Importance of p16 and Ki-67 Expression in the Presence of Human Papillomavirus in Adenocarcinoma of the Uterine Cervix

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Abstract: In recent years, the incidence of cervical adenocarcinoma has increased. The role of human papillomavirus (HPV) infection in cervical carcinoma is well established. The aim of this study was to investigate the possible correlation between the expression of cell cycle-associated proteins and HPV infection in cervical adenocarcinomas. We analyzed the expression of p16, p53, and Ki-67 in 68 cases of in situ and invasive cervical adenocarcinomas by immunohistochemistry. These cases were divided into high and low expression groups based on immunohistochemical staining data. High p16 expression was seen in 29 of 30 (96%) HPV-DNA-positive cases shown to be HPV-DNA positive by polymerase chain reaction (PCR), and in 26 of 27 (96%) cases shown to be HPV-DNA positive by in situ hybridization (ISH). In 50 cases of stage I b and stage II adenocarcinomas, statistical analysis showed a highly significant differences between the high expression of p16 and the HPV-positivity by ISH ($P=0.003$) and PCR ($P=0.001$). High expression of Ki-67 also showed a strong relation with HPV-Positivity by ISH ($P=0.002$). Patients in whom a high expression of p16 was observed had a lower recurrence rate than patients with a low expression of p16 ($P=0.028$). Cases with a high expression of Ki-67 showed a better prognosis than those with a low expression of Ki-67 ($P=0.024$). P16 and Ki-67 are useful biomarkers for HPV-DNA-positive cervical adenocarcinomas, which are easily monitored by immunohistochemical staining.

Key words: Endocervical adenocarcinoma, Human papillomavirus, In situ hybridization, Immunohistochemistry, p16

Introduction

The incidence of adenocarcinoma of the uterine cervix has increased since the 1970’s, particularly among young women in Japan1. Adenocarcinomas currently account for 20-25% of all cervical cancers2. High-risk types of human papillomavirus (HPV) play an important role in the etiology of cervical adenocarcinomas. HPV infection is closely associated with the development of cervical adenocarcinomas, and the role of HPV in adenocarcinomas is well established3. The purpose of this study was to investigate the possible correlation between the expression of cell cycle-associated proteins and HPV infection in cervical adenocarcinomas. We analyzed the expression of p16, p53, and Ki-67 in 68 cases of in situ and invasive cervical adenocarcinomas by immunohistochemistry. These cases were divided into high and low expression groups based on immunohistochemical staining data. High p16 expression was seen in 29 of 30 (96%) HPV-DNA-positive cases shown to be HPV-DNA positive by polymerase chain reaction (PCR), and in 26 of 27 (96%) cases shown to be HPV-DNA positive by in situ hybridization (ISH). In 50 cases of stage I b and stage II adenocarcinomas, statistical analysis showed a highly significant differences between the high expression of p16 and the HPV-positivity by ISH ($P=0.003$) and PCR ($P=0.001$). High expression of Ki-67 also showed a strong relation with HPV-Positivity by ISH ($P=0.002$). Patients in whom a high expression of p16 was observed had a lower recurrence rate than patients with a low expression of p16 ($P=0.028$). Cases with a high expression of Ki-67 showed a better prognosis than those with a low expression of Ki-67 ($P=0.024$). P16 and Ki-67 are useful biomarkers for HPV-DNA-positive cervical adenocarcinomas, which are easily monitored by immunohistochemical staining.

Key words: Endocervical adenocarcinoma, Human papillomavirus, In situ hybridization, Immunohistochemistry, p16
role in the carcinogenesis of cervical adenocarcinomas and are detected in 43–94% of 
adenocarcinomas). HPV oncoproteins E6 and E7 inactivate the tumor suppressor proteins 
p53 and retinoblastoma protein (pRb) that maintain tightly regulated cell proliferation and 
growth. When the control mechanisms of the cell cycle are disturbed, unrestricted cell prolif-
eration results in tumor development. p16 is one of the cyclin-dependent kinase (CdK) inhibitory proteins that decelerate the cell cycle by inactivating the CdKs that phosphorylate 
pRb. Because p16 underlies a negative feedback control by pRb, inactivation of pRb by 
HPV or through mutations may result in an overexpression of p16 and dysregulation of 
CdK4 and CdK6. Ishikawa et al reported that overexpression of p16 may be an indication 
of the pathogenic activity of high-risk types of HPV. In the present study, we have used 
immunohistochemistry to test whether p16, another cell cycle protein (p53), and a cell prolif-
eration marker (Ki-67) are overexpressed in cervical adenocarcinomas, and we examined 
the possible correlation between the overexpression of these proteins and the presence of 
HPV-DNA [measured by in situ hybridization (ISH) and polymerase chain reaction (PCR)]. 
We also analyzed the possible correlation between the expression of these proteins, HPV-
DNA positivity, and the clinicopathological factors in 50 stages Ib and II cases (tumors 
limited to the uterine cervix with or without parametrial invasion) assessed in accordance 
with the staging established by the International Federation of Gynecology and Obstetrics.

