Prognostic Significance of Serum Antibodies to HPV-16 L1 Virus-like Particles in Patients with Invasive Cervical Cancer

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Abstract. Background: Persistent infection with high-risk human papilloma virus (HPV) is a prerequisite for the development of cervical cancer. The prognostic value of HPV-16 capsid antibodies in patients with invasive cervical cancer and its correlation with clinicopathological factors were investigated. Patients and Methods: Serum samples from 150 patients with invasive cervical cancer and 40 healthy female control subjects were analyzed by ELISA for HPV-specific antibodies to HPV-16 L1 virus-like particles (VLPs). Results: HPV-16 L1 antibodies were detectable in 65 out of 150 patients (43.3%) and in 12 out of 40 controls (30.0%). Seropositivity was correlated with prolonged, progression-free (p=0.012) and overall survival (p=0.043). Especially in the early FIGO-stages I and II antibodies to HPV-16 L1, VLPs predicted a better outcome. Conclusion: Antibodies to HPV-16 L1 capsid protein may be of prognostic value for patients with invasive cervical cancer and lack of HPV-16 L1 antibodies may indicate a group of patients with a poor prognosis.

Cervical cancer is the second most frequent cause of death from cancer, throughout the world (1). In many developing countries, it is even the main cause of cancer deaths in women. Although the industrialized countries have succeeded in reducing the incidence and mortality rates, by introducing effective screening tests (such as the Papanicolaou smear), the total number of cases is still very high (2). Every year, 500,000 new diagnoses of cervical cancer are recorded, and some 350,000 patients die of the disease (3). It has been demonstrated that malignant cervical lesions are almost always associated with persistent infection with high-risk types of human papillomavirus (hrHPV), and HPV DNA has been detected using sensitive polymerase chain reaction (PCR) assays, in nearly 100% of tumor specimens (4). Some 15 different types of genital HPV are known to be oncogenic in humans, most notably HPV types 16 and 18, which account for 50-60% and 20% of cases of invasive cervical cancer, respectively (5).

As HPV does not grow effectively in cell culture, systems using HPV virus-like particles (VLPs) have been used to study viral infectivity. The antibody response to HPV is type-specific, and the detection of human papillomavirus capsid antibodies in a patient’s serum, proves that there has been past infection. Seropositivity is connected with a threefold increase in the risk of developing cervical intraepithelial neoplasia or cervical cancer, and, in contrast to DNA-based assays, serological tests are able to identify previous, as well as current HPV infection, as the antibodies against the capsid proteins are detectable for many years after the infection has been cleared (6-8). Several current vaccination studies are using HPV L1 VLP as an immunogenic stimulus for the development of protective antibodies (9). Specific antibodies for HPV-16 L1 have been detected between four months and five years after a primary infection, but some individuals fail to produce anti-HPV L1 antibodies and remain seronegative (10). It has been suggested that seropositivity after an HPV infection is associated with an increased risk of developing cervical dysplasia and progression to cervical cancer, as well as a poor prognosis, in patients with invasive cervical cancer (11).

The purpose of the present study was to assess whether antibodies to HPV-16 L1 VLPs can be regarded as a prognostic marker in patients with cervical cancer, or not. The seroprevalence of antibodies in cervical cancer patients was investigated using a commercially available VLP-based enzyme-linked immunosorbent assay (ELISA). The data obtained were assessed against the histopathological, clinical, and other individual parameters.

Patients and Methods

The population study consisted of 150 patients with invasive cervical cancer, at the International Federation of Gynecology and
Obstetrics (FIGO) stages IB1–IV (aged 24-89 years, median 50.5 years), who were treated in the Department of Obstetrics and Gynecology at Erlangen University Hospital in Germany between 1995 and 2000. The clinical stage was determined at the time of the diagnosis, in accordance with the FIGO staging system for cervical cancer, by a gynecological oncologist and the head of the department. Histological diagnosis was carried out by two gynecological pathologists in the Department of Pathology at Erlangen University Hospital, who were not blinded to the original diagnosis. Blood samples were taken after the diagnosis, before treatment, and during the follow-up, and were stored at –80°C until analysis. To verify the quality of serum samples for the HPV-16 L1 VLP test, a protein stability test using human serum albumin, was carried out.

