Selective Immunohistochemical Staining of Blood and Lymphatic Vessels Reveals Independent Prognostic Influence of Blood and Lymphatic Vessel Invasion in Early-Stage Cervical Cancer

Peter Birner,1 Andreas Obermair, Monika Schindl, Heinrich Kowalski, Gerhard Breitenecker, and Georg Oberhuber

Institute of Clinical Pathology [P. B., H. K., G. B., G. O.] and Department of Gynecology and Obstetrics [A. O., M. S.], University of Vienna, A-1090 Vienna, Austria

ABSTRACT

Lymphovascular space invasion was shown to play a key role in the progression of cervical cancer. Because of the absence of a specific marker for lymphatic vessels, earlier studies could not reliably distinguish between blood and lymphatic vessel invasion. By immunostaining for podoplanin, a novel marker for lymphatic endothelium, and for factor VIII-related antigen, we determined lymphatic and blood vessel invasion in tissue samples of 98 patients with cervical cancer pT1b treated by radical hysterectomy. Eleven (11.2%) specimens showed invasion of blood vessels, 20 (20.4%) showed invasion of lymphatic vessels, and 15 (15.3%) showed invasion of blood and lymphatic vessels. There was a strong association of lymphatic vessel invasion and lymph node involvement (P < 0.001). In univariate analysis, both blood and lymphatic vessel invasion failed to reach a statistically significant influence on overall survival, but a significant influence on disease-free survival was found (P = 0.0002 and P < 0.0001, respectively). In multivariate analysis of disease-free survival, only blood vessel invasion remained statistically significant (P = 0.0457). Lymphatic vessel invasion reached significance when lymph node status was excluded from the model (P = 0.0025). Both lymphatic vessel and blood vessel invasion occur frequently in early-stage cervical cancer. Determination of the vessel status may be of clinical importance because it signifies the risk of recurrent disease.

INTRODUCTION

Although cervical screening has led to a significant decrease in the incidence of cervical cancer in industrialized countries (1), it is still one of the most common female cancers worldwide (2). Fortunately, in developed countries cervical cancer is frequently diagnosed in early stages by cytological screening (3) with most patients first seen with a stage 1 disease. Despite the good prognosis of stage 1 cervical cancer, approximately 20–35% of the patients are expected to die from their disease (4). To better determine the risk of tumors, prognostic factors including lymph node status, depth of invasion, and lymphovascular space involvement have been established (5).

It is widely accepted that the invasion of tumor cells into blood and lymphatic vessels is one of the critical steps for the establishment of metastasis (6). One of the shortcomings of previous morphological studies is the fact that it is almost impossible to exactly differentiate between blood and lymphatic vessel invasion in H&E-stained sections. Therefore, immunohistochemistry has been proposed as an investigative tool (7). Blood vessels can be reliably identified by immunostaining, e.g., for factor VIII-related antigen (7), with only some lymphatic endothelia staining weakly. Until recently, no reliable marker for lymphatic vessels in paraffin-embedded specimens was available (8, 9). With a polyclonal antibody recognizing podoplanin (10), it is now possible to selectively stain lymphatic vessels (11).

Podoplanin is a Mr ~38,000 membrane mucoprotein that was originally detected on the surface of rat glomerular epithelial cells (podocytes) and was found to be linked to flattening of foot processes that occurs in glomerular diseases (12). Podoplanin shows features of a membrane mucoprotein with several conserved O-glycosylation sites. Currently, it is of unknown biological function (10). Because heavily O-glycosylated mucoproteins were identified recently as counterreceptors for selectins that mediate adhesion of inflammatory cells (13), it is possible that podoplanin plays a similar role in lymphatic endothelia (10).

In the present study, we present data on the prognostic value of lymphatic and blood vessel invasion in samples of cervical cancers of Union International Contre Cancer classification pT1b (clinically visible lesion confined to the cervix). Blood and lymphatic vessels were differentiated by immunostaining for podoplanin and factor VIII-related antigen.

