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

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

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For all of the manuscripts Dr. Edyta Pirog designed the study, collected and reviewed the cases, performed all or portion of laboratory testing, analyzed the results and wrote the manuscript.
Chapter 1

Introduction

Aim and outline of the thesis
Introduction

1. Pathobiology of genital tract lesions caused by Human Papillomavirus infection

Human Papillomavirus (HPV) is the single most important pathogen of the lower genital tract. Human Papillomaviruses cause benign and malignant lesions of the cervical, vaginal, vulvar, and penile mucosa and skin.

HPVs are epitheliotropic DNA viruses that are typed based on their DNA sequence and subgrouped into cutaneous and mucosal types, with the latter group further subclassified into low and high oncogenic risk types (1, 2). High oncogenic risk HPVs are currently considered to be the main causative factors of cervical carcinogenesis (2). In addition, high oncogenic risks HPVs have been detected in cancers of the anogenital and oropharyngeal mucosa in both women and men (3-22). These tumors include squamous cell carcinomas of the vagina (3), vulva (3, 4), penis (5-7), anus (8-11), tonsils (12, 13) as well as other oropharyngeal sites (14-22). Low oncogenic risk HPVs cause anogenital condyloma acuminata (23, 24).

Human Papillomavirus infection

Genital HPV infections are extremely common; in the USA there are an estimated 6.2 million new infections annually (25). Most HPV infections are asymptomatic and do not result in epithelial changes, therefore, they are not detected by either visual exam or by cytologic screening tests (Pap tests). Figure 1-1 shows the age-dependent prevalence of HPVs in the US in cervical smears of women with normal Pap test results (26). The high peak of HPV prevalence in 20-24 year-olds is related to sexual début, while the subsequent decrease in prevalence reflects the acquisition of immunity and monogamous relationships (27).
Most HPV infections are transient and are eliminated by the immune response over the course of months (28). Typically, 50% of HPV infections clear within 8 months and 90% of infections are cleared within 2 years (28, 29). The duration of the infection is also related to HPV type. On average, infections with high oncogenic risk HPVs last longer than infections with low oncogenic risk HPVs, 13 months versus 8 months, respectively (28). Persistent infection with high oncogenic risk HPVs increases the risk of development of premalignant lesions and subsequent carcinoma. It is still unclear why only a fraction of women infected with HPV develop epithelial lesions. Immunosuppression, persistent infection and age >30 years have all been well-documented risk factors for the development of symptomatic infection (29).

Mucosal HPVs infect immature, basal cells of the squamous epithelium in areas of epithelial breaks resulting from injury or immature basal and squamous metaplastic cells present at the squamo-columnar junctions (SCJ) (30, 31) (Figure 1-2).
Less often, HPVs infect glandular cells or neuroendocrine cells from the SCJ regions. SCJs are present in the cervical, anal, and oropharyngeal mucosa. Consequently, these mucosal areas are most susceptible to HPV infections. HPVs do not infect the mature superficial squamous cells that cover the ectocervix, vagina, or vulva. Establishment of HPV infection in these sites requires disruption of the surface epithelium which allows the virus to access the immature cell layer at the base of the epithelium (30, 31). The cervix, with its relatively large area of immature squamous metaplastic epithelium, is particularly vulnerable to HPV infection. This is in contrast to vulvar mucosa and skin which is covered by mature squamous cells. These differences in epithelial susceptibility to HPV infection result in different incidence of HPV-related cancers arising in various anatomical sites, and explain high incidence of cervical cancer in women or anal cancer in men who have sex with men and low incidence of vulvar and penile cancers (32, 33).

Although the virus can infect only immature squamous cells, the replication of HPV occurs in maturing squamous cells and results in a cytopathic effect termed “koilocytic atypia” which consists of nuclear atypia and cytoplasmic perinuclear halos (30, 31). In order to replicate, HPV has to induce DNA synthesis in host cells. Since HPV replicates in maturing, already non-
proliferating squamous cells, it has to reactivate the mitotic cycle in cells (30, 31). Experimental molecular genetic studies have shown that HPV activates the cell cycle by interfering with the function of the tumor suppressor proteins, retinoblastoma protein (pRB), and p53 (34). It has been shown that the HPV oncoprotein E7 binds pRB and thereby prevents its association with E2F transcription factors. Rising levels of free E2Fs activate DNA synthesis and cell, as well as viral, replication. Further, HPV oncoprotein E6 decreases the intracellular levels of p53 protein by stimulating its degradation (34). By interfering with pRB and p53 functions, high oncogenic risk HPVs initiate genetic events that may ultimately result in malignancy (Figure 1-3). Oncoproteins of low oncogenic risk HPVs have been shown to have a lower affinity for p53 and pRB, conferring a minimal or nil risk of malignancy (34).