After the initial diagnosis, the patients underwent radical hysterectomy (Piver types II or III), in accordance with the international standards. Primary intracavitary and external beam radiotherapy were administered when the patients declined surgery, were not eligible for surgery, or when FIGO stages III or IV were suspected. After the primary treatment, the patients were regularly examined in the Hospital or by their gynecologists, during a follow-up period, ranging from 0.1 to 12.8 years (mean 4.6 years). Routine check-up examinations used to assess the course of the disease included systemic reviews, Papanicolaou (Pap) smears, pelvic examinations, ultrasonography, tumor-marker testing, chest radiography, and computed tomography or magnetic resonance imaging, when necessary. The patients were classified according to the outcome of the disease: 64% were alive without evidence of disease, 4% were alive with recurrence or metastasis, 30% died of cervical cancer, and 2% died of other causes. The histopathological results, clinical parameters, and treatment measures were recorded using a relational database (Statistical Package for the Social Sciences version 11.0; SPSS, Inc., Chicago, Illinois, USA). Since 1996, a Hybrid Capture II test has been carried out in all patients with cervical cancer to determine their HPV status.

A group of 40 cytologically healthy patients, aged 17–73 years (mean 34 years), were recruited as a control group. Those women visited the gynecological outpatient clinic for routine Pap smears and colposcopy, between 2003 and 2004. The patients did not show any evidence of malignancies, pathological Pap smears, genital infections, or pregnancy. All of the women in the control group tested negative for current HPV infection, using the Hybrid Capture II assay. All of the women provided informed consent to the storage of their serum samples and their use for research purposes.

Serology. For serological assays, an improved commercially available VLP-based ELISA was used (Cytoimmun Diagnostics, Ltd., Pirmasens, Germany). VLPs consisting of HPV-16 L1 capsid proteins were generated in eukaryotic cells. The particles were extracted, purified, and diluted in a storage buffer. Serum samples were analyzed for serum immunoglobulin G (IgG) antibodies directed against HPV-16 L1 capsid antigens. HPV-16 VLPs were used as antigens, VLP-free bovine serum albumin (BSA) was the control antigen, and three defined human HPV-16 IgG serum samples were treated as positive controls. To produce heparin-based ELISAs, microtiter plates (Polyborb; Nunc, Ltd., Wiesbaden, Germany) were coated with heparin–BSA, and free binding sites were blocked with BSA. After incubation and washing with phosphate-buffered saline 0.05% Tween 20 (PBST), the plates were dried, sealed, and stored at 4°C, until use (12). The following steps were required for detection of serum antibodies: 100 μl of HPV-16 VLP were added to the wells for an incubation period of 1 h at 37°C. After three washes with 350 μl PBST, serum diluted 1:50 in PBST supplemented with 10% horse serum (Gibco/Invitrogen, Ltd., Karlsruhe, Germany) were added for an incubation period of 1 h at 37°C. After three washes with 350 μl PBST, 100 μl of a horseradish peroxidase-coupled secondary antibody were added (goat anti-human IgG, 1:10,000 in storage buffer; Jackson ImmunoResearch Europe Ltd., Soham, United Kingdom). After 30 min at 37°C, the wells were washed three times with 350 μl PBST. Trimethylbenzidine substrate, 100 μl, was added to start the development of the assay at 37°C. Ten min later, the reaction was stopped with 100 μl 1N HCl. Optical densities were read on an ELISA reader (BEP 2000 Advance; Dade Behring Marburg, Ltd., Marburg, Germany) at 450 nm. The mean optical density value of VLP-free negative controls, measured in parallel for all serum samples, was subtracted to determine the final absorbance. The cut-off value was calculated as the mean value plus the two standard deviations, including all samples, except the outliers.

Statistics. The patients’ survival times were calculated from the day of surgery to the date of last contact or death. Overall survival and probabilities were estimated using the Kaplan-Meier method. The log-rank test was used to detect differences between survival curves for stratified variables. Univariate and multivariate (stepwise, forward) Cox regression analyses were used to reveal independent prognostic factors and estimate their hazard ratios; 95% confidence intervals were calculated for all relevant factors. Significance was assumed for a p-value smaller than 5%. All data were analyzed using the SPSS version 13.0 (SPSS, Inc., Chicago, Illinois, USA).

Results

This retrospective analysis included serum samples from 150 women with invasive cervical cancer (for patients characteristics see Table I) and from 40 healthy women, who had tested negative for HPV with Hybrid Capture II. The median follow-up period in the cervical cancer group was 77.7 months. The primary treatment was mainly surgery, either alone (75 out of the 150) or in combination with postoperative radiotherapy (27 out of the 150) or chemoradiotherapy (20 out of the 150). Thirteen out of the 150 patients were treated with primary radiotherapy, and eleven patients received primary chemoradiotherapy. Four patients declined any further therapy after the primary diagnosis. All but twelve of the patients were found to be positive for high-risk HPV using the Hybrid Capture II assay; in these twelve patients the test was not yet available at the time of the diagnosis.

Seroresponse for HPV-16 L1 VLPs was detected in preoperative sera from 65 out of the 150 patients (43.3%) and in 12 out of the 40 control patients (30.0%). Correlations between HPV-16 L1 VLP positivity and significant clinicopathological factors are shown in Table II. Seroresponse and seronegative patients were found to be evenly distributed among HPV-16 L1 VLP positive and
negative patients, as well as the FIGO stages and the prognostically important clinicopathological factors, such as lymph-node status, grading, lymphovascular space involvement, and histology.