Materials and Methods

Patients

This study included 68 Japanese women with cervical adenocarcinomas distributed as 
follows: 38 patients had mucinous adenocarcinomas (28 endocervical type, 1 minimal 
development, 2 villoglandular papillary, and 7 intestinal-type adenocarcinomas), 7 patients 
had endometrioid adenocarcinoma, 2 patients had clear-cell adenocarcinoma, 3 patients 
had mixed adenocarcinomas, 8 patients had adenocarcinoma in situ (AIS), and 10 patients 
had adenosquamous carcinoma. The patients were treated in the Department of Obstetrics and 
Gynecology of Showa University Hospital and related hospitals. The patients ranged in age 
from 30 to 77 years (mean, 48 years). The cases were staged according to the International 
Federation of Gynecology and Obstetrics staging system. Eight cases were stage 0, 5 cases 
were stage 1a, 33 cases were stage 1b, 4 cases were stage Iia, 13 cases were stage IIb, 4 
cases were stage IIIb, and 1 case was stage IVb. Of the 13 patients with stage 0 and 1a 
carcinomas, 3 had undergone conization and 10 had had a hysterectomy. Of the 50 patients 
with stage 1b and stage II carcinomas, 42 had undergone radical hysterectomy, 2 had under-
gone subradical hysterectomy, and 4 had undergone simple abdominal total hysterectomy; 
details for the remaining 2 cases are not known. Pelvic lymphadenectomy was performed 
in 46 of the 50 patients with stages 1b and stage II carcinomas. Two of the 5 patients with 
stages III and stage IV carcinomas underwent radical hysterectomy and the other 3 patients 
were given chemotherapy and radiotherapy. Patients were followed until June 2005 and 11 
cases were lost to follow-up. The average follow-up period was 43.6 months (range, 2–180 
months). During follow-up, no death or recurrence was observed in 12 of the 13 patients 
with stage 0 and stage 1a carcinomas; we could not follow-up the remaining patient. We 
could follow up 41 of the 50 patients with stages 1b and stage II carcinomas; 6 of these 
patients died of cancer. Three of 4 patients with stage III and stage IV carcinomas died of 
cancer; we could not follow up the remaining patient.
Table 1. Immunohistochemical profile of antibodies used to stain formalin-fixed, paraffin-embedded sections.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Source</th>
<th>Dilution</th>
<th>Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>DO7</td>
<td>Nichirei, Japan</td>
<td>neat</td>
<td>autoclave</td>
</tr>
<tr>
<td>p16</td>
<td>E6H4</td>
<td>DAKO, Denmark</td>
<td>1: 25</td>
<td>autoclave</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>DAKO, Denmark</td>
<td>1: 50</td>
<td>autoclave</td>
</tr>
</tbody>
</table>

**Tissue specimens**

One block containing the maximum amount of tumor tissue was selected from each of 63 hysterectomy and/or conization specimens, and each of 5 biopsy specimens. For each case, the histological diagnosis was confirmed on a tissue section (4-μm thick) stained with hematoxylin-eosin.

**Immunohistochemistry**

Sections of 4-μm thickness were prepared from the 68 formalin-fixed, paraffin-embedded samples, deparaffinized and rehydrated in graded ethanol solutions, and washed with distilled water. For high-temperature antigen retrieval, sections were autoclaved in 0.01M citrate buffer, pH6, for 15 min. at 121°C. Endogenous peroxidase was blocked with 3% H₂O₂ in water for 5 min. Sections were washed with phosphate-buffered saline (PBS) and incubated with the primary monoclonal antibodies (Table 1) for 60 min. at room temperature. The sections were washed again with PBS, incubated with secondary antibodies (horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies as part of the ChemMate™ En Vision™ Detection Kit, code No. K5007, Dako Diagnostika) for 30 min at room temperature, and developed with 3,3'-diaminobenzidine-tetrahydrochloride (DAB; Dako Diagnostika). Sections were counterstained with hematoxylin, dehydrated, and mounted.