MATERIALS AND METHODS

Patients and Tissues. Formalin-fixed, paraffin-embedded surgical specimens of 98 consecutive patients with invasive cervical cancer, stage pT1b, were examined. Diagnosis was established preoperatively by punch biopsy or cone excision, and all patients were treated with radical hysterectomy and pelvic lymph node dissection. In cases with pelvic lymph node metastases or tumor invasion of the outer third of the uterine cervix, adjuvant radiation therapy was applied postoperatively. Radiation therapy consisted of brachytherapy at a total dose of 42 Gy applied intracavitarily. In patients with positive lymph
nodes, external beam radiation at a total dose of 50 Gy was applied. Tumors were considered bulky when infiltrating the outer third of the cervix or having a diameter of 40 mm or more.

Immunohistochemistry. Rabbit antihuman podoplanin IgG was raised against the recombinant human homologue of the rat M, 43,000 glycoprotein podoplanin as described previously (10). Affinity purification of rabbit serum was performed using nitrocellulose strips containing recombinant protein (14).

Histological slides, 4 μm in thickness, were deparaffinized in xylol. Slides were heated in 0.01 m citrate buffer for 16 min in a microwave oven. After cooling for 20 min and washing in PBS, endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. For immunohistochemical detection of podoplanin, specimens were incubated at room temperature with the polyclonal rabbit antibody in a dilution of 1:2000 for 1 h. Immunohistochemical detection of factor VIII-related antigen was performed on a separate slide from the same block using a polyclonal rabbit antibody (BioGenex, San Ramon, CA) according to a standard protocol (4). Detection of positive staining for both antigens was performed using the ChemMate kit (DAKO, Glostrup, Denmark) and 3-amino-9-ethylcarbazole (BioGenex, San Ramon, CA) for podoplanin immunostaining and diaminobenzidine for factor VIII immunostaining. Counterstaining was performed using hematoxylin. A tissue block of breast cancer with a high microvessel density served as a positive control. The specimen has already been used in previous studies (4, 15). The negative control slide was prepared from the same tissue block. Instead of the primary antibody, a nonimmune serum was applied. All slides were investigated by one single pathologist (P.B.) blinded to the cases to prevent any interindividual classification errors. Lymphovascular space involvement in immunostained slides was considered positive if at least one tumor cell cluster was clearly visible in a decorated lymphovascular space (16). Because factor VIII-related antigen is also weakly expressed on a few lymphatic vessels (17), stained areas were only considered blood vessels, when they were clearly and strongly decorated by the antibody (7). Furthermore, results of factor VIII and podoplanin staining of consecutive sections were compared, allowing exact classification of the labeled vessels. For comparison, lymphovascular space involvement was determined in routinely H&E-stained slides. In this setting, differentiation between blood and lymphatic vessel was not attempted.

Statistical Methods. Correlation of vessel infiltration and lymph node status, histological grading, and tumor size was tested using the χ² test or Mann-Whitney test, as appropriate. In patients with recurrent disease, the influence of lymphatic and/or blood vessel invasion on the localization of recurrent disease (local or distant) was computed by a regression model.

OS² was defined as the period from primary surgery until the death of the patient. Death from any other cause than cervical cancer or survival until the end of the observation period was considered an event to be censored. DFS was defined as period in between the end of the primary therapy and the first evidence of progression of disease. Univariate analysis of survival was performed as outlined by Kaplan and Meier (18). The Cox proportional hazards model was used for multivariate analysis. Age, lymph node status, grading, lymphovascular invasion, and tumor size (bulky versus nonbulky) were entered into Cox regression.

For all tests, P of ≤ 0.05 was considered significant. All Ps given are results of two-sided tests.

RESULTS

The mean age of the patients at the time of diagnosis was 42.8 ± 10.7 years. The median observation time was 85.6 ± 43 months. During this observation period, 30 patients (30.6%) developed recurrent disease (22 local recurrence and 8 distant metastasis) and died. In specimens from 52 (53.1%) patients, no lymphovascular invasion by tumor cells was found. In 11 (11.2%) specimens, an invasion of blood vessels (Fig. 1a), in 20 (20.4%) an invasion of lymphatic vessels (Fig. 1b), and in 15 (15.3%) an invasion of blood and lymphatic vessels were observed. In 33 cases, vessel invasion was already suspected in conventionally H&E-stained sections. In 10 of these specimens, this result could not be verified by immunohistochemistry. In 23 additional cases, immunohistochemistry revealed lymphovascular invasion that had not been found in H&E-stained slides (Table 1). As a result, H&E-stained slides were false positive in 10.2% and false negative in 23.5%. χ² test revealed a significant association of lymphatic vessel infiltration and lymph node involvement (P < 0.001); 65.7% of patients with lymphatic vessel invasion had positive lymph nodes. In contrast, no correlation of blood vessel invasion and lymph node involvement was found (P = 0.182; Table 2).

