Chapter 2

Prevalence of HPV DNA in different histological subtypes of cervical adenocarcinoma

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Prevalence of Human Papillomavirus DNA in Different Histological Subtypes of Cervical Adenocarcinoma

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The prevalence of human papilloma virus (HPV) DNA in different histological subtypes of cervical adenocarcinoma and related tumors was examined using formalin-fixed, paraffin-embedded tissue samples from 105 primary cervical adenocarcinomas and adenosquamous carcinomas. Broad-spectrum HPV DNA amplification and genotyping was performed with the SPF10 primer set and line probe assay (LiPA), respectively. HPV DNA was detected in 82 of 90 (91%) mucinous adenocarcinomas, encompassing endocervical, intestinal, and endometrioid histological subtypes, and in nine of nine adenosquamous tumors (100%). HPV DNA was not detected in any nonmucinous adenocarcinomas including clear cell, serous, and mesonephric carcinomas (0/6). The most common viral types detected in adenocarcinoma were HPV 16 (50%) and HPV 18 (40%), followed by HPV 45 (10%), HPV52 (2%), and HPV 35 (1%). Multiple HPV types were detected in 9.7% of the cases. In conclusion, mucinous adenocarcinomas and adenosquamous carcinomas of the cervix demonstrate a very high prevalence of HPV DNA, similar to that reported for cervical squamous cell carcinoma. Only rare histological variants of cervical adenocarcinoma seem unrelated to HPV infection. (Am J Pathol 2000, 157:1055–1062)

Adenocarcinoma of the cervix (AdCx) accounts for approximately 15% of cervical cancers and has been increasing in incidence during the last few decades, particularly in younger women.1 The etiology of squamous cell carcinoma of the cervix, the most common type of cervical malignancy, is linked to infection with oncogenic types of human papillomavirus (HPV), but the pathogenesis of AdCx is less well understood. Although HPV DNA is consistently detected in >90% of squamous cell carcinomas of the cervix,2 the reported prevalence of HPV DNA in AdCx varies significantly, from 32% to 100%, depending on the detection method used.3–13

A strong association between a sexually transmitted agent (HPV) and the risk of development of cervical squamous cell carcinoma has been clearly established, however, the relationship between HPV and cervical adenocarcinoma remains uncertain.14,15 Only a few, small, epidemiological studies separately examining AdCx have been conducted and the statistical power to detect an association with HPV has been limited.14,15 Epidemiological risk factors for cervical adenocarcinoma include those that correlate with the risk of acquiring HPV infection, such as early age at first sexual intercourse and multiple sexual partners.14,15,17,18 In addition, AdCx was also found to be associated with obesity, a well-known endometrial cancer risk factor.14,15,17,18 Some studies have reported an association of AdCx with the prolonged use of oral contraceptives.14,15,17,18 However, the lack of a protective effect of barrier contraception could be a confounding factor in these studies, because the relationship between AdCx and oral contraceptives disappeared after accounting for HPV infection19 and the use of a diaphragm was found to be inversely related to the risk for AdCx.18

Cervical adenocarcinomas include several different histological types. The majority of tumors are mucinous adenocarcinomas that resemble either endocervical, in-
Histological and molecular characterization of adenocarcinomas of the uterine cervix and endocervical glandular lesions

Kimberly A. Smedley, Susan E. Cooper, William J. Frazee, S. Mara Simor, Michael K. Syrjanen, and Nasser R. J. Malek

Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

Materials and Methods

Clinical specimens

Consecutive cases of in situ and invasive cervical adenocarcinomas and related tumors were retrieved from the archives of the Departments of Pathology at New York Presbyterian Hospital (1978 to 1998) and Lenox Hill Hospital (1990 to 1998), both in New York City; Kyoto Diagnostics LLC, New City, NY (1997); and Laboratory of Reproductive Pathomorphology, Warsaw Medical School, Warsaw, Poland (1998). A total of 73 cases of invasive adenocarcinomas, 23 cases of adenocarcinoma in situ (AIS), two cases of adenoid basal carcinoma, and one case of glassy cell carcinoma were collected. Six cases of adenosquamous carcinoma were selected and included in the study as a positive control group. Nonprimary cervical carcinomas were excluded.

All cases were reviewed and diagnostic groups were assigned and graded according to standard histological criteria. The presence of an associated squamous intraepithelial lesion was recorded. A representative tissue block from each case was selected for HPV analysis. Clinicopathological parameters were obtained from the pathology reports.

DNA extraction

Three, 5-μm sections of formalin-fixed, paraffin-embedded tissue were placed on glass slides after cutting deep into the block. The microtome blade was changed after each case. The tissue sections were deparaffinized and stained with hematoxylin. Tumor tissue was carefullymicrodissected from the adjacent squamous epithelium and stroma using a sterile scalpel blade. Benign cervical stroma away from the tumor was separately scraped from the same slide and processed in parallel as a negative control. The samples were incubated with proteinase K (1 mg/ml) for 18 hours at 56°C and heat inactivated.

HPV DNA detection and typing

Broad-spectrum HPV DNA amplification was performed using the short PCR fragment (SPF10) primer set, as described previously.20 The SPF10 primers amplify a 65-bp fragment from the L1 region of the HPV genome.20,21 The PCR products were analyzed by both 3% agarose gel electrophoresis and HPV DNA enzyme immunoassay (DEIA), a microtiter plate-based hybridization assay (Innogenetics Inc., Alpharetta, GA), as previously described.20 To ensure adequate DNA preparation, PCR amplification of β-globin was performed in a separate reaction using primers PC03 and PC04, resulting in a 96-bp product.24 Samples identified as positive for HPV DNA were genotyped with the INNO-LiPA HPV prototype research assay (LiPA; Innogenetics Inc.).21 In this assay, the HPV PCR product is hybridized to the genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. Twenty-five individual HPV genotypes (HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74) can be identified simultaneously in a single assay. The hybridization and the color reaction, which results in a purple precipitate, were performed automatically in an AutoLiPA device. The results of hybridization were assessed visually by comparing to the standard grid.

Statistical analysis

The differences of the means of the continuous variables were analyzed with the Student’s t-test and the distribution of noncontinuous clinicopathological variables versus HPV status was analyzed with the chi-square test, using the SPSS software package (SPSS Inc., Chicago, IL). P values of <0.05 were used as the cut-off for statistical significance.

Results

Clinicopathological characteristics

The clinicopathological characteristics are presented in Table 1. Patients with adenocarcinoma in situ were almost a decade younger than those with invasive adenocarcinoma (36.3 years versus 45.2 years, P < 0.05). The average age of patients with invasive adenocarcinoma and adenosquamous carcinoma was almost identical (45.2 years versus 45.8 years). Patients with adenoid basal carcinoma, clear cell, and minimal deviation adenocarcinoma were older than the other patients; however, the differences were not statistically significant.

AIS was identified in 52% of patients with invasive mucinous tumors. High-grade squamous intraepithelial lesions were identified in all cases of adenosquamous carcinoma. High-grade squamous intraepithelial lesions...
were also identified in 39.1% of the AIS cases and in 18.1% of mucinous adenocarcinomas. Patients with high-grade squamous intraepithelial lesions were younger than those with no identifiable squamous intraepithelial lesion (39.1 years versus 44.3 years, \( P < 0.05 \)).

**Table 1. Clinicopathological Data**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Average age</th>
<th>Age range</th>
<th>HSIL</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Stage 1</th>
<th>Stage 2</th>
<th>Stage 3</th>
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<tr>
<td>Adenocarcinoma in situ</td>
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<td>19–58</td>
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<td>55</td>
<td>44.1</td>
<td>22–80</td>
<td>18.1</td>
<td>66.6</td>
<td>31.5</td>
<td>1.8</td>
<td>87.1</td>
<td>4.3</td>
<td>8.6</td>
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<td>26–69</td>
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<td>33.3</td>
<td>66.6</td>
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<td>33–69</td>
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<td>75.0</td>
<td>25.0</td>
<td>100.0</td>
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<tr>
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<td>53.5</td>
<td>37–70</td>
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<td>100.0</td>
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<td>75.0</td>
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<td>39</td>
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<td>100.0</td>
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<tr>
<td>All invasive adenocarcinomas</td>
<td>73</td>
<td>45.2</td>
<td>22–80</td>
<td>15.0</td>
<td>61.6</td>
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<td>8.2</td>
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<td>2.8</td>
<td>14.2</td>
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<td>59.5</td>
<td>55–64</td>
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<td>50.0</td>
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<td>100.0</td>
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<tr>
<td>Adenosquamous carcinoma</td>
<td>6</td>
<td>43.0</td>
<td>31–60</td>
<td>100.0</td>
<td></td>
<td>66.6</td>
<td>33.3</td>
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<tr>
<td>All adenosquamous carcinomas</td>
<td>9</td>
<td>45.8</td>
<td>31–64</td>
<td>77.7</td>
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<td>55.5</td>
<td>45.5</td>
<td>100.0†</td>
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</tr>
</tbody>
</table>

AdCx, adenocarcinoma; HSIL, high-grade squamous intraepithelial lesion.

*Clinicopathological stage available in 35 cases.

†Clinicopathological stage available in six cases.

HPV DNA Detection and Typing

\( \beta \)-globin DNA was amplified in all cases and HPV DNA was amplified in 91 of 105 cases, some of which were stored in the paraffin blocks for as long as 20 years. In

**Table 2. HPV DNA Detection and Typing**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>HPV+</th>
<th>HPV16</th>
<th>HPV18</th>
<th>HPV45</th>
<th>Other HPV</th>
<th>Multiple HPV</th>
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</thead>
<tbody>
<tr>
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<td>23</td>
<td>23</td>
<td>100.0</td>
<td>43.4</td>
<td>6</td>
<td>26.0</td>
<td>41^</td>
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<td>Endocervical-type AdCx</td>
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<td>50</td>
<td>90.7</td>
<td>83.3</td>
<td>0</td>
<td>44.0</td>
<td>42.0</td>
</tr>
<tr>
<td>Intestinal-type AdCx</td>
<td>6</td>
<td>5</td>
<td>83.3</td>
<td>0</td>
<td>0</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Endometrial-type AdCx</td>
<td>4</td>
<td>4</td>
<td>100.0</td>
<td>50.0</td>
<td>2</td>
<td>50.0</td>
<td>0</td>
</tr>
<tr>
<td>Minimal deviation AdCx</td>
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<td></td>
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<tr>
<td>All in situ and invasive mucinous adenocarcinomas</td>
<td>90</td>
<td>82</td>
<td>91.1</td>
<td>41.4</td>
<td>30</td>
<td>36.5</td>
<td>8</td>
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<tr>
<td>Clear cell AdCx</td>
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<td>0.0</td>
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<tr>
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<td>Mesonephric AdCx</td>
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<td></td>
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<tr>
<td>All nonmucinous adenocarcinomas</td>
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<td>0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoid basal carcinoma</td>
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<td>100.0</td>
<td>0</td>
<td>0.0</td>
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<td>100.0</td>
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<tr>
<td>Adenosquamous carcinoma</td>
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<td>6</td>
<td>100.0</td>
<td>33.3</td>
<td>3</td>
<td>33.3</td>
<td>16.6†</td>
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<tr>
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<td>9</td>
<td>100.0</td>
<td>4</td>
<td>44.4</td>
<td>3</td>
<td>11.1</td>
</tr>
</tbody>
</table>

AdCx, adenocarcinoma.

*Other HPV types: ^HPV 35, ^HPV 52, ^HPV 31.

†Multiple HPV types: ^HPVs: 16 + 18; 16 + 31; 16 + 53; 16 + 39 + 66; 16 + 11; ^HPVs: 16 + 18; 16 + 33; 18 + 52.
cases with HPV DNA amplification, the presence of HPV-specific sequences was confirmed with the DNA enzyme immunoassay and the individual HPV genotypes were subsequently identified with the LiPA. The cases, in which HPV DNA was not detected, were of various storage ages.

The results of HPV DNA detection in different histological tumor subtypes are summarized in Table 2. The tumor subtypes were grouped as follows: 1) in situ and invasive mucinous adenocarcinomas; 2) nonmucinous adenocarcinomas; and 3) tumors with adenosquamous differentiation. We included the endometrioid type of adenocarcinoma in the mucinous group, as it expresses the same range of mucins as the tumors with endocervical-type histology (see Discussion).

HPV DNA was detected in 82 of 90 in situ and invasive mucinous adenocarcinomas (91.1%), in none of the six nonmucinous adenocarcinomas (0%), and in all nine tumors with adenosquamous differentiation (100%) (Table 2). HPV 16 was the most common viral type identified and was detected in 50% of the HPV-positive adenocarcinomas. These included cases in which HPV 16 was found as the sole viral type (41.5%) and cases with multiple-type HPV infection (8.5%) (Figure 1). HPV 18 was almost equally prevalent and was detected in 40.2% of all HPV-positive adenocarcinomas—as a single HPV type in 36.5% and with other HPV types in 3.7%. HPV 45 was found in 9.7% of HPV-positive adenocarcinomas. HPV 35 and 52 were identified in one tumor each as a single HPV-type infection. Carcinomas with adenosquamous differentiation had a similar HPV-type distribution.

Multiple HPV types were detected in 9.7% of the cases (Table 2 and Figure 1). Multiple-type infection was more frequent in AIS than in endocervical AdCx (21.7% versus 6.0%, \( P < 0.05 \)). In all cases of multiple HPV infection, either HPV 16 or HPV 18 was always detected in addition to other high- or low-risk viral types, which included HPV 11, 31, 33, 39, 52, 53, and 66. (Table 2, footnote). After accounting for multiple-type infections, the ratio of HPV 16 to HPV 18 was essentially 1:1 in endocervical AdCx, endometrioid AdCx, and adenosquamous carcinomas. In AIS, HPV 16 was more than twice as common as HPV 18 (2.5:1).

The average age of the patients with HPV DNA-positive versus HPV DNA-negative tumors (42.8 years versus 44.1 years) was not significantly different (\( P = 0.7 \)). There was no difference in the patients’ average age when stratified by HPV type (HPV 16 versus 18 versus 45). However, patients with multiple HPV infection were significantly younger than patients with a single viral type (33.5 years versus 43.7 years, \( P < 0.05 \)). No association between the tumor grade or stage and the presence of HPV DNA or a particular HPV type was detected.

**Discussion**

Our results demonstrate a very high prevalence of HPV DNA in cervical adenocarcinomas when compared to most previous reports\(^3\)–\(^12\) and similar to that reported for cervical squamous cell carcinoma.\(^2\) The relative difficulty in detecting HPV DNA in adenocarcinomas, in contrast to squamous cell carcinomas, may be attributed to a lower viral load in glandular lesions as compared to squamous lesions. Premalignant and malignant squamous lesions, in particular those associated with HPV 16, contain a large number of episomal viral particles, in addition to integrated HPV sequences.\(^26\) Glandular epithelium does not support productive viral infection and HPV DNA in endocervical neoplasms (notably HPV 18), is usually present in the integrated form.\(^27\) As a result, detection of HPV DNA in adenocarcinomas requires a sensitive detection assay. Further, as the successful amplification of HPV DNA in a PCR assay depends on the presence of intact DNA target sequences, two additional factors may reduce the efficiency of HPV detection: 1) DNA fragmentation as a result of formalin fixation and storage in paraffin; and 2) loss of portions of the viral genome during integration. Integration of HPV DNA may result in deletion of the viral genome containing the sequences targeted in the PCR reaction. In such cases, the detection of HPV DNA in the assay will depend on the presence of intact episomal HPV copies. The absence of an episomal HPV genome in the majority of glandular tumors, as opposed to squamous tumors,\(^27\) may result in a significant underestimation of HPV DNA prevalence in adenocarcinomas.

In this study, HPV DNA amplification was performed using a novel, sensitive, broad-spectrum HPV PCR assay (SPF 10) which allows for the detection of at least 43 known HPV types. The SPF 10 assay significantly diminishes the problems of HPV detection by amplifying only a 65-bp fragment located within the L1 region of the HPV genome. The amplification product is much shorter than the products obtained with other frequently used general

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**Table 2 and Figure 1.** Identification of HPV genotypes using the LiPA. LiPA strips with hybridization bands indicating a single HPV type infection (lane 1, HPV 16, lane 2, HPV 18, lane 3, HPV 45, lane 4, HPV 31, and a multiple HPV type infection (lane 5, HPV 11 + 16, lane 6, HPV 16 + 18, lane 7, HPV 16 + 31, lane 8, HPV 16 + 39 + 66. Note: HPV 18 is reactive with two probes: 18 and c68.
primer sets such as My11/09 (450 bp) or GP 5+/6+ (150 bp).\textsuperscript{28,29} The kinetics of the PCR reaction favor amplification of shorter DNA sequences and consequently, the SPF assay has been shown to be more sensitive than amplification systems using My11/09 or GP 5+/6+ primers.\textsuperscript{20} In addition, a short target sequence is statistically less likely to be affected by either DNA fragmentation or loss during viral integration.

HPV 16 and HPV 18 were the most common viral types identified and occurred with almost equal frequency. This result is similar to that reported by other investigators (Table 3) and highlights a difference from that found in squamous cell carcinomas where the frequency of HPV 16 is much greater than HPV 18.\textsuperscript{2} Other less common HPV types identified were HPV 45, followed by HPVs 52 and 35. Multiple HPV types were detected in 9.7% of the cases and in each, either HPV 16 or 18 was always identified in addition to other viral types. Multiple HPV infection was more frequently present in adenocarcinoma in situ and correlated with a younger age. According to a recent study of a large number of cervical cancers in Morocco, the odds ratio for the development of cervical

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>HPV DNA positive (%)</th>
<th>HPV 16:18 (%:%)</th>
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<tr>
<td>Adenocarcinomas</td>
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<td>78</td>
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<td>70</td>
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<tr>
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<td>91</td>
<td>44:42</td>
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<td>12</td>
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cancer was higher with double HPV infection versus single HPV infection (odds ratio, 1.4 versus 1.0). Further research is required to determine the importance of multiple HPV-type infection.

HPV DNA was identified in >90% of in situ and invasive mucinous adenocarcinomas, which encompass endocervical, intestinal, and endometrioid morphology and account for ~95% of all cervical adenocarcinomas. Several previous studies have also found a high HPV prevalence in cervical mucinous tumors, especially in those with endocervical morphology. Traditionally, endometrioid tumors of the cervix were classified separately from the mucinous cervical tumors. In this study only the tumors that did not show intracellular mucin with the standard hematoxylin and eosin staining were subclassified as endometrioid. Many of the cases, however, had mixed patterns of differentiation with areas of glands with abundant intracellular mucin, areas with modest amounts of mucin, as well as areas with mucin-depleted glands resembling endometrioid-type epithelium. All these cases were subclassified as endocervical subtype of adenocarcinoma. It is thought, however, that many of the tumors classified as endometrioid adenocarcinomas may in fact represent less-differentiated mucinous endocervical-type tumors that have decreased capacity to produce mucin. The arguments in support of this opinion are the following: 1) many of the cases of cervical adenocarcinoma show a spectrum of differentiation from areas with abundant intracellular mucin to mucin-depleted areas resembling endometrioid-type tumors; 2) when examined with histochemical stains, endometrioid-type adenocarcinomas express the same range of mucins, but in lesser quantities, as the mucinous tumors with endocervical-type histology.

Minimal deviation adenocarcinoma (MDA) may be a special category among mucinous cervical tumors. MDA is a rare lesion, accounting for only 1 to 3% of cervical adenocarcinomas, and is occasionally associated with Peutz-Jeghers syndrome and synchronous ovarian mucinous tumors. In our series, only two cases of MDA were available for analysis and both were negative for HPV DNA. This result is consistent with previous reports in which a total of 9 cases were negative for HPV. Recently, Lee et al described loss of heterozygosity of the 19p13.3 chromosomal region in nine sporadic cases of MDA, suggesting the presence of a putative tumor suppressor gene in this area. The clinical association between MDA and Peutz-Jeghers syndrome along with the results of molecular genetic and HPV studies indicates that the pathogenesis of MDA may not be related to HPV infection.

Nonmucinous adenocarcinomas of the cervix are relatively rare neoplasms and only six tumors were available for analysis in this study. All six were negative for HPV DNA, including four clear cell carcinomas (CCCs). CCCs account for 2 to 7% of cervical adenocarcinomas and comprise a heterogeneous group of malignancies. CCCs presenting in young patients and involving the ectocervix are usually associated with diethylstilbestrol (DES) exposure in utero. Other patients have no known risk factors and occur in an older age group. In the largest published series of CCCs, three of 14 tumors were positive for HPV 31 (Table 3). Other investigators have reported a highly variable prevalence of HPV DNA in CCCs (Table 3). Of note, CCC of the cervix has to be differentiated from clear-cell squamous carcinoma and clear-cell adenosquamous carcinoma, as both of the latter tumors are associated with HPV.

Serous AdC is another rare tumor with distinct clinicopathological characteristics including a bimodal age distribution with one peak occurring before the age of 40 and the second peak after the age of 65, coinciding with the peak occurrence of uterine serous carcinoma. The only patient in our series was a 39-year-old woman with a family history of ovarian and peritoneal serous carcinomas, and breast carcinoma. The clinical history in this case suggests the presence of a germline BRCA-1 mutation responsible for the familial breast-ovary cancer syndrome. HPV DNA was not identified in this tumor. Of three cases of serous carcinoma reported previously in the literature, two were HPV DNA-positive and one was HPV-negative (Table 3).

Mesonephric adenocarcinoma is another rare, nonmucinous cervical tumor, which is derived from the mesonephric ducts located deep in the lateral cervical stroma. The single case analyzed in our series was negative for HPV DNA. To our knowledge, no previous reports of HPV DNA detection are available in these tumors.

Carcinomas with adenosquamous differentiation account for 5 to 25% of all cervical cancers. The histological subtypes include adenosquamous (not otherwise specified), adenoid basal, adenoid cystic, glassy cell, and clear-cell adenosquamous carcinoma. In this study and in previously published reports, these tumors have been found to be associated with HPV in a high percentage of cases. Another group of cervical tumors which display focal glandular and squamous differentiation are neuroendocrine carcinomas. These tumors also have a high prevalence of HPV DNA, ranging from 53 to 85%, and are associated with both HPV 16 and 18; however, HPV 18 seems to be the most predominant in the small-cell carcinoma histological subtype.

Our results combined with data from epidemiological, clinicopathological, and molecular studies indicate that squamous cell carcinomas, adenosquamous carcinomas, mucinous adenocarcinomas, and neuroendocrine carcinomas of the cervix share a common pathogenesis that involves infection with oncogenic HPV types. Although little is known about the molecular genetic events involved in the pathogenesis of cervical adenocarcinoma after HPV infection, it is well-established that expression of the high-risk HPV E6 and E7 oncoproteins in keratinocytes (squamous cells) disrupts the function of the cell cycle-regulating proteins p53 and pRB, respectively. It is assumed that the same mechanism of HPV-related carcinogenesis occurs in cervical glandular epithelium.

References


Chapter 3

Detection and typing of Human Papillomavirus (HPV) DNA in penile carcinoma — evidence for multiple independent pathways of penile carcinogenesis.

Rubin MA, Kleter B, Zhou M, Ayala G, Cubilla AL, Quint WGV, Pirog EC.

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Detection and Typing of Human Papillomavirus DNA in Penile Carcinoma

Evidence for Multiple Independent Pathways of Penile Carcinogenesis

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To clarify the role of human papillomavirus (HPV) in penile cancer we evaluated the prevalence of HPV DNA in different histological subtypes of penile carcinoma, dysplasia, and condyloma using a novel, sensitive SPF10 HPV polymerase chain reaction assay and a novel genotyping line probe assay, allowing simultaneous identification of 25 different HPV types. Formalin-fixed, paraffin-embedded tissue samples were collected from the United States and Paraguay. HPV DNA was detected in 42% cases of penile carcinoma, 90% cases of dysplasia, and 100% cases of condyloma. There were significant differences in HPV prevalence in different histological cancer subtypes. Although keratinizing squamous cell carcinoma and verrucous carcinoma were positive for HPV DNA in only 34.9 and 33.3% of cases, respectively, HPV DNA was detected in 80% of basaloid and 100% of warty tumor subtypes. There was no significant difference in HPV prevalence between cases from Paraguay and the United States. In conclusion, the overall prevalence of HPV DNA in penile carcinoma (42%) is lower than that in cervical carcinoma (~100%) and similar to vulvar carcinoma (~50%). In addition, specific histological subtypes of penile cancer—basaloid and warty—are consistently associated with HPV, however, only a subset of keratinizing and verrucous penile carcinomas is positive for HPV DNA, and thus these two tumor groups seem to develop along different pathogenetic pathways. (Am J Pathol 2001, 159:1211–1218)

Penile cancer (PC) is an uncommon disease in the United States and in Europe and has a yearly incidence of 0.29 per 100,000 among whites in the United States.¹ The incidence is an order of magnitude higher in some of the African and South American countries, such as Uganda (incidence of 4.4 per 100,000) or Paraguay (incidence 4.2 per 100,000).²,³ The etiology of PC is not well understood. Traditionally, lack of neonatal circumcision was considered to be the most significant risk factor for PC, however, the causal relationship has never been established. In the most recent detailed epidemiological study, the highest risk for PC was associated with a history of penile rash lasting more than a month (Bowen’s disease?) [relative risk (RR) = 9.4] and a history of genital warts (RR = 5.9).⁴ As compared with these two high-risk factors, the risk of cancer in men uncircumcised or circumcised after the neonatal period was lower: RR = 3.2, and RR = 3.0, respectively. Other risk factors identified in the study included: penile tear (RR = 3.9), difficulty of foreskin retraction (RR = 3.5), more than 30 lifetime sexual partners (RR = 3.4), smoking (RR = 2.8), and smegma (RR = 2.1).⁴ The results suggest a strong association between human papillomavirus (HPV) infection and development of PC.

High oncogenic risk HPVs have been detected in virtually 100% of carcinomas of the uterine cervix and the role of HPV in malignant transformation of the cervical epithelium has been well established.⁵,⁶ In contrast to the
consistent finding of HPV in the cervical tumors, the reported prevalence of HPV in PC is highly variable, from 15 to 71%, depending on the sensitivity of the detection method and the selection of the tumor type. Penile carcinomas include several different histological subtypes. The majority of tumors are well-differentiated, keratinizing squamous cell carcinomas (SCCs) (Figure 1A) that resemble SCCs arising in nongenital skin. The second most common tumor subtype, verrucous carcinoma (Figure 1B), and the less prevalent variants, namely, basaloïd carcinoma (Figure 1C) and warty carcinoma (Figure 1D) arise most frequently on the mucosal surfaces of the anogenital and oropharyngeal regions, and on the penis these tumors most often involve the glans. The histological subtypes of PC are identical to those described in the vulva and it is plausible that penile carcinogenesis parallels the pathogenetic mechanisms of malignant transformation of the vulvar epithelium. The current concept of vulvar carcinogenesis includes at least two independent pathways: one, HPV-related and the other, not associated with HPV infection. Tumors associated with HPV tend to occur in younger women with past history of genital warts or cervical dysplasia and arise from the in situ lesions similar to those found in the cervix. The carcinomas are frequently of basaloïd or warty type. The prevalence of HPV in these tumors ranges from 75 to 100%. Tumors not associated with HPV occur in older women and are typically well-differentiated keratinizing SCCs arising in a background of differentiated vulvar intraepithelial neoplasia and/or lichen sclerosis. The risk factors are unknown.

Few studies of PC have analyzed the different histological subtypes separately. This lack of separation of histological types may have confounded both the results of the HPV studies and the epidemiological findings. The goal of this study was to further investigate the relationship between HPV and PC, by examining the prevalence of HPV DNA in a large series of tumors encompassing a spectrum of histological differentiation. In addition we wanted to analyze the prevalence and the distribution of HPV types in the premalignant penile lesions (penile dysplasia). Benign penile condyloma were included in the study as the HPV-positive control group.

The specific questions that we wanted to answer in this study were: 1) are there one or many pathways of penile carcinogenesis?, 2) are the various histological subtypes of penile carcinoma etiologically related?, 3) are there differences in frequency of various histological tumor subtypes between the high-risk and low-risk geographical regions (Paraguay versus the United States)?, 4) are there differences in HPV prevalence in the tumors from the high-risk and low-risk geographical regions (Paraguay versus the United States)?

HPV DNA amplification was performed using a novel, sensitive, broad-spectrum HPV polymerase chain reaction (PCR) assay (SPF 10 PCR), which permits general HPV DNA amplification of at least 43 known HPV types. HPV genotyping was performed using a novel line probe assay (LiPA). LiPA assay enables simultaneous identification of 25 individual HPV genotypes, allowing efficient detection of single and/or multiple HPV infection. High sensitivity of the assays used in this study was confirmed in a previous investigation in which HPV DNA was detected in 100% of cases of cervical carcinoma.

Materials and Methods

Clinical Specimens

Cases of penile condyloma, dysplasia (including Bowenoid papulosis and Bowen’s disease), and carcinoma were retrieved from the archives of the Pathology Departments at University of Michigan (Ann Arbor, MI), Baylor College of Medicine (Houston, TX), Yale University (New Haven, CT), Universidad Nacional de Asención, (Asuncion, Paraguay), and the Weill Medical College of Cornell University (New York, NY). Twelve cases of condyloma, 30 cases of dysplasia, and 155 cases of PC were collected. All cases were reviewed and diagnostic groups were assigned and graded according to standard histological criteria. A representative tissue block from each case was selected for HPV analysis. Clinicopathological parameters were obtained from the pathology reports.