**Scoring for p16-, p53- and Ki-67-positive cells.**

Immunoreactivity for p16, p53, and Ki-67 was quantified as the percentage of positive tumor cells per 1000 cells counted in different areas of each section by two pathologists (A. S and M. K) independently. With regard to immunoreactivity for p16 and Ki-67, the specimens were divided into a negative or low-expression group with less than 50% positive cells and a high-expression group with more than 50% positive cells. With regard to immunoreactivity for p53, the specimens were divided into a negative or low-expression group with less than 25% cells with positive nuclei and a high-expression group with more than 25% cells with positive nuclei. These categories were determined only according to the percentage of positive cells, regardless of the intensity of the immunohistochemical staining. According to their pattern of staining intensity, the specimens were classified as weakly, moderately or strongly positive for p16 and p53. Thus, tumors were considered weakly positive when focal tumor cells showed partial nuclear and/or cytoplasmic staining, moderately positive when diffuse tumor cells showed partial nuclear and/or cytoplasmic staining, and strongly positive when diffuse tumor cells showed global nuclear and/or cytoplasmic staining.
Detection of HPV-DNA by ISH

We used BENCHMARK® (fully automated slide preparation system; Ventana Medical Systems, Tucson, AZ), which is an automated, catalyzed signal amplification system using biotinylated probes for immunochemical detection of high-risk HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68) and low-risk HPV types (HPV-6, -11, -42, -43, and -44), to detect HPV-DNA in formalin-fixed, paraffin-embedded tissue sections of 68 specimens. Automated processing includes baking, deparaffinization, cell conditioning, staining, and counterstaining. HPV-DNA is indicated by pale blue (low-copy number) to blue-black (high-copy number) staining of the nucleus of infected cells.

Detection of HPV-DNA by PCR

Genomic DNA was extracted from cut sections of formalin-fixed, paraffin-embedded tissue of 58 AIS and invasive cervical adenocarcinoma lesions, and from adenocarcinoma elements of 10 adenosquamous carcinoma lesions. Tumor areas were dissociated manually from the surrounding normal tissue, using by needles to hold the specimen in place. Thus, the tumors selected for the study did not contain benign, atypical, or malignant squamous epithelium. We analyzed the samples by PCR for the presence of HPV-DNA and for the type of HPV present, using primers specific for the E6/E7 region of the HPV-16, -18, and HPV-33. The DNA samples were amplified according to the following PCR protocol: 94°C for 5 min. 40 cycles; 94°C for 30s, 55°C for 2 min, and 72°C for 2 min. And at 72°C for 5 min. Amplified DNA was electrophoresed in 2% agarose gels, and photographed under UV light.

Statistical Analysis

Fisher’s exact test and χ² test were used to assess for a potential association between study variables of interest in 50 of 68 stage Ib and stage II cases. P values 0.05 were considered statistically significant.

Results

Immunohistochemical staining for p16, p53, and Ki-67

Adenocarcinoma and adenosquamous carcinoma cells showed cytoplasmic and nuclear staining for p16 (Fig. 1A). None of the cells in normal endocervical epithelium and stroma showed a high expression of p16. The frequencies of adenocarcinomas and adenosquamous carcinomas positive for p16, p53, and Ki-67 are shown in Table 2. Fifty-three of the 68 cases of adenocarcinomas and adenosquamous carcinomas studied (77.9%) showed a high expression of p16 (mean positive tumor cells, 76.0%; range 50-100%). All of the cases of villoglandular adenocarcinomas and adenosquamous carcinomas, and 7 of 8 (87.5%) AIS cases showed a high expression of p16. In contrast, expression of p16 in the minimal deviation adenocarcinoma specimen was low. There was no statistically significant difference among histological subtypes. Of the 53 (94.3%) cases with a high expression of p16, 50 (94.3%) showed a strongly positive staining intensity for p16. Immunohistochemical staining for p53 was localized in the nuclei of cancer cells (Fig. 1B). High expression of p53 was detected in 25 of the 68 cases studied (36.8%). Adenocarcinomas and adenosquamous carcinomas with a high expression of p53 showed varied weakly, moderately or strongly positive staining intensity for p53. There was no significant difference in p53 staining among histo-
Fig. 1. Immunohistochemical analysis for p16, p53, and Ki-67 in endocervical adenocarcinoma. 
(A) shows a diffuse pattern of strong intensity staining in the nuclei and cytoplasm of cancer cells. 
(B) and (C) show staining for p53 and Ki-67, respectively, in the nuclei of cancer cells. No cytoplasmic 
staining for p53 or Ki-67 was observed (×400).

