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.*