DNA Extraction and β-Globin Amplification

Three, 5-μm thick sections of formalin-fixed, paraffin-embedded tissue were placed on glass slides after cutting deep into the block. The microtome blade was changed after each case. The tissue sections were deparaffinized in xylene and stained with hematoxylin. Tumor tissue was dissected from the adjacent squamous epithelium and stroma using a sterile scalpel blade. The samples were digested with proteinase K (1 mg/ml) in a volume of 0.25 ml at 56°C for 18 hours. Proteinase K was heat inactivated at 95°C for 10 minutes and 10 μl of DNA aliquot was used directly for PCR. To ensure adequate DNA quality, PCR amplification of the β-globin gene was performed in a separate reaction using primers PC03 and PC04, resulting in a 96-bp product. In 13 of 155 (8%) cancer cases no β-globin amplification was achieved. Repeated digestion was ineffective in these cases. These results indicated marked DNA degradation and the cases were excluded from further analysis (11 keratinizing, 1 basaloïd, and 1 verrucous carcinoma).

HPV DNA Detection and Typing

Broad-spectrum HPV DNA amplification was performed using the short PCR fragment (SPF10) primer set. The SPF10 primers amplify a 65-bp fragment from the L1 region of the HPV genome. The primer sequences and exact HPV PCR conditions were described previously. The PCR products were run on a 3% agarose gel and the 65-bp product was visualized with ethidium bromide staining. Additional confirmation of the presence of amplified HPV-specific sequences was performed using HPV DNA enzyme immunoassay, a microtiter plate-based hybridization assay. The exact HPV DNA enzyme immunoassay conditions were described previously.
Figure 1. Main histological subtypes of penile carcinoma analyzed in the study. 

A: Keratinizing SCC: infiltrating tumor characterized by nests and tongues of malignant squamous epithelium with prominent central keratin pearls.

B: Verrucous carcinoma: exophytic tumor characterized by papillary architecture and pushing invasive border; malignant squamous epithelium is well differentiated and no koilocytic atypia is present.

C: Basaloid carcinoma: infiltrating tumor characterized by nests and cords of immature malignant squamous epithelium with areas of central necrosis; foci of keratin pearls may be also present.

D: Warty carcinoma: exophytic/infiltrating tumor characterized by papillary architecture and marked cytological atypia with prominent koilocytic features.
Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

All HPV-negative cases were confirmed by the second PCR assay using standard DNA concentration as well as 10\textsuperscript{−}H11003 diluted DNA sample to exclude the presence of PCR inhibitors. Appropriate positive and negative PCR controls were run with all reactions.

Samples identified as positive for HPV DNA were genotyped with the INNO-LiPA HPV prototype research assay.\textsuperscript{16} Twenty-five individual HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, and low-risk HPV: 6, 11, 34, 40, 42–44, 53, 54, 74) can be identified simultaneously in a single assay. In this assay, 10 μl of denatured HPV PCR product was hybridized to the genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. After the washing step, the products of hybridization were detected by a color reaction with alkaline phosphatase-streptavidin conjugate and substrate (5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium), which results in a purple precipitate. The exact assay conditions were described previously.\textsuperscript{16} The results of hybridization were assessed visually by comparing to the standard grid (Figure 2).

**Results**

**Clinicopathological Data**

The study group included 106 cases of keratinizing SCC (33% grade 1 tumors, 48% grade 2 tumors, and 18% grade 3 tumors), 12 cases of verrucous SCC, 15 cases of basaloid SCC, 5 cases of warty SCC, 2 cases of clear cell SCC, 1 case of sarcomatoid SCC, and 1 case of a metastatic penile SCC in which the primary lesion was not available for a review. Benign cases consisted of a group of penile dysplasia (n = 30) and penile condylomas (n = 12). The average age of patients with cancer was 61.2 years (range, 31 to 94 years), with dysplasia was 58.1 years (range, 27 to 85 years), and with condyloma was 50.6 years (range, 34 to 82 years).

There was no significant difference in the distribution of tumor subtypes between the cases from Paraguay and the United States (Table 1).

**HPV DNA Detection and Typing**

Only the cases with positive β-globin DNA amplification were included in the study. HPV DNA was amplified in 99 of 182 benign and malignant cases, some of which were stored in the paraffin blocks for as long as 20 years. HPV DNA amplification was confirmed with the HPV DNA enzyme immunoassay and the individual HPV genotypes were subsequently identified with the LiPA. The cases, in which HPV DNA was not detected, were of various storage ages.

<table>
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<th></th>
<th>United States</th>
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<td>1</td>
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<td>100.0</td>
<td>88</td>
<td>100.0</td>
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</table>

*Chi-square test for the difference in the frequency of the tumor subtype between the series from Paraguay and the United States.

\textsuperscript{†}No statistically significant difference.

scc, Squamous cell carcinoma.
HPV DNA was detected in 100% of condylomas, 90% cases of dysplasia, and 42% of penile carcinomas. The results of HPV DNA detection in the different histological categories are summarized in Tables 2 and 3. All but one case of condyloma were associated with the low-risk viral types, with HPV 6 being the most prevalent type. In contrast, penile dysplasia was associated with high-risk HPVs in 81.5% of cases, and HPV 16 was most frequently detected. In PC, there were significant differences in HPV prevalence among the different histological tumor subtypes. Keratinizing and verrucous carcinoma were positive for HPV DNA in 34.9 and 33.3% of the cases, respectively. Basaloid and warty PCs were positive for HPV DNA in 80 and 100% of the cases, respectively. The difference in HPV prevalence between these two groups was statistically significant (chi-square test, \( P < 0.05 \)).

HPV 16 was the most common viral type identified in PC (60% of HPV+ cases) and was detected in 29 tumors as a single HPV type (48.3%) and as multiple-type infection in 7 tumors (11.6%) (Table 3 and Figure 2). Other HPV types were relatively less common. The types that were detected as a single viral infection included: HPV 45 (\( n = 4 \)), HPV 35 (\( n = 3 \)), HPV 18 (\( n = 2 \)), HPV 52 (\( n = 2 \)), HPV 68 (\( n = 2 \)), HPV 31 (\( n = 1 \)), HPV 53 (\( n = 1 \)), and HPV 6 (\( n = 1 \)). Other high-risk HPVs (51, 54, and 70) were detected in multiple-type infections with HPV 16. Undetermined HPV types (X types) were present in 7.8% of the cases. Multiple viral types were detected in 16.6% of HPV-positive tumors (Table 2). There was no statistically significant difference in HPV prevalence/type distribution between the cases from Paraguay and the United States (Table 4).

The average age of the patients with HPV DNA-positive versus HPV DNA-negative tumors (58.3 years versus 61.2 years) was not significantly different (Student's t-test, \( P > 0.05 \)). No association between the tumor grade and HPV positivity was identified.

### Table 2. HPV DNA Detection in Penile Condyloma, Dysplasia, and Carcinoma

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<th>Diagnosis</th>
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<th>Multiple HPV types</th>
<th>X types</th>
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<td>n%</td>
<td>n%*</td>
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<td>90.00</td>
<td>18.50</td>
<td>81.50</td>
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<td>92.80</td>
<td>16.41</td>
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<td>25.00</td>
<td>50.00</td>
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<td>80.00</td>
<td>11.00</td>
<td>91.70</td>
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<td>5.00</td>
<td>100.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Clear cell scc</td>
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<td>1.00</td>
<td>50.00</td>
<td>0.00</td>
<td>100.00</td>
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<tr>
<td>Sarcomatoid scc</td>
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<td>0.00</td>
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<tr>
<td>Metastatic scc</td>
<td>1</td>
<td>1.00</td>
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<td>0.00</td>
<td>0.00</td>
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<tr>
<td>All cancer cases</td>
<td>142</td>
<td>60.00</td>
<td>42.20</td>
<td>1.60</td>
<td>70.00</td>
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</table>

*Percent of HPV-positive cases.

### Table 3. HPV Types Identified in Penile Condyloma, Dysplasia, and Carcinoma

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<tr>
<th>Diagnosis</th>
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<th>HPV 6</th>
<th>HPV 11</th>
<th>HPV 16</th>
<th>HPV 18</th>
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<th>HPV 45</th>
<th>HPV 52</th>
<th>HPV 68</th>
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<tr>
<td></td>
<td>( n )</td>
<td>( n % )</td>
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<td>( n % )</td>
</tr>
<tr>
<td>Condyloma</td>
<td>12</td>
<td>9.0</td>
<td>75.0</td>
<td>2.0</td>
<td>16.7</td>
<td>1.0</td>
<td>8.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>27</td>
<td>6.0</td>
<td>22.2</td>
<td>1.0</td>
<td>7.7</td>
<td>11.0</td>
<td>40.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>3.7</td>
</tr>
<tr>
<td>All HPV-positive benign cases</td>
<td>35</td>
<td>19.0</td>
<td>38.5</td>
<td>7.7</td>
<td>12.0</td>
<td>30.8</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>10.3</td>
</tr>
<tr>
<td>Keratinizing scc</td>
<td>37</td>
<td>4.0</td>
<td>10.8</td>
<td>0.0</td>
<td>9.0</td>
<td>19.0</td>
<td>51.3</td>
<td>1.0</td>
<td>2.7</td>
<td>2.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Verrucous scc</td>
<td>4</td>
<td>1.0</td>
<td>25.0</td>
<td>0.0</td>
<td>1.0</td>
<td>25.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Basaloid scc</td>
<td>12</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>10.0</td>
<td>83.0</td>
<td>1.0</td>
<td>83.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Warty scc</td>
<td>5</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>5.0</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Clear cell scc</td>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Metastatic scc</td>
<td>1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>All HPV-positive cancer cases</td>
<td>60</td>
<td>5.0</td>
<td>8.3</td>
<td>0.0</td>
<td>36.0</td>
<td>60.0</td>
<td>2.0</td>
<td>3.3</td>
<td>3.0</td>
<td>4.0</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*The percentages of all HPV types detected in a histological subtype may add to more than 100% because of multiple infections.

Other HPV types: A = HPV 31, 33, 39, 45, 51, 54, 58, 66; B = HPV 51, 70, 74; C = HPV 53; D = HPV 31, 54, 70. X types: unknown HPV types.
sensitive, broad-spectrum HPV PCR assay (SPF 10), which allows for the detection of at least 43 known HPV types. The SPF 10 assay significantly diminishes the problems of HPV detection in formalin-fixed tissue by amplifying only a 65-bp fragment located within the L1 region of the HPV genome. The amplification product is much shorter than the products obtained with other frequently used general primer sets such as My11/09 (450 bp) or GP 5+/6+ (150 bp).\textsuperscript{15,20} The kinetics of the PCR reaction favors amplification of shorter DNA sequences and consequently, the SPF assay has been shown to be more sensitive than amplification systems using My11/09 or GP 5+/6+ primers.\textsuperscript{16} In addition, a short target sequence is statistically less likely to be affected by either DNA fragmentation or loss during viral integration. A high sensitivity of this technique was confirmed in the previous investigation in which HPV DNA was detected in 100% of cases of cervical carcinoma.\textsuperscript{6}

The overall prevalence of HPV DNA in PC detected in this study was 42.2%. This result is similar to the results of previously published studies (Table 5). However, we have observed great differences in HPV prevalence depending on the histological subtype of PC. Although basaloid and warty carcinomas were found to be consistently associated with HPV presence, only a subset of keratinizing and verrucous PCs was positive for HPV DNA. The most common viral type identified in PC was HPV 16, which was detected in 60% of HPV+ cancers. This result is similar to those reported by other investigators in which HPV 16 was found in 65 to 74% of HPV-positive tumors.\textsuperscript{6,21,22} In this study we did not find a significant difference between HPV prevalence in tumors from Paraguay and the United States; furthermore, no significant difference in the distribution of the histological tumor subtypes was found. We therefore conclude, that pathogenetic pathways of penile carcinogenesis are likely to be similar in the high-risk and the low-risk geographical regions.

In the cervix, all main histological subtypes of carcinoma: SCC, adenocarcinoma, and adenosquamous carcinoma are associated with HPV infection.\textsuperscript{6,11} In contrast, SCCs of the vulva seem to have multiple pathogenetic pathways. Basaloid and warty carcinomas are consistently associated with high-risk HPVs.\textsuperscript{12–14} Well-differentiated keratinizing SCCs and verrucous carcinomas have a low prevalence of HPV DNA.\textsuperscript{12–14} Rare cases of verrucous carcinoma of the vulva have been described to be associated with low oncogenic risk HPVs.\textsuperscript{24,25} The results of our current study and previously published reports indicate that the etiology and the pathogenetic pathways of PC may parallel the pathogenetic pathways of vulgar, but not cervical carcinoma. First, the overall prevalence of HPV DNA in PC (42.2%) is lower than that in cervical carcinoma (100%)\textsuperscript{6,6} and similar to that reported for vulvar carcinoma (50%).\textsuperscript{14} Second, the correlation between HPV DNA detection and histological tumor subtypes is similar in vulvar and PC. In our study two histological subtypes of PC: basaloid and warty were found to be positive for HPV DNA in almost 100% of cases and associated with HPV 16. This result is consistent with previously published reports. Cubilla and colleagues\textsuperscript{6} reported detection of HPV 16 in 9 of 11 (81%) cases of basaloid and 3 of 5 (60%) cases of warty SCC of the penis.\textsuperscript{9} Interestingly, basaloid carcinomas of the anus and the head and neck mucosa were also described as the specific histological tumor subtypes with high prevalence of HPV.\textsuperscript{26–28} Verrucous carcinomas of the penis analyzed in this study were most commonly HPV-negative and of a total of 26 cases reported in the multiple publications only 3 were found to be positive for

---

Table 4. Prevalence of Different HPV Types in Penile Cancers from Paraguay and the United States

<table>
<thead>
<tr>
<th>HPV phylogenetic groups* detected in penile carcinomas</th>
<th>Paraguay</th>
<th>United States</th>
<th>Chi-square&lt;sup&gt;†&lt;/sup&gt; P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any HPV positive</td>
<td>20</td>
<td>40</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>A10: HPV 6,11,74</td>
<td>3</td>
<td>4</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>A9: HPV 16,31,33,35,52</td>
<td>12</td>
<td>32</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>A7: HPV 18,39,45,68,70</td>
<td>4</td>
<td>6</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>A5: HPV 51,69</td>
<td>1</td>
<td>1</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>Other HPVs</td>
<td>3</td>
<td>4</td>
<td>&gt;0.05&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Phylogenetic viral groups as classified in Mayers and colleagues.\textsuperscript{34}

†Chi-square test for the difference between the frequency of HPV types in the cases from Paraguay and the United States.

No statistically significant difference.

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Table 5. Reported Prevalence of HPV DNA in Penile Carcinoma in the Largest Published Studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Detection method</th>
<th>n</th>
<th>HPV-positive, %</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubin et al 2001</td>
<td>PCR</td>
<td>142</td>
<td>42</td>
<td>United States and Paraguay</td>
</tr>
<tr>
<td>Picconi et al 2000</td>
<td>PCR</td>
<td>38</td>
<td>71</td>
<td>Argentina</td>
</tr>
<tr>
<td>Gregoire et al 1996</td>
<td>PCR</td>
<td>117</td>
<td>22</td>
<td>United States and Paraguay</td>
</tr>
<tr>
<td>Cupp et al 1995</td>
<td>PCR</td>
<td>42</td>
<td>55</td>
<td>United States</td>
</tr>
<tr>
<td>Chan et al 1994</td>
<td>PCR</td>
<td>41</td>
<td>15</td>
<td>Hong-Kong</td>
</tr>
<tr>
<td>Maden et al 1993</td>
<td>PCR</td>
<td>67</td>
<td>49</td>
<td>United States and Canada</td>
</tr>
<tr>
<td>Iwasawa et al 1993</td>
<td>PCR</td>
<td>111</td>
<td>63</td>
<td>Japan</td>
</tr>
<tr>
<td>McCance et al 1986</td>
<td>Southern blot</td>
<td>53</td>
<td>51</td>
<td>Brasil</td>
</tr>
</tbody>
</table>
HPV DNA (12%), and all were positive for low-risk HPVs.10,21,29–32

The reported prevalence of HPV DNA in penile dysplasia or penile intraepithelial neoplasia ranges from 75 to 100%.33 Results of our study (90% HPV +) are consistent with the previous reports. High-risk HPVs were detected in 81.5% of these cases, with HPV 16 being the most prevalent viral type. These findings suggest that penile intraepithelial neoplasia or penile dysplasia, as defined currently, is a precursor lesion to only a subset of tumors, which include basaloid and warty carcinomas. The precursor lesion for keratinizing SCC or verrucous carcinoma is not well established. Squamous cell hyperplasia and/or lichen sclerosus frequently coexist with keratinizing SCC and verrucous carcinoma, however the significance of this association remains to be determined.17

Table 6 summarizes the hypothetical pathways of penile carcinogenesis after combining the results of the current and previous investigations. There are still multiple unknowns that await further research.

Numerous molecular genetic studies have provided strong evidence that HPV is an oncogenic virus. HPV was found to inactivate some of the mechanisms regulating the cellular mitotic cycle. By doing this, the virus launches a cascade of uncontrolled genetic events that may lead to malignant transformation of the host cell. Specifically, HPV has been shown to interfere with the functions of retinoblastoma protein and p53 tumor suppressor protein. Inactivation of retinoblastoma protein keeps the cell in a perpetual proliferative state. The fidelity of cellular DNA may result in malignant transformation of the cell because of accumulation and propagation of DNA errors. However, as the cell cycle is maintained by redundant, multilevel mechanisms, it is thought that more than five different alterations of the major regulatory proteins are required before the cell acquires full malignant potential. And thus, although HPV has been firmly established as a causative factor of many cancers, HPV infection alone is insufficient to cause malignancy. The results of the epidemiological studies clearly indicate that numerous factors, including lack of neonatal circumcision, foreskin injuries, and cigarette smoking contribute to penile carcinogenesis. The exact molecular mechanisms by which these factors increase the risk of PC remain to be determined.

Table 6. Hypothetical Model of Multiple Pathways of Penile Carcinogenesis

<table>
<thead>
<tr>
<th>Etiologic factor</th>
<th>Cancer precursor</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Δ) and/or HPV</td>
<td>(?)</td>
<td>Keratinizing squamous cell carcinoma ~30% HPV +</td>
</tr>
<tr>
<td>(Δ) and/or HPV</td>
<td>(?)</td>
<td>Verrucous carcinoma ~30% HPV +</td>
</tr>
<tr>
<td>HPV and (?)</td>
<td>Penile dysplasia = penile intraepithelial neoplasia</td>
<td>Basaloid carcinoma and warty carcinoma 80–100% HPV +</td>
</tr>
</tbody>
</table>

References

Molecular markers and HPV detection in the diagnosis of lower genital tract lesions


Chapter 4

Prevalence of mucosal and cutaneous Human Papillomaviruses in different histologic subtypes of vulvar carcinoma.

*de Koning MNC, Quint WGV, Pirog EC.*

Prevalence of mucosal and cutaneous human papillomaviruses in different histologic subtypes of vulvar carcinoma

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¹DDL Diagnostic Laboratory, Voorburg, The Netherlands and ²Department of Pathology, Weill Medical College of Cornell University, New York-Presbyterian Hospital, New York, NY, USA

Two independent pathways of vulvar carcinogenesis have currently been identified, one related to infection with mucosal human papillomaviruses (HPVs) and a second related to chronic inflammatory or autoimmune processes. The goal of the study was to examine a possible role of cutaneous HPVs from the beta genus in vulvar carcinogenesis and to evaluate the distribution of intratypic variants of HPV 16 in HPV 16-positive vulvar cancer. Consecutive cases of vulvar carcinoma were retrieved from the files and included the following histologic subtypes: keratinizing (n = 21), basaloid (n = 7), warty (n = 1), mixed basaloid–warty (n = 4), verrucous (n = 4), keratoacanthoma (n = 1), basal cell carcinoma (n = 1). All tumors were microdissected and tested for 25 beta HPV types and 25 mucosal HPV types. Cases identified as positive for HPV 16 were further tested for intratypic variants. All cases were immunostained for p16INK4a. Beta HPVs were not detected in any of the tumors. Mucosal HPVs were detected in all but one basaloid/warty carcinomas; of these, nine cases (82%) were positive for HPV 16, including five European subtypes, one African subtype, one North American subtype and two indeterminate subtypes. Two of four verrucous carcinomas were positive for HPV 5. Mucosal HPVs were not detected in keratinizing carcinomas, keratoacanthoma and basal cell carcinoma. All cases of basaloid/warty carcinomas, but none of the remaining tumors, overexpressed p16INK4a protein. Our data do not support a role of beta HPVs in the pathogenesis of vulvar carcinoma. The study reaffirms the role of mucosal HPVs, in particular that of HPV 16, in the pathogenesis of basaloid and warty tumor subtypes. The HPV 16 intratypic variation showed correlation with patients’ ethnic background. P16INK4a immunostaining seems to be a sensitive and specific marker of vulvar carcinomas positive for oncogenic mucosal HPVs.

Keywords: vulvar carcinoma; HPV; P16INK4a

The pathogenesis of vulvar carcinoma is not entirely understood. Results of epidemiologic, clinicopathologic and virologic studies are in support of two independent pathways of vulvar carcinogenesis, one related to infection with oncogenic mucosal human papillomaviruses (HPVs) and a second related to a chronic inflammatory and/or autoimmune process involving vulvar mucosa and skin.¹⁻⁶

Vulvar cancers associated with oncogenic mucosal HPVs tend to occur in young women with past history of genital warts, cervical dysplasia and/or immunosuppression. These carcinomas typically belong to basaloid or warty histologic subtypes and develop from a well-characterized in situ lesion, namely, classic vulvar intraepithelial neoplasia grade 3 (VIN3).⁷ Approximately 80–90% of HPV-positive vulvar cancers are associated with HPV16 and nearly all remaining tumors are positive for HPV18 and HPV33.⁴⁻⁶,⁸⁻⁻¹³

Vulvar carcinomas not associated with mucosal HPVs occur in older women. These well-differentiated keratinizing squamous cell carcinomas histologically resemble conventional squamous cell carcinomas occurring in non-genital skin.⁴ However, unlike the usual squamous cell carcinomas, vulvar cancers are not arising in the setting of actinic keratosis. In the vulva, these tumors develop in a background of squamous cell hyperplasia, a chronic inflammatory condition, or long-lasting lichen sclerosus, a lesion with strong links to autoimmune diseases. Although patients with lichen sclerosus
have an average 4.5% cumulative risk of vulvar squamous cell carcinomas (reviewed in Carlson 1998, Table 6). Neither lichen sclerosus nor squamous cell hyperplasia are universally accepted to be premalignant lesions. Differentiated VIN or VIN simplex is recognized as an immediate precursor of invasive keratinizing squamous cell carcinomas in the vulva. Since differentiated VIN is almost invariably seen in association with squamous cell hyperplasia and lichen sclerosus, it is thought to represent a histologically distinct phase of malignant transformation in a non-neoplastic epithelial disorder, such as squamous cell hyperplasia or lichen sclerosus. The consecutive molecular events of malignant transformation in lichen sclerosus, squamous cell hyperplasia and differentiated VIN leading to invasive squamous cell carcinomas are currently unknown. A report of allelic imbalance in squamous cell hyperplasia and lichen sclerosis supports the hypothesis that both conditions pose a risk for neoplasia despite the lack of morphologic evidence of atypia. Rare cases of lichen sclerosus and adjacent squamous cell carcinomas with identical p53 gene mutations have been reported. Overall, however, p53 gene mutation is an infrequent and rather late event in vulvar carcinogenesis.

As reviewed by Nindl et al., there is evidence supporting a role for cutaneous HPV types belonging to the beta-genus (betaPVs) (Table 1) in the pathogenesis of non-melanoma skin cancer. Originally, betaPVs have been found in benign and malignant skin lesions from patients with a rare hereditary disease, epidermodysplasia verruciformis. These individuals develop multifocal skin warts and maculae that have a high rate of progression to squamous cell carcinomas, in particular on sun-exposed sites. Specific betaPVs, that is HPV5 and HPV8, have been suggested as possible high risk types in these patients. Subsequently, studies of betaPV types showed their presence in squamous cell carcinomas of immunosuppressed individuals and in skin cancers of immunocompetent individuals. Cutaneous HPVs, however, are ubiquitous and are persistently present on the surface of normal skin and in plucked hairs. Whether the detection of betaPVs in squamous cell carcinomas reflects their oncogenic potential or whether it represents merely a persistent cutaneous infection that occurs in a large percentage of the population is still unclear.

The groups of vulvar squamous cell carcinomas that may be associated with cutaneous HPVs are keratinizing and verrucous carcinomas. These tumors usually develop in a background of squamous cell hyperplasia and lichen sclerosus, conditions treated with topical corticosteroids producing local immunosuppression. Some of the keratinizing squamous cell carcinomas show areas of cytologic atypia with high resemblance to koilocytic change. Furthermore, the characteristic feature of verrucous carcinoma is a prominent exophytic growth resembling a skin wart. It is not inconceivable that this pattern of growth may reflect a proliferation stimulated by cutaneous HPVs. While testing for cutaneous HPVs in keratinizing and verrucous vulvar squamous cell carcinomas has not been reported previously, these tumor subtypes have been previously tested for mucosal HPVs showing either absence or low prevalence of HPV DNA. Some studies report the presence of HPV6 and HPV11 in rare cases of verrucous carcinoma. Since keratinizing and verrucous vulvar squamous cell carcinomas have never been evaluated for the presence of cutaneous HPVs, the first goal of our study was to test these tumors for the presence of cutaneous HPV types from the beta genus and for mucosal HPVs using the SPF10–LiPA25 system detecting 25 low and high oncogenic risk mucosal HPVs.

**Table 1** The different genera from the family Papillomaviridae that contain HPV types and whether they infect mucosal or cutaneous epithelia is shown.

<table>
<thead>
<tr>
<th>Genus</th>
<th>HPV type</th>
<th>Tissue tropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphapapillomavirus</td>
<td>6, 11, 16, 18, 26, 30, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84, 85, 86, 87, 89 and 90</td>
<td>Mucosal</td>
</tr>
<tr>
<td>Betapapillomavirus</td>
<td>2, 3, 10, 27, 28, 29, 57, 77, 78 and 94</td>
<td>Mucosal and cutaneous</td>
</tr>
<tr>
<td>Betapapillomavirus</td>
<td>5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93 and 96</td>
<td>Cutaneous</td>
</tr>
<tr>
<td>Gammapapillomavirus</td>
<td>4, 48, 50, 60, 65, 88 and 95</td>
<td>Cutaneous</td>
</tr>
<tr>
<td>Mupapillomavirus</td>
<td>1 and 63</td>
<td>Cutaneous</td>
</tr>
<tr>
<td>Nupapillomavirus</td>
<td>41</td>
<td>Cutaneous</td>
</tr>
</tbody>
</table>

The cutaneous HPV types investigated in this study belong to the beta genus.
Recently, upregulation of p16INK4a, a tumor suppresor protein, has been identified as a sensitive marker of active E7 oncoprotein expression in \textit{in situ} and invasive carcinomas caused by oncogenic mucosal HPVs.\textsuperscript{36–38} P16INK4a immunostaining of such lesions allows for a visual verification of the extent of tissue involved by oncogenic mucosal HPVs. To validate the results of HPV DNA detection by PCR and to exclude the cases with detection of skin surface ‘HPV passengers’, all cases in our study were immunostained for p16INK4a protein.

Oncogenic properties of HPVs mainly depend on two viral oncoproteins, E6 and E7, which interfere with the functions of the host cell p53 tumor suppressor protein and the retinoblastoma protein, respectively. Intratypic variations of HPV16 E6 and E7 genes have been described in which the gene sequence variation results in oncoprotein variants with a transforming potential, which differs from that of the prototype (reviewed by Giannoudis et al\textsuperscript{39} and Bernard et al\textsuperscript{40}). The second goal of this study was to examine the intratypic variation of HPV16 in vulvar carcinomas.

Materials and methods

Case Selection and Review

Institutional review board approval was obtained for this study. Consecutive cases of vulvar squamous cell carcinomas diagnosed between December 1990 and December 2005 were retrieved from the files of the Pathology Department at the Weill Medical College of Cornell University New York, NY, USA. A total of 39 cases were identified, including keratinizing squamous cell carcinoma (n = 21), verrucous (n = 4), basaloid (n = 7), warty (n = 1), mixed basaloid–warty (n = 4), keraoacanthoma (n = 1) and basal cell carcinoma (n = 1). The cases were re-reviewed to confirm the diagnosis and subclassification into the histologic subtypes. The standard diagnostic criteria were used.\textsuperscript{1} Cases of re-excision or recurrences were excluded from the study.

Tissue Dissection and DNA Preparation

Genomic DNA was prepared from two to three 4 μm sections from each case using standard methods. Briefly, the slides were deparaffinized and tissue was scraped with a sterile blade. To check for contamination representative tumor sections were microdissected into three separate samples:

1. deep tumor portion, consisting of deep nests with a minimum of 2 mm distance from the tumor surface,
2. superficial tumor portion, consisting of 2 mm superficial layer,
3. stroma, a negative dissection control.