Figure 1-3 Schematic representation of the cell cycle changes caused by HPV infection.

Infections of the squamous epithelium by mucosal HPVs most commonly result in productive infections (Figure 1-4 a) whereby the virus completes its life cycle by packaging replicated DNA into the capsid proteins (L1). This is followed by disruption of the cytoskeleton facilitated by oncoprotein E4 and release of the infected nuclei into the environment (30, 31). Rarely, in immature squamous metaplasia, HPV cannot complete its cycle, resulting in
transforming/abortive infection. In such cases, the expression of E7 is markedly upregulated and extends from its normal basal location to the superficial layers of the epithelium. In such cases viral DNA replication and packaging is significantly diminished and viral DNA may become integrated within the host genome. High levels of E7 and inhibition of pRB stimulate cellular proliferation and expansion of immature basal squamous cells towards the epithelial surface (Figure 1-4 b and c) (30, 31).

Figure 1-4 Schematic representation of HPV life cycle: a – productive infection with completion of life cycle in the superficial layers of the epithelium (high expression of E4, L1 and high viral DNA content), b and c – transforming/abortive infection with inefficient viral replication and high cellular proliferation (expanded expression of E7, decreased expression of E4, L1 and lower viral DNA content) (reproduced with permission from J Clin Virol 2005, 32S, S7) Copyright 2005 Elsevier.

In addition to infecting squamous cells, HPVs may occasionally infect glandular, reserve or neuroendocrine cells present in the cervical mucosa and cause their malignant transformation resulting in adenocarcinomas, adenosquamous and neuroendocrine carcinomas. These tumor subtypes are less common since glandular, reserve and neuroendocrine cells do not support productive HPV infections (35).
Classification of genital lesions caused by HPV infection

VULVA/PENIS
- Condyloma acuminatum
- Vulvar/penile intraepithelial neoplasia (low and high grade squamous intraepithelial lesions)
- Vulvar/penile squamous cell carcinoma: basaloid and warty subtype

VAGINA
- Condyloma acuminatum
- Vaginal intraepithelial neoplasia (low and high grade squamous intraepithelial lesions)
- Vaginal squamous cell carcinoma

CERVIX
- Condyloma acuminatum
- Cervical intraepithelial neoplasia (low and high grade squamous intraepithelial lesions)
- Cervical carcinoma: squamous cell carcinoma, endocervical adenocarcinoma, adenosquamous carcinoma, neuroendocrine carcinoma.

Condyloma acuminatum
Condylomata acuminata are benign lesions that involve skin or mucosal surfaces and display a distinctive warty appearance (Figure 1-5 A). Typically, these lesions are multiple and multifocal involving vulvar, penile, and perianal regions as well as the vagina and, less commonly, the cervix. Condylomata acuminata are caused by low oncogenic risk HPVs, principally types 6 and 11, and are only occasionally due to high oncogenic risk HPVs (23, 24). On histologic examination, they consist of branching, papillary cores of stroma covered by thickened squamous epithelium with viral cytopathic effect. Condylomata represent productive viral infections where HPV replicates and completes its life cycle in mature superficial squamous epithelium causing distinct cytologic changes, known as “koilocytic atypia”. These are characterized by nuclear enlargement, hyperchromasia, coarseness of chromatin, variations in
nuclear sizes and shapes and cytoplasmic perinuclear halos which indicate disruption of the cytoskeleton prior to release of the virus (Figure 1-5 B). The degree of viral cytopathic change depends on the duration of the lesion; koilocytic atypia may be quite prominent in recent infection but diminishes in regressing infections. Condylomata acuminata are not considered to be premalignant lesions (36).

Figure 1-5 A. Condyloma acuminatum showing branching, papillary cores of stroma covered by thickened squamous epithelium (x 100); B. Koilocytosis characterized by nuclear enlargement, hyperchromasia, coarseness of the chromatin, variations of nuclear sizes and shapes and cytoplasmic perinuclear halo (x 200).

