
Ancillary Techniques in the Histopathologic Diagnosis of Squamous and Glandular Intraepithelial Lesions of the Uterine Cervix

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1. Introduction

Squamous cell carcinoma, adenocarcinoma and adenosquamous carcinoma comprise the most common cancers of the uterine cervix. Cervical cancer is one of the common cancers in women, especially in certain parts of the world, as Sub-Saharan Africa, Central America, South Central Asia and Melanesia [1]. In several countries the incidence of cervical cancer was reduced after the introduction of effective screening methods and prevention programs, initially based on the Papanicolaou smear (Pap test), and it is expected to diminish much further with the introduction of vaccination against human papilloma virus (HPV) [2-4].

In recent years papilloma virus has been linked to these cancers through a significant amount of scientific data, derived from epidemiologic, clinical, experimental and molecular studies [5-10]. A large number of scientific reports during the last three decades led to an explosion of information regarding the role of HPV in lower genital tract carcinogenesis, thus paving the way for the introduction of effective vaccines against the virus, expected to diminish the incidence of both HPV-related carcinomas and precursor lesions in forthcoming years [2,3,5]. This has affected in several ways the histopathologic diagnostic approach to cervical carcinomas and their precursor lesions, including the classification and terminology of the latter. Although several important questions remain, we are now able to examine HPV-related morphologic alterations from a different perspective that encompasses parts -although limited- of this new information, in an effort to achieve more efficient and precise recognition of precancerous lesions.

In the field of cervical squamous precursor lesions the basic concept underlying the Cervical Intraepithelial Neoplasia (CIN) terminology, which refers to a single disease spectrum, has

been questioned and gradually replaced by various approaches trying to face the whole group of HPV-related histopathologic abnormalities. In the field of glandular precursor lesions adenocarcinoma in situ is considered the precursor to most invasive cervical adenocarcinomas, while the concept of glandular “dysplasia” is being evaluated [11-15].

Morphology represents a gold standard for lesion diagnosis, since histologic and/or cytologic examination allows in most cases the recognition of viral cytopathic effects and precancerous epithelial alterations; however it can be hampered by inter- and intra-observer variability. In this context, several biomarkers have been investigated for their potential utility in assisting the histopathologic classification of preinvasive lesions and facilitating their distinction from non-HPV induced alterations [16-21]. Human papillomavirus-related intracellular interactions formed the basis for the identification of markers that may assist in this distinction, including cellular proteins targeted directly by viral oncoproteins, and markers related to the cell cycle, which is disturbed by multiple actions of the virus. Additionally, it is expected that the correlation of slight cellular alterations with new sensitive methods of HPV-detection might lead to the identification of different groups of lesions of clinical significance, as well as to the correct application of current morphologic criteria [21-23].

The application of immunohistochemistry and in situ hybridization techniques in the histopathologic diagnosis of cervical intraepithelial alterations of both squamous and glandular epithelium will be presented in this chapter. The immunohistochemical markers that are currently in use in several laboratories worldwide, as well as some new promising biomarkers will be included. Scientific background for each of these markers and special indications for their application will be summarized. A synoptic review of the pertinent literature will be presented, in an effort to summarize the existing data and the remaining questions at both the practical and the theoretical level.

2. Precursor lesions of cervical carcinoma

Precursor lesions of both squamous cell carcinoma and adenocarcinoma are well defined, according to our current understanding of cervical neoplasia. However, years of scientific observations and research preceded the recognition of these lesions. Observations concerning spatial and/or temporal relationship between invasive carcinomas and non-invasive, intraepithelial alterations of the uterine cervix had been repeatedly reported in the past, as early as the end of the 19th century [24-27]. These observations resulted in the recognition of precancerous alterations of cervical epithelium, for which different terms and definitions have been used in the following years.

The terms carcinoma in situ and dysplasia have been in use for several decades, in order to describe non-invasive, intraepithelial lesions showing cytologic abnormalities akin to those of invasive carcinoma. In the late 1960's the concept of one disease spectrum was introduced, based on observed similarities between groups of lesions, which were considered as different grades of the same disease process, termed cervical intraepithelial neoplasia / CIN [28,29]. This

terminology became the most widely used for the next decades and is currently in use in many laboratories worldwide.