DNA was released from the samples by incubating them with 100 μl proteinase K solution (1 mg/ml) for 16 h at 56 °C. Following heat inactivation at 95 °C for 10 min, 10 μl of the supernatant was used for PCR. Appropriate positive and negative controls were incorporated during DNA preparation and subsequent testing to monitor test performance.

BetaPV Genotyping with the PM-PCR RHA Method

BetaPV detection and genotyping was carried out with the PM-PCR reverse hybridization assay (RHA) method (The skin (beta) HPV prototype research assay; Diassay BV, Rijswijk, The Netherlands) as described earlier by de Koning et al.\textsuperscript{33} The method was designed for the identification of the 25 established betaPV types (ie, HPV genotypes 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93 and 96). The PM-PCR, generating a biotinylated amplimer of 117 bp from the E1 region, was carried out with all precautions to avoid contamination as described by the manufacturer. Briefly, PM-PCR was performed in a final reaction volume of 50 μl, containing 10 μl of the isolated DNA, 2.5 mM MgCl\textsubscript{2}, 1 × GeneAmp PCR Buffer II, 0.2 mM deoxynucleoside triphosphates, 1.5 U AmpliTaq Gold DNA polymerase and 10 μl of the PM primer mix. The PCR was performed by a 9 min preheating step at 94 °C, followed by 35 cycles of amplification comprising 30 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C. The PCR is ended by a final elongation step of 5 min at 72 °C. As a positive PCR control, a betaPV plasmid clone was included at an amount of approximately 100 times the limit of detection of the assay. Identification of the amplified HPV types was performed by analyzing the amplimers with the RHA.

Mucosal HPV DNA Amplification and Genotyping

Broad-spectrum HPV DNA amplification and mucosal HPV genotyping was performed using the SPF\textsubscript{10}–LiPA\textsubscript{25} system (SPF\textsubscript{10} HPV LiPA, version 1; manufactured by Labo Bio-Medical Products, Rijswijk, The Netherlands) as described previously.\textsuperscript{34,35} Briefly, SPF\textsubscript{10} PCR amplifies a 65-base pair fragment from the L1 region of the HPV genome. The reaction was performed in a total volume of 50 μl containing 10 μl of isolated DNA, 1 × GeneAmp PCR Buffer II, 0.2 mM deoxynucleoside triphosphates, 15 pmol of each of the forward and reverse primers, and 1.5 U of AmpliTaq Gold DNA polymerase. AmpliTaq Gold was activated by incubation at 94 °C for 9 min. HPV DNA was amplified in 40 cycles of 30 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C, and a final extension of 5 min at 72 °C. The use of biotinylated reverse primers enabled the capture of the reverse strand of the amplimer onto streptavidin-coated microtiter plates. The captured amplimers were denatured by alkaline treatment, and detected by a defined
cocktail of digoxigenin-labeled probes. This method is designated the HPV DEIA, which provides an optical density value and is able to detect more than 50 HPV types. Amplimers from positive samples can be used to identify 25 individual HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70, and low-risk HPV: 6, 11, 34, 40, 42–44, 53, 54, 74) simultaneously in the RHA.

**HPV16 Variant Testing**

Cases identified as positive for HPV16 were further tested for intratypic subtypes and variants by PCR and subsequent analysis of the sequences of the E6/E7 region by reverse hybridization. The following nucleotide positions were assessed according to the HPV16 reference sequence (accession number K02718): 131, 132, 143, 145, 178, 286, 289, 335, 350, 403 and 532. This method allowed for determination of the European subtype with variants Asian, 350G, 350T, and 131G, the African subtype with variants African 1 and African 2 and the North American/African 1 and verrucous carcinoma accounted for 21/39 (54%) of the cases, whereas combined basaloid, warty and mixed basaloid–warty carcinomas added up to 12/39 (31%) of cases (Table 2). The average age of the patients with basaloid, warty (Figure 1a and b) and mixed basaloid–warty carcinomas was 58 years. The patients with keratinizing and verrucous carcinomas (Figure 1c and d) were on average two decades older (average age 76 years) and the average age difference between the two groups was statistically significant, $P = 0.0004$.

P16\(^{Nk4a}\) Immunohistochemistry

P16\(^{Nk4a}\) immunohistochemical staining was performed on 4 \(\mu\)m sections of formalin-fixed, paraffin-embedded specimens. The sections were subjected to heat-induced antigen retrieval and incubated in an automated stainer with p16\(^{Nk4a}\) antibodies obtained from ClNtec\textsuperscript{a}Histology Kit (MTM Laboratories, Westborough, MA, USA). The sections were subsequently stained using Bond Polymer Detection System (Vision Biosystem, Norwell, MA, USA) developed with diaminobenzidine chromogen and counterstained with hematoxylin. The staining was performed with respective negative and positive controls. The result of the staining was graded as following: 0, negative or weak nuclear and cytoplasmic ‘blush’; 1, moderate to strong intensity nuclear and cytoplasmic staining with diffuse or patchy distribution. According to the manufacturer’s instructions, weak ‘blush’ staining should be interpreted as a negative result.

**Results**

Clinicopathologic Characteristics of Cases

A total of 39 cases of vulvar carcinoma were identified during the study period. Typical keratinizing squamous cell carcinoma accounted for 21/39 (54%) of the cases, whereas combined basaloid, warty and mixed basaloid–warty carcinomas added up to 12/39 (31%) of cases (Table 2). The average age of the patients with basaloid, warty (Figure 1a and b) and mixed basaloid–warty carcinomas was 58 years. The patients with keratinizing and verrucous carcinomas (Figure 1c and d) were on average two decades older (average age 76 years) and the average age difference between the two groups was statistically significant, $P = 0.0004$.

All cases of basaloid/warty carcinomas were associated with classic VIN3 (Table 2). Sixty-eight percent (17/25) of combined keratinizing and verrucous carcinomas were associated with squamous cell hyperplasia and differentiated VIN; in addition, three of these cases also showed lichen sclerosus. The remaining cases were associated with a single lesion: squamous cell hyperplasia, 3/25 (12%); differentiated VIN, 3/25 (12%) and lichen sclerosus, 2/25 (8%). A single case of keratoacanthoma was associated with squamous cell hyperplasia and differentiated VIN. No non-neoplastic vulvar disorder was identified adjacent to a case of basal cell carcinoma.

Some cases of basaloid carcinomas showed focal morphologic features overlapping with keratinizing carcinoma, and vice versa. For instance, 5 of 12 cases (42%) of basaloid and warty carcinomas showed focal areas of keratin pearl formation (Figure 2a) and 7 of 21 cases (33%) of moderately differentiated keratinizing carcinoma showed areas of tumor nests with scant basophilic cytoplasm and...
Inconspicuous keratinization, resembling basaloid carcinoma (Figure 2b). In addition, 7 of 21 cases of keratinizing squamous cell carcinomas showed areas of cytologic atypia resembling koilocytic change, mimicking warty carcinoma (Figure 2c); in two of such cases adjacent differentiated VIN also showed similar ‘pseudo-koilocytic’ atypia (Figure 2d).

The two tumor groups, however, showed distinct differences in nuclear chromatin distribution. Keratinizing/verruccous tumors had relatively pale nuclei with vesicular chromatin and prominent nucleoli, while basaloid/warty tumors showed hyperchromatic nuclei with coarse, stippled chromatin and inconspicuous nucleoli.

**Detection of betaPV and Mucosal HPVs**

Cutaneous betaPVs were not detected in any of the tumor cases (Table 2). All but one case (case 1) of basaloid, warty and mixed basaloid–warty carcinomas were positive for mucosal HPVs. Microdissection and SPF10–LiPA25 analysis of case 1 was repeated twice and confirmed the original result. Nine cases were positive for HPV16 and two tumors were positive for HPV33 and HPV33 plus HPV51, respectively (Table 2). Analysis of intratypic HPV16 variation revealed five European subtypes, one North American variant and one African1 variant (Table 2). In two cases, the exact subtype/variant could not be determined by

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DVIN, differentiated vulvar intraepithelial neoplasia; VIN3, vulvar intraepithelial neoplasia grade 3.
Figure 1 Main histologic subtypes of vulvar carcinoma: (a) basaloid carcinoma, (b) warty carcinoma, (c) keratinizing carcinoma, (d) verrucous carcinoma (hematoxylin and eosin, (a, b) original magnification × 200, (c, d) original magnification × 100).
the RHA system. The patients with European subtypes were of either Caucasian or Hispanic racial background, the patient with the African1 variant was an Afro-American and the background of the patient with North American variant was not on record. Of two patients with undetermined

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**Figure 2** Overlapping features in subtypes of vulvar carcinoma: (a) keratin pearls in basaloid carcinoma, (b) pseudo-basaloid nests in keratinizing carcinoma, (c) pseudo-koliocytic atypia in keratinizing carcinoma, (d) pseudo-koliocytic atypia in differentiated VIN (hematoxylin and eosin, (a–d) original magnification ×200).
variants, one was Caucasian and one was Afro-American.

Mucosal HPVs were not detected in any of the keratinizing squamous cell carcinomas, keratoacanthoma or basal cell carcinoma; however, two of four verrucous carcinomas were positive for HPV6.

Comparison of the results of HPV DNA amplification between the three microdissected samples from the same case enabled, by repeat analysis, identification of six cases with amplification contamination: in three cases, tumor surface showed contamination with HPV6 (n = 1) and HPV16 (n = 2), and three cases showed post-dissection contamination with HPV66. All PCR assays were controlled by appropriate positive and negative controls. No aberrant results were observed.

\[ P16^{NK^4a} \text{ Immunostaining} \]

All cases of basaloid, warty and mixed basaloid–warty carcinomas and adjacent VIN3 stained diffusely and strongly with p16\(^{NK^4a}\). None of the verrucous, keratinizing squamous cell carcinomas, keratoacanthoma and basal cell carcinoma, adjacent squamous cell hyperplasia, lichen sclerosus or differentiated VIN showed significant p16\(^{NK^4a}\) positivity with the exception of focal ‘blush’ staining, which was identified in 20% of the cases. Weak ‘blush’ result of p16\(^{NK^4a}\) immunostaining should be interpreted as a negative result, as explained in the Materials and methods section.

Positive p16\(^{NK^4a}\) staining showed excellent correlation with the detection of high-risk mucosal HPVs with the exception of one case: case number 1 in Table 2. In this case, the tumor was of basaloid subtype and was associated with classic VIN3 in situ lesion. Based on these histopathologic features, the lesion was expected to be positive for high-risk mucosal HPV; in addition, both the tumor and VIN3 was diffusely and strongly positive for p16\(^{NK^4a}\).

**Discussion**

Cutaneous betaPVs were not detected in any of the vulvar cancer cases in the current study. While this was a first investigation of betaPVs in vulvar carcinoma that will require further corroboration, results of our initial findings suggest that betaPVs are not involved in pathogenesis of vulvar squamous cell carcinomas. The results of this study, however, provide further validation to the hypothesis of two pathogenetic pathways in vulvar carcinogenesis, one of which is related to infection with high-risk mucosal HPVs. In our study all, but one, basaloid and warty carcinomas were found to be positive for mucosal HPVs, with HPV16 alone accounting for 82% of positive cases. The single HPV-negative basaloid carcinoma most likely represented a false-negative error of the PCR assay, based on histopathologic features and presence of p16\(^{NK^4a}\) overexpression, although the PCR assay tested for more than 50 HPV types.

The results of studies of HPV16 variants in cervical mucosa demonstrated that non-European HPV16 variants confer a higher risk of prevalent and incident high-grade cervical dysplasia. In addition, results of a case-controlled study from Mexico indicated a higher risk of invasive cervical carcinoma conferred by Asian American–North American variants (odds ratio 27.0), as compared to European HPV16 variants (odds ratio 3.4). The current study is a first report on intratypic variation of HPV16 in vulvar carcinoma. The HPV16 intratypic variation correlated with patients’ ethnic background and showed a pattern of distribution similar to that reported for cervical squamous cell carcinoma in North America. In contrast to previous studies demonstrating increased frequency of non-European HPV16 variants in invasive squamous cell carcinomas of the cervix, we did not observe such relationship in our limited sample of HPV16-positive vulvar cancers. Mucosal HPVs were not detected in keratinizing carcinomas, keratoacanthoma or basal cell carcinoma. This result is in agreement with previous studies that used different analytical techniques and demonstrated either no or only low prevalence of mucosal HPVs in these tumors. Interestingly, two of four verrucous carcinomas in our study were found to be positive for HPV6. Since HPV6 is a low oncogenic risk HPV type, lesions caused by this HPV are not expected to show overexpression of p16\(^{NK^4a}\) and, with in situ hybridization assays being insensitive, no independent verification of viral presence in these tumors is available. While in the majority of larger studies of vulvar and penile verrucous carcinomas no HPV DNA has been detected, rare cases of verrucous carcinoma positive for low-risk mucosal HPV types have been reported. These latter reports faced two criticisms: possible detection of ‘passenger HPVs’ and misclassification of giant condyloma of Buschke–Lowenstein as verrucous carcinoma. In our study, we made the best attempt to dissect deep tumor tissue to avoid contamination from surface HPVs; in addition, a correct diagnosis has been established as no koilocytic atypia has been identified in any of the cases and the tumors were associated with in situ lesions, including squamous cell hyperplasia and differentiated VIN. There is a long-standing controversy with regard to pathogenesis of verrucous carcinoma and its relationship, or lack of thereof, to giant condyloma of Buschke–Lowenstein. Aside from subtle architectural differences, the main distinguishing feature between these two entities is the presence of koilocytic atypia in giant condyloma and absence of koilocytosis in verrucous carcinoma. However, it is also well documented that a significant proportion of genital condylomata shows minimal or no koilocytic atypia at all. Given the overlapping histologic
features, some authorities regard giant condyloma of Buschke–Lowenstein and verrucous carcinoma as a spectrum of the same process. Since condylomata acuminate do not regress spontaneously and, unless excised, will persist for years or even decades, showing decreasing koilocytosis and proliferation while developing increasing superficial keratinization (unpublished observations42), it is not inconceivable that verrucous carcinoma may evolve from a persistent, irritated condyloma. Low oncogenic risk HPV may initiate the growth process, while subsequent chronic epithelial irritation may contribute to a gradual evolution of malignant phenotype. A strong argument against the connection between giant condylomas and verrucous carcinomas is the fact that patients with giant condylomas are on average 30 years younger than patients with verrucous carcinomas (35–46 years old vs 65–75 years old)49,53.

The current study shows a precise separation between the two main groups of vulvar cancers: basaloid/warty and keratinizing/verrucous carcinomas, and we have found an accurate correlation between the histologic tumor subtype, adjacent in situ lesion, the presence or absence of oncogenic in situ mucosal HPVs and p16INK4a overexpression.12,13,15,54 The reasons for this apparent lack of correlation in some cases may be multifold: (1) the presence of mixed morphologic features in the different areas of the tumor, resulting in imprecise classification (eg presence of keratin pearls in basaloid carcinoma, presence of pseudo-koilocytes or inconspicuous keratin pearls in keratinizing carcinoma); (2) detection of ‘passenger HPV’ resulting in non-correlating positive result of HPV detection and negative result of p16INK4a staining; (3) failure of HPV DNA amplification resulting in non-correlating negative result of HPV detection and positive result of p16INK4a staining; (4) additionally, a recent study from our laboratory showed that false-positive interpretative error of ‘blush’ p16INK4a immunostaining could result in a lack of correlation with detection of high oncogenic risk HPVs in cervical mucosa.55

The overlapping histologic features in different tumor subtypes were analyzed in detail in a recent study by Santos et al.13 In the series of vulvar carcinomas described by Santos et al, keratin pearls were identified in over a half of basaloid carcinomas and in all warty and keratinizing carcinomas. In addition, ‘koilocytic atypia’ was identified in 43% of HPV-negative keratinizing carcinomas. Cases with mixed histologic features were also identified in our study: keratin pearls were found focally or diffusely in 42% of basaloid/warty carcinomas, while pseudo-koilocytic atypia and pseudo-basaloid nests were found in 33% of keratinizing carcinomas. Both the correlation between the overall tumor morphology and adjacent in situ lesion and nuclear chromatin distribution were very helpful in determining tumor subclassification in the cases with overlapping histologic features. Keratinizing/verrucous tumors exhibited pale vesicular chromatin and prominent nucleoli, while basaloid/warty tumors showed dark, coarse, granular chromatin and inconspicuous nucleoli. Results of our study also show that p16INK4a immunostaining may be used as an objective adjunct test to confirm the diagnosis in challenging cases.

Some pathologists rely on p53 immunostaining to identify differentiated VIN and HPV-negative vulvar cancer. P53 immunoexpression, however, is neither sensitive nor specific marker of differentiated VIN,56 neither it correlates with the actual p53 gene mutation in benign or malignant vulvar squamous epithelium.57,58 These two problems with p53 immunostaining have been widely illustrated by numerous previous publications11,15,19,20,56–58 and in the authors own experience p53 immunostain is not useful in differential diagnosis of HPV-negative vulvar lesions.

The presented data do not support a role for betaPVs in vulvar carcinoma but reaffirm the role of high-risk mucosal HPV types in the pathogenesis of basaloid and warty vulvar carcinoma. Our results corroborate previous findings of Santos et al13 that p16INK4a immunostaining is a sensitive and specific marker of HPV-positive vulvar carcinomas, useful in subclassifying tumors with mixed or overlapping histologic features.

Acknowledgements

We thank J Lindeman and Labo Bio-Medical Products BV (Rijswijk, The Netherlands) for providing the RHA strips.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

Molecular markers and HPV detection in the diagnosis of lower genital tract lesions


Chapter 5

Low grade vulvar and vaginal intraepithelial neoplasia: correlation of histologic features with Human Papillomavirus DNA detection and MIB-1 immunostaining.

Logani S, Lu D, Quint W, Ellenson L, Pirog EC.

Low-Grade Vulvar and Vaginal Intraepithelial Neoplasia: Correlation of Histologic Features with Human Papillomavirus DNA Detection and MIB-1 Immunostaining

Sanjay Logani, M.D., Danielle Lu, M.D., Wim G. V. Quint, Ph.D., Lora H. Ellenson, M.D., Edyta C. Pirog, M.D.

Department of Pathology, Emory University School of Medicine, Atlanta, GA (SL); Department of Pathology, Washington University School of Medicine, St. Louis, MO (DL); Academic Medical Center, Amsterdam, The Netherlands (WGVQ); and Department of Pathology, Weill Medical College of Cornell University, NY, NY (LHE, ECP)

Histologic criteria of low-grade vulvar/vaginal intraepithelial neoplasia (VIN1/VAIN1) are well established; however, a significant interobserver variability in diagnosing VIN1/VAIN1 has been reported. The goal of this study was to evaluate the utility of MIB-1 immunostaining as an adjunct test to increase the diagnostic accuracy in equivocal cases of VIN1/VAIN1. The second goal was to examine the distribution of low- and high-oncogenic risk human papillomaviruses (HPVs) in VIN1/VAIN1 lesions. Consecutive vulvar/vaginal biopsies originally diagnosed as VIN1/VAIN1 (n = 43) or benign (n = 20) were reviewed by two pathologists to obtain a consensus diagnosis. The diagnosis was further confirmed with HPV testing using Short PCR Fragment 10 and Line Probe Assay. MIB-1 immunostaining was performed, and positive staining was defined as a cluster of two or more stained nuclei in the upper two thirds of the epithelial thickness. After verification of the diagnosis using the consensus histologic review and HPV detection as an objective confirmatory test, 31% of cases originally diagnosed as VIN1/VAIN1 were identified as being overdiagnosed. The sensitivity and the specificity of MIB-1 staining for identifying VIN1/VAIN1 were 0.96 and 0.90, respectively. Seventy percent of VIN1 cases were associated with low-risk viral types. In contrast, the majority (84%) of VAIN1 cases were associated with high-risk HPVs. In conclusion, MIB-1 staining is sensitive and specific for identifying VIN1/VAIN1, helpful in verifying the diagnosis in equivocal cases.

KEY WORDS: HPV, Low-grade vulvar intraepithelial neoplasia, Low-grade vaginal intraepithelial neoplasia, Ki-67, MIB-1, VAIN1, VIN1.

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Human papillomavirus (HPV) infection of vulvar skin and mucosa may clinically present either as exophytic condyloma (CON-A) or as a flat lesion currently termed vulvar intraepithelial neoplasia (VIN). The International Society for the Study of Vulvar Disease (ISSVD) (1) and the World Health Organization (2) endorsed the VIN terminology for noninvasive neoplastic vulvar lesions with subdivisions into VIN1 (mild dysplasia), VIN2 (moderate dysplasia), and VIN3 (severe dysplasia) depending on the level of involvement of the epithelium by the immature dysplastic cells. The ISSVD classification helped to standardize the terminology between different laboratories and eliminated numerous descriptive clinical diagnoses such as Bowen’s disease, Bowenoid papulosis, erythroplasia of Queyrat, and dystrophy with atypia, among others. Even though VIN terminology is useful and has been quickly adopted, there are still certain diagnostic problems. First, it is sometimes difficult to distinguish VIN2 from VIN3. In many cases the distinction is so subjective that some laboratories choose to combine the two grades and render the diagnosis as “high-grade vulvar intraepithelial neoplasia (VIN2–3)” or “high-grade squamous intraepithelial lesion (VIN2–3)” in parallel to the diagnostic scheme used for cervical lesions. Second, diagnosis of VIN1 has poor interobserver and intraobserver reproducibility. The diagnostic difficulty arises...
from subtle features of HPV cytopathic effect (koilocytosis) in the vulvar skin/mucosa, and lesser degree of nuclear atypia, as compared with the case of cervical low-grade squamous intraepithelial lesions (LSIL). In cases where nuclear atypia is minimal, the main differential diagnosis of VIN1 is “normal” skin/mucosa. In addition, presence of pseudokolocytes in benign skin/mucosa may cause overdiagnosis. These diagnostic problems are clearly illustrated by a study of van Beurden et al. (3), in which 44% of vulvar biopsies originally diagnosed as “normal” received a diagnosis of VIN1 upon review, and 40% of cases originally diagnosed as VIN1 were reclassified as “normal.” In another study (4), an audit of cases originally diagnosed as VIN1 was performed by the dermatopathologist and the surgical pathologists. In only 2 of 21 cases (9.5%) was the original diagnosis validated; the diagnosis in the remaining cases was revised to squamous cell hyperplasia (28%), psoriasis (24%), lichen planus (14%), and vesicular/bullous diseases (14%).

The pathobiology of VIN1 has not been well studied, and association with specific HPV genotypes is not known. In contrast, other vulvar lesions have been extensively investigated. Vulvar condyloma acuminatum is a benign growth that has been shown to harbor low-risk HPVs, most commonly HPV6 or HPV 11 (5, 6). VIN3, on the other hand, is a premalignant lesion associated with high-oncogenic risk HPVs, specifically HPV 16 (3, 7, 8). It is not clear, however, whether VIN1 is biologically related to benign condyloma, or if it is a precursor of VIN2–3.

Vaginal lesions associated with HPV infection are similarly challenging, and both underdiagnosis or overdiagnosis may result from subtle features of koilocytic atypia or presence of pseudokolocytes, respectively. In a study by Nuovo et al. (9), low-grade vaginal lesions were tested for HPV DNA by PCR, and only 63% of cases were found to be HPV positive, suggesting overdiagnosis in the remaining cases. The investigators observed that perilucinal haloes and mild nuclear atypia were not predictive of HPV DNA detection.

The purpose of this study was to evaluate the utility of MIB-1 immunostaining as an adjunct test to increase the diagnostic accuracy in equivocal cases of low-grade vulvar as well as vaginal intraepithelial neoplasia (VIN1/VAIN1). Next, we wanted to determine the distribution of low- and high-oncogenic risk HPVs in VIN1/VAIN1 to acquire better understanding of the pathogenesis of these lesions. Finally, we were interested in identifying any subgroups within VIN1/VAIN1 cases based on either histologic or virologic characteristics. In particular, we wanted to determine whether dividing low-grade vaginal/vulvar intraepithelial neoplasia into two separate categories of “mild dysplasia” and “flat condyloma” has any biological significance.

Because any study of VIN1/VAIN1 potentially may be flawed by inclusion of non-neoplastic cases because of subjectivity of histologic diagnosis, our first objective was to identify the gold standard VIN1/VAIN1 and benign cases. The archival cases of VIN/VAIN and benign cases, including squamous cell hyperplasia and lichenoid lesions, were reviewed, and a consensus histologic diagnosis was obtained. The consensus diagnosis was further confirmed with HPV testing. In such validated gold standard cases, we determined the sensitivity and specificity of MIB-1 immunostaining for verifying the diagnosis. Finally, we performed genotyping of HPV DNA detected in VIN1/VAIN1 lesions using VIN3 cases as a positive control.

MATERIALS AND METHODS

Case Selection

The surgical pathology files of the Departments of Pathology at the Weill Medical College of Cornell University and Washington University School of Medicine were searched from 1997 to 2000 to identify successive vulvar/vaginal biopsies with the diagnosis of either benign or low-grade vulvar and vaginal intraepithelial neoplasia (VIN1 and VAIN1) and high-grade vulvar intraepithelial neoplasia (VIN3). The diagnoses were rendered by numerous faculty members with varying years of diagnostic experience. The study group consisted of 43 cases of combined VIN1 (n = 23) and VAIN1 (n = 20). The negative control group consisted of 20 benign cases, including squamous cell hyperplasia (n = 9); lichen planus (n = 4); and benign squamous mucosa (n = 7). The positive control group consisted of 6 cases of VIN3.

Verification of Histologic Diagnosis

To verify the histologic diagnoses, all cases were reviewed by two pathologists (SL and ECP) using standard criteria (10) to obtain a consensus diagnosis that was further correlated with the results of HPV testing. The study included only VIN/VAIN cases in which the consensus diagnosis was confirmed by positive HPV result (considered gold-standard positive cases) and benign cases in which the consensus diagnosis was confirmed by negative HPV result (considered as gold-standard negative cases). Cases of differentiated VIN were not included in the study.

MIB-1 Immunohistochemistry

MIB-1 immunohistochemical staining was performed on 4-μm sections of formalin-fixed,
paraffin-embedded specimens. The sections were subjected to heat-induced antigen retrieval and incubated in an automated stainer with MIB-1 antibody (Zymed Laboratories, Inc., San Francisco, CA) at a dilution of 1:300, stained with diaminobenzidine chromogen, and counterstained with hematoxylin. Presence of parabasal epithelial staining was used as a positive control. A presence of a cluster of two or more stained nuclei in the upper two thirds of the epithelial thickness was interpreted as a positive result (5).

DNA Preparation
Genomic DNA was prepared from two to three 4-μm sections from each case using standard methods (5). Briefly, the slides were deparaffinized and stained with hematoxylin, and tissue was scraped with a sterile needle. The samples were incubated with 250-μL proteinase K (1 mg/mL) in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.5% Tween 20 for 18 hours at 56°C. After heat inactivation at 95°C for 10 minutes, 10 μL of the supernatant was used for PCR. The entire tissue preparation was carried out in a specially dedicated laboratory to avoid PCR product carryover. Adequate DNA quality was established by PCR amplification of β-globin gene, resulting in a 96-base pair product (11).

HPV DNA Amplification
Broad-spectrum HPV DNA amplification was performed using the Short PCR Fragment (SPF 10) primer set, as described previously (12). SPF 10 PCR amplifies a 65-base pair fragment from the L1 region of the HPV genome. Briefly, HPV DNA amplification was performed in a total volume of 50 μL containing 10 μL of isolated DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.0 mM MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 200 μM of each deoxynucleotide triphosphate, 15 pmol of each of the forward and reverse primers (sequence as in Kleter et al. [12]), and 1.5 U of AmpliTaq Gold (Perkin Elmer). AmpliTaq Gold was activated by incubation at 94°C for 9 minutes. HPV DNA was amplified in 40 cycles of 30 seconds at 94°C, 45 seconds at 52°C, and 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. Each experiment was performed with separate positive (plasmid HPV DNA) and negative (H₂O) controls. PCR products were analyzed using 3% agarose gel electrophoresis. All HPV-negative cases were confirmed by the second PCR assay using standard DNA concentration as well as 10× diluted DNA sample to exclude presence of PCR inhibitors.

HPV DNA Genotyping
Samples identified as positive for HPV DNA were genotyped with the HPV-Line Probe Assay (Innogenetics Inc., Alpharetta, GA). Twenty-five individual HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70; low-risk HPV: 6, 11, 34, 40, 42–44, 53, 54, and 74) can be identified simultaneously in a single assay. The exact assay conditions were described previously (13). Briefly, 10 μL of denatured HPV PCR product was hybridized (60 min at 50°C) to genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. After the washing step, the products of hybridization were visualized in a color reaction using alkaline phosphatase-streptavidin conjugate, 5-bromo-4-chloro-3-indolylphosphate, and nitroblue tetrazolium, which results in a purple precipitate. The results were assessed by aligning the strips with the standard grid (Fig. 1).