Cervical intraepithelial neoplasia (low and high grade squamous intraepithelial lesions)

The terminology and classification of cervical squamous dysplasia has evolved over time and terms from different classification systems are currently used interchangeably (37). The oldest classification system used “dysplasia” terminology with a three-tier grading system of mild, moderate, and severe dysplasia (Table 1). This was followed by a three-tier “cervical intraepithelial neoplasia” (CIN) classification, where mild dysplasia was renamed CIN1, moderate dysplasia as CIN2, and severe dysplasia as CIN3. Since patient management is two tiered, observation versus surgical treatment, the three tier classification system has recently been simplified to a two tiered system to facilitate clinical follow-up. CIN1 was renamed low grade
**squamous intraepithelial lesion** (LSIL) and CIN2 and CIN3 were combined into **high grade squamous intraepithelial lesion** (HSIL) (37).

**Table 1.** Classification systems for premalignant cervical lesions

<table>
<thead>
<tr>
<th>Dysplasia/Carcinoma-in-situ</th>
<th>Cervical Intraepithelial Neoplasia (CIN)</th>
<th>Squamous Intraepithelial Lesion (Current Classification)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mild dysplasia</td>
<td>CIN 1</td>
<td>Low grade squamous intraepithelial lesion (LSIL)</td>
</tr>
<tr>
<td>moderate dysplasia</td>
<td>CIN 2</td>
<td>High grade squamous intraepithelial lesion (HSIL)</td>
</tr>
<tr>
<td>severe dysplasia carcinoma-in-situ</td>
<td>CIN 3</td>
<td>High grade squamous intraepithelial lesion (HSIL)</td>
</tr>
</tbody>
</table>

CIN1 and some CIN2 lesions represent productive viral infections without significant disruption or alteration of the host cell cycle (31, 34). Most CIN1 and some CIN2 regress spontaneously, with only a small percentage progressing to CIN3. CIN1 do not progress directly to invasive carcinoma and, for this reason, CIN1 is not considered to be a premalignant lesion (34). CIN2 and CIN3 represent a progressive deregulation of the cell cycle by HPV and result in increased cellular proliferation, decreased or arrested epithelial maturation, and a lower rate of viral replication compared to CIN1. Derangement of the cell cycle in CIN3 may become irreversible and lead to a fully transformed malignant phenotype (31, 34).

**Figure 1-6 A and B** illustrates CIN1 and CIN3. The diagnosis of CIN is based on identification of the viral cytopathic effect, koilocytic atypia, or nuclear atypia, as previously described. The grading of CIN is based on the expansion of the immature cell layer from its normal, basal location. The immature basal cells extend to the lower one-third of the epithelium in CIN 1, to the middle-third in CIN 2, and to the top-third in CIN3 (37).
Vulvar, penile and vaginal intraepithelial neoplasia

The histologic features of vulvar, penile and vaginal squamous dysplasias are similar to those of cervical intraepithelial neoplasia and the terminology parallels the cervical classification: vulvar intraepithelial neoplasia 1 (VIN1), penile intraepithelial neoplasia 1 (PIN1), vaginal intraepithelial neoplasia 1 (VAIN1) categories represent benign productive viral infections, and VIN2-3, PIN2-3 and VAIN2-3 represent lesions with progressive, premalignant potential (38, 39).

Vulvar carcinoma

Carcinoma of the vulva is an uncommon malignant neoplasm which is approximately one eighth as frequent as cervical cancer. Squamous cell carcinoma (SCC) is the most common histologic type and is divided into two groups: keratinizing and basaloid/warty carcinomas. Keratinizing squamous cell carcinomas develop in a background of chronic inflammatory
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Disease and account for approximately 70% of all cases. The basaloid and warty carcinomas, which are related to infection with high oncogenic risk HPVs, account for an additional 30% of cases (40).

Invasive basaloid and warty carcinomas develop from precancerous in-situ lesions called classic vulvar intraepithelial neoplasia 3 (classic VIN3) (41, 42). On histologic examination, basaloid carcinomas (Figure 1-7 A) demonstrate an infiltrating tumor characterized by nests and cords of small, tightly packed malignant squamous cells lacking maturation and resembling immature squamous cells from the basal layer of normal epithelium. The tumor may also have foci of central necrosis. Warty carcinoma is characterized by an exophytic, papillary architecture composed of highly mature, keratinized epithelium with prominent koilocytic atypia (Figure 1-7 B) (40).

Figure 1-7 A. Basaloid carcinoma composed of cords of small, tightly packed malignant squamous cells lacking maturation (x 200); B. Warty carcinoma with papillary architecture, prominent keratinization and marked koilocytic atypia (x 200). (reproduced with permission from Mod Pathol 2008;21:334-344)

Keratinizing squamous cell carcinomas of the vulva frequently arise in patients with long-standing lichen sclerosus or squamous cell hyperplasia and histologically resemble SCCs
arising in non-genital skin. These tumors are composed of invasive nests and tongues of malignant squamous epithelium with prominent central keratin pearls (Figure 1-7 C) (40). The immediate premalignant lesion is referred to as differentiated vulvar intraepithelial neoplasia (differentiated VIN) or VIN simplex and is characterized by marked atypia of the basal layer of the epithelium with normal epithelial maturation and differentiation in the superficial layers. The etiology of differentiated VIN is unknown. Since it is frequently seen in a background of lichen sclerosus or squamous cell hyperplasia, it is postulated that chronic epithelial irritation may contribute to gradual evolution of this malignant phenotype (41).

Figure 1-7 C. Keratinizing squamous cell carcinoma with prominent central keratin pearls (x 100); D. Verrucous carcinoma displaying papillary architecture and prominent keratinization but lacking koilocytic atypia (x 100). (reproduced with permission from Mod Pathol. 2008;21:334-344)

Rare variants of vulvar carcinoma include verrucous carcinoma (Figure, 1-7 D), a fungating tumor clinically resembling condyloma acuminatum (43). Although the tumor is well differentiated and does not infiltrate into underlying structures or penetrate vascular channels, the
base of the lesion shows a pushing and expansive tumor growth. The association with HPV is uncertain (43). The tumor shows indolent local growth and metastases are very rare.

**Penile carcinoma**

The histologic subtypes of penile carcinoma are similar to those described in the vulva, however, their precursor lesions have been less well characterized (39). The majority of tumors are well differentiated, keratinizing SCCs which may develop within penile skin or mucosa. The verrucous, basaloid and warty carcinomas arise most frequently on penile mucosal surfaces and often involve the glans (44, 45).

**Vaginal carcinoma**

Primary carcinoma of the vagina is an extremely uncommon cancer. Almost all tumors are squamous cell carcinomas associated with high oncogenic risk HPVs. The main risk factor is prior carcinoma of the cervix or the vulva and 1% to 2% percent of patients with cervical carcinomas eventually develop vaginal squamous cell carcinoma. VAIN3 is a precursor lesion of squamous cell carcinoma of the vagina (46).

**Cervical carcinoma**

Squamous cell carcinoma is the most common histologic type of cervical cancer and accounts for approximately 80% of cancer cases (47). As previously described, CIN3 is an immediate precursor of cervical SCC. Cervical adenocarcinoma accounts for approximately 15% of cervical cancer cases (Table 2) (48). The most common, usual type of cervical adenocarcinoma develops from a precursor lesion, adenocarcinoma in-situ. Adenosquamous and neuroendocrine carcinomas are rare cervical tumors representing less than 5% of cancer cases (35). Adenosquamous tumors develop from adenosquamous carcinoma in-situ. The neuroendocrine carcinomas are aggressive, fast growing tumors, typically presenting at an advanced stage and their precursor lesions have not yet been identified (35). High oncogenic HPVs have been detected in virtually all cervical squamous cell carcinomas, adenosquamous, and neuroendocrine carcinomas, as well as in a subset of adenocarcinomas (35, 47, 49).
Table 2. Histologic subtypes of cervical carcinoma.

I. Squamous cell carcinomas
   - keratinizing /nonkeratinizing
II. Adenocarcinomas
   - endocervical (usual type)
   - endometrioid
   - intestinal
   - minimal deviation (adenoma malignum)
   - clear cell
   - serous
   - mesonephric
III. Adenosquamous carcinomas
   - adenosquamous
   - adenoid basal (adenoid basal epithelioma)
   - glassy cell
IV. Neuroendocrine carcinomas
   - carcinoid tumor
   - large cell neuroendocrine carcinoma
   - small cell neuroendocrine carcinoma

On histologic examination, SCC are composed of invasive nests and tongues of malignant squamous epithelium, either keratinizing or non-keratinizing, invading the underlying cervical stroma (Figure 1-8 A).