Fig. 2. ISH analysis for HPV in endocervical adenocarcinoma. Dot-like or diffuse reaction products in 
the nucleus indicate the presence of HPV-DNA (×1000).
Table 2. Immunohistochemical staining for p16, p53, and Ki-67 HPV-DNA positivity in cervical adenocarcinoma (n = 68)

<table>
<thead>
<tr>
<th>Histological types</th>
<th>n</th>
<th>P16 low</th>
<th>P16 high</th>
<th>P53 low</th>
<th>P53 high</th>
<th>Ki-67 low</th>
<th>Ki-67 high</th>
<th>HPV (ISH) (-)</th>
<th>HPV (ISH) (+)</th>
<th>HPV (PCR) (-)</th>
<th>HPV (PCR) (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invasive adenocarcinoma</td>
<td>50</td>
<td>14</td>
<td>36</td>
<td>28</td>
<td>22</td>
<td>15</td>
<td>35</td>
<td>28</td>
<td>22</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>Endocervical</td>
<td>28</td>
<td>9</td>
<td>19</td>
<td>13</td>
<td>15</td>
<td>8</td>
<td>20</td>
<td>17</td>
<td>11</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Minimal deviation</td>
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<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal glandular</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
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<tr>
<td>Intestinal</td>
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<td>3</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>5</td>
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<tr>
<td>Clear cell</td>
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<td>2</td>
<td>0</td>
<td>2</td>
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<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Mix</td>
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<td>1</td>
<td>2</td>
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<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>AIS</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>1</td>
<td>5</td>
<td>3</td>
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<tr>
<td>Adenosquamous</td>
<td>10</td>
<td>0</td>
<td>10</td>
<td>9</td>
<td>1</td>
<td>2</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>15</td>
<td>53</td>
<td>43</td>
<td>25</td>
<td>19</td>
<td>49</td>
<td>41</td>
<td>27</td>
<td>38</td>
<td>30</td>
</tr>
</tbody>
</table>

Detection of HPV-DNA by ISH & PCR

ISH analysis demonstrated the presence of high-risk HPV types in 27 of the 68 tumors studied. A positive reaction was characterized by a single or multiple dot-like to diffuse signals in the nuclei of cancer cells, but not in normal epithelial or stromal cells on the same slides (Fig. 2). Low-risk HPV types were not detected in any of the cases studied. Twenty-six of the 27 HPV-DNA-positive cases (96.2%) displayed high p16 expression, with moderately or strongly positive staining intensity. The one HPV-DNA-positive case with low expression of p16 and weakly positive staining intensity was a case of poorly differentiated endometrioid-type adenocarcinoma. Fourteen of the 41 HPV-DNA-negative cases (34.1%) showed low p16 expression. On PCR analysis, HPV-16 was detected in 9 (33.3%) and HPV-18 in 13 (48.1%) of the 27 tumors shown by ISH to be positive for HPV-DNA. One of the HPV-18-positive cases was also positive for HPV-33. HPV-16, HPV-18, or -33 were not detected in the remaining five cases shown to be HPV-DNA positive by ISH. There was no statistically significant correlation between HPV-DNA positivity and the histological subtype in the ISH or PCR studies. Table 3 shows the relationship between HPV-DNA positivity and immunohistochemical data for the 68 cases studied. There was a significant association between high expression of p16 and HPV-DNA positivity assessed by ISH \((P = 0.003)\) and PCR \((P = 0.001)\). There was also no significant association between the high expression of Ki-67 and HPV-DNA positivity assessed by ISH \((P = 0.002)\).

Table 4 shows the association between study variables such as immunoreactivity for p16, p53 and Ki-67, HPV-DNA positivity, clinical stage, recurrence, prognosis, lymphovascular invasion, and lymph node metastasis in 50 stage Ib and stage II cases. There was a significantly higher association with lower recurrence rates in cases with a high p16 expression.
Table 3. Relationship between HPV-DNA and immunohistochemical data for adenocarcinomas and adenosquamous carcinomas (n = 68).

<table>
<thead>
<tr>
<th>HPV-DNA positivity (n = 68)</th>
<th>p16</th>
<th>p53</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td>low</td>
<td>high</td>
<td>p</td>
<td>low</td>
</tr>
<tr>
<td>ISH method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>14</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>positive</td>
<td>1</td>
<td>26</td>
<td>0.003</td>
</tr>
<tr>
<td>PCR method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>14</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>positive</td>
<td>1</td>
<td>29</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 4. Result of immunoreactivity for p16, p53 and ki-67, HPV-DNA positivity in relation to clinicopathological parameters in stage Ib and stage II cases.

<table>
<thead>
<tr>
<th>Clinicopathological parameters (n)</th>
<th>p16</th>
<th>p53</th>
<th>Ki-67</th>
<th>HPV-DNA (ISH)</th>
<th>HPV-DNA (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low</td>
<td>high</td>
<td>p</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Recurrence (42)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>30</td>
<td>0.028</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>Prognosis (41)**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>5</td>
<td>30</td>
<td>0.077</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Dead</td>
<td>3</td>
<td>3</td>
<td>&gt;0.999</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Vascular invasion (48)***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>23</td>
<td>21</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>No</td>
<td>5</td>
<td>13</td>
<td>&gt;0.999</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Lymph node metastasis (46)****</td>
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<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>3</td>
<td>10</td>
<td>11</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>No</td>
<td>8</td>
<td>25</td>
<td>&gt;0.999</td>
<td>19</td>
<td>14</td>
</tr>
</tbody>
</table>

* Recurrence was unknown in 8 of 50 cases.
** Prognosis was unknown in 9 of 50 cases.
*** Two cases could not undergo operative therapy.
**** Pelvic lymphadenectomy was performed in 46 of 50 cases.

than in cases with a low p16 expression (P = 0.028). Cases with a high expression of Ki-67 had a better prognosis than the low-expression cases (P = 0.024). No statistically significant association was found between the extent of p53 expression and clinical status. Recurrence rates were significantly lower in HPV-DNA-positive cases than in HPV-DNA-negative cases, with positivity assessed by ISH (P = 0.034) and PCR (P = 0.003). HPV-DNA-positive cases (assessed by PCR) had a significantly better prognosis than HPV-DNA-negative cases (P = 0.006).

Discussion

Numerous recent studies have shown that p16 is a useful diagnostic marker for cervical adenocarcinoma 10-12). In the present study, 77.9% of the adenocarcinoma and adenosquamous carcinoma cases analyzed showed a high expression of p16. As recently reported,
a high expression of p16 protein is also a useful marker for HPV infection. Khleif et al reported that HPV-16 E7 is responsible for the increment of p16 levels, and that the expression of p16 mRNA is markedly induced by E2F\textsuperscript{13}. They postulated that the accumulation of E2F might induce the high expression of p16 through negative feedback. Ansari-Lari et al reported that overexpression of p16 was seen in all 14 cases of HPV-positive adenocarcinoma they studied\textsuperscript{10}. Similarly, a high percentage of HPV-positive cases showing marked overexpression of p16 with moderate to strong staining intensity in both the nuclei and cytoplasm was shown by other studies\textsuperscript{4,5,11,12}. In the present study, we found that 96% of the HPV-DNA-positive adenocarcinomas and adenosquamous carcinomas studied had a high expression of p16 with moderately to strongly staining intensity. Immunohistochemical staining for p16 may be a useful tool in identifying in situ and invasive adenocarcinomas that are HPV-DNA positive.

There was no significant relationship between the histological type and p16 expression in this study (Table 2). We also found that the minimal deviation adenocarcinoma that we studied stained weakly for p16 and was HPV-DNA negative by ISH and PCR. Other studies have reported the absence or very low prevalence of HPV in this subtype\textsuperscript{3,4}. These results suggested that the development of minimal deviation adenocarcinoma might not be related to HPV infection. In contrast, villoglandular adenocarcinoma showed both a high expression of p16 with strong staining intensity, and detection of HPV-DNA positivity. Jones et al demonstrated the presence of HPV infection in 12 cases of villoglandular adenocarcinoma\textsuperscript{14}. In the present study, the two cases of villoglandular adenocarcinoma studied showed positivity for HPV-DNA by ISH and PCR. These results suggest that HPV-DNA might be more prevalent in this subtype than in other types of cervical adenocarcinoma. However, these cervical adenocarcinoma subtypes are so rare (minimal deviation adenocarcinomas account for only 1 to 3% of cervical adenocarcinomas) that more cases need to be studied to define the relationship between minimal deviation or villoglandular adenocarcinoma and HPV-DNA positivity.