RESULTS
Clinical Data
The average age of patients with the diagnosis of VIN1 and VAIN1 was 45.1 years (range, 18–91) and 43.7 years (range, 18–81), respectively. History of intraepithelial neoplasia (either vulvar or cervical) was present in 25% of VIN1 and 19% of VAIN1 cases. A concurrent high-grade vulvar/vaginal intraepithelial neoplasia was identified in 28% of

![FIGURE 1. HPV genotyping using Line Probe Assay. Lane 1, HPV11; Lane 2, HPV35 and HPV42; Lane 3, HPV16 and HPV51.](image-url)
VIN1 and 10% of VAIN1 cases. The average age of the patients with VIN3 was 43.3 years (range, 27–65). History of vulvar or cervical intraepithelial neoplasia was present in all cases. The average age of the patients with benign diagnosis was 61 years (range, 46–91). History of vulvar or cervical intraepithelial neoplasia was not recorded in any of the cases, and concurrent VIN3 was present in one case.

Identification of Gold-Standard VIN1/VAIN1 Cases

Thirty of 43 cases (69%) originally diagnosed as VIN1/VAIN1 were confirmed as VIN1/VAIN1, and the remaining 13 (31%) were identified as overdiagnosis and reclassified as benign based on the result of consensus review and negative HPV testing (Table 1). The reclassified cases included 11 originally diagnosed as VIN1 and 2 originally diagnosed as VAIN1. The revised diagnoses for these cases included squamous cell hyperplasia (1 case), lichen planus (2 cases), and benign squamous mucosa with reactive changes (10 cases). All cases originally diagnosed as VIN3 or benign were validated as such.

MIB-1 Immunostaining in Gold-Standard VIN1/VAIN1, VIN3, and Benign Cases

MIB-1 positivity was identified in 29 of 30 gold-standard VIN1/VAIN1 cases (Table 1), 6 of 6 VIN3 cases, and 2 of 33 gold-standard benign cases (Fig. 2). The two MIB-1–positive benign cases had a diagnosis of squamous cell hyperplasia. The sensitivity and the specificity of MIB-1 positivity for identifying VIN1/VAIN1 lesions was 0.96 and 0.90, respectively. There was no difference in MIB-1 staining pattern between cases positive for low-risk and high-risk HPVs.

HPV DNA Genotyping in Gold-Standard VIN1, VAIN1, and VIN3 Cases

The results of HPV genotyping are presented in Table 2 and Figure 1. Seventy percent of VIN1 cases were associated with low-risk HPVs, and 84% of VAIN1 cases were associated with high-risk HPVs. All 6 cases of VIN3 were associated with high-risk HPVs. Mixed HPV type infection was identified in 20% of VIN1/VAIN1 and 30% of VIN3 cases, respectively.

“Flat Condyloma” Versus “Mild Dysplasia”

All gold-standard VIN1/VAIN1 cases were subclassified—using a consensus review—into “flat condyloma” (a lesion with koilocytic atypia but no evidence of dysplasia; n = 15) or mild dysplasia (a lesion with koilocytic atypia in the superficial layers of the epithelium, loss of maturation with associated nuclear hyperchromasia, pleomorphism, and cellular crowding confined to the lower one third of the epithelium; n = 15). The average age of the patients in both groups was 45 years. The frequency of past history of HPV infection was 40% in the flat condyloma group versus 53% in the mild dysplasia group (P > .05 by χ²). The ratio of low-risk to high-risk HPVs was 1:2.7 in the flat condyloma group and 1:2.2 in the mild dysplasia group (P > .05 by χ²). The MIB-1 staining pattern in both groups was identical.

DISCUSSION

The histologic criteria to diagnose VIN1 are elaborated in the ISSVD classification of vulvar disorders, but the application of these criteria has considerable interobserver variability, and the diagnosis of VIN1 and VAIN1 continues to be a challenge when based on histopathologic criteria alone. In this study we have audited cases with the original diagnosis of VIN1/VAIN1 using a consensus histologic review and HPV DNA detection as an objective confirmatory test. We found that 31% of cases were overdiagnosed as VIN1/VAIN1. The medical and social ramifications of this finding are of great importance. Because VIN1/VAIN1 is related to the sexual transmission of a potentially oncogenic virus, women for whom the diagnosis is rendered face significant therapeutic, sexual, and social consequences. Although verification of the accuracy of the diagnosis could be performed with demonstration of HPV DNA by PCR, this technique requires special expertise and may not be always available. MIB-1 positivity has been extensively used as a surrogate marker of dysplasia in cervical biopsies. Notably, it has been used to differentiate pseudokoiocytic changes and atrophy from dysplasia (14, 15) and has been used to evaluate cauterized margins in cervical cone biopsies (16). In this study we evaluated the utility of the stain as an adjunct test for accurate diagnosis of VIN1/VAIN1.

MIB-1 is a monoclonal antibody recognizing Ki-67 nuclear nonhistone protein expressed

** Presence of two or more stained nuclei in the upper two-thirds of the epithelial thickness was interpreted as a positive result.

**G o l d s t a n d a r d d i a g n o s i s w a sb a s e do nt h e r e s u l t s o fc o n s e n s u sr e-

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Gold Standard Diagnosis</th>
<th>MIB-1 Positive**</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIN1/VAIN1</td>
<td>30</td>
<td>VIN1/VAIN1</td>
<td>29</td>
</tr>
<tr>
<td>VIN1/VAIN1</td>
<td>13</td>
<td>BENIGN</td>
<td>0</td>
</tr>
<tr>
<td>BENIGN</td>
<td>20</td>
<td>BENIGN</td>
<td>2</td>
</tr>
<tr>
<td>VIN3</td>
<td>6</td>
<td>VIN3</td>
<td>6</td>
</tr>
</tbody>
</table>

* Gold standard diagnosis was based on the results of consensus review and HPV testing.
** Presence of two or more stained nuclei in the upper two-thirds of the epithelial thickness was interpreted as a positive result.
throughout the mitotic cycle, with the exception of the G₀ phase. Immunostaining with MIB-1 or polyclonal anti–Ki-67 antibodies yields the same results (17). In normal vulvar skin and vulvar/cervical mucosa, MIB-1 positivity is found exclusively in the parabasal nuclei of the squamous epithelium (3, 5, 18–22). HPV infection results in increased proliferative activity of the squamous cells and extension of MIB-1–positive cells to the upper layers of the epithelium (5, 21, 22). Presence of MIB-1–positive cells above the parabasal epithelial layer has been described in both VIN1 (3), and VIN2–3 (3, 18, 20, 23); however, these results were not correlated with HPV testing. Previous studies of vulvar condylomata and LSIL of the cervix showed a complete correlation between detection of HPV DNA and MIB-1 positivity that was defined as presence of MIB-1–positive cells in the upper two thirds of the epithelial thickness (5, 14). In the current study, we used the same definition of MIB-1 positivity and found an excellent correlation between HPV detection and MIB-1 positivity. The sensitivity of MIB-1 immunostaining for identification of VIN1/VAIN1 was 0.96.

The differential diagnosis of VIN1 includes normal skin/mucosa and, among the most common disorders, squamous cell hyperplasia, lichen planus, psoriasis, and differentiated VIN. We detected positive MIB-1 staining in the upper two thirds of the epithelium in 2 of 9 cases of squamous cell hyperplasia. None of 4 cases of lichen planus were positive for MIB-1 in our study. The specificity of MIB-1 for detection of VIN1/VAIN1 was 0.90. In addition, previous reports described only basal/parabasal MIB-1 staining in psoriasis (19, 24) and differentiated VIN (23). These results indicate that MIB-1 is a specific marker of vulvar/vaginal dysplasia; however, in cases where the differential diagnosis includes squamous cell hyperplasia, HPV testing may be preferable for validation of the diagnosis.

Even though interpretation of MIB-1 positivity is highly reproducible, regardless of the pathologist’s experience, there are few instances that may result in false-positive interpretation of the staining. Tangential sectioning through the stromal papillae may result in apparent presence of positive nuclei in superficial layers of the epithelium. Examination of the overall tissue architecture may help to evade this potential interpretative error. In cases of vulvitis/vaginitis or lichenoid lesions, MIB-1–positive lymphocytes may be present throughout the epithelial thickness. High-power examination helps to identify these cells as nonepithelial.

The second goal of our study was to determine the distribution of low- and high-oncogenic risk HPV in VIN1/VAIN1 to acquire insight into the pathobiology of these lesions. We found that VIN1 was most commonly associated with low-risk HPVs (70%). This result suggests that flat VIN1 lesions are related to exophytic condylomata acuminata which are positive for low-risk HPVs in 77–88% of cases (5–7). For the same reason, it is unlikely that VIN1 is a precursor of VIN3, which is consistently associated with high-risk HPVs. Interestingly, 84% of VAIN1 lesions were found to be positive for high-risk HPVs, similar to cervical LSILs, which have been reported to be associated with high-risk HPVs in 83% of cases (25).

### TABLE 2. HPV DNA Genotyping in Gold Standard VIN1, VAIN1, and VIN3 Cases

<table>
<thead>
<tr>
<th>Gold Standard Diagnosis</th>
<th>( n )</th>
<th>Low:High Risk HPV Ratio*</th>
<th>HPV Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIN1</td>
<td>11</td>
<td>70:30</td>
<td>Low risk: HPV6 (( n = 4 )), HPV11 (( n = 1 )), HPV44 (( n = 1 )), HPV74 (( n = 1 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High risk: HPV31 (( n = 1 )), HPV68 (( n = 1 )), Mixed high/low risk types: HPV16 + HPV51 (( n = 1 )), HPV unknown type (( n = 1 ))</td>
</tr>
<tr>
<td>VAIN1</td>
<td>19</td>
<td>16:84</td>
<td>Low risk: HPV42 (( n = 1 )), HPV44 (( n = 1 )), HPV53 (( n = 1 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>High risk: HPV16 (( n = 2 )), HPV56 (( n = 2 )), HPV18 (( n = 3 )), HPV31,59,68 (( n = 1 ) each)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mixed high/low and high/high risk types: HPV16 + 66, HPV16 + 53 + 68, HPV35 + 42, HPV 42 + 56, HPV56 + 66 (( n = 1 ) each)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HPV unknown type (( n = 1 ))</td>
</tr>
<tr>
<td>VIN3</td>
<td>6</td>
<td>0:100</td>
<td>High risk: HPV16 (( n = 3 )), HPV58 (( n = 1 ))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mixed high/low risk types: HPV16 + 6, HPV18 + 52 + 56 + 74 (( n = 1 ) each)</td>
</tr>
</tbody>
</table>

* Cases of mixed high/low risk HPV infection were counted as high risk cases.
lates with the detection of HPV in both vulvar and vaginal low-grade squamous intraepithelial lesions with high sensitivity and specificity. Furthermore, MIB-1 immunostaining is routinely available, inexpensive, and reproducible. Our results clearly demonstrate that MIB-1 immunostaining is a beneficial adjunctive test that may be an important element of quality assurance in rendering diagnoses in equivocal cases.

REFERENCES

Chapter 6

Diagnostic accuracy of cervical low grade squamous intraepithelial lesions is improved with MIB-1 immunostaining.

*Pirog EC, Baergen RN, Soslow RA, Tam D, DeMattia AE, Chen YT, Isacson C.*

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Diagnostic Accuracy of Cervical Low-Grade Squamous Intraepithelial Lesions Is Improved With MIB-1 Immunostaining

Edyta C. Pirog, M.D., Rebecca N. Baergen, M.D., Robert A. Soslow, M.D., Diane Tam, M.D., Amy E. DeMattia, M.D., Yao-Tseng Chen, M.D., Ph.D., and Christina Isacson, M.D.

There is considerable interobserver variation in the diagnosis of low-grade squamous intraepithelial lesion that involves mature squamous epithelium. Our aim was to evaluate the utility of MIB-1 immunostaining as an adjunct test to increase diagnostic accuracy. Consecutive cervical biopsies originally diagnosed as normal (n = 26) or low-grade squamous intraepithelial lesion (n = 23) were reviewed by three pathologists to obtain a consensus diagnosis. MIB-1 immunostaining was performed, and positive staining was defined as a cluster of at least two stained nuclei in the upper two thirds of the epithelial thickness. Human papillomavirus (HPV) DNA detection was performed using a polymerase chain reaction assay. All cases were subsequently reclassified as low-grade squamous intraepithelial lesion (LSIL) or normal (NL) when two or three of three gold standard criteria were satisfied (LSIL gold standard criteria = consensus diagnosis of LSIL, HPV+, MIB-1+; NL gold standard criteria = consensus diagnosis of NL, HPV−, MIB-1−). Using the gold standard diagnoses, we have identified that 14 normal cases (36%) were originally overdiagnosed as LSIL, and one LSIL case (10%) was originally underdiagnosed as normal. All MIB-1-positive cases were HPV+ and identified as LSIL in the consensus review. All MIB-1-negative cases were NL by gold standard criteria. The sensitivity (1.0) and the specificity (1.0) of MIB-1 staining for identifying LSIL were superior to the sensitivity (0.9) and the specificity (0.8) of HPV testing. In conclusion, MIB-1 is a highly sensitive and specific marker for identifying low-grade squamous intraepithelial lesion and is helpful in verifying the diagnosis of equivocal cases.

Key Words: Low-grade squamous intraepithelial lesion—MIB-1—Ki67—HPV.

Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

SILs. The pattern of MIB-1 staining in SILs differs significantly from that of normal cervical epithelium. In normal cervix MIB-1-positive cells are confined to the parabasal cell layer. In cervical SILs MIB-1 expression is increased in the parabasal areas with extension of the positive cells into the intermediate and superficial epithelial layers. In our previous studies of vulvar epithelium we have found an excellent correlation between HPV DNA detection and the presence of MIB-1-positive nuclei in the upper two thirds of the epithelial thickness, and we refer to these cases as “MIB-1 positive.”

In this study we have retrospectively reviewed a series of cervical biopsies with original diagnoses of normal/benign changes NL or LSIL, and correlated the histologic diagnoses with the results of MIB-1 staining and PCR detection of HPV DNA. All cases were subsequently reclassified according to the gold standard criteria consisting of a consensus histopathologic review and two objective tests (HPV detection, MIB-1 staining). All cases were reclassified when two of three, or all three, gold standard criteria were satisfied (Fig. 1). After establishing the gold standard diagnoses for the cases we could then determine which adjunct test had higher sensitivity and specificity for validating the accurate LSIL diagnosis.

The rationale for combining three separate criteria to establish the gold standard diagnoses was as follows:

1. Consensus histopathologic diagnosis alone is subjective.
2. With HPV testing alone we expected false-positive cases due to subclinical HPV infection of normal cervical mucosa and false-negative cases when tissue sections cut for PCR analysis no longer contained the lesion (further reviewed by Shroyer et al.).
3. Increased MIB-1 staining was expected in LSIL, but not in normal cervix or cases with subclinical HPV infection; however, the pattern of the increased staining (qualitative vs quantitative), as well as specificity and sensitivity of this marker are still not established.

MATERIALS AND METHODS

Case Retrieval

The surgical pathology files of the New York Presbyterian Hospital from the year 1996 were used to identify successive cervical biopsies with the diagnosis of either normal (encompassing benign cervical mucosa, ± acute cervicitis, ± chronic cervicitis) or LSIL (CIN 1). The diagnoses were rendered by numerous Department of Pathology faculty members with varying years of diagnostic experience. Forty-nine separate cases were selected to include normal (n = 26) and LSIL (n = 23).

49 consecutive cervical biopsies with original diagnosis of:

- LSIL, n = 23 and normal, n = 26
- were reclassified when 2 or 3 out of 3 GOLD STANDARD criteria were satisfied

LSIL criteria:
- consensus dx +
- HPV +
- MIB-1 +

normal criteria:
- consensus dx -
- HPV -
- MIB-1 -

RESULT

- LSIL, n = 10
- normal, n = 39

- 14 normal cases were originally overdiagnosed as LSIL (36%)
- 1 LSIL case was originally underdiagnosed as normal (10%)

FIG. 1. The outline of the study design and results.

MIB-1 Immunostaining

Serial 4-μm unstained sections from formalin-fixed, paraffin-embedded tissue blocks were used. Immunohistochemical staining was performed with MIB-1 antibody (Immunotech, Westbrook, ME, USA) at a dilution of 1:300 after microwave-antigen retrieval. The sections were stained with diaminobenzidine chromogen and counterstained with hematoxylin. Sections of human tonsil were used as a positive control, and a negative control without primary antibody was prepared on each case. The parabasal cells of the squamous epithelium served as an internal positive control.

Based on our previous experience on the correlation between MIB-1 staining with HPV DNA detection and previous reports on MIB-1 staining patterns in the cervix, MIB-1 staining was defined as positive when a cluster of at least two strongly stained epithelial nuclei was present in the upper two thirds of the epithelial thickness anywhere within the lesion.

HPV DNA Detection

Genomic DNA was extracted from two 4-μm sections from each case and amplified by PCR with the
MY09/MY11 consensus primers, as previously described.\textsuperscript{14} The PCR products were run on a 2% agarose gel, and the 450-base pair product was visualized with ethidium bromide staining. Adequate DNA quality was established by PCR analysis with primers for an esol gene.\textsuperscript{14} All HPV-negative cases were confirmed by a second PCR assay. Appropriate positive and negative PCR controls were run with all reactions.

Consensus Review

The hematoxylin and eosin sections from each case were reevaluated independently by three gynecologic pathologists blinded to both the original diagnosis and other pathologists’ review. A consensus diagnosis was defined as an agreement between at least two reviewers.

Statistical Analysis

The concordance between the gold standard diagnosis and HPV positivity, MIB-1 positivity, and consensus review was determined by kappa statistical analysis using the SPSS 6.1 software package. Kappa values of 1.0, 0.75, 0.50, and 0.25 were used as a threshold for excellent, good, moderate, and poor concordance, respectively.

RESULTS

The outline of the study and the summary of the results are presented in Figure 1 and Table 1. After combining the results of blinded consensus histopathologic review, HPV testing, and MIB-1 immunostaining, the cases were reclassified into gold standard diagnoses using the criteria as presented in Figure 1 and described in detail in Materials and Methods. Using the gold standard diagnoses, we have identified that 14 normal cases were originally overdiagnosed as LSIL (36%) and one LSIL case was originally underdiagnosed as benign (10%).

Among gold standard LSIL cases, 10 of 10 (100%) were identified as LSIL in the consensus review, 9 of 10 (90%) were HPV positive, and 10 of 10 (100%) were MIB-1 positive. Among gold standard normal cases, 35 of 39 (90%) were identified as normal in the consensus review, 7 of 39 (18%) were HPV positive, and 0 of 39 (0%) were MIB-1 positive.

All MIB-1-positive cases were HPV+ and identified as LSIL in the consensus review. All MIB-1-negative cases were identified as gold standard NL. This establishes that our qualitative criterion for MIB-1 positivity (a cluster of at least two strongly stained epithelial nuclei present in the upper two thirds of the epithelial thickness) was appropriate. The sensitivity and specificity of MIB-1 staining for identifying LSIL are shown in Table 2.

One of 10 gold standard LSIL cases was negative for HPV DNA but positive with MIB-1 and identified as LSIL in the consensus review (false-negative HPV PCR result). Further, 18% of gold standard normal cases were positive for HPV DNA. This rate is consistent with the rate of subclinical HPV infections of the cervix. The sensitivity and specificity of HPV testing for identifying LSIL are shown in Table 2.

The consensus review diagnoses correlated with the gold standard diagnoses in 45 of 49 cases. The four remaining cases, which were both HPV negative and MIB-1 negative (and thus defined as normal by gold standard criteria), were independently diagnosed as LSIL by two pathologists and as normal by one pathologist. Upon subsequent multihead-scope review by all pathologists, the cases were determined to be overdiagnosed. The sensitivity and specificity of a consensus review for identifying LSIL are shown in Table 2.

DISCUSSION

Criteria for the histologic diagnosis of SILs on tissue sections are well established. However, the application of these criteria is subject to individual interpretation, and a low interobserver agreement for diagnosing low-grade lesions (kappa = 0.55) has been reported.\textsuperscript{4,18} This is unfortunate because histology is the basis upon which treatment of cervical disease is based. Identifying an objective test to aid histologic diagnosis could prove beneficial for both pathologists and patients.

We retrospectively evaluated a series of cervical biopsies originally diagnosed as LSIL or normal/benign using a review of three pathologists and detection of HPV DNA and MIB-1 staining. After establishing the gold standard diagnoses for the cases we then determined that MIB-1 immunostaining and a consensus review had higher sensitivity for accurately identifying LSIL than HPV testing and that MIB-1 had the higher specificity than either consensus review or HPV testing (Table 2). Therefore, our study indicates that MIB-1 immunostaining is a beneficial adjunctive test in the evaluation of low-grade lesions of the cervix and helps to establish the

### TABLE 1. Results of the consensus review, HPV detection, MIB-1 immunostaining and subsequent “gold standard diagnoses” of 49 cervical biopsies

<table>
<thead>
<tr>
<th>Case no.</th>
<th>No.</th>
<th>Consensus diagnosis</th>
<th>HPV</th>
<th>MIB-1</th>
<th>Gold standard diagnosis</th>
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<td>1–9</td>
<td>9</td>
<td>LSIL</td>
<td>+</td>
<td>+</td>
<td>LSIL</td>
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<tr>
<td>10</td>
<td>1</td>
<td>LSIL</td>
<td>−</td>
<td>+</td>
<td>LSIL</td>
</tr>
<tr>
<td>11–14</td>
<td>4</td>
<td>LSIL</td>
<td>−</td>
<td>−</td>
<td>NL</td>
</tr>
<tr>
<td>15–42</td>
<td>28</td>
<td>NL</td>
<td>−</td>
<td>−</td>
<td>NL</td>
</tr>
<tr>
<td>43–49</td>
<td>7</td>
<td>NL</td>
<td>+</td>
<td>−</td>
<td>NL</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; LSIL, low-grade squamous intra-epithelial lesions; NL, normal.
Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

presence of a lesion with great accuracy. The advantage of MIB-1 staining over HPV testing lies not only in its higher specificity (the staining is negative in subclinical HPV infections), but MIB-1 staining is also a routinely available, low-cost laboratory technique.

Several other studies have evaluated MIB-1 immunostaining in LSILs; however, only a few of these studies included HPV DNA analysis for a rigorous verification of LSIL diagnoses. In many of these previous studies, the differences in MIB-1 staining between the normal cervical mucosa and LSIL were determined in a quantitative way, by calculating the percentage of MIB-1-positive nuclei to all nuclei, by either manual counting or by computer image analysis. In addition to being cumbersome and time-consuming, this approach also does not allow for a clear separation of normal cases from...

FIG. 2. Microphotographs of cervical biopsies: hematoxylin and eosin stain (left), with respective MIB-1 immunostain (right). (A) Gold standard diagnosis = LSIL. Original diagnosis = LSIL. HPV positive. MIB-1 positive (positive nuclei present in the upper two thirds of the epithelial thickness). (B) Gold standard diagnosis = normal. Original diagnosis = LSIL. HPV negative. MIB-1 negative (positive nuclei present only in the parabasal layer). (C) Gold standard diagnosis = normal. Original diagnosis = LSIL. HPV negative. MIB-1 negative (positive nuclei present only in the parabasal layer).
LSILs, as some of the reports show an overlap in MIB-1 scores determined for the full epithelial thickness.²,⁷

In our previous studies of vulvar epithelium⁹,¹⁴ we have found an excellent correlation between HPV DNA detection and the presence of MIB-1-positive nuclei in the upper two thirds of the epithelial thickness, and we used this criterion to classify cases as MIB-1 positive. The results of the current study parallel these previous findings as all cases with MIB-1-positive nuclei in the upper two thirds of the epithelial thickness were found to be positive for HPV DNA and were identified as LSIL in the consensus review. This indicates that our simple, fast, “eyeball” qualitative criterion for MIB-1 positivity was appropriate.

Although “eyeball” interpretation of MIB-1 positivity is highly reproducible, regardless of the pathologist’s experience (kappa >0.9, results not shown), there are few instances that may result in false-positive interpretation of the staining. Tangential sectioning through the stromal papillae may result in the presence of positive nuclei in apparently superficial layers of the epithelium. Quick inspection of the overall tissue architecture may help to evade this potential interpretative error. In cases of cervicitis, MIB-1-positive lymphocytes may be present throughout the epithelial thickness. High-power examination helps to identify these cells as nonepithelial. There are several limitations of MIB-1 use. MIB-1-positive cells may be present in the upper layers of the epithelium in the immature squamous epithelium, squamous metaplasia, or in an area of cervical erosion (repair). Positive MIB-1 staining in these cases may not be associated with HPV presence.

Our retrospective correlation of the original diagnoses with the gold standard diagnoses indicated that there was a trend to overdiagnose cervical biopsies. Many benign mimics of LSIL have been described, including pseudo-koilocytosis,⁸ epithelial repair,¹⁹ and inflammatory/reactive changes.¹⁵ The above-referenced articles include useful didactic microphotographs of the mimics. The lesions overdiagnosed in our series were equivocal cases, some of which were similar to the described mimics. As much as 36% of the original LSIL cases were identified as overdiagnosis based on the negative results of MIB-1 staining, HPV detection, and the consensus histologic review. The medical and social ramifications of this finding cannot be overemphasized. As LSIL is related to a sexual transmission of the potentially oncogenic virus, the patient to whom this diagnosis is rendered is facing significant therapeutic, reproductive, sexual, and social consequences.³,¹³

Results of previous studies and our current study indicate that even stringent morphologic criteria fail to reliably predict the presence of HPV in both vulvar and cervical lesions.¹,⁷,¹³ Although verification of the accuracy of the diagnosis could be performed with demonstration of HPV DNA by PCR, this technique requires special expertise and may not be readily available.

MIB-1 immunohistochemistry, however, is an easy, reproducible, and relatively inexpensive test. MIB-1 positivity is associated with the detection of HPV in both cervical and vulvar lesions with high sensitivity and specificity.⁹,¹⁴ Our results clearly demonstrate that positive MIB-1 staining can serve as an objective test and may be an important element of quality assurance in rendering diagnoses.

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Molecular markers and HPV detection in the diagnosis of lower genital tract lesions


Chapter 7

MIB-1 immunostaining is a beneficial adjunct test for the accurate diagnosis of vulvar condyloma acuminatum.

Pirog EC, Chen YT, Isacson C.

MIB-1 Immunostaining Is a Beneficial Adjunct Test for Accurate Diagnosis of Vulvar Condyloma Acuminatum

Edyta C. Pirog, M.D., Y.-T. Chen, M.D., Ph.D., and C. Isacson, M.D.

The histopathologic diagnosis of vulvar condyloma acuminatum is often based on architectural features that are not specific for human papillomavirus (HPV) infection. Because HPV-associated lesions show increased cellular proliferation, the authors evaluated the usefulness of MIB-1 immunostaining as an aid in the differential diagnosis of cases equivocal for condyloma. The MIB-1 immunostaining pattern was correlated with HPV DNA detection in condyloma acuminatum (CON-A; n = 15), “consistent with condyloma” (c/w CON-A; n = 26), fibroepithelial polyp (FEP; n = 14), and squamous papilloma (n = 10). HPV was detected in 100% of the CON-A cases, and all cases demonstrated MIB-1-positive nuclei in the upper two thirds of the epithelial thickness. With this definition of MIB-1 positivity, there was complete concordance between MIB-1 positivity and HPV detection for all cases (kappa = 0.88). Of the cases c/w CON-A, 17 of 26 (65%) were positive for both MIB-1 and HPV, and could be reclassified as CON-A, whereas 35% were identified as an overdiagnosis based on negative results. In addition, two cases of FEP were MIB-1 and HPV positive, and thus were identified as an underdiagnosis. These results suggest significant overdiagnosis of cases equivocal for condyloma, and indicate that MIB-1 immunostaining is a beneficial adjunctive test when the morphologic features are suggestive but not diagnostic for CON-A.

Key Words: Condyloma acuminatum—Vulva—MIB-1—HPV.


Histopathologic confirmation of vulvar condyloma acuminatum (CON-A) implies human papillomavirus (HPV) infection and confers an increased risk for synchronous or subsequent HPV-associated lesions elsewhere in the female genital tract.5,21 Although koilocytosis is considered to be a pathognomonic feature of HPV-associated lesions, this finding is often not present in vulvar condylomata. Instead, the diagnosis is based on nonspecific architectural features such as papillomatosis, acanthosis, parakeratosis, and dyskeratosis. Many pathologists choose to diagnose these histologically equivocal lesions as “consistent with condyloma” (c/w CON-A). Assessment of diagnostic accuracy could be performed with HPV detection by either in situ hybridization (ISH) or polymerase chain reaction (PCR); however, these techniques have limited clinical use because of their low sensitivity and high cost, respectively.

Because HPV infection is associated with increased proliferative activity of the squamous epithelium, we evaluated the usefulness of immunohistochemical staining with MIB-1, a marker of cell proliferation, as a diagnostic adjunctive test in the differential diagnosis of benign exophytic vulvar lesions. The study included a group of equivocal cases diagnosed as c/w CON-A as well as two reference groups expected to be either HPV positive (condylomas) or HPV negative (fibroepithelial polyps [FEPs] and squamous papillomas [SQPs]). MIB-1 immunohistochemical results were correlated with the detection of HPV DNA using ISH and PCR.