The recognition of the important role[s] of human papillomavirus in cervical carcinogenesis gradually led to different approaches, concerning the whole group of HPV-related lesions and their classification, based on more recent biologic and molecular data [11,13,30,31]. Among them the distinction between two basic biological entities was included: specifically, between a productive viral infection and an intraepithelial neoplastic process. Several questions remain to be answered; however, on a practical level, patient management remains an important basis that influences and often guides choices of terminology.

In the last decades a binary system of classification based on The Bethesda System [TBS] has been mainly in use by cytopathologists; however, several histopathology laboratories worldwide have also adopted similar terminology. A binary system is more consistent with the current knowledge concerning HPV-related disease and is considered to form a basis for improved communication between gynaecologists and pathologists [31]. Moreover, the intermediate group (IN2) has not been shown to comprise a reproducible diagnostic category among pathologists.

2.1. Precursor lesions of squamous cell carcinoma

The terms squamous intraepithelial lesion (SIL) of the uterine cervix or cervical intraepithelial neoplasia (CIN) encompass a group of alterations of squamous epithelium that usually occur in or close to the transformation zone and are related to HPV.

Abnormal proliferation and maturation of squamous cells and nuclear atypia, including enlargement, pleomorphism, irregular nuclear borders, and change in chromatin texture, are the characteristics of these intraepithelial lesions. In one group of lesions the observed cellular alterations reflect mainly viral cytopathic effect, corresponding to koilocytic atypia. This is characterized by an abnormal appearing nucleus surrounded by an irregularly shaped cytoplasmic halo with a sharp edge. In these lesions atypia is more conspicuous in the maturing squamous cells, with mild alterations of the basal-parabasal cell morphology. In other groups of lesions cellular atypia is conspicuous in all cell layers: both middle/upper and lower epithelial layers (Fig.1)

Low-grade squamous intraepithelial lesions (LSILs) exhibit differences in density, size and staining of the maturing squamous cells, often accompanied by binucleated cells, cytoplasmic halos, and/or changes in epithelial thickness (Fig.1a) [14]. High-grade squamous intraepithelial lesions (HSILs) exhibit conspicuous nuclear atypia in all epithelial layers, with nuclear crowding, high nuclear:cytoplasmic ratio, loss of normal polarity, irregular nuclear membranes, and increased mitoses, which can be atypical [32]. In these lesions koilocytosis may be identified or not. There is significant basal/parabasal atypia, with little or no cytoplasmic maturation in the middle-upper layers of the epithelium, and mitotic activity extends to these epithelial layers (Fig.1c).

The differential diagnosis includes mainly: (a) reactive epithelial changes, (b) immature metaplastic changes, and (c) postmenopausal/atrophic epithelia, which may all mimic

squamous intraepithelial lesions. The distinction of these alterations from HPV-related lesions is based on well-defined morphologic criteria; however, in certain lesions the distinction is less straightforward, and ancillary techniques can be of help, leading to a more precise diagnosis and increased diagnostic reproducibility. Regressing LSILs may also cause a diagnostic problem [33]. On the other hand, ruling out invasion can be difficult in certain high-grade lesions. The next parts of the present chapter are going to describe the ancillary techniques, which allow for a more precise diagnosis in some of the problematic cases.

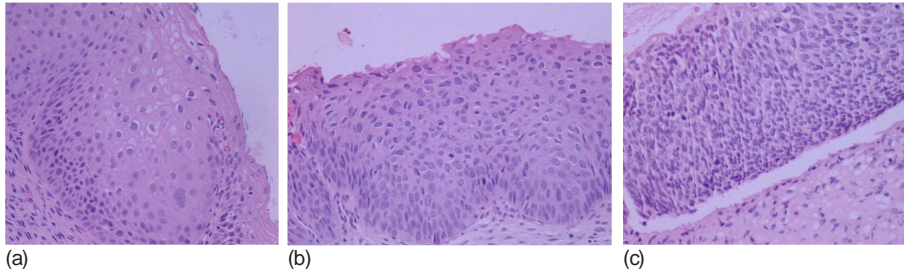


Figure 1. a-c). The above lesions represent a spectrum of alterations in cervical biopsies, ranging from low-grade to high-grade lesions.

2.2. Precursor lesions of cervical adenocarcinoma

The precursor lesion of cervical adenocarcinoma, that is adenocarcinoma in situ (AIS), was introduced as a concept in 1953 and is now acknowledged to be the precursor to most invasive cervical adenocarcinomas [34]. AIS is less common than SIL, with a ratio of AIS/HSIL ranging in most series between 1:26 and 1:237 [33].