In the univariate survival analysis, the detection of antibodies against HPV-16 L1 VLPs was found to be significantly associated with longer disease-free survival \((p=0.012, \text{Figure } 1)\) and longer overall survival \((p=0.044, \text{Figure } 2)\). The mean overall survival time was 90.6 months in the HPV-16 L1 VLP-positive group (95% CI, 79.8 to 100.2 months) and 76 months (95% CI, 67.2 to 86.2 months) in the HPV-16 L1 VLP-negative group, while the median was not reached in none of the groups. The mean recurrence-free survival time was calculated as 89.3 months in HPV-16 L1 VLP-positive patients (95% CI, 78.8 to 99.9 months) and 75.0 months in HPV-16 L1 VLP-negative patients (95% CI, 63.9 to 86.1 months). Sixteen out of the 65 seropositive patients (24.6%) died of the disease, in comparison to 36 out of the 85 seronegative patients (42.4%); 47.1% of the seronegative patients had recurrent disease, in comparison with 25.1% in the seropositive group \((p=0.004)\). In the early FIGO stages I and II, seven out of the 46 patients in the seropositive group (13.2%), but 18 out of the 48 patients in the seronegative group (27.3%) died of the disease \((p=0.048)\). Five years after diagnosis, 79.6% of the HPV-16 L1 VLP-positive patients were still alive, in comparison with 61.4% of the HPV-16 L1 VLP-negative patients. Patients with a negative HPV-16 L1 VLP status, thus, having a significantly less favorable prognosis than women with antibodies to HPV-16 L1 VLPs, corresponded to an adjusted hazard ratio of 1.81 (Table III).

However, in the multivariate analysis, using a stepwise forward Cox regression model for the univariate significant parameters, such as age, FIGO stage, lymph-node status, lymphovascular space involvement, grading and HPV-16 L1 VLP status regarding overall survival, only a positive lymphnode status was found to be significantly correlated with survival (Hazard ratio 6.062; 95% CI 2.3-11.3, \(p=0.001\)), while all other factors failed to show significant differences.
Discussion

In this analysis, detection of antibodies to HPV-16 L1 VLPs was associated with a better recurrence-free and the overall survival in patients with cervical cancer. Particularly in the early FIGO stages I and II, overall survival and recurrence-free survival were statistically significantly better in the seropositive group than in seronegative patients.

A prerequisite for the synthesis of antibodies against a specific antigen is exposure of the antigen to specific B lymphocytes and successful interaction between the B cells and T helper cells. Antibodies against HPV-16 L1 VLPs are highly type-specific. Detection of these antibodies has been shown to be clear proof of HPV-16 infection, showing previous HPV infections better than DNA-based tests, and identifying earlier HPV infection, even after clearance of the HPV DNA (13).

In accordance with findings previously reported in the literature, antibodies to HPV-16 L1 VLPs have been found in 46% of the patients with cervical cancer and 30% of the control patients (21). Particularly in the early FIGO stages, patients with antibodies to HPV-16 L1 VLPs were found to be less likely to develop a recurrence or die of the cervical neoplasia.

The FDA-approved Hybrid Capture II test has been used to detect 13 different high-risk types of HPV, rather than a PCR test for differentiating between single virus types. This means that the possibility that other high-risk types of HPV in addition to HPV-16, such as HPV-18, HPV-31 and others, might have been associated with the development of the cervical carcinomas, could not be excluded. On the other hand, the only commercially available HPV L1 VLP ELISA that is type-specific for HPV-16, has been used. For this reason, it could not be clearly demonstrated whether the