MATERIALS AND METHODS

Case Selection

Consecutive cases of exophytic vulvar lesions were retrieved from the files of the Department of Pathology, New York Presbyterian Hospital, for 1996 through 1997. A representative hematoxylin and eosin section from each case was chosen and reviewed by two independent pathologists (E.C.P. and C.I.) who were masked to the original diagnosis and without knowledge of the HPV detection and immunohistochemical results. In the event of a discordant diagnosis, the case was reviewed jointly.
and a consensus opinion was determined. Diagnostic groups were assigned according to the following histologic definitions, including CON-A (n = 15), c/w CON-A (n = 26), FEP (n = 14), and SQP (n = 10):

- Condyloma acuminatum (CON-A): Definitive koilocytosis (nuclear atypia encompassing enlargement, hyperchromasia, pleomorphism, and membrane irregularity plus cytoplasmic cavitation) and architectural features, including papillomatosis, acanthosis, hyperkeratosis, and/or parakeratosis
- Consistent with condyloma (c/w CON-A): Marked papillomatosis, acanthosis, hyperkeratosis, and/or parakeratosis, but without definitive koilocytosis
- Fibroepithelial polyp (FEP): The presence of a discernible fibrovascular core covered by squamous epithelium with mild papillomatosis, acanthosis, hyperkeratosis, and minimal parakeratosis
- Squamous papilloma (SQP): A slender fibrovascular core covered by nonkeratinized squamous epithelium with mild to moderate papillomatosis.

**MIB-1 Immunohistochemistry and Quantitation of the MIB-1 Index**

Immunohistochemical staining was performed on 4-µm sections of formalin-fixed, paraffin-embedded specimens. The sections were subjected to microwave antigen retrieval and were incubated in an automated stainer with MIB-1 antibody (Immunotech, Westbrook, ME, USA) at a dilution of 1:50, stained with diamino-benzidine chromogen, and counterstained with hematoxylin. Sections of human tonsil were used as a positive control, and a negative control without primary antibody was performed with each case. The parabasal cells of the epithelium served as an internal positive control.

Quantitation of MIB-1 immunostaining was performed by counting separately the positively and negatively stained nuclei in the lower third, middle third, and upper third of the full-thickness epithelium in the region of highest staining. At least two visual fields were counted per layer using a 40× objective with a 10× eyepiece. Approximately 400 nuclei were evaluated in each case. Only moderate (2+) to strong (3+) staining intensity was scored as positive. The MIB-1 index was expressed as the percentage of positively stained nuclei to all epithelial nuclei counted in each layer.

**HPV ISH**

ISH was performed with the PathoGene HPV In Situ Typing Assay (Enzo Diagnostics, New York, NY, USA) according to the manufacturer’s instructions, using 4-µm sections of formalin-fixed, paraffin-embedded specimens. Three type-specific HPV DNA probe groups were used: 6/11 (low risk), 31/33/51 (intermediate risk), and 16/18 (high risk). Hybridization was performed at 37°C for 16 hours. The staining was scored as positive when a reaction product was detected in the squamous epithelial nuclei. In cases that demonstrated staining with more than one probe group in the same region, the stronger staining group was scored as positive. A positive tissue control, and positive and negative probe controls were provided with the kit and were run with each reaction.

**HPV PCR Detection and Typing**

Genomic DNA was extracted from three 5-µm sections from each case, and adequate DNA quality was established by successful PCR amplification of a 250-base pair (bp) amplicon derived from human gene NY-ESO-1. A separate PCR was performed using HPV L1 consensus primers (MY09/MY11–biotinylated) according to the SHARP Signal System protocol (Digene, Silver Spring, MD, USA) with the following modifications: 4.0 mM magnesium chloride, 0.25 U/µl AmpliTaq Gold polymerase, 1 minute annealing at 55°C, and 2 minutes extension at 72°C. The PCR products were run on a 2% agarose gel, and the approximately 450-bp product was visualized with ethidium bromide staining. All PCR products were then evaluated with the enzyme-linked immunosorbent assay-based SHARP Signal System using HPV ribonucleic acid (RNA) probe A (low risk: 6/11/42/43/44) and HPV RNA probe B (intermediate/high risk: 16/18/31/33/35/39/45/51/52/56/58) according to the manufacturer’s specifications. In the event of detection of PCR product with both probes, the PCR products were diluted 1:100 and 1:1000, and were tested again. Cases still positive for both probe groups after the 1:1000 dilution were considered to be true dual positives as opposed to probe cross-reactivity. A second PCR assay and SHARP Signal System analysis confirmed all HPV-negative cases. Appropriate positive and negative PCR controls were used with all reactions including negative DNA extraction controls.

**Statistical Analysis**

The concordance between MIB-1 immunohistochemical expression and the detection of HPV by ISH and PCR was determined by kappa statistical analysis using the SPSS software package (version 6.1; Prentice Hall, Upper Saddle River, NY, USA). Kappa values of 1.0, 0.75, 0.40, and 0.20 were used as a threshold for complete, excellent, good, and poor concordance, respectively.

**RESULTS**

**HPV Detection and Typing**

HPV detection and typing results are shown in Table 1. HPV DNA was detected in 100% of the lesions with...
definite koilocytosis (CON-A). In addition, HPV DNA was detected in 65% of the c/w CON-A cases and in 17% of the combined SQP and FEP cases. Low-risk HPV types were identified in 32 of the 36 HPV-positive cases (89%), including three cases with mixed low- and intermediate/high-risk viral infection. Intermediate/high-risk viral types alone were identified in three cases, and in one case the HPV type could not be determined with the SHARP assay despite amplification of the correct size PCR amplicon.

There was excellent concordance between HPV detection and typing by ISH and PCR (kappa = 0.76). Twenty-five cases were positive by both ISH and PCR, and 29 cases were negative by both assays. Nine cases were negative by ISH but were positive by PCR for low-risk HPV types. Two cases were positive for intermediate- and high-risk HPVs by ISH but were negative by PCR, suggesting either loss of a portion of viral genome containing the PCR target sequence or loss of lesional tissue with further tissue sectioning.

MIB-1 Immunostaining

Representative immunohistochemical MIB-1 staining patterns are shown in Figure 1, and the results of quantitative MIB-1 evaluation are shown in Table 2. MIB-1 immunostaining in all CON-A cases demonstrated prominent, diffuse parabasal expression with extension of the positive cells into the middle and upper thirds of the epithelial thickness (Fig. 1B). The percentage of positively labeled cells (MIB-1 index) decreased from the lower third to the upper third of the full-thickness epithelium. In contrast, all SQP cases displayed a pattern of MIB-1 immunostaining consisting of a single or double row of positive cells in the parabasal location (Fig. 1D), similar to that reported for normal vulva. In none of these cases were positive cells present in the middle or upper thirds of the epithelial thickness. A similar pattern of staining was found in the majority of FEPs, with the exception of two cases in which positive nuclei were present in all epithelial layers. Both of these cases were HPV positive (as described later). Based on these findings, the evaluation of MIB-1 staining could be simplified to "positive" when a group of at least two epithelial cells with moderately to strongly stained nuclei were present in the upper two-thirds of the epithelium anywhere within the lesion. Using this definition, the staining pattern among c/w CON-A cases was MIB-1 positive in 65% (Fig. 1F) and was negative in 35% (Fig. 1H).

Correlation Between MIB-1 Immunostaining and HPV Detection

The correlation between MIB-1 immunostaining and HPV detection is shown in Table 3. There was complete concordance between MIB-1 positivity and detection of HPV DNA by both ISH (kappa = 0.76) and PCR (kappa = 0.88). The sensitivity/specificity of MIB-1 positivity for HPV DNA detection versus ISH was 1.00/0.79 and versus PCR was 0.94/0.94. Furthermore, MIB-1 positivity was more sensitive for HPV detection (0.94) than ISH versus PCR (0.71). The areas with positive MIB-1 staining overlapped with the same area found to be HPV positive by ISH; however, MIB-1 staining was more diffuse with extension throughout the full thickness of the epithelium, whereas ISH showed more prominent staining in the superficial epithelial layers.

All cases of CON-A were MIB-1 positive, and all of these cases were positive for HPV DNA by combined ISH and PCR results. Of the 26 cases diagnosed as c/w CON-A, 17 (65%) were MIB-1 positive, and the same cases were found to be positive for HPV DNA by PCR. Six of these 17 cases were negative for HPV by ISH, which is most likely attributable to the relatively lower sensitivity of this detection method. Two cases of FEP were found to be positive for both MIB-1 and HPV by PCR. These cases were considered retrospectively to be

TABLE 1. HPV DNA detection and typing by ISH and PCR

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>HPV-positive* (%)</th>
<th>ISH—virus types</th>
<th>PCR—virus risk groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-A (n = 15)</td>
<td>15 (100)</td>
<td>11/12/31</td>
<td>Low Int/high Mixed‡ Mixed†</td>
</tr>
<tr>
<td>c/w CON-A (n = 26)</td>
<td>17 (65)</td>
<td>10/0/0/0/0</td>
<td>1 11/12/31/32/33/35/39/45/51/52/56/58</td>
</tr>
<tr>
<td>FEP (n = 14)</td>
<td>3 (21)</td>
<td>1/0/0/0/0</td>
<td>2/0/0/0/0</td>
</tr>
<tr>
<td>SQP (n = 10)</td>
<td>1 (10)</td>
<td>0/0/0/0/0</td>
<td>1/0/0/0/0</td>
</tr>
</tbody>
</table>

HPV, human papillomavirus; ISH, in-situ hybridization; PCR, polymerase chain reaction; CON-A, condyloma acuminatum; c/w CON-A, consistent with condyloma acuminatum; FEP, fibroepithelial polyp; SQP, squamous papilloma.

* Combined ISH and PCR results.
† Mixed infection of HPV 6/11 and 16/18.
‡ Mixed infection of low- and intermediate/high-risk HPVs.
§ Unknown HPV type.
underdiagnosed. Lastly, one case of FEP and one case of SQP were positive for HPV DNA by PCR, but were negative for both MIB-1 and HPV by ISH. In these cases, detection of HPV DNA was interpreted as the presence of a latent, nonproductive HPV infection, often not associated with increased proliferative activity of the host cells.

Overall, if MIB-1 positivity was used for verification of the diagnosis of CON-A, 17 of 26 of the c/w CON-A cases (65%) could be reclassified definitively as CON-A, and 35% could be identified as an overdiagnosis based on the negative results. Conversely, this method also demonstrated an underdiagnosis of condyloma in two of 14 cases originally classified as FEPs.
DISCUSSION

Vulvar condylomata are markers for sexually transmitted disease. Although not considered to be premalignant lesions, they are important because their diagnosis reflects an HPV-related process with both clinical and social implications. Many vulvar condylomata do not display unequivocal evidence of HPV cytopathic effect (koilocytosis), and their diagnosis is based on non-specific architectural features. The reported prevalence of HPV DNA in these questionable lesions varies from 19% to 68%, indicating that a substantial proportion may be overdiagnosed. In a detailed review of cases "equivocal for condyloma," Nuovo et al. were unable to identify histologic features that could help to distinguish HPV-positive and HPV-negative lesions. Because recent studies have demonstrated that immunostaining with MIB-1, a marker of cell proliferation, can be useful in identifying HPV-related intraepithelial lesions of the cervix and vulva, we hypothesized that MIB-1 immunostaining could be a useful adjunctive test in the differential diagnosis of wart-like lesions of the vulva. In the current study we evaluated a series of 65 exophytic vulvar lesions with MIB-1 immunohistochemical staining and correlated the results with detection of HPV DNA by both ISH and PCR.

HPV DNA was detected in all 15 cases with definite koilocytosis (CON-A), as well as in 17 of 26 cases (65%) of c/w CON-A. In addition, HPV DNA was identified in three of 14 cases (21%) of FEP and in one of 10 cases (10%) of SQP. The majority of the HPV-positive cases (89%) contained low-risk HPV types 6 and 11. Intermediate- and/or high-risk HPVs accounted for the remaining cases. Our results are consistent with previous reports of HPV DNA detection in questionable lesions. With sensitive PCR tests, HPV DNA is identified in almost 100% of vulvar condylomata, and low-risk HPV types are identified in 82% to 92% of cases.

All CON-A cases demonstrated MIB-1-positive cells in the upper two-thirds of the epithelial thickness and in the same region as HPV DNA detected by ISH. In contrast, MIB-1-positive cells were not seen above the parabasal cell layer in any of the 10 cases of SQP or in 12 of 14 cases of FEP. Analogous patterns of MIB-1 distribution have been observed in HPV-positive and HPV-negative cervical mucosa. In normal cervix, MIB-1-positive cells are confined to the parabasal cell layer. The percentage of the MIB-1-positive cells is low and ranges between 7% and 14%. In cervical squamous intraepithelial lesions, MIB-1 expression is increased in the parabasal areas (MIB-1 index >15%) with diffuse extension of the positive cells into the intermediate and superficial epithelial layers with increasing lesion grade. In the few studies that have examined the proliferative activity of vulvar squamous epithelium, the pattern of MIB-1 immunostaining mirrors the findings seen in cervical squamous mucosa. MIB-1 expression in normal vulvar skin/mucosa is limited to the basal cell layer. In three reported cases of condyloma there was a partial diffuse pattern of expression with many basal/parabasal cells and occasional cells in the mid and upper epithelium showing MIB-1 reactivity.

Our results confirm and extend these previous reports. Lesions other than CON-A may be associated with increased MIB-1 immunostaining. Both high-grade vulvar intraepithelial neoplasia (VIN) and squamous carcinoma have been reported to have positive nuclei extending from the basal to the upper layers in an intense, diffuse pattern. Psoriasis and lichen sclerosus, and most cases of squamous hyperplasia stain similar to normal squamous epithelium; however, only a small number of cases of squamous hyperplasia have been studied, and in one report there was a mild increase in proliferative activity adjacent to squamous carcinoma. In the current study we evaluated the correlation between the MIB-1 immunostaining patterns and HPV positivity in CON-A and in wart-like vulvar lesions that may resemble CON-A clinically and microscopically; namely, SQPs and FEPs. We did not examine lesions with cytologic atypia suggesting a preneoplastic change.

### TABLE 2. Quantitative MIB-1 indices in the diagnostic groups of exophytic vulvar lesions

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Full-thickness squamous epithelium</th>
<th>Lower third</th>
<th>Middle third</th>
<th>Upper third</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-A (n = 15)</td>
<td>39 (±10)</td>
<td>37 (±16)</td>
<td>22 (±20)</td>
<td></td>
</tr>
<tr>
<td>c/w CON-A (n = 26)</td>
<td>23 (±12)</td>
<td>8 (±12)</td>
<td>3 (±7)</td>
<td></td>
</tr>
<tr>
<td>FEP (n = 14)</td>
<td>18 (±10)</td>
<td>3 (±9)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>SQP (n = 10)</td>
<td>12 (±7)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

MIB-1 index, the percentage of positively stained nuclei to all epithelial nuclei counted in each layer; SD, standard deviation; CON-A, condyloma acuminatum; c/w CON-A, consistent with condyloma acuminatum; FEP, fibroepithelial polyp; SQP, squamous papilloma.

### TABLE 3. Correlation between positive MIB-1 staining and HPV DNA detection

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>MIB-1 positive</th>
<th>HPV positive by ISH</th>
<th>HPV positive by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON-A (n = 15)</td>
<td>15</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>c/w CON-A (n = 26)</td>
<td>17</td>
<td>65</td>
<td>11</td>
</tr>
<tr>
<td>FEP (n = 14)</td>
<td>2</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>SQP (n = 10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MIB-1 positive, a group of at least two stained nuclei in the upper two thirds of the epithelial thickness; HPV, human papillomavirus; ISH, in-situ hybridization; PCR, polymerase chain reaction; CON-A, condyloma acuminatum; c/w CON-A, consistent with condyloma acuminatum; FEP, fibroepithelial polyp; SQP, squamous papilloma.
(VIN); flat, inflammatory lesions/ulcers; or other exo-
physic lesions such as condylomata lata because their
microscopic appearance is usually readily distinguish-
able from CON-A.

Overall, there was complete concordance between
MIB-1 positivity and detection of HPV by both ISH and
PCR. Furthermore, MIB-1 positivity was more sensitive
for HPV detection than ISH versus PCR. This finding
may be explained by the fact that detection of HPV by
ISH requires a high viral DNA copy number, whereas
even a low viral load may stimulate cell proliferation.
Two of 65 cases (3%), one case each of FEP and SQP,
were MIB-1 negative, ISH negative, and positive for
HPV by PCR. We interpret these cases as subclinical
HPV infection. Subclinical HPV infection has been de-
tected in 3% to 9% of benign vulvar mucosa/skin

Our study demonstrates that limiting the diagnosis of
condyloma to only those lesions with koilocytotic atypia
will result in underdiagnosis, and categorizing equivocal
wart-like lesions of the vulva as c/w CON-A is associ-
ated with substantial overdiagnosis. Even stringent mor-
phologic criteria fail to predict reliably the presence
of HPV in both vulvar and cervical lesions.\textsuperscript{1,15} Although verification of the accuracy of the diagnosis could be
performed with demonstration of HPV DNA by either
ISH or PCR, these techniques require technical expertise
and may not be readily available. MIB-1 immunohisto-
chemistry, on the other hand, is an easy, reproducible,
and relatively inexpensive test. MIB-1 positivity is asso-
ciated with the detection of HPV in exophytic vulvar
lesions with high sensitivity and specificity. Our results
suggest that positive MIB-1 immunohistochemistry can
serve as an objective method for expanding the criteria
for condylomata and may be an important element of
quality assurance in rendering diagnoses. Vulvar con-
dylomata are sexually transmitted, and the patient to whom
this diagnosis is rendered may face marked clinical and
social consequences.\textsuperscript{8,16,18,19,21} Among the cases of c/w
CON-A, 17 of 26 (65%) were positive for both MIB-1
and HPV, and could be reclassified as CON-A, whereas
35% were identified as an overdiagnosis based on the
negative results. In addition, two cases of FEP were
MIB-1 and HPV positive, and thus were identified as an
underdiagnosis. These results suggest substantial over-
diagnosis of cases equivocal for condyloma.

The etiology of the HPV-negative and MIB-1-
negative cases diagnosed as c/w CON-A is unclear. It has
been suggested that these cases might represent kerati-
nized papillomas, epidermal nevi, or exaggerated vulvar
rugal folds, and perhaps would be best termed “hyper-
keratotic papillomas” in the absence of increased prolif-
erative activity.\textsuperscript{11,15} Because the etiology of these lesions
is not determined, the patients should be followed; how-
ever, without the stigmatising diagnosis of CON-A.

In conclusion, positive MIB-1 staining, as defined by
a group of at least two cells with stained nuclei in the
upper two thirds of the epithelial thickness, is associated
with the detection of HPV. These results suggest that
MIB-1 immunostaining is a beneficial addition to diag-
nosing CON-A when the morphologic features are sug-
gestive but not diagnostic for the lesion. Furthermore,
MIB-1 immunohistochemistry may be useful as an inde-
pendent reference standard for improving the diagnosis
of equivocal wart-like vulvar lesions.

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Chapter 8

Proliferative activity of benign and neoplastic endocervical epithelium — correlation with HPV DNA detection.

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Proliferative Activity of Benign and Neoplastic Endocervical Epithelium and Correlation with HPV DNA Detection

Edyta C. Pirog, M.D., Christina Isacson, M.D., Mathias J. Szabolcs, M.D., Bernhard Kleter, Ph.D., Wim Quint, Ph.D., and Ralph M. Richart, M.D.

Summary: Recent studies have indicated that the use of the MIB-1 immunostaining may be useful in distinguishing endocervical neoplasia from benign nonneoplastic lesions. We sought to investigate this finding further with a specific emphasis on the common benign processes that may result in a nonspecific increase of MIB-1 staining.

In this study we quantified the MIB-1 immunostaining in the mucinous endocervical epithelium (n=45) and in tubal metaplasia (n=28) during the proliferative and secretory phases (hormonal influence), in the mucinous endocervical epithelium in cases of cervicitis (inflammation) (n=10), in cases with a history of a recent biopsy (regeneration) (n=8), in the endocervical glands adjacent to a squamous intraepithelial lesion (human papilloma virus [HPV] infection) (n=63), and in in situ and invasive cervical adenocarcinomas (n=30). All cases with increased MIB-1 staining were subsequently tested for the presence of HPV DNA. The range of MIB-1 staining in the benign endocervical epithelium was from 0% to 48% and in the neoplastic epithelium from 25% to 84%. MIB-1 staining below 10% always reflected a benign process and MIB-1 staining higher than 50% was always associated with a neoplasia. Rare benign cases (tubal metaplasia during the proliferative phase, glands adjacent to squamous intraepithelial lesions, and cases with a history of a recent biopsy) had increased MIB-1 index, which overlapped with the neoplastic cases. In conclusion, MIB-1 is a useful marker of endocervical neoplasia, although in rare cases an overlap between benign and neoplastic cases may exist. Key words: MIB-1—Ki-67—Endocervix—Tubal metaplasia—Cervical adenocarcinoma—Adenocarcinoma in situ.

Differentiation of adenocarcinoma in situ (AIS) and invasive adenocarcinoma of the cervix (EAD) from benign endocervical lesions may be difficult due to subtle morphological features in some endocervical neoplasms. These diagnostic difficulties led us to search for a specific and reproducible marker for endocervical neoplasia. Recent studies have indicated that the antibody MIB-1 directed against the antigen Ki-67, a marker of mitotically active cells, may be useful in distinguishing endocervical malignancies from benign mimics, such as tubal metaplasia (1), florid microglandular hyperplasia (2,3), and atypical glandular hyperplasia (2).

In this study we quantified MIB-1 immunostaining in the endocervical epithelium during the proliferative and secretory phases of the menstrual cycle (hormonal effect), in cases of cervicitis (inflammation), in cases with a history of a recent biopsy (regeneration), endocervical polyps (benign growth), and in the endocervical glands adjacent to a squamous intraepithelial lesion (human papilloma virus [HPV] infection). We compared the proliferative activity in these benign conditions with that of neoplastic endocervical epithelium (AIS and EAD). Because more than 90% of cervical adenocarcinomas are positive for HPV DNA (4), all cases with increased...
MIB-1 staining were subsequently tested for the presence of HPV to determine whether the increased proliferative activity correlated with HPV infection.

METHODS

Case Selection
Formalin-fixed, paraffin-embedded tissue specimens were obtained from the archives of the Department of Pathology, College of Physicians and Surgeons of Columbia University. The following tissue sections were collected: normal endocervix in the secretory phase (n=22), normal endocervix in the proliferative phase (n=23), tubal metaplasia of the endocervix in the secretory phase (n=13), and tubal metaplasia of the endocervix in the proliferative phase (n=15). These sections were obtained from hysterectomy specimens with a diagnosis of leiomyoma. Only the cases with tubal metaplasia in the area of the squamocolumnar junction (versus the upper endocervix) were selected. The timing of the phase of the menstrual cycle was determined using endometrial dating. Furthermore, sections of normal endocervix with inflammation (n=10), normal endocervix with a history of a recent biopsy (within the preceding 12 weeks) (n=15), benign endocervical polyps (n=8), normal endocervix adjacent to a low-grade squamous intra-epithelial lesion (SIL) (n=35), and normal endocervix adjacent to a high-grade SIL (n=28) were obtained from cervical cone or punch biopsy specimens. Sections of 9 cases of adenocarcinoma in situ and 21 cases of cervical adenocarcinoma, including 18 endocervical and 3 endometrioid adenocarcinomas, were obtained from cervical cone biopsies or hysterectomy specimens.

MIB-1 Immunohistochemistry and Quantitative Image Analysis
MIB-1 immunohistochemical staining was performed on 4-μm tissue sections. Briefly, after deparaffinization, the endogenous peroxidase was blocked with hydrogen peroxide in methanol and the sections were microwaved for antigen retrieval. The slides were incubated in an automated stainer with MIB-1 antibody (Immunotech, Westbrook, ME) at a dilution of 1:300, stained with diaminobenzidine chromogen, and counterstained with hematoxylin. The parabasal cells of the squamous epithelium served as an internal positive control. A negative control without primary antibody was included in each run.

Quantitation of the percentage of positive nuclei was determined using the CAS image analysis system. At least 400 glandular nuclei were evaluated in each specimen. A MIB-1 index, computed by the software, was expressed as the percentage of MIB-1 positive nuclei to all epithelial nuclei counted.

HPV DNA Detection
A test for HPV DNA was performed on 10 randomly selected cases of normal endocervix (proliferative phase) and all benign and neoplastic cases with a MIB-1 index >10%. In all these cases, the glandular epithelium was carefully microdissected from the adjacent squamous epithelium and the connective tissue. The microdissection was performed with a sterile needle under the visual control of an inverted microscope. In cases in which the SIL was present, the entire area of the squamous epithelium was covered with nail polish to prevent accidental scraping of the squamous cells during the dissection of the glandular epithelium. To ensure that no contamination from the squamous epithelium occurred, stroma adjacent to the dissected glands was separately microdissected and the sample was used as a negative dissection control. Between one and three 4-μm sections were used for each case. DNA was extracted using standard methods.

Broad-spectrum HPV DNA amplification was performed using a novel short polymerase chain reaction (PCR) fragment (SPF10) primer set as described previously (5). The SPF primers allow for amplification of a 65-base pair fragment located within the L1 region of the HPV genome. The PCR products were run on a 3% agarose gel and the 65-base pair product was visualized with ethidium bromide staining. All HPV-negative cases were confirmed by a second PCR assay. To determine DNA quality, PCR amplification of β-globin gene was carried out in a separate reaction. Appropriate positive and negative PCR controls were used with all reactions, including negative DNA extraction controls. Simultaneous identification of 25 different HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66 and low-risk: 6, 11, 34, 40, 42–44, 53, 54, 59, 68, 70, and 74) was performed by reverse hybridization using a line probe assay (LiPA) as described previously (6).

RESULTS
The mean MIB-1 index (the percentage of positively staining nuclei) in benign and neoplastic endocervical epithelium is shown in Table 1. The distribution of the MIB-1 index values and the correlation with HPV DNA detection is presented in Figure 1.

MIB-1 in Benign Endocervical Epithelium
The MIB-1 index in the normal mucinous endocervical epithelium was very low (<5%), and there was no
significant difference between the secretory (EC-SE) and the proliferative phases (EC-PR). Tubal metaplasia (TM) of the endocervix in the secretory phase (TM-SE) and a majority of the cases of tubal metaplasia in the proliferative phase (TM-PR) had an index similar to that of mucinous epithelium. However, in five cases of TM-PR the index was >10% and in one case it overlapped with the value range of the neoplastic epithelium (Fig. 1). The MIB-1 index in endocervical polyps and in cases of cervicitis was low (<5%). Among cases with a recent biopsy (within the preceding 12 weeks), four cases had an MIB-1 index >10% and two of these cases overlapped with the malignant cases. Five cases of benign endocervical glands adjacent to SILs had diffusely increased staining with the index >10%, and one case fell into the value range of neoplastic cases.

### MIB-1 in Neoplastic Endocervical Epithelium

The mean MIB-1 index in the neoplastic epithelium (AIS and EAD) was more than 50-fold higher than that of the benign endocervical epithelium (Fig. 1 and 2). The MIB-1 index in AIS was not significantly different from EAD (p>0.05). The MIB-1 index of three endometrioid EADs (57%, 61%, and 84%) fell in the broad range of values of endocervical EADs (25% to 84%).

### HPV DNA Detection in Benign and Neoplastic Endocervical Epithelium

Ten randomly selected cases of normal endocervix (EC-PR) were negative for HPV DNA. Among the cases with an increased MIB-1 index (>10%), no HPV DNA was detected in tubal metaplasia (n=5) or cases with a previous biopsy (n=4). HPV DNA was detected in four of five cases of endocervical epithelium with increased MIB-1 adjacent to SIL: in one case adjacent to LSIL (HPV 16) and in three cases adjacent to HSIL (HPV 16, HPV 31, and HPV 58). There was no cytological atypia of the glandular epithelium in any of these cases.

HPV DNA was also detected in all cases of AIS and in all but one EAD case. Interestingly, the HPV-negative

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**TABLE 1.** The MIB-1 index in benign and neoplastic endocervical epithelium

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>MIB-1 Index</th>
<th>MIB-1 Index</th>
<th>MIB-1 Index Range</th>
<th>p**</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC secretory phase</td>
<td>22</td>
<td>0.6</td>
<td>0.0–1.8</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>EC proliferative phase</td>
<td>23</td>
<td>0.8</td>
<td>0.0–4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM secretory phase</td>
<td>13</td>
<td>0.9</td>
<td>0.0–6.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TM proliferative phase</td>
<td>15</td>
<td>5.6</td>
<td>0.0–31.7</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>EC inflammation</td>
<td>10</td>
<td>1.3</td>
<td>0.3–4.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>EC polyp</td>
<td>8</td>
<td>1.0</td>
<td>0.0–1.9</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>EC history of recent biopsy</td>
<td>15</td>
<td>7.6</td>
<td>0.0–32.1</td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>EC next to a SIL</td>
<td>63</td>
<td>2.3</td>
<td>0.0–48.4</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma in situ</td>
<td>9</td>
<td>57.5</td>
<td>45.6–72.8</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>21</td>
<td>52.1</td>
<td>25.0–84.0</td>
<td>&lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

EC, normal mucinous endocervical epithelium; TM, tubal metaplasia of the endocervix; SIL, squamous intraepithelial lesion.