Figure 1-8 A. Cervical squamous cell carcinoma with invasive nests of keratinized malignant epithelium (x 200); B. Endocervical adenocarcinoma, usual type, composed of invasive mucinous glands (x 200).
The most common type of cervical adenocarcinoma is a prototypical endocervical adenocarcinoma, also termed “usual type” (49). It is characterized by proliferation of malignant endocervical glands lined by cells with large, hyperchromatic nuclei, and variable amounts of intracytoplasmic mucin (Figure 1-8 B). Less common subtypes of cervical adenocarcinomas include other mucinous tumors, such as intestinal, endometrioid, and minimal deviation adenocarcinoma, and non-mucinous tumors including mesonephric, clear cell, and serous adenocarcinomas (49). The intestinal subtype of cervical adenocarcinomas is characterized by the presence of goblet cells (Figure 1-9 A). Endometrioid cervical tumors of the cervix have scant, but densely eosinophilic cytoplasm (Figure 1-9 B), resembling adenocarcinomas of the endometrium.

**Figure 1-9** A. Endocervical adenocarcinoma, intestinal type, with characteristic goblet cells (x 200); B. Endocervical adenocarcinoma, endometrioid type, with scant, densely eosinophilic cytoplasm (x 200).

Minimal deviation adenocarcinoma is a malignancy with minimal cytologic atypia of the invasive mucinous glands, which can present significant diagnostic challenge (Figure 1-10 A). Mesonephric adenocarcinomas originate from the mesonephric embryonic remnants and typically develop within the deep portion of the lateral cervical walls. These tumors may show a wide range of architectural patterns, however, most characteristically they are composed of small cystic spaces with eosinophilic secretions (Figure 1-10 B).
Figure 1-10  A. Minimal deviation adenocarcinoma, an invasive tumor with minimal cytologic atypia (x 400); B. Mesonephric adenocarcinoma composed of small cystic spaces with eosinophilic secretions (x 400).

Clear cell and serous adenocarcinomas of the cervix resemble their endometrial and ovarian counterparts. Clear cell carcinomas of the cervix have a characteristic papillary, tubulocystic or solid growth pattern with tumor cells which demonstrate pale cytoplasm and markedly atypical nuclei (Figure 1-11 A). Serous carcinomas are composed of papillary fronds or complex glands lined by cuboidal cells with deeply eosinophilic-purple cytoplasm and markedly atypical nuclei (Figure 1-11 B).

Figure 1-11  A. Cervical clear cell carcinoma with solid growth of cells with pale cytoplasm and markedly atypical nuclei (x 200). B. Cervical serous adenocarcinoma with papillary fronds lined by cells with deeply eosinophilic-purple cytoplasm and markedly atypical nuclei (x 200).
Adenosquamous carcinomas are tumors composed of malignant epithelium with areas of glandular and squamous differentiation. Neuroendocrine cervical carcinomas typically have an appearance similar to small cell carcinomas of the lung. In contrast to lung tumors, which are not related to HPV infection, cervical small cell carcinomas are positive for high oncogenic risk HPVs (50).

2. Molecular markers of HPV-related genital lesions

Histopathologic interpretation of tissue sections forms the basis for the appropriate management of patients with genital HPV-related lesions. Since genital dysplasia is related to sexual transmission of a potentially oncogenic virus, a patient with this diagnosis faces significant therapeutic, reproductive, sexual, and social consequences (51). Over-diagnosis can lead to needless anxiety as well as unnecessary treatments and iatrogenic complications such as cervical incompetence, chorioamnionitis, and premature birth. Despite well-defined criteria, there is considerable interobserver variation in the diagnosis of condylomas, low and high grade squamous intraepithelial lesions, adenocarcinoma in-situ and microinvasion in squamous cell carcinomas (52-54). In equivocal cases, verification of the morphologic impression with an objective test may be necessary for accurate diagnosis.

HPV testing and molecular markers for diagnosis of condyloma, low and high squamous intraepithelial lesions and adenocarcinoma in situ.

HPV DNA detection may be used to confirm the diagnosis of condyloma, dysplasia or carcinoma (55). However, due to the high cost of labor-intensive techniques, HPV detection in tissue sections is still not routinely performed. HPV detection in formalin-fixed, paraffin-embedded tissue is best accomplished with commercially available tests, like SPF10-LiPA PCR (version 1; Labo Bio-Medical Products, Rijswijk, The Netherlands), while the commercially available in-situ hybridization tests are generally not as sensitive and specific (56). Subclinical HPV infections, where histologically normal tissue tests positive for HPV DNA, create a shortcoming for HPV testing by decreasing its specificity (55). However, a new technology of combination of laser-capture microscopy (LCM) with a PCR system for detecting and typing
HPV can help to avoid such false positive errors resulting from viral presence on the tissue surface or in the surrounding mucus. In addition, LCM-PCR can be used to determine exact HPV type in a specific tissue location, and assign HPV type to histologic changes in multiple HPV-type infections (55a).

Since HPV testing on tissue sections may not be routinely available in many pathology laboratories, molecular markers of HPV infection including Ki-67 and p16, have been identified.

**Ki-67** is a nuclear nonhistone protein expressed throughout the mitotic cycle with the exception of the G0 phase. Since HPV infection is associated with an increase in proliferation of the squamous and glandular epithelium, it has been suggested that immunostaining with anti-Ki-67 antibody may be useful in the diagnosis of SIL and endocervical neoplasia (57, 58). The testing may be performed with either polyclonal anti–Ki-67 antibodies or with MIB-1, a monoclonal antibody recognizing a Ki-67 epitope yielding the same staining results. In normal squamous mucosa or skin, Ki-67 positivity is found exclusively in the nuclei of parabasal squamous epithelium (57, 58). In cases with productive or transforming HPV infections, there is increased mitotic activity of the keratinocytes with extension of the proliferating cells into the intermediate and superficial epithelial layers (57, 58). In such cases Ki-67 positive nuclei are seen in the parabasal area, as well as, in the superficial 2/3 of the epithelium (Figure 2-1A). In adenocarcinoma in-situ, there is a diffuse increase in Ki-67 staining in the affected glands.

**P16**, a cyclin kinase inhibitor, is a regulatory protein that inhibits the cell cycle by preventing phosphorylation of retinoblastoma tumor suppressor protein (pRB). In cells infected with hrHPV, there is a functional overexpression of p16 mediated by E2F transcription factors. Despite high levels of p16, the hrHPV-infected cells continue to proliferate because pRB, the target of p16 inhibitory activity, is inactivated by the E7 HPV oncoprotein (59). P16 has been shown to be a sensitive marker of cells with active expression of E7 oncoprotein. A 2-tier scoring system is used to evaluate p16 staining. No staining or discontinuous, patchy staining pattern is considered a negative result (60). A positive result is recognized as diffuse and strong nuclear and cytoplasmic staining (Figure 2-1B) (60). A strong and diffuse p16 immunostaining was reported in 97%-100% of cervical squamous cell carcinomas and adenocarcinomas and in 92%-100% cases of HSIL (Figure 2-1B) and adenocarcinoma in-situ (58-62). P16, however, has limited value as a marker of LSILs since on average the stain is positive in less than 40% of cases (62).
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Figure 2-1 A. Positive Ki-67 immunostaining in HSIL: Ki-67 positive nuclei are seen in the upper two-thirds of the epithelial thickness (x 200); B. Positive p16 immunostaining in HSIL: diffuse and strong nuclear and cytoplasmic staining is present (x 200) (reproduced with permission from Arch Pathol Lab Med 2007;131:1346). Copyright 2007 CAP.

Molecular markers of microinvasion

In order to aid the diagnosis of microinvasion in cervical carcinomas, there has been an interest in the utilization of stains for basement membrane (BM) components. However, initial studies showed that a well formed BM was present in a proportion of invasive tumors and even in metastatic lesions (63, 64). Well differentiated tumors were more likely to show positive staining for BM components, while poorly differentiated tumors were more likely to loose the ability to produce BM and lacked staining (65). Although many tumors were shown to produce BM, definitive BM gaps were described at the invasive tumor fronts or in areas of microinvasion (Figure 2-2) (63-65).

In order to explain these conflicting observations, Liotta (66) suggested that tumor nests proceed through cycles of growth surge with BM destruction and budding of the tumor cells into the surrounding stroma followed by quiescence and new BM formation. The mere presence or absence of basement membrane components is not sufficient evidence for the presence or absence of invasion.
**Figure 2-2** Double immunostaining for cytokeratin (red) and collagen IV (black) in microinvasive vulvar carcinoma (x 100), a gap in the basement membrane is visualized through which the neoplastic squamous cells are seen invading the underlying stroma.

However, visualization of the neoplastic epithelial cells migrating through the BM gaps into the stroma provides convincing evidence of stromal invasion. Using double immunostaining for cytokeratin and basement membrane components such as collagen IV or laminin may be of interest in evaluation of early invasion in cervical and vulvar carcinomas (**Figure 2-2**).
Aim and outline of the thesis

This thesis investigates the prevalence of HPV in lower genital tract lesions and establishes the role of molecular markers as adjunct tests for accurate diagnosis of HPV-related lesions.

1. HPV detection and genotyping in the lower genital tract lesions.

HPV detection and genotyping in cervical adenocarcinoma

The etiology of squamous cell carcinoma of the cervix (SCCx), the most common type of cervical malignancy, is linked to infection with high oncogenic risk HPVs. However, the pathogenesis of cervical adenocarcinoma (AdCx) is not as well characterized. Although HPV DNA is consistently detected in almost 100% of SCCx (67), the reported prevalence of HPV DNA in AdCx varies significantly, from approximately 50% to over 90%, depending upon detection methods (68, 69). Cervical adenocarcinomas include several distinct histologic subtypes: endocervical, intestinal, endometrioid, minimal deviation, serous, clear cell and mesonephric (40). Many of the studies involving endocervical adenocarcinomas have not analyzed the different histologic subtypes separately. Lack of separation of histologic types may have confounded the results of HPV studies. To further investigate the relationship between HPV and cervical adenocarcinoma, we examined a large number of tumors encompassing a broad spectrum of morphological differentiation, including mucinous and non-mucinous adenocarcinomas and related tumors with adenosquamous differentiation. HPV genotyping was performed using SPF 10 PCR and LiPA tests. The results are presented in Chapter 2.

HPV detection and genotyping in penile carcinoma

The pathogenesis of penile carcinoma (PC) has not been clearly established. The reported prevalence of HPV in PC is highly variable, from 15 to 71%, depending on the sensitivity of the detection method and selection of tumor subtype (5-7). Another puzzling epidemiologic detail is the incidence of PC showing marked geographic differences. The incidence is lowest in Western Europe and North America and highest in South America and Africa (70). In order to investigate the relationship between HPV and PC, we examined a large number of cases collected in North
and South America (United States and Paraguay, respectively) encompassing main histologic tumor subtypes. We wanted to answer several questions in this study: 1) What are the pathways of penile carcinogenesis?; 2) Are the various histological subtypes of penile carcinoma etiologically related?; 3) Are there differences in frequency of the various histological tumor subtypes between high- and low-risk geographical regions (Paraguay versus the United States)?; 4) Are there differences in HPV prevalence in tumors from high- and low-risk geographical regions (Paraguay versus the United States)? The results are presented in Chapter 3.

**HPV detection and genotyping in vulvar carcinoma**

Results of epidemiologic, clinico-pathologic and virologic studies support at least two independent pathways of vulvar carcinogenesis. The first is related to infection with oncogenic mucosal HPVs and the second, although still not well characterized, is related to chronic inflammatory processes involving vulvar mucosa and skin. Vulvar cancers associated with oncogenic mucosal HPVs typically belong to basaloid or warty histologic subtypes and are almost invariably associated with HPV16 (71, 72). The pathogenesis of vulvar carcinomas not associated with mucosal HPVs is still not established. There is recent evidence supporting a role for cutaneous HPV types belonging to the beta-genus (betaPVs) in the pathogenesis of SCC of skin (73, 74). The goal of the study was to examine a possible role of cutaneous HPVs from the beta genus in vulvar carcinogenesis, evaluate the distribution of viral genotypes in vulvar cancers positive for mucosal HPVs, and correlate the detection of mucosal HPVs with overexpression of p16 protein in tumor sections. The results are presented in Chapter 4.

**HPV detection and genotyping in vulvar intraepithelial neoplasia 1 (VIN1) and vaginal intraepithelial neoplasia 1 (VAIN1)**

The pathobiology of vulvar and vaginal intraepithelial neoplasia 1 (VIN1/VAIN1) has not been well studied and the association with specific HPV genotypes is not known. Other vulvar and vaginal lesions have been extensively investigated and it has been established that vulvar/vaginal condyloma acuminatum are benign growths that have been shown to harbor low risk HPVs, most commonly HPV 6 or HPV 11 (23, 24). VIN3/VAIN 3, on the other hand, are premalignant lesions associated with high oncogenic risk HPVs, specifically HPV 16 (4, 46). The goal of our study, using HPV genotype analysis, was to determine whether VIN1/VAIN1 are
biologically related to benign condyloma, or if they are precursors of VIN3/VAIN 3. The results are presented in Chapter 5.