Table III. Univariate Cox proportional hazard analysis of pathologic criteria on overall survival.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>No. of deaths</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50 yrs</td>
<td>80</td>
<td>17</td>
<td>1.00</td>
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<td></td>
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<tr>
<td>&gt;50 yrs</td>
<td>70</td>
<td>35</td>
<td>2.34</td>
<td>1.53-4.89</td>
<td>0.001</td>
</tr>
<tr>
<td>Lymph node negative</td>
<td>70</td>
<td>7</td>
<td>1.00</td>
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<td></td>
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<tr>
<td>positive</td>
<td>47</td>
<td>25</td>
<td>7.65</td>
<td>3.29-17.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Lymph vascular space invasion (LVI) negative</td>
<td>65</td>
<td>6</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>positive</td>
<td>51</td>
<td>20</td>
<td>4.10</td>
<td>2.68-6.267</td>
<td>0.001</td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>93</td>
<td>26</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>51</td>
<td>21</td>
<td>1.43</td>
<td>1.081-1.915</td>
<td>0.013</td>
</tr>
<tr>
<td>Anti-HPV16 L1 positive</td>
<td>85</td>
<td>16</td>
<td>1.00</td>
<td></td>
<td></td>
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<tr>
<td>negative</td>
<td>65</td>
<td>36</td>
<td>1.81</td>
<td>1.008-3.277</td>
<td>0.047</td>
</tr>
<tr>
<td>FIGO-Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>74</td>
<td>12</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>46</td>
<td>14</td>
<td>2.13</td>
<td>0.985-4.610</td>
<td>0.049</td>
</tr>
<tr>
<td>III + IV</td>
<td>30</td>
<td>26</td>
<td>4.14</td>
<td>2.832-6.073</td>
<td>0.001</td>
</tr>
</tbody>
</table>
HPV-16 L1 VLP-negative patients had not had an antibody response, or they had had a response to a different type of HPV high-risk capsid protein. Despite this, the group of seropositive patients were shown to have a significantly better survival period than the HPV-16 L1 VLP-negative women.

It has been shown that during the viral life cycle, the HPV L1 capsid protein was produced at a rate that was dependent on the severity of the HPV infection (14). While the HPV L1 capsid protein is only rarely found in nonsuspicious Pap smears, it has been abundantly produced in mild to moderate dysplasia. Due to the disturbed viral-cellular interaction, however, it has been produced only in rare cases of severe dysplasia and has not been produced in carcinomas (14). As the capsid protein is not produced in cervical carcinomas, the VLP-specific antibodies measured must have been generated several years beforehand, since it has been expected that progression from mild and moderate dysplasia to cervical cancer had taken up to 10 years (15). It has been suggested that a positive capsid antibody response is an indicator of prolonged exposure to the replicating HPV virus, since a persistent HPV infection is a prerequisite for a detectable antibody response (16). It has been shown previously that HPV VLP antibody positivity was associated with the progression of cervical cancer precursors to invasive cervical cancer (17), while direct detection of the L1 protein in cervical smears was associated with high regression rates. Griesser and coworkers, as well as our own group, have shown that mild to moderate dysplasias, that are negative for HPV L1 capsid protein, are significantly more likely to progress than HPV L1-positive cases (18).

Park et al. have detected antibodies to HPV-16 VLPs in 39% of patients with cervical intraepithelial neoplasia and in 56% of cervical cancer patients, while no difference between tumor stages was found – a finding that was consistent with the results of the present study. No differences were found in their series between adenocarcinoma and squamous-cell cancer, either (19). A decrease in the levels of antibodies to HPV-16 L1 VLP was observed in patients with complete responses after therapy, and the possibility of using HPV-16 L1 VLP antibody levels, as a tumor marker for cervical cancer, was raised.

Silins has found only a trend toward better survival in patients with HPV-16 capsid antibodies, which did not attain statistical significance. It was concluded that the size of their study population might have been too small to detect a significant difference. No correlation for the antibody response to oncoproteins E6 and E7 was found (20).

In a study similar to the present one, Heim and colleagues have found that 28% of 68 patients with invasive cervical cancer had HPV-16 L1 IgG antibodies (21). In the group of patients with HPV-16 DNA-positive tumors, only 40% had been found to have antibodies to HPV-16 L1 VLPs, a figure that has also been reported by other groups. Heim et al. have reported a significant positive correlation for overall survival, but not for disease-free survival, in seropositive HPV-16 L1 VLP patients, in both the univariate and multivariate analyses. A failure to generate an HPV-specific humoral immune response, resulting in seronegativity to HPV-16 L1 VLPs might be the reason for the impaired prognosis in these patients, as has been shown in animal models (22).

The present study has confirmed these findings in a larger group of patients and has also been able to identify patients with a poorer prognosis, by assessing HPV-16 L1 VLP antibodies. It can be hypothesized that the seronegative patients had reduced HPV-specific immune competence, so that the virus might have been able to escape both the humoral and the cellular defense mechanisms, resulting in an impaired ability of the immune system to control the HPV-induced tumor. The lack of statistical significance in the multivariate analysis, while the univariate analysis has demonstrated significance for overall survival and progression-free survival, might again have been attributed to the relatively small study population. These findings, as well as the observations reported by Heim et al., have shown that there is a need to study the prognostic significance of antibodies to HPV-16 L1 VLPs further, in a larger study population.

It can be concluded from the present study that patients who are seronegative for HPV-16 L1 virus-like particles, particularly in the early FIGO stages, represent a group of women with a poor prognosis who may need a more aggressive therapeutic approach, such as adjuvant chemotherapy, in the treatment of their disease.

References


Received February 9, 2006
Revised October 12, 2006
Accepted October 17, 2006