*MIB-1 index = percentage of MIB-1 positive nuclei to all epithelial nuclei.

** ANOVA test for the difference between the means versus EC-proliferative phase.

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**FIG. 1.** Distribution of MIB-1 index values and results of HPV DNA detection. All cases with MIB-1 >10% were tested for HPV DNA presence; cases in rectangles were found to be HPV-positive. EC, normal mucinous endocervical epithelium; TM, tubal metaplasia of the endocervix; SE, secretory phase; PR, proliferative phase.

**FIG. 2.** MIB-1 immunostaining in AIS and adjacent benign endocervical glands (hematoxylin counterstain).
case also had the lowest MIB-1 index value (25%) (Fig. 1).

**DISCUSSION**

Recent studies have indicated that MIB-1 immunostaining may be useful in distinguishing endocervical neoplasia from benign endocervical lesions (2–4,7,8). Table 2 summarizes previous reports of the quantitative analysis of MIB-1/Ki-67 positivity in the endocervix. In addition to these references, Cina et al. (2) described Ki-67 staining as negligible to low (<10%) in normal endocervix (n = 4), florid microglandular hyperplasia (n = 2), and atypical glandular hyperplasia (n = 2) and as moderate to high (>11%) in AIS (n = 4) and EAD (n = 7).

We have sought to investigate these findings further with a specific emphasis on the common benign processes in the endocervix that may result in a nonspecific increase in MIB-1 staining. We have observed that rare cases of tubal metaplasia of the endocervix, cases of benign-appearing endocervical glands adjacent to an SIL, and cases with a history of recent biopsy may show increased MIB-1 staining that overlaps with the lower end of the values observed in the malignant endocervical epithelium. Nonetheless, a MIB-1 index <10% always reflected a benign process and the values >50% were always associated with a malignancy.

Tubal metaplasia is a very common finding in the endocervix and has been reported to be present in over 30% of hysterectomy specimens (9). TM typically consists of three cell types: ciliated, nonciliated, and intercalated cells, but with the predominance of the intercalated cells the process is sometimes referred to as ciliated cell metaplasia, and with the predominance of the nonciliated cells it may be termed tuboendometrioid or endometrioid metaplasia. The histologic diagnosis of TM is usually straightforward; however, in some cases of TM the epithelial nuclei are stratified, enlarged, and have a coarse chromatin pattern. In such cases, especially when a single cell type predominates, the differential diagnosis between TM and AIS may be very difficult. In addition, a nonspecific myxoid stromal alteration surrounding the metaplastic glands may occasionally be present (10), raising the possibility of an invasive carcinoma. As demonstrated by the current and the previous studies, MIB-1 immunostaining may be a helpful adjunctive test in such cases, but only when the staining is either low (<10%) or very high (>50%). TM has been shown to stain positively for vimentin, much the same as endometrial epithelium, and vimentin immunostaining has been suggested as a specific marker distinguishing TM from AIS (11). However, since cases of endometrioid or tubal-type AIS arising in TM have been described (12), the value of this stain may be limited. An additional test that may potentially be useful in differentiating equivocal cases of TM from AIS is HPV testing using a sensitive assay. As indicated by the results of the current and previous studies, TM is not associated with HPV infection, whereas more than 90% of cervical adenocarcinomas, both endocervical and endometrioid subtypes, are positive for HPV DNA (4).

The MIB-1 immunostaining has to be interpreted with caution in cases with a history of recent biopsy, because the regenerative activity of the endocervical epithelium may be brisk, resulting in a high MIB-1 index. In addition, benign-appearing endocervical glands adjacent to SILs may occasionally have increased mitotic activity. These cases may represent an occult HPV infection of the endocervical epithelium, because 80% of such cases in our series were positive for HPV DNA. However, the true significance of this finding remains uncertain. Although the glandular epithelium in these cases was carefully microdissected and separately microdissected stroma served as a negative control, the possibility of HPV contamination from the adjacent squamous epithelium cannot entirely be ruled out. Two more definitive approaches were attempted to verify the results: laser capture microdissection and *in situ* hybridization. Unfortunately, laser microdissection did not yield amplifiable DNA and HPV *in situ* hybridization assays, which were tested in our laboratory and which could help in localization of the virus, did not reach the appropriate sensitivity (results not shown). Until a more refined technique becomes available, we can only speculate that the finding represents a subclinical HPV infection of the glandular epithelium. Because glandular epithelium does not support productive HPV infection, no viral cytopathic effect equivalent to koilocytosis is observed in the endocervix.

### Table 2. Reported percentage of MIB-1–positive cells in benign and neoplastic endocervical epithelium

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Mean Staining (%)</th>
<th>Staining Range (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal endocervix</td>
<td>52</td>
<td>NA</td>
<td>0–8</td>
<td>1</td>
</tr>
<tr>
<td>Normal endocervix</td>
<td>8</td>
<td>2.4</td>
<td>0.8–4.3</td>
<td>3</td>
</tr>
<tr>
<td>Normal endocervix</td>
<td>10</td>
<td>0.34</td>
<td>NA</td>
<td>13</td>
</tr>
<tr>
<td>Cervicitis</td>
<td>8</td>
<td>5.7</td>
<td>1–13</td>
<td>3</td>
</tr>
<tr>
<td>Microglandular hyperplasia</td>
<td>8</td>
<td>8.5</td>
<td>2.9–18.4</td>
<td>3</td>
</tr>
<tr>
<td>Tubal metaplasia</td>
<td>27</td>
<td>NA</td>
<td>0–21</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma <em>in situ</em></td>
<td>10</td>
<td>23.5</td>
<td>NA</td>
<td>13</td>
</tr>
<tr>
<td>Adenocarcinoma <em>in situ</em></td>
<td>8</td>
<td>NA</td>
<td>15–88</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>13</td>
<td>NA</td>
<td>24–96</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>10</td>
<td>80</td>
<td>57–96</td>
<td>3</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>20</td>
<td>49.7</td>
<td>20–75</td>
<td>14</td>
</tr>
</tbody>
</table>

NA, not available.
Currently, AIS and invasive adenocarcinoma are the only recognized morphologic manifestation of HPV infection of the endocervix. The existence of a precursor lesion to AIS (“glandular dysplasia”) is uncertain, and no objective morphologic or molecular criteria correlating with the clinical outcome have been established.

In conclusion, MIB-1 may be a helpful marker in distinguishing endocervical malignancies from benign mimics, although in rare cases an overlap between benign and neoplastic cases may exist. Nevertheless, a MIB-1 index below 10% reflects a benign process and a MIB-1 index higher than 50% is indicative of a neoplasm.

REFERENCES


Chapter 9

P16 and Ki-67 immunostaining in atypical immature squamous metaplasia of the uterine cervix: correlation with Human Papillomavirus detection.

*Iaconis L, Hyjek E, Ellenson LH, Pirog EC.*


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p16 and Ki-67 Immunostaining in Atypical Immature Squamous Metaplasia of the Uterine Cervix

Correlation With Human Papillomavirus Detection

Lori Iaconis, MD; Elizabeth Hyjek, MD; Lora H. Ellenson, MD; Edyta C. Pirog, MD

Objective.—To examine whether a combination of immunostaining for p16 and Ki-67 could be used to stratify AIM cases into 3 categories: benign, cases with nondiagnostic atypia, and high-grade squamous intraepithelial lesion (HSIL).

Design.—The study consisted of 37 cases of AIM, 23 cases of benign cervical mucosa (NEG), and 36 cases of HSIL. All cases were tested for high-risk human papillomaviruses using SPF 10 polymerase chain reaction and immunostaining for p16 and Ki-67.

Results.—All cases of HSIL were positive for both p16 and Ki-67. Seven cases of AIM (19%) displayed a pattern of immunostaining identical to HSIL, and these most likely represent a spectrum of HSIL. A total of 54% of cases of AIM were negative for both p16 and Ki-67, consistent with benign reactive atypia. Two AIM cases (5%) were negative for p16 and positive for Ki-67 in the area adjacent to an ulcer, representing regeneration. Finally, 22% of AIM cases were positive for p16 and negative for Ki-67; such cases may represent a precursor of HSIL, or, alternatively, a regressing HSIL.

Conclusion.—The combination of immunostaining for p16 and Ki-67 is helpful in limiting the number of cases with nondiagnostic atypia of the cervix.

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A typical immature squamous metaplasia (AIM) of the cervix is a loosely defined entity characterized by immature metaplastic cells with mild cytotologic atypia. Since recognition of mild atypia is poorly reproducible, cases carrying the diagnosis of AIM have been shown to include a spectrum ranging from bona fide high-grade squamous intraepithelial lesion (HSIL) to benign reactive changes or atrophy.1–3

Atypical immature squamous metaplasia was first described by Crum at al1 in 1983 and then further characterized in 1999 as a lesion “which exceeded the limits of the typical metaplasia. Features typical of this group of epithelial alterations include immature squamous cells, which contain greater degrees of nuclear atypia or nuclear crowding, increased mitotic activity, and absence of normal differentiation, yet maintain the growth pattern of immature metaplastic epithelium.”2(p1161) Such a defined category included a spectrum of atypical immature squamous proliferations, and the authors used 3 morphologic features—namely, nuclear crowding, nuclear atypia, and cytoplasmic maturation—to subclassify cases of AIM into 3 groups: (1) “favor reactive,” (2) not otherwise specified (NOS), and (3) “favor HSIL.” There was a good interobserver reproducibility for subclassification of cases as favor reactive (κ = 0.8); however, the agreement was poor (0.23) and fair (0.41) for the NOS and favor HSIL cases, respectively. The prevalence of human papillomavirus (HPV) DNA in categories with consensus diagnoses of favor reactive, NOS, and favor HSIL was 77%, 28.6%, and 37.5%, respectively. The study highlighted 2 issues: low reproducibility of the histologic diagnosis of AIM and uncertainty of its clinical significance, since as many as two thirds of the cases subclassified as favor HSIL were found to be HPV negative.

In a study by Geng et al,3 high-risk HPVs (hrHPVs) were detected in 67% of cases diagnosed as AIM. Of HPV-positive cases, 80% of patients had a concurrent or subsequent diagnosis of HSIL. In contrast, only 16% of patients with HPV-negative AIM had a follow-up or concurrent diagnosis of HSIL. Results of that study confirmed that AIM represents a spectrum of lesions, from HPV-negative, benign, immature squamous metaplasia to hrHPV-positive cases representing either HSIL or an HSIL precursor. The authors recommended HPV testing as a diagnostic tool to separate benign cases from potentially preneoplastic lesions.4 Currently, however, only polymerase chain reaction (PCR) can be used to reliably detect hrHPVs in formalin-fixed tissue sections, and this technique is not routinely available in pathology laboratories. In addition, it requires a special certification for use in
clinical testing. While HPV in situ hybridization and anti-HPV capsid immunostaining are both insensitive, immunostaining for Ki-67 and p16 are novel tests that are currently being evaluated as surrogate markers of HPV infection.

Ki-67, a marker of proliferation, has been shown to be a sensitive and specific marker of HPV infection in mature squamous epithelia and is useful for confirmation of the diagnosis in equivocal low-grade squamous intraepithelial lesions (LSILs) of the cervix and vulva.4–6 Ki-67, however, may be positive in HPV-negative squamous metaplasia or regenerating epithelium, and therefore positivity of this marker in immature squamous epithelium is not specific for HPV infection. Not surprisingly, staining for Ki-67 in AIM showed variable results, with a wide range of positivity and significant overlap between HPV-positive and HPV-negative cases.3

p16, a cyclin kinase inhibitor, is a cell cycle regulatory protein that inhibits the cell cycle by preventing the phosphorylation of retinoblastoma (Rb) tumor suppressor protein. It has been shown that in cells infected with hrHPV, there is a functional overexpression of p16 mediated by E2F transcription factors.7 Despite high levels of p16, however, the hrHPV-infected cells continue to proliferate, because Rb, the target of p16 inhibitory activity, is inactivated by the E7 HPV oncoprotein.2,8

p16 has been shown to be a sensitive marker of cells with active expression of E7 oncoprotein. A strong and diffuse p16 immunostaining was detected in 97% to 100% of cervical squamous cell carcinomas and adenocarcinomas9–12 and 92% to 100% of cases of HSIL.10–12 However, p16 is not a sensitive marker of LSILs; on average, the stain is positive in less than 50% of cases with this diagnosis (range, 37%–72%).10–13

Although p16 is a sensitive marker of hrHPV infection in the cervical mucosa, it is not entirely specific for HPV infection or dysplastic/neoplastic process. For example, it has been shown that benign tubal metaplasia and benign ciliated cells of the endocervix both stain strongly with p16.11 In addition, benign endometrium and endometrioid adenocarcinomas not related to HPV infection show p16 positivity.15,16 Nonetheless, positive p16 staining appears to be a surrogate marker of hrHPV infection in AIM.

We undertook this study to determine the sensitivity and the specificity of p16 immunostaining for detection of hrHPV infection in AIM. In addition, we wanted to examine whether a combination of immunostaining for Ki-67 and p16 may be helpful in subclassifying cases of AIM into benign and preneoplastic categories.

MATERIALS AND METHODS

Case Selection

The surgical pathology records of the Department of Pathology, Weill Medical College of Cornell University, were searched from 2002 to 2005 to identify successive cervical biopsies with the diagnosis of "cervicitis," "atypical immature squamous metaplasia," or "high-grade squamous intraepithelial lesion." The study group consisted of 37 cases of AIM, the negative (NEG) control group consisted of 23 cases of benign cervical mucosa with inflammation and reactive changes, and the positive control group consisted of 36 cases of HSIL. In order to verify the histologic diagnoses, all cases were re-reviewed by 2 pathologists (L.I., and E.C.P) to obtain a consensus diagnosis. The diagnostic criteria for AIM were described by Crum et al11 and Park et al11 and quoted here in the Introduction. The standard diagnostic criteria for HSIL were used as described by Wright et al17 in the Blaustein textbook of gynecologic pathology. AIM was diagnosed in 24 cervical punch biopsies and 13 cone biopsies. The average age of the patients was 33 years (range: 16–68 years). Previous, concurrent, and 2-year follow-up diagnoses of patients with AIM were obtained from the departmental records.

All cases were tested for the presence of hrHPVs and were stained for p16 and Ki-67 as described below. Cases in which the lesion disappeared on deeper sections from the paraffin block were not included in the study.

HPV DNA Amplification and Genotyping

Genomic DNA was prepared from two to three 4-μm sections from each case using standard methods. Briefly, the slides were deparaffinized, and tissue was scraped with a sterile blade. The samples were incubated with 250 μl proteinase K (1 mg/ml) in 50mM Tris-HCl (pH 8.0), 1mM EDTA, and 0.5% Tween 20 for 18 hours at 56°C. Following denaturation at 95°C for 30 minutes, 10 μl of the supernatant was used for PCR. Adequate DNA quality was established by PCR amplification of β-globin gene, resulting in a 96-base pair product.20 Broad-spectrum HPV DNA amplification was performed using the Short PCR Fragment (SPF) 10 primer set, as described previously.14 SPF 10 PCR amplifies a 65-base pair fragment from the L1 region of the HPV genome. The reaction was performed in a total volume of 50 μl containing 10 μl of isolated DNA, 10mM Tris-HCl (pH 9.0), 50mM KCl, 2.0mM MgCl2, 0.1% Triton X-100, 0.01% gelatin, 200μM of each deoxynucleoside triphosphate, 15 pmol of each of the forward and reverse primers, and 1.5 units of AmpliTaq Gold (Perkin Elmer, Boston, Mass). AmpliTaq Gold was activated by incubation at 94°C for 9 minutes. HPV DNA was amplified in 40 cycles of 30 seconds at 94°C, 45 seconds at 52°C, and 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. Each experiment was performed with separate positive (plasmid HPV DNA) and negative (H2O) controls. PCR products were analyzed using 3% agarose gel electrophoresis. Samples identified as positive for HPV DNA were genotyped with the HPV-Line Probe Assay (LIPA; Inogenetics, Ghent, Belgium).21 Twenty-five individual HPV genotypes (high-risk HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 70; low-risk HPV: 6, 11, 34, 40, 42–44, 53, 54, 74) can be identified simultaneously in a single assay. The exact assay conditions were described previously.21 Briefly, 10 μl of denatured HPV PCR product was hybridized (60 min at 50°C) to genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. Following the washing step, the products of hybridization were visualized in a color reaction using alkaline phosphatase-streptavidin conjugate, 5-bromo-4-chloro-3-indoylphosphate, and nitroblue tetrazolium, which results in a purple precipitate. The results were assessed by aligning the strips with the standard grid.

Ki-67 and p16 Immunohistochemistry

Immunohistochemical staining for p16 was performed on 4-μm sections of formalin-fixed, paraffin-embedded specimens. The sections were subjected to heat-induced antigen retrieval and incubated in an automated stainer with p16 antibody (Dako, Glostrup, Denmark; as of January 2007 manufactured by MTM Laboratories AG, Heidelberg, Germany)22 at a dilution of 1:25, and were stained with diaminobenzidine chromogen and counterstained with hematoxylin. The staining was performed with respective negative and positive controls. The staining was graded as 0 = negative, = weak nuclear or cytoplasmic (p16) or moderate to strong-intensity nuclear and cytoplasmic staining with diffuse or patchy distribution.
**Table 1. Results of Human Papillomavirus (HPV) Testing**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>hrHPV* (No. (%))</th>
<th>hrHPV* (No. (%))</th>
<th>hrHPV* (No. (%))</th>
<th>hrHPV* (No. (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSIL</td>
<td>36 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NEG</td>
<td>23 (17)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AIM</td>
<td>37 (20)</td>
<td>1 (3)</td>
<td>16 (43)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* hr indicates high risk; lr, low risk; HSIL, high-grade squamous intraepithelial lesion; NEG, negative; and AIM, atypical immature squamous metaplasia.

**Table 2. Correlation Between High-Risk Human Papillomavirus (hrHPV) Detection and p16 Positivity**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>hrHPV* p16*</th>
<th>hrHPV* p16*</th>
<th>hrHPV* p16*</th>
<th>hrHPV* p16*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSIL</td>
<td>36 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NEG</td>
<td>23 (17)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AIM</td>
<td>37 (20)</td>
<td>1 (3)</td>
<td>16 (43)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* HSIL indicates high-grade squamous intraepithelial lesion; NEG, negative; and AIM, atypical immature squamous metaplasia.

**Table 3. Correlation Between Human Papillomavirus (HPV) Detection and p16 and Ki-67 Positivity**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>HPV</th>
<th>p16* Ki-67*</th>
<th>p16* Ki-67*</th>
<th>p16* Ki-67*</th>
<th>p16* Ki-67*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSIL (n = 36)</td>
<td>HR</td>
<td>36 0 0 0 1</td>
<td>16 1 1 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>NEG (n = 23)</td>
<td>LR</td>
<td>0 0 0 2 0 0 1 0 0</td>
<td>15 1 1 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>AIM (n = 37)</td>
<td>HR</td>
<td>7 8 4 1 1</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
</tbody>
</table>

* HSIL indicates high-grade squamous intraepithelial lesion; HR, high risk; NEG, negative; LR, low risk; and AIM, atypical immature squamous metaplasia.

**RESULTS**

**Results of HPV Detection**

The results of HPV testing are shown in Table 1. High-risk HPVs were detected in all cases of HSIL, 17% of NEG cases, and 54% of cases of AIM.

**Results of p16 Immunostaining**

All cases of HSIL showed strong, diffuse positivity for p16. The staining was both cytoplasmic and nuclear, and in most cases it spanned the full thickness of the epithelium (Figure 1, A), whereas in the remaining cases it involved the lower two thirds of the epithelial thickness, with the strongest staining at the basal layer (Figure 1, B).

In NEG and AIM cases, immunostaining for p16 showed variation of staining intensity, including strong and diffuse staining, patches and single cells with faint blush staining, or entirely negative staining. According to the manufacturer instructions, blush staining should be interpreted as a negative result. Only 1 NEG case showed strong, diffuse positive staining, and this case was also positive for hrHPV. A total of 5 NEG cases showed focal faint blush staining; 1 case showed full-thickness faint cytoplasmic staining (Figure 1, C), and in 4 cases there were single p16-positive cells dispersed throughout the epithelial thickness (Figure 1, D). None of these cases were positive for hrHPVs or Ki-67.

Of the AIM cases, 15 were strongly, diffusely positive for p16. In addition, 8 cases showed faint blush, but only 2 of them were positive for hrHPV, and none of these cases were positive for Ki-67. These findings confirmed that weak blush staining does not correlate with a significant detection of hrHPV. The blush, however, was quite common and overall was identified in 30% of NEG and 22% of AIM cases. Blush staining has to be recognized as a potential pitfall of interpretation of a p16 staining result. The k value for interobserver agreement of p16 stain interpretation in AIM was 0.89.

**Correlation Between hrHPV Detection and p16 Positivity—Specificity and Sensitivity of p16 as a Marker of hrHPV Infection**

The correlation between hrHPV detection and p16 positivity is summarized in Table 2. We did not identify any cases positive for p16 and negative for hrHPV in any of the diagnostic groups (with strong, diffuse p16 staining considered as a positive result). This indicates a complete specificity of p16 as a marker of hrHPV in all 3 diagnostic categories.

In the group of HSIL cases, there was a complete correlation between hrHPV detection and p16 positivity. The sensitivity of p16 as a marker of hrHPV in HSIL was 1.

However, p16 was not an entirely sensitive marker of hrHPV in NEG and AIM cases. In both groups, 13% of cases were found to be positive for hrHPV but negative for p16. In all of these cases, the patients had a previous or a concurrent diagnosis of cervical dysplasia. Given the complete sensitivity of p16 as a marker of hrHPV in HSIL, it is plausible that NEG and AIM cases that are positive for hrHPV but negative for p16 represent asymptomatic HPV presence without an active expression of E7 oncoprotein. The sensitivity of p16 as a marker of hrHPV in AIM was 0.75.

**Correlation Between HPV Detection and p16 and Ki-67 Positivity**

The examples of negative and positive results of Ki-67 immunostaining are shown in Figure 1, E and F, respectively, and the correlation between HPV detection and p16 and Ki-67 positivity is provided in Table 3. A cluster of 2 or more strongly stained nuclei in the upper two thirds of the epithelial thickness was defined as a positive result.

The vast majority of HSIL cases demonstrated diffuse, full-thickness positivity for Ki-67; however, 4 (11%) of 36 cases showed only rare clusters of Ki-67–positive cells in the upper layers of the epithelium, barely fulfilling the criterion of positivity.
Molecular markers and HPV detection in the diagnosis of lower genital tract lesions

All cases of HSIL showed positivity for both p16 and Ki-67 (Table 3). All NEG cases were negative for both p16 and Ki-67, with the exception of 1 case positive only for Ki-67 in the area adjacent to a mucosal ulcer and another case positive only for p16 and showing minimal cytologic atypia. Seven cases of AIM displayed a pattern of immunostaining identical to HSIL, and these cases most likely represent a spectrum of HSIL with mild cytologic atypia (Figure 2, A and B). Eight cases were positive for p16 and negative for Ki-67. In the view of normal proliferative activity, the neoplastic potential of such cases is uncertain; these cases may represent either a precursor of HSIL or, alternatively, a regressing HSIL (Figure 2, C and D). Two AIM cases were positive only for Ki-67 in the area adjacent to a mucosal ulcer, representing regeneration with reactive atypia (Figure 2, E and F). Finally, 20 cases of AIM were negative for both p16 and Ki-67; therefore, these cases are thought to represent a benign reactive atypia or an atypia related to atrophy (Figure 2, G and H). Four of these p16- and Ki-67-negative cases were positive for hrHPV, and such cases most likely represent asymptomatic HPV presence without expression of E7 oncoprotein, because a similar percentage of hrHPV-positive, p16- and Ki-67-negative cases was also identified in the NEG group.

Based on the results of immunostaining, 59% (22/37) of...
Figure 2. Examples of different subcategories of atypical immature squamous metaplasia. Cases A through D were positive for high-risk human papillomavirus (hrHPV) by polymerase chain reaction (PCR); cases E through G were negative for hrHPV by PCR. A, p16-positive, Ki-67-positive case. B, p16-positive, Ki-67-positive case. C, p16-positive, Ki-67-negative case. D, p16-positive, Ki-67-negative case. E, p16-negative, Ki-67-positive case. F, p16-negative, Ki-67-positive case. G, p16-negative, Ki-67-negative case. H, p16-negative, Ki-67-negative case (hematoxylin-eosin stain, original magnification ×400 [A through H]).
AIM cases in our study could be reclassified as benign considering the negative result of p16 immunostaining, and another 19% (7/37) of cases could be reclassified as HSIL or favor HSIL based on positive staining for both p16 and Ki-67, limiting the number of cases with nondiagnostic atypia by approximately 80% (Figure 3).

At the end of the study, the results of HPV testing and Ki-67 and p16 immunostaining were correlated with the original hematoxylin-eosin sections of cases of AIM. The histologic features seen on the routine sections were very similar between the different subgroups of AIM. Many of the AIM cases positive for HPV, p16, and Ki-67 (Figure 2, B) appeared to be histologically indistinguishable from cases of AIM that were HPV, p16, and Ki-67 negative (Figure 2, G). This morphologic similarity underscores the importance of objective markers of HPV infection and dysplasia for establishing accurate diagnosis.

Follow-up of Patients With Diagnosis of AIM

Previous diagnoses, concurrent diagnoses, and 2-year histologic and cytologic follow-up of patients with diagnosis of AIM are summarized in Table 4.

Table 4. Previous and Concurrent Diagnoses and 2-Year Follow-up of Patients With Diagnosis of Atypical Immature Squamous Metaplasia*

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>NEG</th>
<th>LSIL</th>
<th>HSIL</th>
<th>ASC-US</th>
<th>Not Available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previous diagnosis</td>
<td>0</td>
<td>5</td>
<td>15</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Concurrent diagnosis</td>
<td>27</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Follow-up diagnosis</td>
<td>20</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>

*N EG indicates negative; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; and ASC-US, atypical squamous cells of undetermined significance.

COMMENT

The results of our study indicate that nearly two thirds of cases of AIM could be reclassified as benign based on negative p16 staining, and another one fifth could be reclassified as HSIL/favor HSIL based on positivity for both p16 and Ki-67, therefore markedly reducing the number of cases interpreted as nondiagnostic atypia. The significance of AIM lesions identified as p16 positive but negative for Ki-67 is uncertain. Such cases have active expression of viral oncoproteins yet exhibit normal proliferative activity. It is plausible that high levels of p16 may exert inhibiting effects on residual, unsequestered Rb, overriding mitotic drive stimulated by E7 and E6 oncoproteins. Consequently, such lesions may represent early stages of HSIL or, alternatively, regressing HSIL. In a previous study by Geng et al, 80% of patients with HPV-positive AIM had concurrent or subsequent HSIL. No LSILs or ASC-US were recorded on follow-up. Based on these follow-up data, the authors suggested that HPV-positive cases of AIM with low/moderate proliferative activity may represent precursors of HSIL. In our study group, however, 40% of AIM cases were preceded by a diagnosis of HSIL, whereas only 1 case of HSIL (3%) was identified in a follow-up; thus, results from our study may suggest that AIM represents HSIL regression. A shortcoming of our study, aside from the small sample of AIM cases, is that one third of the index AIM cases were cervical cone biopsies; therefore, these lesions were completely excised. Duggan et al reported that follow-up of 32 AIM cases included 50% LSIL, 15.5% HSIL, 25% benign, and 9.5% nondiagnostic atypia. The authors have previously suggested that AIM “is a type of LSIL involving immature squamous metaplasia,” based on similar patterns of weak, focal p16 positivity. This view, however, is not universally shared, because the morphologic features of AIM clearly place it in the spectrum of HSIL-related lesions.

Another interesting finding in our study is that 11% of HSIL cases demonstrated very strong p16 positivity but only very focal Ki-67 staining, indicating a presence of a small subset of HSILs with low proliferative activity. A similar observation has been described recently by Qiao et al. Cases like this may represent first stages of HSIL regression. It has been previously estimated that approximately 30% of HSIL cases regress spontaneously, with an average follow-up of 2 years. The process of HSIL regression has not been elucidated on the molecular level. Baak et al observed that HSIL cases with benign follow-up (no lesion found on subsequent biopsy) had higher immunohistochemical expression of either p53 or Rb protein, as opposed to persistent HSIL lesions. Neither the pattern...
of Ki-67 nor p16 immunostaining was significantly different between the two groups of HSIL in that study.\textsuperscript{28}

Identification of Ki-67 and p16 as specific and sensitive biomarkers of intraepithelial neoplasia and HPV infection was a giant step toward improving diagnostic accuracy of the lesions of the lower genital tract; however, interpretation of the special stains requires some degree of experience. For example, p16 immunostaining results in frequent, weak blush staining that does not correlate with detection of hrHPV. In our study, the blush was quite common and present in 30\% of NEG and 22\% of AIM cases. Blush staining has to be recognized as a potential pitfall that may result in false-positive interpretative error. In the experience of our laboratory, interpretation of Ki-67 stain is easier than that of p16, since only rarely do we encounter borderline positive Ki-67 cases. Still, 11\% of HSIL lesions in this study showed only rare clusters of Ki-67–positive cells in the upper layers of the epithelium. Without careful examination of the epithelium using high-power magnification, these Ki-67–positive cells may have not been spotted, resulting in false-negative interpretative errors.