Adenocarcinoma in situ is characterized by glands with nuclear hyperchromasia and atypia, increased nuclear:cytoplasmic ratio, pseudostratification or stratification, mitoses and apoptotic bodies (Fig.2). It may coexist with SIL and can also be multifocal. It may show a variety of cellular differentiation, and several subtypes have been described, including endocervical, endometrioid, intestinal, tubal, and stratified [34].

The diagnosis of glandular dysplasia has been used for intraepithelial alterations of glandular epithelium less pronounced than AIS. However, it has low reproducibility, and it has been suggested that this term should no longer be used in the clinical setting [35], especially since glandular epithelium does not support a productive infection by HPV [33]. It has been suggested that problematic endocervical glandular atypias should be evaluated with special studies [34,35]. The term cervical glandular intraepithelial neoplasia (CGIN), with high grade CGIN equating to AIS, is being used in several laboratories [20].

Cervical endometriosis, tubal and endometrioid metaplasia, and reparative changes have to be distinguished from AIS. Arias-Stella reaction, atypia due to irradiation, atypical forms of microglandular hyperplasia, as well as other viral infections, specifically Cytomegalovirus and

Herpesvirus, may occasionally pose problems of differential diagnosis. Ancillary techniques can be of help in these cases, as described in the next parts of the present chapter.

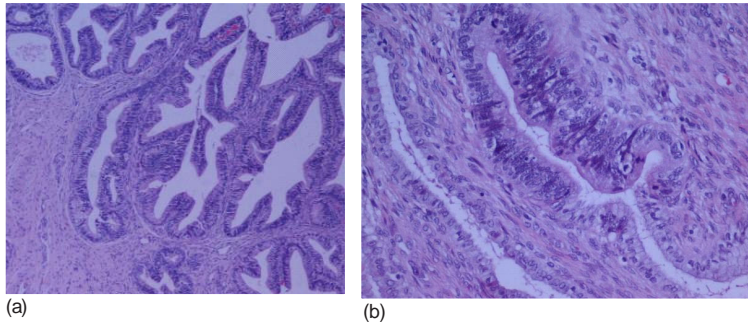


Figure 2. a-b). AIS. Continuity with benign cervical epithelium is obvious in [b].

3. HPV in carcinogenesis

Human papillomavirus is estimated to comprise a causal agent in 5% of human cancers and is associated with more human cancers than any other virus [36]. Among them, it is associated with the vast majority of cervical cancer cases. In contrast to several other infectious agents, which act as indirect carcinogens by inducing immunosuppression or by preventing apoptosis, high-risk HPVs (HR-HPVs) act mainly as direct carcinogenic factors [3]. Persistent infection by HR-HPVs correlates with increased risk of cervical cancer. However, infection by low-risk HPV types (LR-HPVs), carries a negligible risk of malignant progression. Additionally, other factors, related to the host or the environment, contribute to the development of neoplasia.

Several studies have revealed the complex intracellular interactions, which take place among oncoproteins encoded by human papillomaviruses and their cellular target proteins [3,37-39]. Their complexity is reflected in the long interval between infection and invasive carcinoma detection, often spanning a period of 15 to 25 years [3]. These interactions have offered to investigators the opportunity to study important cellular pathways related to the carcinogenic process, while several participating proteins have been studied for their possible use as markers of HPV infection in biopsy or cytology specimens. These biomarkers are presented in the next part of the present chapter.

3.1. HPV in carcinomas of the anogenital tract

Cervical cancer represents today a relatively well-studied prototype of a human tumor related to a viral infection, as well as a model for multi-step carcinogenesis. The revealed strong association led to the suggestion that human papillomavirus is not only the main cause of cervical cancer, but also a necessary cause [6].

In addition to cervical cancers, a significant percentage of vaginal, vulvar, penile, anal and perianal carcinomas are HPV-positive [7,40-42], while a fraction of carcinomas in other sites of the human body has also been linked to high-risk [HR] HPV infections. Percentages of HPV positivity observed in carcinomas of the anogenital area are presented in Table 1.

Vaginal carcinomas	60-91%
Vulvar carcinomas	50%
Penile carcinomas	30-50%
Anal and perianal carcinomas	60-94%

Table 1. Percentage of HPV detection in carcinomas of the anogenital region other than cervical carcinoma [4,7].

The distinction of HPV types into low- and high-risk is based on their association with carcinomas, and this distinction is sometimes challenging, especially in the case of rare/weakly carcinogenic viral types. The most common HPV types detected in cervical carcinomas include HPV 16, 18, 45, 31, 33, 52, 58, and 35, belonging to the high-risk group [43-45]. Low-risk viral types may confer risk reflecting an “at risk behavior” [14].

The fraction of cervical squamous cell carcinomas attributable to HPV16 and HPV18, which comprise the two most common high-risk viral types, is estimated at about 70%, while the respective fraction of cervical adenocarcinomas is 86%. As expected, in low-grade squamous intraepithelial alterations the respective percentages differ, since a significant number of these lesions contain HPVs which do not belong to the most common high-risk types.

3.2. Human papillomavirus oncoproteins and their main interactions with cellular pathways

HPVs are epitheliotropic double-stranded DNA viruses, whose replication is dependent on the terminally differentiating epithelial tissue. Their circular genome includes several open reading frames (ORFs) encoding for proteins which control early (E) and late (L) viral functions (E1, E2, E4, E5, E6, E7, L1, and L2) [46].

High-risk mucosal HPVs encode three transforming proteins: E5, E6 and E7, which exhibit multiple biological activities. These have been extensively studied in the last few decades; however, several aspects remain to be elucidated [47-48].

HPV E5 is able to transform mouse fibroblasts and keratinocytes in culture [49]. It is believed to contribute to early stages of carcinogenesis and works in concert with E6 and E7 [50-51]. These latter proteins, which often act synergistically, are necessary for the induction and maintenance of the transformed phenotype. They inhibit the function of tumor suppressors p53 and pRb, respectively, whereas their expression enables cells to bypass normal cell cycle checkpoints.

E6 and E7 are required for both the development of precursor lesions of cervical carcinoma, and for maintaining the malignant phenotype of cervical cancer cells [3]. E6 and E7 proteins play critical roles, being able to immortalize human keratinocytes and induce cell proliferation

and transformation. High-risk HPV E6 proteins target p53 for proteasomal degradation through association with the cellular ubiquitin ligase E6AP [48,52]. Low-risk HPV E6 proteins can also associate with E6AP; however, high-risk HPV proteins target p53 for ubiquitination. Activation of telomerase is another important facet of E6 functions, which is augmented by E7-induced interactions.

HPV E7 proteins interact with the retinoblastoma tumor suppressor protein, pRB, which controls S-phase entry through association with E2F transcription factor family members. They also interact with the related pocket proteins, p107 and p130. High-risk HPV E7 targets pRB for proteasomal degradation, while low-risk HPV E7 binds pRB with lower efficiency (approximately 10-fold lower) than the former [47,53]. E7 proteins cause aberrant activation of cdk2 (cyclin-dependent kinase 2), which is associated with cyclins E and A, as well as cdk inhibitors, mainly p21^{CIP1} and p27^{KIP1}. E7 expression results in dysregulated expression of cyclins E and A [48,54]. Through its multiple interactions, E7 can uncouple keratinocyte differentiation from cell cycle progression and retain differentiating keratinocytes in a DNA synthesis competent state.

The above interactions form the basis for the application of some important biomarkers used nowadays in many laboratories worldwide. These include p16, a cyclin-dependent kinase inhibitor, often exhibiting increased expression in HPV-related intraepithelial lesions, as well as cyclin E, as will be discussed in the following. Furthermore, proliferation markers, like Ki67, show increased/altered expression, in comparison to non-HPV-infected epithelium of the uterine cervix.

In addition, HR-HPV E6 and E7 proteins cooperate to generate mitotic defects and aneuploidy through induction of supernumerary centrosomes and multipolar mitoses in epithelial cells [55], while genomic instability results in the addition of molecular alterations. The detection of abnormal mitoses is a useful morphologic indicator of high-risk HPV-associated lesions [32].

Finally, integration of HPV genome into host chromosomes is an important event in cervical carcinogenesis [56,57]. Integration occurs frequently during malignant progression and may result in dysregulation of E6/E7 expression due to disruption of E2, with associated loss of the inhibitory E2 action.

Except for the above interactions, several other factors contribute to the development of neoplasia, and these are related to the host or the environment. Smoking, the use of oral contraceptives, high parity and Chlamydia are associated with a relative risk of 2 to 4 [7,9,58-60]. Immunity plays an important role, and this is reflected in data concerning cervical lesions in HIV-infected individuals and in transplant-recipients.

4. Immunohistochemical stains in the diagnosis of cervical intraepithelial lesions

The role of the pathologist examining a cervical biopsy suspicious for an intraepithelial lesion consists of: a) confirming or excluding the presence of a lesion, b) excluding other entities

entering the differential diagnosis, and, c) if the diagnosis of an intraepithelial lesion is confirmed, distinguishing a low-grade from a high-grade alteration. Histopathological diagnosis of intraepithelial lesions is based on well-defined criteria, as discussed above. However, in certain cases distinguishing both low- and high-grade lesions from their mimics may pose problems [14,61], even to experienced gynecologic pathologists. Florid reactive changes and metaplastic patterns may present histopathologic features, which may cause difficulties in the distinction from HPV-induced alterations. Attempts have been made to redefine the traditional criteria for lesion diagnosis, while other efforts aimed at the adoption of new, more objective methods, which might support the former [22,23,62-64]. However, studies attempting to correlate HPV presence and replication to certain cytohistologic alterations are becoming less frequent and/or fruitful.

In recent years immunohistochemistry (IHC) is an important adjunct in many diagnoses of surgical pathology, since it may reveal certain characteristics of cells and tissues, which cannot be evaluated by morphology alone. Cervical biopsies are not an exception. Molecular studies have revealed markers that might be of utility in the diagnosis of squamous and glandular intraepithelial alterations, including cellular proteins targeted directly by viral oncoproteins, and markers related to the cell cycle, which is disturbed by multiple actions of the virus, as summarized in the above paragraphs.

4.1. Immunohistochemical stains in the diagnosis of squamous intraepithelial lesions (SILs)

The immunohistochemical stains that are currently in use in several laboratories worldwide, as well as some new promising markers are presented in the following paragraphs. The terms low grade squamous intraepithelial lesion (LSIL) and high grade squamous intraepithelial lesion (HSIL) will be used interchangeably with CIN1 and CIN2/3, respectively.

4.1.1. p16

One extensively studied marker is p16^{INK4A} (hereafter referred to as p16), a cyclin-dependent kinase inhibitor, which decelerates the cell cycle and functions as a tumor suppressor, while having a role in cellular senescence. p16 affects pRb-mediated regulation of the G1/S transition [65-68]. The expression of p16 is altered in several human tumors by deletions, mutations, or methylation. Germ-line mutation carriers are predisposed to a high risk of pancreatic and breast cancers [69].

Increased expression of p16 is often observed in HPV-related intraepithelial lesions and this is mainly attributed to the presence of a feedback loop, which depends on the status of retinoblastoma protein (pRb) and the potential of high-risk HPV E7 protein to inactivate the latter [48,65,70]. Thus, it could be regarded as a marker of E7 activity. Despite the presence of high levels of p16 in SILs, its suppressor function is not normally exerted.

Several groups of investigators have examined immunohistochemically the expression of p16 in cervical squamous intraepithelial lesions and its possible correlations with lesion grade, HPV types and/or lesion “progression” (reviewed by Kostopoulou et al. [17]). Indeed, p16 is one of the best studied markers in gynaecologic pathology. However, percentages of immu-

nohistochemical positivity vary among different studies, as presented in Table 2. In the latter, studies published in the last ten years and including more than 100 cases of squamous intraepithelial lesions in histopathologic specimens are summarized [71-85], and the reported percentages of p16 immunopositivity are presented, together with the criteria and the antibodies used by the authors. As shown in the Table, different criteria have been used for p16 immunoreactivity evaluation, with some authors focusing only on diffuse immunopositivity, some reporting any type of immunostaining, and others reporting nuclear and cytoplasmic staining separately. Importantly, some authors interpret focal positivity as a false-positive reaction. Positivity in the studies presented below varied from 5.6% to 100% for LSIL and from 45.2% to 100% for HSIL (Table 2). The percentage of immunopositivity observed in negative for SIL (NS) epithelia also varied between 0% and 32.7%.