In patients with recurrent disease, no significant influence (P = 0.12) of lymphatic and/or blood vessel invasion on the localization of recurrent disease (local or distant) was found in a regression model, in which also lymph node status was included. Both blood vessel and lymphatic vessel invasion failed to attain a statistically significant influence on overall survival in univariate (P = 0.2165 and P = 0.7583, respectively; log-rank test) and multivariate (Table 3) analysis. Log-rank test revealed a significant influence of blood vessel as well as lymphatic vessel invasion on DFS in univariate analysis (P = 0.0002 and P < 0.0001, respectively). In multivariate analysis of DFS, only blood vessel invasion remained statistically significant (Table 3). In multivariate analysis that did not include lymph node status, lymphatic vessel invasion (P = 0.0025) and bulky disease (P = 0.02) but not blood vessel invasion (P = 0.1017) reached statistical significance (Table 3). The five-year DFS rate was 80.6% in patients without blood vessel invasion, whereas it was only 46% in patients with blood vessel invasion (Fig. 2a). Five-year DFS rate in patients without lymphatic vessel invasion was 84.1%, whereas in patients with lymphatic vessel invasion, it was 48.2% (Fig. 2b).

DISCUSSION

In this study, data on the prognostic influence of blood and lymphatic vessel invasion in early-stage cervical cancer are presented. For the first time exact differentiation between these

---

² The abbreviations used are: OS, overall survival; DFS, disease-free survival.
two types of vessels was performed by applying factor VIII and podoplanin immunostaining. Podoplanin is a recently developed highly specific marker for lymphatic vessels, which has been used to classify tumors of vessel origin (10).

Our study revealed that 35.7% of the tumors showed lymphatic vessel invasion. This is in good correlation with the findings of Sakuragi et al. (6), who found lymphatic vessel invasion in 37.2–67.6%, depending on the stage of the disease. In 26.5% of cases, we observed blood vessel invasion, which is slightly more than in the study by Sakuragi et al. (6), who observed blood vessel invasion in 21% of samples in H&E-stained specimens (6). One shortcoming of the work of Sakuragi et al. (6) was, however, that differentiation between vessel types was determined by normal light microscopy using histomor-

Table 1  Correlation between lymphovascular invasion assessed by immunostaining and in H&E-stained specimens

<table>
<thead>
<tr>
<th>Lymphovascular invasion based on immunostaining</th>
<th>Lymphovascular invasion in H&amp;E-stained specimens</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>10</td>
<td>42</td>
</tr>
<tr>
<td>Blood vessels</td>
<td></td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Lymphatic vessels</td>
<td></td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Blood and lymphatic vessels</td>
<td></td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2  Association of immunohistochemically assessed vessel invasion with lymph node involvement

<table>
<thead>
<tr>
<th>Types of vessels infiltrated</th>
<th>No. of patients with positive lymph nodes</th>
<th>No. of patients with negative lymph nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Lymphatic vessels</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Blood and lymphatic vessels</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
phology. Therefore, these data had to be confirmed by studies using markers that are specific for lymphatic vessels, as also suggested by the authors themselves (6).

Other investigators studied lymphovascular space involvement in H&E-stained sections without differentiation between blood and lymphatic vessel invasion. Whereas some found lymphovascular space involvement as a prognostic factor in multivariate analysis (19, 20), others did not (21, 22). We suggest that, at least in part, the lower capability to detect invasion of microvessels in H&E-stained specimens and a possible influence of the investigator’s skill on the rate of detection of lymphovascular invasion might explain these differing results.

The comparison of specimens with or without lymphovascular space invasion detection in H&E-stained sections with findings in immunohistochemistry revealed that ~33% of cases were incorrectly classified in our study. Comparison with data in the literature revealed that the percentage of lymphovascular space invasion (33.7%) is comparable with that observed by others in H&E-stained sections. Although in various studies results it ranged from 6% (23) to 63% (24), lymphovascular space invasion was usually found in 20–30% (25). Our results show that data based on lymphovascular space invasion determined in H&E-stained sections have to be interpreted with care.

Sakuragi et al. (6) reported a significant influence of blood vessel invasion on OS, but no influence of lymphatic vessel invasion on OS was found (6). Analysis of DFS had not been performed by these authors (6). In our study, multivariate analysis revealed that both blood and lymphatic vessel invasion did not have a significant influence on OS, but blood vessel invasion was significantly associated with shorter DFS. This is of particular interest, because in our patient population all patients who developed recurrence also died of their disease. Lymphatic vessel invasion was significantly associated with lymph node involvement. This explains the finding that lymphatic vessel invasion lost its prognostic significance in multivariate analysis, including lymph node status. We showed here for the first time that in a setting with unknown lymph node status, lymphatic vessel invasion becomes an independent prognostic factor, with patients showing a significantly shortened DFS despite the early stage of cervical cancer. This is of potent clinical importance. In cases where primary irradiation instead of radical hysterectomy of early-stage cervical cancer is intended or lymph node status could not be evaluated, immunostaining for podoplanin might deliver additional information because of the strong correlation between lymphatic vessel invasion and lymph node involvement and might serve as a basis for further therapeutic decisions.

In conclusion, both lymphatic vessel and blood vessel invasion occur frequently in early-stage cervical cancer. Deter-

| Table 3 Multivariate analysis of OS and DFS in 98 patients with cervical cancer stage pT1b |
|-----------------------------------------------|----------------|----------------|
| Significance | 95% confidence interval | Relative risk |
| OS | Blood vessel invasion | \( P = 0.3937 \) | 1.04–9.56 | 3.15 |
| | Lymphatic vessel invasion | \( P = 0.4319 \) | 1.02–5.15 | 2.29 |
| | Lymph node involvement | \( P = 0.0428 \) | 1.02–5.15 | 2.29 |
| | Tumor size | \( P = 0.9739 \) | | |
| | Age | \( P = 0.9739 \) | | |
| | Grading | \( P = 0.0405 \) | 1.02–2.85 | 1.71 |
| DFS | Blood vessel invasion | \( P = 0.0457 \) | 1.02–5.15 | 2.29 |
| | Lymphatic vessel invasion | \( P = 0.1645 \) | 1.02–5.15 | 2.29 |
| | Lymph node involvement | \( P = 0.108 \) | 1.02–5.15 | 2.29 |
| | Tumor size | \( P = 0.0821 \) | 1.02–5.15 | 2.29 |
| | Age | \( P = 0.4519 \) | 1.02–5.15 | 2.29 |
| | Grading | \( P = 0.301 \) | 1.02–5.15 | 2.29 |

DFS (without lymph node status)

Blood vessel invasion | 0.1017 |
Lymphatic vessel invasion | 0.0025 | 1.59–8.88 | 3.7625 |
Tumor size | 0.02 | 1.16–5.74 | 2.5809 |
Age | 0.7021 |
Grading | 0.2759 |

Fig. 2 a, cumulative DFS of patients with cervical cancer without blood vessel invasion (A) and with blood vessel invasion (B). b, cumulative DFS of patients with cervical cancer without lymphatic vessel invasion (A) and with lymphatic vessel invasion (B).
mination of the vessel status is considered to be of clinical importance because both blood and lymphatic vessel invasion are markers for high risk of recurrence.

ACKNOWLEDGMENTS

We thank Andrea Güthlin-Dörfler for expert technical assistance. The continuous support of D. Kerjaschki was highly appreciated.

REFERENCES

Selective Immunohistochemical Staining of Blood and Lymphatic Vessels Reveals Independent Prognostic Influence of Blood and Lymphatic Vessel Invasion in Early-Stage Cervical Cancer

Peter Birner, Andreas Obermair, Monika Schindl, et al.