References

Chapter 10

Double immunostaining for cytokeratin and basement membrane components is useful for detection of microinvasion in vulvar and cervical intraepithelial neoplasia.

*Rush D, Hyjek E, Baergen R, Ellenson LH, Pirog EC.*


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Detection of Microinvasion in Vulvar and Cervical Intraepithelial Neoplasia Using Double Immunostaining for Cytokeratin and Basement Membrane Components

Demaretta Rush, MD; Elizabeth Hyjek, MD; Rebecca N. Baergen, MD; Lora H. Ellenson, MD; Edyta C. Pirog, MD

Context.—Identification of early invasion in vulvar intraepithelial neoplasia 3 (VIN 3) and cervical intraepithelial neoplasia 3 (CIN 3) may be difficult with the use of routine hematoxylin-eosin staining. Presence of obscuring inflammation and tangential tissue sectioning are the most common diagnostic pitfalls.

Objective.—To examine the utility of double immunostaining for cytokeratin–collagen IV or cytokeratin-laminin in the detection of early invasion in VIN 3 and CIN 3.

Design.—The study group consisted of 10 cases of “VIN 3, suspicious for invasion” and 10 cases of “CIN 3, suspicious for invasion.” The negative control group consisted of VIN 3 (n = 15) and CIN 3 (n = 10). The positive control group consisted of cases of invasive vulvar carcinoma (n = 11) and invasive cervical carcinoma (n = 25). All cases were double immunostained for cytokeratin and collagen IV and, in a separate reaction, for cytokeratin and laminin. The continuity of the basement membrane and the presence of stromal invasion were assessed in the stained sections.

Results.—The staining for collagen IV and laminin yielded identical results. A well-defined, continuous basement membrane was visualized in all cases of VIN 3 and CIN 3. A discontinuous or absent basement membrane was observed around the malignant cells on the invasive tumor front in all cases of vulvar and cervical carcinoma. In 2 of 10 cases of VIN 3, suspicious for invasion and in 4 of 10 cases of CIN 3, suspicious for invasion definitive foci of microinvasion were identified with the use of double immunostaining. A well-defined, continuous basement membrane was present in the remaining cases “suspicous for invasion.”

Conclusion.—Double immunostaining for cytokeratin–collagen IV or cytokeratin-laminin is useful for evaluation of early invasion in equivocal cases of VIN 3 and CIN 3. (Arch Pathol Lab Med. 2005;129:747–753)

Microinvasive squamous carcinoma of the uterine cervix is defined by the International Federation of Gynecology and Obstetrics (FIGO) as an invasive squamous lesion with a maximum depth of invasion of less than 3 mm and less than 7 mm in the lateral extension (FIGO stage IA1). In the vulva, superficial invasion is defined by FIGO as a depth of invasion of less than 1 mm from the tip of the closest dermal papillae (FIGO stage IA). Detection of areas of minimal invasion in intraepithelial neoplasia of these sites is of critical importance. Although the prognosis of minimally invasive disease in these sites is good, with recurrence and metastases occurring extremely rarely, the presence of invasion in these cases warrants a more aggressive management and follow-up plan than does noninvasive disease. Patients with superficial invasion of the uterine cervix must be treated, at a minimum, by cold knife cone with documentation of complete removal of the lesion with clear margins and are offered simple hysterectomy as well. Patients with superficial invasion of the vulva must likewise be treated by wide local excision with removal of the entire lesion and documentation of clear margins. In both cases such treatment is necessary to ensure that no associated area of more serious disease is missed. Moreover, in any tumor, the identification of any amount of invasion, however small, puts the patient at a risk, however small, for metastasis. Cases with invasion as small as 0.8 mm in the cervix have presented with widespread metastases, and patients with minimal invasion must be advised of this risk and followed up closely.

The detection of microscopic foci of invasion in intraepithelial neoplasia of the vulva and cervix can be difficult by light microscopy. The problem is often compounded by the presence of dense obscuring inflammatory infiltrate in these lesions and by the small size of the biopsy specimens and artifacts associated with newer, shallower cervical excisions such as the loop electrosurgical excision procedure.

With the advent of immunohistochemical analysis, there was an interest in the use of stains for the basement mem-
brane (BM) components as an adjunct to light microscopy in the diagnosis of superficial invasion in various carcinomas. Yet the use of this technique to investigate the presence of BM in situ and invasive lesions of various tissues has produced conflicting results. Some of the studies have reported areas of discontinuous BM in cases of cervical intraepithelial neoplasia 3 (CIN 3) or carcinoma in situ of the skin, whereas a well-formed BM was identified in a proportion of invasive cervical and skin carcinomas. In addition, metastatic foci of squamous cell carcinoma in the lymph nodes were found to be surrounded by BM. Some of the authors concluded that immunohistochemical staining for the elements of BM may be of limited value in cases with questionable stromal invasion. Other studies, however, reported the presence of continuous BM in all cases of CIN 3. A more precise analysis of BM in carcinoma in situ of the skin revealed that some of the reported breaks were present in the areas of regression of the lesion, where BM was reduplicated and the breaks were seen in the “old” BM. In addition, diminutive BM breaks were observed in the areas of marked inflammation where mononuclear cells could be seen crossing through BM into the epithelium. However, no migration of the dysplastic epithelial cells in the opposite direction was identified in these cases. Detailed studies of BM in invasive carcinomas of the cervix and skin revealed that most tumors retained the capacity for BM synthesis, since collagen IV and laminin were identified surrounding invasive and metastatic tumor nests. Well-differentiated tumors were more likely to show positive staining for BM components, whereas poorly differentiated tumors were more likely to lack staining. Although many tumors were shown to produce BM, definitive BM gaps were described at the invasive tumor fronts or in the areas of microinvasion.

As demonstrated in the previous studies, the mere presence or absence of BM components is not sufficient evidence of the presence or absence of invasion. However, a visualization of the dysplastic epithelial cells breaching the BM gaps into the stroma, using double immunostaining for cytokeratin (CK) and BM components, may provide convincing evidence of stromal invasion. Recently, a technique for double immunostaining with CK and smooth muscle actin has been developed in our department for detection of early invasion in breast carcinomas. In the current study, we sought to determine if a similar technique, using double labeling for CK and collagen IV or CK and laminin, might be useful in assessing the presence or absence of invasion in problematic cases of CIN and vulvar intraepithelial neoplasia (VIN).

MATERIALS AND METHODS

Case Selection

The surgical pathology files of the Department of Pathology, Weill Medical College of Cornell University, were searched from 1997 through 2002 to identify successive vulvar and cervical specimens. The study group consisted of 10 cases of VIN 3 with areas suspicious for invasion and 10 cases of CIN 3 with areas suspicious for invasion. The negative control group consisted of 15 cases of VIN 3 and 10 cases of CIN 3. The positive control group consisted of superficially invasive vulvar carcinoma (n = 1), invasive vulvar carcinoma (n = 10), microinvasive cervical squamous cell carcinoma (n = 15), and invasive cervical squamous cell carcinoma (n = 10).

Immunohistochemical Analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded 4-µm tissue sections in TecHMate automated immunostainer (Ventana Medical Systems Inc, Tucson, Ariz) using anti-laminin monoclonal antibody (clone LAM-90, Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom), anti-collagen IV antibody (clone CIV 22, DakoCytomation, Carpinteria, Calif), and anti-human CK monoclonal antibody (clone MNF 116, DakoCytomation). The immunostaining consisted of 2 steps; in the first step, after antigen retrieval in 0.1% trypsin at 37°C for 25 minutes, the sections were incubated with anti-laminin antibody at a dilution of 1:100 for 1 hour or with anti-collagen IV antibody at a dilution of 1:200 for 1 hour, followed by a horseradish peroxidase–labeled mouse Envision Plus detection system (DakoCytomation) for 30 minutes. Peroxidase reaction was developed using diaminobenzidine liquid chromogen (DakoCytomation), resulting in a dark brown color of the BM. In the second step, sections were incubated with anti-C (MNF 116) antibody at a dilution of 1:600 for 50 minutes, followed by secondary goat anti-mouse biotinylated antibody, and finally followed by the avidin-biotin complex alkaline-phosphatase detection system (Ventana Medical Systems) with BT Red reagent substrate incubated using diaminobenzidine liquid chromogen resulting in a bright red staining of the epithelium. Sections were then counterstained with hematoxylin and mounted. There is an excess of secondary antibodies in the first step of staining using horseradish peroxidase–labeled mouse Envision Plus detection system, which results in complete saturation of respective epitopes on the primary mouse antibodies, and therefore we did not observe any cross-reactivity between the secondary anti-mouse antibodies used in the second step and the primary mouse antibodies used in the first step of staining. The technical aspects are discussed in detail in the article by Prasad et al.

RESULTS

Comparison of Double Immunostaining for CK–Collagen IV and CK–Laminin

The immunostaining for CK using alkaline-phosphatase complex with BT Red reagent substrate resulted in red staining of the epithelium, whereas immunostaining for laminin or collagen IV using diaminobenzidine liquid chromogen resulted in dark brown staining of the BM (Figure 1, A). There were no significant differences between the results of the staining for CK–collagen IV or CK–laminin. The staining for laminin resulted in a visualization of a slightly thicker membrane. Rarely, laminin showed cytoplasmic staining in the basal cells of the normal or dysplastic epithelium. The archival cases stored more than 10 years showed inconsistent staining for both collagen IV and laminin. In cases less than 10 years old, the staining was consistent; however, in few cases the pretreatment digestion time required adjustment because of variation of adequacy of specimen fixation. Long digestion of tissue sections sporadically caused some epithelial cells to become discohesive and displaced on the slide; the displaced cells were easily identified as an artifact, because they were in a different plane of focus from the rest of the tissue.

Double Immunostaining in the Negative Control Cases: VIN 3 and CIN 3

A well-defined, continuous BM was visualized in all cases of VIN 3 and CIN 3 (Figure 1, B). The staining intensity for collagen IV or laminin in these cases was identical to that of normal cervical and vulvar mucosa (Figure 1, A). In cases with inflammation at the epithelial-stromal junction, inflammatory cells were seen migrating through...
Figure 1. Control cases. Double immunostaining for cytokeratin and collagen IV. A, Normal vulvar epithelium (original magnification x100). B, Vulvar intraepithelial neoplasia 3 (original magnification x100). C, Invasive vulvar carcinoma, no basement membrane staining (original magnification x100). D, Invasive vulvar carcinoma, focal basement membrane staining (original magnification x100). E, Verrucous carcinoma (original magnification x100), pushing front with continuous basement membrane staining. F, Verrucous carcinoma (original magnification x400), single tumor cells below the pushing tumor front. G, Microinvasive cervical carcinoma (original magnification x40).
the micropores in BM. Small size, scant cytoplasms, and lack of red CK staining clearly identified these cells as nonepithelial. In the areas of marked acute and chronic inflammation, focal duplication of the BM was occasionally observed; however, the continuity of the main membrane was always preserved in these areas. Tangential or parallel sectioning through the BM resulted in an apparent widening of the membrane with slight decrease in the staining intensity.

**Double Immunostaining in the Positive Control Cases: Vulvar and Cervical Carcinoma**

A discontinuous or absent collagen IV and laminin staining was observed around the malignant cells on the invasive tumor front in all cases of vulvar and cervical carcinoma, both invasive and microinvasive. In some cases, the entire tumor failed to stain (Figure 1, C—invasive carcinoma that lacked collagen IV cuff). In most cases, however, focal continuous collagen IV and laminin staining was present, especially around larger, more centrally located tumor nests (Figure 1, D—continuous collagen IV staining around the larger tumor nest and no staining around the smaller tumor nest below). In 2 cases of verrucous carcinoma of the vulva, a well-defined, continuous BM was visualized on the pushing tumor front (Figure 1, E); however, after a thorough examination of multiple tumor sections, small foci of tumor nests and single tumor cells devoid of BM were identified in the stroma below the pushing front (Figure 1, F). The CK stain was particularly helpful in detecting these areas. In some cases of microinvasive carcinoma of the cervix, there was intense inflammatory infiltrate present in the stroma, obscuring the invasive epithelial nests and impeding the assessment of the tumor dimensions. Again, the CK stain was helpful in highlighting the invasive tumor within the inflammatory infiltrate (Figure 1, G), facilitating more accurate measurement of the tumor size.

**Double Immunostaining in Equivocal Cases of CIN and VIN Suspicious for Invasion**

In 2 of 10 cases of “VIN 3, suspicious for invasion” and in 4 of 10 cases of “CIN 3, suspicious for invasion,” definitive foci of microinvasion were identified with the use of double immunostaining, and these cases were reclassified as superficially invasive or microinvasive carcinoma. A well-defined, continuous BM was visualized in the remaining cases suspicious for invasion. Figure 2, A, illustrates a case of CIN 3 suspicious for invasion with a routine hematoxylin-eosin stain. Intense inflammatory infiltrate obscures the epithelial-stromal junction of the rete ridge indicated by the arrowhead. The CK—collagen IV stain (Figure 2, B) of the same area demonstrates no collagen IV staining around the suspicious focus compared with a well-defined, continuous staining around the adjacent rete ridge. Figure 2, C, shows a hematoxylin-eosin stain of a case of VIN 3 suspicious for invasion. The CK—collagen IV stain of the same focus (Figure 2, D) visualized a large gap in the BM, with epithelial cells transgressing into the stroma. Figure 3, A through D, illustrate further examples of microinvasive foci in cases of VIN 3 suspicious for invasion identified with the use of CK—collagen IV immunostain. An unsuspected capillary lymphatic space invasion was identified using the stain (Figure 3, D), with a red-staining squamous cell present in the lumen of the capillary channel. This small tumor cell was interpreted as endothelial on the routine hematoxylin-eosin section.

**COMMENT**

The process of cancer invasion has been extensively studied at the ultrastructural level. The micoinvasion in the cervical carcinoma at the level of electron microscopy was described by Kudo et al. In areas of invasion, the basal lamina of BM disappeared, and pseudopod-like cytoplasmic protrusions of the cancer cells were seen in a direct contact with the stroma. The cytoplasmic protrusions of the invading cells contained abundant 70- to 90-nm vesicles, some of which were open directly to the extracellular matrix of the stroma. The vesicles were not observed in carcinoma in situ adjacent to intact BM. The authors suggested that the substances contained in the vesicles may play a role in BM destruction. In addition, the cells traversing the BM gaps showed accumulation of actin filaments in the pseudopod protrusions. These local aggregates of cytoskeletal structures, not observed in adjacent carcinoma in situ, were thought to facilitate an amoeba-like movement of the cancer cells. The destruction of the BM with migration of the cancer cells through BM gaps has been observed universally at the invasive tumor fronts in cancers that arise from different epithelial types. On the other hand, however, BM deposition around tumor nests has been also widely described. A plausible explanation of these seemingly conflicting observations was proposed by Liotta, whose findings were further corroborated by the works of Cam et al and Ehrmann et al. It appears that cancer nests proceed through cycles of growth surge with BM destruction and stromal invasion followed by quiescence and BM re-formation. During the quiescent phase, BM remains relatively intact until a new surge of growth, during which BM is focally dissolved and newly formed tumor buds grow out of the old nest. The degree of BM production varies among different tumor types.

The current study is the first, to our knowledge, to describe double immunostaining for CK and BM components in evaluation of microinvasion in CIN and VIN. Since evaluation of BM continuity with the use of single antibody for collagen IV or laminin may not reliably visualize the areas of microinvasion, we have developed a technique of double immunostaining where the addition of CK staining distinctly highlights the areas of epithelial cells that cross the BM boundaries.

The findings reported herein confirm and expand those reported previously using a single antibody for collagen IV or laminin. Collagen IV and laminin was found to be present, discontinuous, or absent around nests of malignant cells in invasive tumors. As reported in previous studies, our study has found that the larger and more central tumor nests tend to have better formed BM, whereas the nests and the single tumor cells on the invasive front lack BM or show discontinuous staining. In a case of a specific subtype of vulvar carcinoma, a verrucous carcinoma, the BM was almost entirely intact; however, a few small tumor nests and single cells devoid of BM were identified focally below the pushing tumor border. This is in keeping with other studies of similar tumors of the head and neck characterized by a pushing type of border. The retention of BM in verrucous carcinoma correlates with relatively good clinical outcome. Verrucous carcinomas are known for their indolent, localized, superficial
growth, devoid of metastatic potential. This observation further confirms that dissolution of BM facilitates destructive stromal invasion and metastatic spread of cancers.

Although several previous studies have described focal discontinuity of the BM in in situ lesions, others reported intact BM and none of the cases of CIN 3 or VIN 3 reviewed in this study showed significant BM disruption. A migration of inflammatory cells through the micropores...
in BM was evident in the areas of inflammation, but the pores were smaller than the size of the epithelial cells; furthermore, staining for CK did not reveal epithelial cell movement across BM. The process of leukodiapedesis, although similar to that of cancer cell invasion, involves only a focal and limited proteolytic degradation of BM components.19,20

To our knowledge, only one prior study4 reported the use of collagen IV immunostaining to reevaluate cases in which previous diagnosis, using light microscopy only, had been inconclusive as to the presence or absence of invasion. In that study, 6 of 15 cervical biopsy specimens were reclassified as showing definitive invasion after correlation of the routine sections with the results of immunohistochemical analysis. In our study, 4 of 10 inconclusive cervical biopsy specimens and 2 of 10 inconclusive vulvar biopsy specimens were shown to have areas of definitive invasion. These findings indicate that a definitive diagnosis as to the presence or absence of microinvasion can be achieved in equivocal cases. The use of immunohistochemical analysis to demonstrate the absence of invasion in areas in question can spare the patient and clinician unnecessary procedures and anxiety. Likewise, the confirmation of invasion in problematic cases can facilitate and speed the proper and definitive treatment.

The use of the anti-CK antibody in our study provided some additional useful information. In one of the cases, the use of CK staining allowed identification of a focus of lymphatic invasion not evident on light microscopy, which would have also been missed had only BM staining been used. The CK immunostaining also visualized the invading single tumor cells in verrucous carcinomas in which the BM was almost entirely intact. Finally, CK staining helped to highlight the invasive tumor cells obscured by severe inflammation. Thus, the extent of the invasive component, which is of prognostic significance, may be more accurately assessed in some cases based on CK immunostaining.

Finally, the use of immunohistochemical techniques for the evaluation of invasion is particularly suited to these cases in which minimal invasion is suspected. Our study demonstrates, as have many previous studies that examined the expression of BM by various tumors, that invasive tumors can indeed produce BM. Thus, tumors that have already developed extensive invasion may yet express intact or relatively intact BM around some nests. Fortunately, such cases rarely pose a diagnostic dilemma.

In conclusion, the results of the current and previous studies indicate that invasive tumor is not defined simply by the absence of BM, and the presence of intact BM surrounding tumor is not proof that a lesion is not invasive. Rather, what defines invasion is the ability of tumor cells to traverse the BM, which initially contains them. This is the process manifested in minimally invasive disease, which may be precisely visualized by the technique described herein.

References


Chapter 11

Discussion and conclusions
Discussion and conclusions

- **HPV detection and genotyping in the lower genital tract lesions**

Cervical adenocarcinoma

The relative and absolute incidence of cervical adenocarcinoma has been increasing in recent years in both industrialized and developing countries (1, 2). As a result, there has been a growing interest in researching tumoral pathogenesis of cervical adenocarcinoma. For many decades, detection of HPV in cervical adenocarcinomas posed a technical difficulty due to a relatively low viral load in neoplastic glandular epithelium that did not support productive HPV infections (3). Only recently, thoroughly designed broad-spectrum PCR primers have facilitated amplification of HPV DNA with high sensitivity and allowed wide-spectrum HPV genotyping (4, 5). In our study (Chapter 2), HPV DNA was detected in 82 of 90 adenocarcinomas (91%), encompassing endocervical, intestinal, and endometrioid histologic subtypes, and in 9 of 9 adenosquamous carcinomas (100%). After stratification into in-situ and invasive lesions, HPV DNA was detected in 100% of adenocarcinomas in-situ (AIS) and 80.8% of invasive adenocarcinomas. HPV DNA was not detected in any of the 6 non-mucinous adenocarcinomas including clear cell, serous, and mesonephric subtypes. In addition, 2 cases of minimal deviation adenocarcinoma were negative for HPV.

Our study was the first to demonstrate that mucinous adenocarcinomas (with the exception of minimal deviation adenocarcinoma) and adenosquamous carcinomas of the cervix have a very high prevalence of HPV DNA, similar to that reported for cervical squamous cell carcinoma. In addition, our results indicate that there are rare histologic variants of cervical adenocarcinoma, such as minimal deviation, clear cell, serous, and mesonephric adenocarcinoma that appear unrelated to HPV infection.

Studies of HPV in adenocarcinomas prior to our publication (reviewed in Chapter 2) were hindered by low sensitivity assays, with the exception of the study by Tenti et al (6), but our report was followed by a series of larger studies (reviewed in Ref. 7, Table 2) using HPV detection techniques with greater sensitivity. The study by Zielinski et al (8) was the most comprehensive and compared HPV detection and type distribution in AIS and invasive...
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The analysis in other publications was limited to either invasive adenocarcinomas (9-11) or to the detection of only HPV16 and 18 (12, 13). The results of these follow-up studies were similar to our results, showing a high prevalence of HPV in adenocarcinomas (100% in AIS and 88-97% in invasive tumors) and a narrow spectrum of HPV genotypes limited mainly to HPV16 and HPV18. Studies by Zielinski et al (8), Castellsague et al (9), An et al (10) and Schwartz et al (11) reported detection of HPV16 and/or HPV18 in 76% to 89% of invasive adenocarcinomas. In our study (Chapter 2) HPV16 and/or HPV18 were found in 86% cases of invasive adenocarcinoma and 92% cases of adenocarcinoma in-situ. HPV45 (12%) and HPV52 (2%) were other single HPV genotypes identified in invasive adenocarcinomas and HPV45 (4%) and HPV35 (4%) were additional single HPV genotypes detected in AIS.

Due to the high percentage of HPV positivity in AIS and invasive tumors, HPV testing can be used as a screening tool. The relatively narrow spectrum of HPV genotypes detected in these tumors (mainly HPV16, 18 and 45) is very promising for the high efficacy of current prophylactic vaccination programs. A bivalent HPV vaccine targeting HPV16 and HPV18 has been also shown to offer cross-protection against type HPV45, HPV 31 and HPV33 (14). A quadrivalent HPV vaccine does not appear to have a significant protective effect against type 45 but reduces infections with types HPV31, 33, 52, 39, 59, 35 and 58 (15). Due to this cross-protection, currently available HPV vaccines are likely to prevent close to 100% of HPV-positive cervical adenocarcinomas.

The studies of minimal deviation adenocarcinoma and non-mucinous cervical adenocarcinomas (clear cell, serous, and mesonephric) that followed our report have confirmed our initial finding that the pathogenesis of these rare types of cervical tumors may not be related to HPV infection (16, 17). Minimal deviation adenocarcinoma (MDA, “adenoma malignum”) has been recently identified as a malignancy preceded by a benign precursor lesion termed lobular endocervical glandular hyperplasia/pyloric gland metaplasia (LEGH) producing gastric-type mucins (16, 18). Some cases of MDAs are associated with Peutz-Jeghers syndrome, an autosomal dominant disorder caused by germline mutation of STK11, a serine threonine kinase gene. In addition, somatic mutations of STK11 are identified in over half of the sporadic cases of MDA (19). Since the precursor lesions for clear cell, serous and mesonephric adenocarcinoma are unknown, and LEGH (precursor of MDA) is difficult to detect by cytologic screening, the prevention for these 4 subtypes of adenocarcinoma will still pose a challenge, with no role for
HPV testing and the role of cytologic screening limited to detection of invasive tumor at the possibly lowest stage. MDA and clear cell carcinoma account for 1%-2% and 2%-7% of all adenocarcinomas, respectively, while serous and mesonephric adenocarcinomas are exceedingly rare. Overall, non-HPV related tumors comprise less than 10% of cervical adenocarcinomas.

**Penile carcinoma**

Our study (Chapter 3) was the first to comprehensively analyze and compare HPV detection in different subtypes of penile cancers including four main histologic types namely, keratinizing, verrucous, basaloid, and warty squamous cell carcinomas. Precise tumor classification and use of high sensitivity HPV DNA detection assays unequivocally demonstrated multiple independent pathways in penile carcinogenesis. Overall, HPV DNA was detected in 42% cases of penile carcinoma, 90% cases of dysplasia, and 100% cases of condyloma. There were significant differences in HPV prevalence in different histologic cancer subtypes. The highest HPV detection rate was observed in warty and basaloid carcinomas, 100% and 80%, respectively (difference not statistically significant) and much lower in keratinizing and verrucous carcinomas, 34.9% and 33.3% respectively (difference not statistically significant). The difference in HPV prevalence between warty and basaloid versus keratinizing and verrucous carcinomas was statistically significant (p<.05). There were no significant differences in HPV DNA prevalence between tumor cases obtained from the high incidence region (Paraguay) and the low incidence region (USA). Overall, the prevalence of HPV DNA in penile carcinoma was lower than that in cervical carcinoma and similar to that in vulvar carcinoma. In addition, specific histological subtypes of penile cancer, warty and basaloid, were consistently associated with HPV. However, only a subset of keratinizing and verrucous penile carcinomas was positive for HPV DNA. These findings support multiple pathogenetic pathways in penile carcinogenesis; HPV-dependent for warty and basaloid tumors and a subset of keratinizing and verrucous carcinomas, and HPV-independent pathways for the majority of keratinizing and verrucous tumors.

The results of older studies on penile carcinogenesis were obscured by the lack of separation of different histologic tumor subtypes. The overall HPV prevalence in penile carcinomas reported in previous and subsequent studies ranged from 22% to 71% (20-23) with an average of 45% (24), which is similar to our study (42%). The results of worldwide meta-
analysis of HPV detection in penile cancer by Beckes et al (24) compiling 30 studies with 1266 cases showed significantly higher HPV detection rates in warty-basaloid carcinomas (66.3%) compared with keratinizing and verrucous tumors (47.8% and 22.4%). These results were consistent with our findings. The limitation of the meta-analysis was the lack of uniform histopathologic classification of the cases. Pooled HPV genotype analysis by Beckes et al (24) showed that the most common HPV types detected in penile cancers were HPV16 (30.8%), HPV6 (6.7%) and HPV18 (6.6%). All other HPV types had a prevalence of less than 2%, and included HPV28, 22, 45, 74, 53, 11, 33, 70, 52, 68, 34, 54, 31, 35, 31, 35, 51, 59, 56, and 58 (24). Our study showed a narrow spectrum of HPV genotypes in warty-basaloid carcinomas (HPV16 -75%, HPV35– 5% and HPV31– 5%) and a wide spectrum of genotypes in keratinizing carcinomas with HPV16 (17.9% of cases) being the most common. Other genotypes that were detected as a single viral infection included HPV45 (3.7%), HPV52 (3.7%), HPV68 (3.2%), HPV35 (1.8%), HPV18 (0.9%). Overall, we have identified HPV16 and HPV18 in 25.5% and 1.4% of all cancer cases, respectively. Based on the results of our study and meta-analysis by Beckes et al (24), it may be expected that current HPV vaccines could prevent between 25%-40% of penile cancer cases.

The pathogenesis of penile carcinomas not related to HPV infection, which includes 60%-75% of all cases, is still not well understood. Ferreux at al. (25) examined elements of the p16/cyclinD/RB pathway in penile cancer and reported that 15% of HPV-negative tumors showed hypermethylation of the p16 gene promoter and an additional 10% of tumors overexpressed polycomb gene product, BMI-1, known to downregulate p16 expression. Further, somatic mutations of p53 gene and deregulation of p14/MDM2/p53 pathway have been identified in HPV-negative penile cancers (26). It is possible that several independent gene alterations will be identified as a cause of malignant transformation within this group of tumors.

**Vulvar carcinoma**

A retrospective review of consecutive cases of vulvar carcinomas diagnosed at New York Hospital between 1990 and 2005 (Chapter 4) showed 68% to be keratinizing squamous cell carcinomas, 31% warty and basaloid and 10% verrucous carcinomas. The overall HPV positivity was 33%, with HPV16, HPV33+51 and HPV6 accounting for 23%, 5%, and 5% of all cases, respectively. Mucosal HPVVs were detected in all but one (10/11) of warty and basaloid
carcinomas. Of these HPV positive cases, 82% were positive for HPV16 and 18% for HPV 33+ 51. In addition, two of four verrucous carcinomas were positive for HPV type 6. Mucosal HPVs were not detected in any of the keratinizing carcinomas. In the immunohistochemical analysis, all cases of warty and basaloid carcinomas, but none of the remaining tumors, showed overexpression of p16 protein. All cases were tested for the presence of the 25 most common beta-PVs, and were found to be negative. The results of our study reaffirmed the role of mucosal HPVs, and in particular that of HPV16, in the pathogenesis of warty and basaloid vulvar cancer. In addition, p16 immunostaining was shown to be a sensitive and specific marker of vulvar carcinomas positive for oncogenic mucosal HPVs. A possible association between low oncogenic risk HPVs and development of verrucous carcinoma will require further studies. Beta-PVs were shown to be unlikely pathogens in vulvar carcinogenesis.