2. Role of molecular markers in the diagnosis of HPV-related lesions

Viral and molecular markers of low grade squamous intraepithelial lesions (VAIN1, VIN1 and CIN1)

The subjectivity of diagnosing low grade squamous lesions relies upon identification of koilocytic atypia. Presence of “pseudokoilocytes” is a well recognized diagnostic pitfall (75). “Pseudo-koilocytes” are benign squamous cells with perinuclear halo caused by intracytoplasmic glycogen vacuoles that mimic the cytoskeleton disruption caused by HPV. In addition, “pseudokoilocytes” may show mild variation of nuclear sizes and shapes due to reactive cellular changes. Together, these benign cytoplasmic and nuclear changes may lead to overdiagnosis of LSIL (52, 75). The correlation between HPV DNA detection and Ki-67 positivity in verification of the diagnosis of VAIN1/VIN1 and CIN1 is described in Chapter 5, and Chapter 6, respectively.

Viral and molecular markers of condyloma acuminatum

The differential diagnosis of condyloma acumiantum includes fibroepithelial polyp (skin tag) and squamous papilloma, neither of which is caused by HPV infection (23). Subjectivity of diagnosing condyloma acuminatum relates to identification of true HPV cytopathic effect, i.e. koilocytic atypia. Since koilocytic atypia in condylomas is usually subtle and may be difficult to distinguish from the reactive nuclear changes seen in irritated fibroepithelial polyps or squamous papillomas, the distinction between these lesions may be very difficult in some cases. The correlation between HPV DNA detection and Ki-67 (MIB-1) proliferation marker in the diagnosis of condyloma acuminatum are described in Chapter 7.

Viral and molecular markers of cervical adenocarcinoma

Differentiation of adenocarcinoma in-situ and invasive adenocarcinoma of the cervix from benign endocervical lesions may be challenging due to the subtle morphological features of some endocervical neoplasms (76, 77). These diagnostic difficulties led us to search for a
specific and reproducible marker for endocervical neoplasia. The goal of our study was to compare the proliferative activity of neoplastic endocervical epithelium of adenocarcinoma in-situ and invasive adenocarcinoma with benign endocervical epithelium. To assess the specificity of MIB-1 staining in the diagnosis of endocervical neoplasia, we quantified MIB-1 immunostaining in benign endocervical epithelium under different benign conditions to assess the effect of various factors on epithelial proliferation. We examined the influence of hormones (cases from the proliferative and secretory phase of the cycle), inflammation (cases of cervicitis), regeneration (cases with a history of a recent biopsy), benign growth (endocervical polyps), and HPV infection (endocervical glands adjacent to high-grade squamous intraepithelial lesion). The results of immunostaining were correlated with HPV DNA detection by PCR/LiPA. The data is presented in Chapter 8.

Viral and molecular markers of high grade squamous intraepithelial lesions

The most common differential diagnosis of HSIL is benign immature squamous metaplasia. The most problematic cases are the ones with mild cytologic atypia, referred to as atypical immature squamous metaplasia (AIM) (78, 79). Since recognition of mild atypia is not well reproducible, cases diagnosed as AIM have been shown to include a spectrum ranging from bona fide HSIL to benign reactive changes or atrophy (77, 78). The goal of our study was to determine the sensitivity and specificity of p16 immunostaining as a marker of high oncogenic risk HPV infection in AIM. In addition, we wanted to examine whether immunostaining for Ki-67 and p16 may be helpful in reclassifying cases of AIM into HSIL and benign categories. The study is presented in Chapter 9.

Molecular diagnostic markers of microinvasive squamous cell carcinoma

The detection of microscopic foci of invasion in HSIL of the vulva and cervix can be difficult with routine hematoxylin-eosin staining (80). The problem is often confounded by the presence of dense, obscuring inflammatory infiltrates in these lesions. The visualization of neoplastic epithelial cells migrating through the basement membrane gaps into the stroma provides convincing evidence of stromal invasion. A technique of double immunostaining for cytokeratin and collagen IV/laminin has been developed and assessed for its utility in evaluation of early invasion in cervical and vulvar carcinomas. The results are presented in Chapter 10.
REFERENCES


40. Pirog E. Pathology of vulvar neoplasms. Surgical Pathology Clinics. 2010;00:000.