Ishikawa et al reported that overexpression of p16 was not associated with tumor recurrence\textsuperscript{4}. These data contrast with our finding that high expression of p16 in the tumor correlated strongly with low disease recurrence (Table 4). Schorge et al reported that p16 does not appear to have a major role in the progression from in situ to invasive cervical carcinoma\textsuperscript{11}. Nakao et al observed a dramatic enhancement of p16 in a HPV-16 / 18-immortalized model of in situ adenocarcinoma\textsuperscript{9}. This overexpression did not increased further after malignant transformation of the cells to an invasive carcinoma. These data suggest that a high expression of p16 may be observed at the early stage of the carcinogenic process, but it is not associated with the progression of cancer. The activity of p16 may be related to the low recurrence.

Data from studies of correlation between Ki-67 and prognostic factors in cervical carcinoma are contradictory. Ho et al reported that for stage I cervical squamous cell carcinomas, the 10-year survival rates in patients with a high expression of Ki-67 are apparently worse than those in patients with a low expression of Ki-67\textsuperscript{15}. In the present study, we found an association between HPV-DNA positivity, as demonstrated by ISH, and a high expression of Ki-67. Cases with a high expression of Ki-67 had a better prognosis than the low-expression cases. Shiohara et al reported that lymph node metastasis is an independent prognostic factor for an unfavorable outcome, and that lymph node metastasis and lymph vascular
permeation are negatively correlated with Ki-67 expression in stage Ib and stage II cervical squamous cell carcinomas\(^6\). They also reported that the cell cycle status of tumor cells during invasion or metastasis is usually quiescent, with the cells in G0 or early G1 phases in which Ki-67 is negative. Therefore, the negative correlation between Ki-67 expression and lymph node metastasis or lymph vascular permeation may indicate that many quiescent tumor cells are involved in invasion or metastasis. The precise reason for this discrepancy is unknown. However, HPV-associated cervical adenocarcinomas with invasive or metastatic potentials may have less growth activities than adenocarcinomas without invasive or metastatic potentials. We need to collect data on additional cases in order to discuss correlations between overexpression of Ki-67 and prognosis in cervical adenocarcinoma.

In our study, overexpression of p53, seen in 36.8% of the cervical adenocarcinomas studied, was not significantly associated with HPV-DNA positivity. Overexpression of p53 has been linked to a point mutation in the p53 gene\(^17\). Thus, Helland et al reported a high frequency of p53 mutations (42%) in cervical cancers, and a significant association between p53 mutation and increased p53 expression\(^18\). However, some contradictory studies have demonstrated that p53 mutations are infrequent in primary cervical carcinomas, and shown that overexpression of p53 as detected by immunohistochemistry is not predictive of a somatic mutation in the p53 gene. Castren et al demonstrated that wild-type p53 may accumulate in response to DNA damage which normally leads to growth arrest or programmed cell death\(^19\). Sahu et al reported that rearrangement of p53 correlates with increases in p53 expression at the mRNA and protein levels\(^17\). There is no clear explanation for overexpression of p53 in the absence of a mutation in the p53 gene\(^19\). However, overexpression of p53 may not only result from a mutation, but may also be a response to DNA damage and other unknown factors.

In this study, the incidence of HPV-DNA-positivity in the specimens studied was low, when compared to previous studies. According to some ISH studies, high-risk HPV types can be detected in 33.6-75% of cervical adenocarcinomas\(^8,20-23\). We suggest two reasons for the low incidence observed in our study. First, the HPV-DNA in the specimens was present in small quantity and/or was of poor quality because of prolonged or inadequate fixation in formalin, or because the use of fixatives other than neutral buffered formalin significantly reduces the likelihood of obtaining a positive signal with ISH; this may apply to older specimens from our laboratory. Second, we cannot rule out that the poor sensitivity of the techniques used in this study contributed to the low incidence of HPV-DNA positivity.

In summary, we have investigated the presence of HPV-DNA and the expression of proteins involved in cell cycle control or associated with cell proliferation, in cervical adenocarcinomas. There was a strong correlation between the presence of HPV-DNA and a high expression of p16 or Ki-67. In addition, patients with a high expression of p16 had lower recurrence rates than patients with a low expression of p16, and patients with a high expression of Ki-67 had a better prognosis than patients with a low expression of Ki-67. Immunohistochemical analysis for p16 and Ki-67 is an easy procedure which might be a good screening tool for HPV infection. Demonstration of high p16 or Ki-67 expression in the specimens by immunohistochemistry was indicative of a lower recurrence rate or a better prognosis for cervical adenocarcinomas.
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