Two recent meta-analysis by Smith et al (27) and de Vuyst et al (28) pooled the results of HPV detection in 1379 and 1873 cases of vulvar carcinoma, respectively, and showed an average 40% HPV positivity in all cancer cases. These results are consistent with our findings (33%). De Vuyst et al (28) demonstrated that warty-basaloid carcinomas were positive for HPV in 69.4% of cases, keratinizing carcinomas in 13.2% of cases, and squamous cell carcinomas not otherwise specified in 48.2% of cases. The results were similar to these by Smith et al (27) who found 85.9% HPV positivity in warty-basaloid carcinomas and 37.8% HPV positivity in keratinizing carcinomas and carcinomas not otherwise specified. The results of our study (Chapter 4) parallel other reports of high HPV prevalence in warty-basaloid tumor subtypes, however, do not confirm HPV detection in keratinizing vulvar cancers (27, 28), and they are also not consistent with our own results of HPV detection in keratinizing penile carcinomas (Chapter 3). We have not detected mucosal HPVs in any of the keratinizing vulvar carcinomas and this was further supported by negative p16 immunostaining in these tumors. The discrepant results between our study and other reports may be related to methodological variations in tumor subclassification and differences in sample preparation for HPV detection. For example, in our study of vulvar cancers all cases were collected within a single institution and therefore the entire case was available for re-review and tumor subclassification. In the study of penile cancers (Chapter 3), the cases were collected from multiple institutions and only one representative slide was available for review, while no slide review was performed for the meta-analysis reports (17 and 19), which may have contributed to inaccuracies in tumor subclassification and confounded the
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results. In addition, in our study of vulvar cancers (Chapter 4), the tumor tissue was microdissected and only deep portions of the tumor were used for HPV analysis. In contrast, in the penile cancer study (Chapter 3), the entire tumor section was processed for HPV testing. Since penile tumors were not microdissected it is possible that some HPV positivity reflected HPV presence on the surface of the penile skin/mucosa. To our knowledge, tumor microdissection prior to HPV testing was not performed in any of the previous studies of vulvar or penile cancers. The importance of tissue microdissection for accurate HPV detection should be further examined and considered in the future HPV studies.

According to meta-analysis by Smith et al (27) HPV 16 was detected in 29.3% of all vulvar tumors, followed by HPV18 (5.6%) and HPV33 (3.3%). Analysis by de Vuyst et al (28) had similar findings (HPV16=32.2%, HPV33=4.5%, HPV18=4.4%). All other HPV types accounted for less than 1% of cases. The results of our genotyping analysis are similar to these reports. Based on results from our study and previous reports of HPV prevalence in vulvar cancers, it is expected that current vaccines may prevent between 25% and 40% of vulvar cancer cases.

Low grade squamous intraepithelial lesions of the vulva and vagina

The results of our study of HPV genotyping in cases of VIN1 and VAIN1 (Chapter 5), demonstrated that although these two lesions have similar histologic features, they represent unrelated conditions with different viral associations. Our results showed that 70% of cases of VIN1 were associated with low oncogenic risk HPVs (HPV6, 11, 44 and 74) which suggests that the majority of cases of flat VIN1 lesions are related to, or may be a precursor of exophytic condylomata acuminata, which are positive for low risk HPVs in 77-88% of cases (Chapter 7). For similar reasons, it is unlikely that VIN1 is a precursor of VIN3, which was shown to be associated exclusively with high risk HPVs (28). In contrast, 84% of VAIN1 lesions were found to be positive for high oncogenic risk HPVs (HPV16, 56, 18, 31, 35, 59, 68). This is similar to cervical CIN1 which has been reported to be associated with high risk HPVs in over 80% of cases. HPV16 and/or 18 accounted for 36.8% of VAIN1 cases and 9.0% of VIN1 cases. HPV6 and/or 11 accounted for 45.4% of VIN1 cases.

Our study was the first to compare HPV genotypes in low grade vulvar and vaginal squamous intraepithelial lesions. Results of our study were confirmed by a follow up study by
Srodon et al (29) which included a larger number of cases. Their study also showed significantly higher prevalence of high risk HPVs in VAIN1 (76% positivity) in comparison to VIN1 (42% positivity). Recent meta-analysis by De Vuyst et al (28) further corroborated our findings showing that the most common HPV types in VIN1 were HPV6 (22.4%), HPV16 (9.8%) and HPV11 (9.0%) while HPV16 predominated (23.4%) in VAIN1 among other high risk HPVs, with no detection of HPV6 or 11. It may be expected that bivalent HPV vaccine will prevent up to 25-40% of VAIN1 and approximately 10% of VIN1 cases, while quadrivalent vaccine is expected to prevent similar percentages of VAIN1 in addition to up to 55% of VIN1 cases.

Results of our study suggest that VIN1, a lesion associated predominantly with low-oncogenic risk HPVs, does not require follow up. However, VAIN1 has the potential to progress to a high-grade squamous intraepithelial lesion in immunosuppressed patients and such patients should be followed with regular colposcopic examinations.

- **Role of molecular markers in diagnosis of HPV-related lesions**

**Ki-67 as a marker of low grade vulvar, vaginal, cervical squamous intraepithelial lesions and vulvar condyloma.**

In our studies of low grade vulvar, vaginal and cervical intraepithelial lesions and vulvar condyloma (Chapter 5, 6, and 7) we used a diagnostic gold standard consisting of consensus histologic review in correlation with HPV detection. The control cases and the study cases were re-classified according to the gold standard, and Ki-67 immunostaining was performed on all cases. The positive result was defined as the presence of a cluster of at least two strongly-stained epithelial nuclei in the upper two-thirds of the epithelium. With such definition of Ki-67 positivity, we have found almost complete concordance between gold standard diagnosis of low grade dysplasia/condyloma, detection of HPV DNA in tissue sections and positive Ki-67 immunostaining. The sensitivity of Ki-67 as a marker of VIN1/VAIN1, CIN1 and vulvar condyloma was 0.96, 1 and 1, respectively. The specificity of Ki-67 as a marker of VIN1/VAIN1, CIN1, and condyloma was equally high, 0.9, 1 and 1, respectively.

Our results are concordant with previous reports of increased Ki-67 positivity in dysplastic squamous epithelium as compared to benign genital mucosa (30-33). The novelty of our approach was to qualify the Ki-67 result as positive with any positivity identified in the
upper two-thirds of the epithelial thickness. Prior reports have quantified differences between normal and dysplastic epithelium by manually calculating the percentage of Ki-67 positive nuclei in a given field. Quantifying Ki-67 staining in the entire epithelial thickness is not only cumbersome and time consuming, but also it never produced results that allowed for clear separation of non-neoplastic cases from dysplasia or condylomata, and did not correlate with results of HPV detection (30).

As we re-reviewed cases with original diagnoses of VIN1, VAIN1, CIN1, and condyloma using our diagnostic gold standard, we have identified a significant trend for overdiagnosis. Of the consecutive biopsies diagnosed by pathologists with varying experience, overdiagnosis was identified in 31% to 36% of cases in all diagnostic categories (Chapter 5, 6, and 7). The main diagnostic pitfall was the occurrence of “pseudokoilocytes”. Underdiagnosis, on the other hand, was relatively rare and was found in less than 10% of cases in all categories. Use of Ki-67 as an objective marker of low grade dysplasia has improved the diagnostic accuracy in our laboratory and a subsequent study of 500 consecutive cervical biopsies diagnosed as CIN1 showed only 0.6% of overdiagnosis (3 of 500 cases) (manuscript in preparation).

**Recommendations**

Ki-67 positivity defined as the presence of positive nuclei in the upper two-thirds of the epithelial thickness was shown to be a sensitive and specific marker of vulvar, vaginal and cervical low grade squamous intraepithelial lesions, and condyloma acuminatum. The stain may be used in equivocal cases to confirm the histologic diagnosis. Use of Ki-67 is especially recommended for pathologists with limited diagnostic experience because of significant potential of overdiagnosis. Even though interpretation of Ki-67 positivity is highly reproducible, regardless of the pathologist experience, there are several instances that may result in a false-positive interpretation.

- Positive Ki-67 staining may not be specific for dysplasia in immature or regenerating epithelium. Ki-67 positive cells may be present in the upper layers of the epithelium in benign immature squamous metaplasia, on the edge of an ulcer or erosion, and in vulvar squamous cell hyperplasia. Positive Ki-67 staining in such cases may not be reflective of HPV presence.
- In cases of cervicitis or vulvitis, Ki-67 positive lymphocytes may be present throughout the epithelial thickness. High-power examination helps to identify these cells as nonepithelial.
**Ki-67 as a marker of endocervical adenocarcinoma in-situ and invasive adenocarcinoma**

In our quantitative analysis of Ki-67 expression in normal endocervix, benign glandular lesions and endocervical adenocarcinoma (Chapter 8) we have found that Ki-67 index below 20% always reflected a benign process, while Ki-67 index higher than 50% was indicative of neoplasia. The range of positivity in adenocarcinoma in situ was 45% to 73% and 25% to 84% in invasive adenocarcinoma. Normal endocervical epithelium showed range of positivity from 0% to 5%, however, rare benign cases, such as endometriosis, tubal metaplasia during proliferative phase and cases of regenerating epithelium showed increased proliferative activity of up to 32%, overlapping with neoplastic cases.

Our results are concordant with previous reports of increased Ki-67 staining in endocervical adenocarcinoma (34-36). After publication of our study, novel markers of endocervical neoplasia were identified, namely, positive staining for p16 (35, 36) and negative staining for estrogen and progesterone receptors (36). The latter two stains are especially useful in cases in which the differential diagnosis includes endometriosis and tubo-endometrioid metaplasia.

**Recommendations**

Ki-67 was also shown to be a sensitive and a specific marker of endocervical neoplasia: Ki-67 index below 20% reflects a benign process, while Ki-67 index higher than 50% indicates neoplasia (AIS or invasive adenocarcinoma).

- In rare cases benign changes of endocervical epithelium may show increased Ki-67 labeling, and therefore Ki-67 immunostaining is best supplemented by immunostaining for estrogen and progesterone receptors.

**Utility of p16 and Ki-67 in the diagnosis of high-grade cervical squamous intraepithelial lesion and atypical immature squamous metaplasia**

Atypical immature squamous metaplasia (AIM) of the cervix is a term used for cases with atypia that is suggestive, but not diagnostic, of HSIL. Such cases are problematic in regards
to recommending patient treatment and follow up. In our study of AIM (Chapter 9) we used cases of HSIL as positive controls. All HSIL cases were found to be positive for high-risk HPV DNA, and were also positive for p16 and Ki-67 immunostains. P16 positivity was defined as strong and diffuse staining involving either the entire thickness or lower two-thirds of the epithelium. The definition of Ki-67 positivity was the same as described above (Chapter 5, 6 and 7). In our study, one-fifth (19%) of cases of AIM showed positivity for HPV DNA, p16 and Ki-67 in the pattern identical to HSIL and therefore these cases appeared to represent a spectrum of HSIL, and could be reclassified as such. However, half of all cases (54%) were negative for HPV, p16 and Ki-67, thus represented a benign reactive atypia. Two AIM cases (5%) were negative for HPV and p16, but positive for Ki-67 in the area adjacent to an ulcer, consistent with regenerative atypia. Finally, 22% of AIM cases were positive for HPV and p16, and negative for Ki-67; such cases could represent a precursor of HSIL or, alternatively, a regressing HSIL. Our study has demonstrated that the combination of immunostaining for p16 and Ki-67 may be helpful in establishing the diagnosis in cases with histologic features borderline between HSIL and reactive/regenerative atypia and guide the recommendations for patient’s treatment and follow up.

Our study and previous reports have confirmed that p16 is a highly sensitive and specific marker of HSIL (37-39). However, positive p16 immunostaining is not entirely specific for HPV-related dysplasia in cervical glandular epithelium. For example, benign tubo-endometrioid and ciliated cell metaplasia of the endocervix both stain strongly for p16 (38). Furthermore, interpretation of p16 immunostaining requires some degree of experience; benign cases may show weak blush staining that does not correlate with detection of high oncogenic risk HPVs. In our study, blush was identified in 30% of benign cases.

Recently, the utility of Ki-67 and p16 immunostaining has been extended from application for histologic diagnosis to use in cytopathologic diagnosis of cervical smears. Double immunostaining for Ki-67 and p16 has been developed where the nucleus is immunolabeled red with anti-Ki-67 antibodies and the cytoplasm labeled dark-brown with anti-p16 antibodies. It has been shown that identification of double-positive cells (Ki-67 positive and p16 positive) in cervical smears has a 0.90 sensitivity and 0.95 specificity for detection of CIN2-3, or cancer (40).
Recommendations

Ki-67 and p16 were shown to be sensitive and specific markers of cervical high-grade squamous intraepithelial lesion, helpful in confirming the diagnosis in equivocal cases. Use of these stains may reduce the number of cases diagnosed as non-diagnostic atypia (AIM) by almost 80%.

Interpretation of p16 immunostaining requires some degree of experience and caution should be used to accurately recognize potential interpretative pitfalls:

- Weak blush p16 staining does not correlate with HPV DNA detection and should be recognized as a potential pitfall that may result in false positive interpretation.
- P16-positive benign tubo-endometrioid and ciliated cell metaplasia may be present on the surface of the squamous epithelium.
- P16 should not be used to diagnose LSIL in mature squamous epithelium due to low sensitivity (approx. 30%-50%).

Utility of epithelial and basement membrane markers for diagnosis of microinvasive squamous cell carcinoma of the cervix and vulva

Our study of double-immunostaining for cytokeratin and collagen IV/laminin in microinvasive vulvar and cervical squamous cell carcinomas (Chapter 10) showed accurate visualization of areas of invasion with these stains. The immunostaining for either of the basement membrane component, collagen IV or laminin, yielded the same results. A well-defined and continuous basement membrane (BM) was delineated in all cases of VIN3 and CIN3, however, a migration of malignant squamous cells through a discontinuous or absent basement membrane was observed in the invasive tumor fronts in all cases of vulvar and cervical carcinoma. Of interest, cases of verrucous carcinomas of the vulva showed continuous BM throughout the pushing tumor front with only rare, single tumor cells seen below the basement membrane. This finding is consistent with indolent local growth and lack of lymph node or distant metastasis in this type of tumor. Out of 20 cases initially diagnosed as “suspicious for invasion” using routine hematoxilin-eosin stain, 6 cases showed definitive foci of microinvasion clearly highlighted with the double immunostain, which also facilitated precise measurement of the depth of invasion for tumor staging.
Microinvasion in cervical carcinoma at the level of electron microscopy was described by Kudo et al (41). In areas of invasion, there was a disappearance of basal lamina of BM and pseudopod-like cytoplasmic protrusions of the cancer cells were seen in direct contact with the stroma. The cytoplasmic protrusions of the invading cells contained vesicles, some of which were opened onto the extracellular matrix; such vesicles were not observed in HSIL adjacent to intact BM. The authors suggested that the substances contained in the vesicles might play a role in BM destruction. In addition, the cells traversing the BM gaps showed accumulation of actin filaments in the pseudopod protrusions. These local aggregates of cytoskeletal structures, not observed in adjacent carcinoma-in situ, were thought to facilitate an amoeba-like movement of the cancer cells. Using double immunostaining in our study helped to visualize this process on the light microscopy level.

Since transition from noninvasive to invasive carcinoma is linked to the proteolytic destruction of the basement membrane, there is an interest in the diagnostic utility of proteolytic markers (41-44). Plasminogen plays an important role in early invasion because it has both the capacity to degrade extracellular matrix proteins, including basement membrane, and activate metalloproteases. Plasminogen is converted to active plasmin by either urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA). Two plasminogen activator inhibitors, PAI-1 and PAI-2, have been identified. Expression of uPA, and PAI-1 was shown to be upregulated in oral and pulmonary SCC. Several reports have described diagnostic utility of PAI-1, urokinase inhibitor and laminin immunostaining as markers of early invasion in squamous cell carcinoma and mucinous adenocarcinoma (42-43), however, in our laboratory we were not able to reproduce these results.

**Recommendations**

**Double-immunostaining for cytokeratin and collagen IV or laminin is useful for accurate visualization of microinvasion in cervical and vulvar carcinoma.**

- The staining is best used in cases where minimal, early invasion is suspected since already established invasive tumor nests may develop a secondary BM.
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Chapter 12

Summary
Summary

- **HPV detection and genotyping in the lower genital tract lesions**

  Our study of **HPV DNA detection in endocervical adenocarcinomas** (Chapter 2) demonstrated for the first time that mucinous adenocarcinomas (with exception of minimal deviation adenocarcinoma) and adenosquamous carcinomas of the cervix have a very high prevalence of HPV DNA, similar to that reported for cervical squamous cell carcinoma. In addition, our results suggested that rare histologic variants of cervical adenocarcinoma, such as minimal deviation, clear cell, serous and mesonephric adenocarcinoma were unrelated to HPV infection. In our study HPV DNA was detected in 82 of 90 mucinous adenocarcinomas (91%), encompassing endocervical, intestinal and endometrioid histologic subtypes, and in 9 of 9 adenosquamous carcinomas (100%). HPV DNA was not detected in any of 6 non-mucinous adenocarcinomas including clear cell, serous and mesonephric subtype. In addition, 2 cases of minimal deviation adenocarcinoma (MDA) were negative for HPV. Our results showed both a high prevalence of HPV in adenocarcinomas and a narrow spectrum of HPV genotypes limited mainly to HPV16 and HPV18. In our study HPV16 and/or HPV18 were found in 86% cases of invasive adenocarcinoma and 92% cases of adenocarcinoma in-situ (AIS). HPV45 (12%) and HPV52 (2%) were other single HPV genotypes identified in invasive adenocarcinomas and HPV45 (4%) and HPV35 (4%) were additional single HPV genotypes detected in AIS. High percentage of HPV positivity in AIS and invasive tumors allows for HPV testing as a screening tool while the relatively narrow spectrum of HPV genotypes detected in these tumors is very promising for high efficacy of current prophylactic vaccination programmes. Since the precursor lesions for clear cell, serous and mesonephric adenocarcinoma are unknown, and precursor of MDA is difficult to detect by cytologic screening, the prevention for these four subtypes of adenocarcinoma will still pose a challenge. Overall, non-HPV related tumors comprise less than 10% of cervical adenocarcinomas.

  In our study of **HPV detection in penile tumors** (Chapter 3) we comprehensively analyzed and compared HPV prevalence in four main histologic subtypes of penile carcinoma including keratinizing, verrucous, basaloid and warty squamous cell carcinoma. Overall, HPV DNA was detected in 42% cases of penile carcinoma, 90% cases of dysplasia, and 100% cases of
condyloma. The highest HPV detection rate was observed in warty and basaloid carcinomas (100% and 80%, respectively) and much lower in keratinizing and verrucous carcinomas (34.9% and 33.3% respectively). There were no significant differences in HPV DNA prevalence between tumor cases obtained from high incidence region (Paraguay) and low incidence region (USA). Overall, the prevalence of HPV DNA in penile carcinoma was lower than that in cervical carcinoma and similar to that in vulvar carcinoma. In addition, specific histological subtypes of penile cancer — warty and basaloid — were consistently associated with HPV, however, only a subset of keratinizing and verrucous penile carcinomas was positive for HPV DNA. These findings indicate multiple pathogenetic pathways in penile carcinogenesis, HPV-driven for all warty and basaloid tumors and a subset of keratinizing and verrucous carcinomas, and HPV-independent pathway for majority of keratinizing and verrucous tumors. Our study showed a narrow spectrum of HPV genotypes in warty-basaloid carcinomas (HPV16 - 75%, HPV35– 5% and HPV31– 5%) and a wide spectrum of genotypes in keratinizing carcinomas with HPV16 (17.9% of cases) being the most common. Overall, we have identified HPV16 and HPV18 in 25.5% and 1.4% of all cancer cases, respectively. Based on results of our study it may be expected that current HPV vaccines may prevent between 25 to 40% of penile cancer cases. The pathogenesis of penile carcinoma not related to HPV infection, which applies to 60%-75% of all cases, is still not well understood; alterations of p16/cyclinD/RB and p14/MDM2/p53 pathways have been identified by others in proportion of these tumors.

Our study of mucosal HPV and beta-PV (cutaneous HPV) detection in vulvar carcinoma consisted of 39 cases and included keratinizing, verrucous, basaloid and warty tumor types (Chapter 4). The overall positivity for mucosal HPVs was 35.9% with HPV16 and HPV33+51 accounting for 23% and 5% of all cases, respectively. Mucosal HPVs were detected in all but one (10/11) of warty and basaloid carcinomas. Of these tumors, 82% were positive for HPV16 and 18% for HPV types 33+51. Two of four verrucous carcinomas were positive for HPV type 6. Mucosal HPVs were not detected in any of the keratinizing carcinomas. In the immunohistochemical analysis, all cases of warty and basaloid carcinomas but none of the remaining tumors showed overexpression of p16 protein. In addition, all cases were tested for the presence of 25 most common beta-PVs, and were found to be negative. The results of our study reaffirmed the role of mucosal HPVs, and in particular that of HPV16, in the pathogenesis of warty and basaloid vulvar cancer. In addition, p16 immunostaining was shown to be a sensitive
and specific marker of vulvar carcinomas positive for oncogenic mucosal HPVs. A possible association between low oncogenic risk HPVs and development of verrucous carcinoma will require further studies. Beta-PVs were shown to be unlikely pathogens in vulvar carcinogenesis. Based on results of our study it is expected that current vaccines may prevent between 25 to 40% of vulvar cancer cases.

The results of our study of HPV genotyping in cases of VIN1 and VAIN1 demonstrated that although these two lesions have similar histologic features, they represent unrelated conditions with different viral association (Chapter 5). Our results showed that 70% of cases of VIN1 were associated with low oncogenic risk HPVs (HPV6, 11, 44 and 74). This suggests that majority of cases of flat VIN1 lesions are related to, or may be a precursor of exophytic condylomata acuminata, which are positive for low risk HPVs in 77-88% of cases. For the same reason, it is unlikely that VIN1 is a precursor of VIN3, which was shown to be associated exclusively with high risk HPVs. In contrast, 84% of VAIN1 lesions were found to be positive for high oncogenic risk HPVs (HPV16, 56, 18, 31, 35, 59, 68), similar to cervical CIN1, which has been reported to be associated with high risk HPVs in over 80% of cases. HPV16 and/or 18 accounted for 36.8% of VAIN1 cases and 9.0% of VIN1 cases. HPV6 and/or 11 accounted for 45.4% of VIN1 cases. Results of our study suggest that VIN1, a lesion associated predominantly with low-oncogenic risk HPVs does not require a follow up, however, VAIN1 may have a potential of progression to a high-grade squamous intraepithelial lesion in immunosuppressed patients and such patients should be followed with regular colposcopic exams.

**Role of molecular markers in diagnosis of HPV-related lesions**

In our studies of low grade vulvar, vaginal and cervical squamous intraepithelial neoplasia and vulvar condyloma we have demonstrated that Ki-67 is a sensitive and specific marker of low grade dysplasia and condyloma (Chapter 5, 6 and 7). For our studies we have first established gold standard diagnoses using consensus review in correlation with HPV detection. After the cases were re-classified according to gold standard, Ki-67 immunostaining was performed on all cases. The positive result was defined as presence of a cluster of at least two strongly-stained epithelial nuclei in the upper two-thirds of the epithelial thickness. With such definition of Ki-67 positivity, we have found almost complete concordance between
positive Ki-67 immunostaining, detection of HPV DNA in tissue sections and gold standard diagnosis of low grade dysplasia/condyloma. The sensitivity of Ki-67 as a marker of low grade vulvar/vaginal and cervical lesions, and vulvar condyloma was 0.96, 1 and 1, respectively. The specificity of Ki-67 as a marker of low grade vulvar/vaginal and cervical dysplasia, and condyloma was equally high, 0.9, 1 and 1, respectively. An audit of consecutive biopsies stained with routine hematoxilin-eosin stain and diagnosed as VIN1, VAIN1, CIN1, and condyloma by pathologists with varying diagnostic experience revealed overdiagnosis in 31%-36% of cases.

Recommendations

Ki-67 positivity defined as the presence of positive nuclei in the upper two-thirds of the epithelial thickness was shown to be a sensitive and specific marker of vulvar, vaginal and cervical low grade squamous intraepithelial lesions, and condyloma acuminatum. The stain may be used in equivocal cases to confirm the histologic diagnosis. Use of Ki-67 is especially recommended for pathologists with limited diagnostic experience because of significant potential of overdiagnosis.

In quantitative analysis of Ki-67 expression in normal endocervix, benign glandular lesions and endocervical adenocarcinoma we have found that Ki-67 index below 20% always reflected a benign process, while Ki-67 index higher than 50% was always indicative of neoplasia (Chapter 8). The range of positivity in adenocarcinoma in situ was 45% to 73% and 25% to 84% in invasive adenocarcinoma. Normal endocervical epithelium showed range of positivity from 0% to 5%, however, rare benign cases, such as endometriosis, tubal metaplasia during proliferative phase and cases of regenerating epithelium demonstrated increased proliferative activity of up to 32%, and thus overlapping with neoplastic cases.

Recommendations

Ki-67 was also shown to be a sensitive and a specific marker of endocervical neoplasia: Ki-67 index below 20% reflects a benign process, while Ki-67 index higher than 50% indicates neoplasia (AIS or invasive adenocarcinoma).

Atypical immature squamous metaplasia (AIM) of the cervix is a term used for cases with atypia suggestive but not diagnostic of HSIL. In our study we have examined the utility of p16 and Ki-67 in the diagnosis of high-grade cervical squamous intraepithelial lesion and
atypical immature squamous metaplasia (Chapter 9). All cases of HSIL, which were used as positive control, were positive for high-risk HPV DNA, p16 and Ki-67 immunostains. P16 positivity was defined as strong, diffuse staining involving either the whole thickness or lower two-thirds of the epithelium. The definition of Ki-67 positivity was a presence of at least two strongly-stained epithelial nuclei in the upper two-thirds of the epithelial thickness. One-fifth (19%) of cases of AIM showed positivity for HPV, p16 and Ki-67 in the pattern identical to HSIL and therefore these cases appear to represent a spectrum of HSIL and may be reclassified as such. Over a half (54%) of AIM cases were negative for HPV, p16 and Ki-67, thus representing a benign reactive atypia. Two AIM cases (5%) were negative for HPV and p16 but positive for Ki-67 in the area adjacent to an ulcer, consistent with regenerative atypia. Finally, one-fifth (22%) of AIM cases were positive for HPV and p16, and negative for Ki-67; such cases may represent a precursor of HSIL or, alternatively, a regressing HSIL.

Recommendations

Ki-67 and p16 were shown to be sensitive and specific markers of cervical high-grade squamous intraepithelial lesion, helpful in confirming the diagnosis in equivocal cases. Combination of immunostaining for p16 and Ki-67 may be helpful in establishing the diagnosis in cases with features borderline between HSIL and reactive/regenerative atypia and may guide the recommendations for further treatment and follow up. The use of the stains may reduce the number of cases of non-diagnostic atypia (AIM) by almost 80%.

In our study of double-immunostaining for cytokeratin and collagen IV/laminin in microinvasive vulvar and cervical squamous cell carcinoma we have identified that the staining was very useful for accurate visualization of areas of invasion (Chapter 10). The immunostaining for either of the basement membrane component - collagen IV or laminin yielded the same results. A well-defined and continuous basement membrane was delineated in all cases of VIN3 and CIN3, however, a migration of malignant squamous cells through a discontinuous or absent basement membrane was observed on the invasive tumor fronts in all cases of vulvar and cervical carcinoma. Of interest, cases of verrucous carcinoma of the vulva showed continuous BM throughout the pushing tumor front with only rare, single tumor cells seen below the basement membrane. Out of 20 cases initially diagnosed as “suspicious for
invasion” using routine hematoxilin-eosin stain, 6 cases showed definitive foci of microinvasion clearly highlighted with the double immunostain, which also facilitated precise measurement of depth of invasion for pathologic tumor staging. The staining is only suited for these cases in which minimal, early, invasion is suspected since already established invasive tumor nests may develop a secondary BM.

Recommendations

Double-immunostaining for cytokeratin and collagen IV or laminin is useful for accurate visualization of microinvasion in cervical and vulvar carcinoma.
RESUME
Dr. Edyta Catalina Pirog received her MD in 1989 from Medical University of Warsaw, Poland, graduating with the highest honors. She was awarded a Mayor of Warsaw Scholarship for Achievements in Research in the area of gynecologic pathology and came to the United States to continue research pursuits. Dr. Pirog enrolled in two consecutive postdoctoral fellowships - at Emory University School of Medicine and University of Kentucky College of Medicine, respectively. Subsequently, Dr. Pirog completed residency training in pathology at Columbia University, followed by fellowships in gynecologic pathology at Columbia University and at Weill Medical College of Cornell University. Upon completion of her training Dr. Edyta Pirog was appointed Instructor, then Assistant Professor, and currently Associate Professor of Pathology and Laboratory Medicine at Weill Medical College.

Dr. Edyta Pirog is a diagnostic pathologist with special expertise in gynecologic pathology and gynecologic cytopathology. Dr. Edyta Pirog is pursuing research in the field of Human Papillomavirus (HPV) infections and pathobiology of HPV-related lesions of the lower anogenital tract.

PUBLICATIONS

Original papers:


Invited review papers and book chapters:


