td>NS*</td>
</tr>
<tr>
<td>Positive</td>
<td>106</td>
<td>40.6 (±4.8)</td>
<td>19.6 (±4.6)</td>
<td></td>
</tr>
<tr>
<td>Percentage of uPA-positive tumor cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–5%</td>
<td>24</td>
<td>33.3 (±9.6)</td>
<td>25.0 (±8.8)</td>
<td>NS</td>
</tr>
<tr>
<td>6–25%</td>
<td>26</td>
<td>50.0 (±9.8)</td>
<td>33.3 (±10.8)</td>
<td></td>
</tr>
<tr>
<td>26–50%</td>
<td>27</td>
<td>40.7 (±9.5)</td>
<td>11.7 (±7.3)</td>
<td></td>
</tr>
<tr>
<td>51–75%</td>
<td>33</td>
<td>30.3 (±8.0)</td>
<td>14.1 (±7.8)</td>
<td></td>
</tr>
<tr>
<td>76–100%</td>
<td>20</td>
<td>35.0 (±10.7)</td>
<td>16.0 (±9.2)</td>
<td></td>
</tr>
<tr>
<td>uPA staining intensity</td>
<td></td>
<td></td>
<td></td>
<td>0.0271</td>
</tr>
<tr>
<td>No staining</td>
<td>24</td>
<td>33.3 (±9.6)</td>
<td>25.0 (±8.8)</td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>90</td>
<td>44.4 (±5.2)</td>
<td>22.5 (±5.2)</td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>16</td>
<td>12.5 (±8.3)</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

* NS, not significant.

Table 4  Ranking of different prognostic parameters in 130 SCCs of the esophagus

<table>
<thead>
<tr>
<th>Prognostic parameter</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVI</td>
<td>0.0001</td>
</tr>
<tr>
<td>uPA expression</td>
<td>0.0027</td>
</tr>
<tr>
<td>pT category</td>
<td>0.0034</td>
</tr>
<tr>
<td>pN category</td>
<td>0.0428</td>
</tr>
<tr>
<td>Tumor size</td>
<td>NS*</td>
</tr>
<tr>
<td>Tumor grade</td>
<td>NS</td>
</tr>
<tr>
<td>BVI</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, not significant.

Fig. 2  a, overall survival in 130 esophageal cancer patients with (uPA positive) or without (uPA negative) uPA expression (log-rank test; not significant). b, overall survival in 130 esophageal cancer patients with weak or absent (uPA weakly positive or absent) or intense (uPA strongly positive) uPA expression (log-rank test; P = 0.0271).

Able outcome in patients with SCCs of the esophagus. The prognostic impact was independent of other prognostic parameters, such as pT category, pN category, tumor size, histological grade, BVI, and LVI. Therefore, our results concur with the theory of the putative role of uPA in disease progression as a key point in the process of cancer invasion and metastasis (30).

In our series of 150 SCCs of the esophagus, uPA expression could be demonstrated immunohistochemically in the majority of all tumors. The proportion of uPA-expressing tumors verified in our study is within the range that was found in other tumor types, such as cervical (81%; Ref. 17), ovarian (100%; Ref. 16), and gastric (65.6%; Ref. 31) cancer. The uPA staining of tumor cells observed in this study was in general diffuse and cytoplasmic, as was also shown in various other human neoplasms (21, 32–35).

In contrast to other current methods for the assessment of uPA expression in tumor tissues (e.g., Western blot and ELISA), immunohistochemistry provides the opportunity to directly analyze which types of cells (e.g., tumor cells or stroma cells) are positive or negative for uPA expression in a tissue sample. Furthermore, immunohistochemical staining of paraffin-embedded tumor samples facilitates prognostic studies because it allows the analysis of large archival series of one tumor type.

The validity of immunohistochemistry has recently been shown by direct comparison with quantitative methods (ELISA) of uPA measurement (17).

Recent survival studies identified uPA as a prognostic factor predicting unfavorable outcome of patients with breast (13), gastric (14), colorectal (15), ovarian (16), and cervical (17) cancer, as well as malignant melanomas (18) and soft-tissue sarcomas (19), in univariate survival analysis. In cases in which multivariate survival analysis was performed, however, none of the above studies could verify uPA expression as independent prognostic factor. In the present study, both univariate and multivariate survival analyses were performed, verifying uPA expression as a strong independent prognostic variable in SCC of the esophagus, with the staining intensity being the stratifying parameter.
factor for prognostic purposes. This might reflect an overexpression of uPA in tumor cells resulting in an accumulation of the enzyme over the level that is present in other tumor cells or the normal squamous epithelium, showing a weak constitutive uPA expression. A possible mechanism leading to overexpression of uPA in tumor cells might be the modulation of the plasminogen activator by inflammatory mediators. In several tumor cell lines, an up-regulation of uPA expression can be induced in vitro by inflammatory cytokines, such as IL-1α (36), IL-1β (36-38), IL-6, and tumor necrosis factor α (38, 39). In vivo, cytokines and growth factors may have their source in the tumor-infiltrating lymphocytes and macrophages, which are regularly found in the stroma of malignant tumors. The amount of stromal inflammatory cell reaction has been shown to have a significant influence on the prognosis of patients with breast cancer (40), cervical cancer (41), and laryngeal dysplasia (42) and indirectly in esophageal neoplasia (43). Accordingly, we were previously able to demonstrate that the degree of inflammatory cell response is an independent prognostic parameter in esophageal cancer (26). In this study, a positive correlation has been found between an increasing density of the inflammatory infiltrate and a more favorable outcome of esophageal cancer patients. However, the degree of inflammatory cell response does not seem to influence the uPA expression in tumor cells because we did not find any correlation between these two parameters in the present study.

Alternatively, the strong cytoplasmic uPA staining intensity of the tumor cells could be caused by increased and rapid internalization of inactive uPA:PAI-1 and uPA:PAI-2 complexes (11, 12). Both mechanisms, either individually or combined with each other, might result in increased availability of active uPA on the cell surface and thus determine the capacity of the respective tumor cells for invasion and metastasis (30).

In some tumor types, an intense uPA staining of the noncancerous elements of tumor tissue (i.e., stromal fibroblast-like cells and macrophages) was observed in addition to the staining of the tumor itself (32, 44, 45). Moreover, in cervical cancer of the uterus and colorectal neoplasia, stromal cells were identified as the predominant sites of uPA production, indicating that in these tissues, uPA is produced by the stromal and not by carcinomatous cells (17, 46). In line with these previous reports, we also observed an intense uPA staining of the noncancerous elements of tumor tissue. However, because stromal fibroblast-like cells and macrophages showed an equal uPA positivity in all tumors, analysis of this staining pattern was unsuitable for prognostic purposes. This result is consistent with recent findings in laryngeal carcinomas, where stromal fibroblast-like cells and macrophages also showed uPA positivity in all tumors, independent of the histological grading and lymph node involvement (28).

In conclusion, in our study, uPA immunoreactivity with a different staining intensity was found in most SCCs of the esophagus. Moreover, it was proved that the evaluation of the intensity of the uPA expression in the tumor tissue provides additional prognostic information in this tumor type. It can be regarded as important prognostic parameter for patients who underwent potentially curative resection for SCCs of the esophagus not only for a more precise identification of high-risk patients but also as a criterion that will be helpful in identifying candidates for adjuvant therapy in future clinical trials.

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