In one recent study [17] the two basic patterns of immunoreactivity, that is focal and diffuse, were further subdivided into groups as following: Focal positivity was subdivided into cases with occasional positive cells, dispersed or in small groups, observed either (a) mainly in the lower epithelial layers, or (b) above the basal/parabasal layer. Diffuse positivity in the horizontal plane involved either (a) all epithelial layers, or (b) only the basal, parabasal and intermediate layers, without extending to the upper third of the epithelium. In HSIL only diffuse positivity was encountered, observed in 24/25 cases (96%) (Figure 3a). In LSIL 41/55 cases (74.5%) showed some type of positivity, most commonly focal/sporadic (Figure 3b). Interestingly, a difference in HPV type distribution was observed between the two patterns of sporadic/focal positivity, involving lower vs intermediate/upper epithelial layers, and probably reflecting an earlier sporadic expression of E7 in certain lesions [17]. LSILs associated with high-risk or probable high-risk HPV types showed positivity for p16 in 25/35 cases (71.4%). Study of the pertinent literature revealed that a percentage of LSILs testing positive for HR-HPV by PCR or HC2 does not exhibit any p16 immunopositivity. Indeed, the percentage of p16 positivity reported for HR-HPV positive LSILs varied from 32.4% to 94.4% [17,78,86]. Furthermore, strong p16 positivity in LSIL cases appeared to be independent of HPV punctate signal pattern by ISH, in a study by Kalof et al [86].

Additionally, as shown in the Table, in several studies often appears a small group of HSILs that do not show any p16 immunoreactivity. The above observations lead to the conclusion that a negative or equivocal p16 immunostain should be carefully evaluated in conjunction with the histopathologic findings and should not be used as the main criterion for diagnosis.

In three large series of the literature reporting more than 200 cases (SILs and controls) each [71,79,81], as summarized in [17], sensitivity of p16 for the detection of SIL varied from 76.6% to 94.8%, with a value of 83.7% calculated in the total number of their cases, while specificity varied from 77.1% to 92.1%, with a value of 84.6% calculated for the total number of cases. In the same studies the positive predictive value varied from 75.7% to 94.1%. In the study by Kostopoulou et al. [17] sensitivity of p16 immunopositivity for the detection of SIL was 81.2% and specificity 85%, while the positive predictive value was 95.6%. In the study by van Niekerk et al. sensitivity and specificity for HSIL were estimated at 90% and 85%, respectively.

Reference	LSIL	HSIL	NS	Evaluation	Antibody
[71]	56.6%	84.5%	11.5%	N and C ≥5% cells	E6H4 (MTM)
[72]	35%	81.2%	0%	N and/or C	Polyclonal (Abcam)
[73]	74.7	100%	ND	N and C ≥5% cells in lower third	CINtec p16 Kit (DakoCytomation)
[74]	37.2%	45.2%	3.2%	N and/or C	E6H4 (MTM)
[75]	72%	94.7%	32.7%	Any reactivity	E6H4 (MTM)
[76]	74.1%	96.1%	7.0%	N and/or C	JC8 (Biocare Medical)
[77]	100%	98.7%	0%	N or C	p16 (Pharmingen)
[78]	24.5%	87.5%	0%	Moderate and strong	E6H4 (MTM)
[79]	90.9%	100%	7.9%	C and N ≥5% cells	Ab7 16P07 (Neomarkers)
[80]	71.4%	100%	6%	Continuous basal and parabasal	p16 Histology Kit (Dako)
[81]	57.1%	96.9%	22.9%	N and C ≥5% cells in each layer	E6H4 (DakoCytomation)
[82]	50%	96.2%	0%	C and N	CINtec p16 Kit (Dako)
[83]	5.6%	96.7%	ND	Diffuse, >1/3 of epithelium	Ab-4, 16P04 (Lab Vision)
[84]	26.7%	79.7%	0%	N and C ≥5% cells	p16 (NeoMarkers)
[85]	44%	99%	11%	Lower ¼ of epithelium	E6H4 (DakoCytomation)

LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; NS: negative for SIL; N: nuclear; C: cytoplasmic; ND: no data

^a Only studies including more than 100 cases of squamous intraepithelial lesions in histopathologic specimens are presented.

Table 2. Percentages of immunopositivity for p16 in studies reported in the last ten years^a

In conclusion, the results of the above mentioned studies point towards the use of p16 immunostain in conjunction with histopathologic evaluation, since the latter remains the “gold standard” in lesion diagnosis. Addition of a consecutive p16-stained slide to the hematoxylin-eosin (HE) stained slides has been shown to improve significantly interobserver agreement for both punch and cone biopsies [18,83,87]. It is of note that a high level of agreement has been observed among pathologists in calling a staining pattern diffusely positive [18]. Moreover, p16 immunostaining may help in the identification of occult lesions [88] and of unusual high-grade lesions not easily recognized in hematoxylin-eosin stained slides [89]. The differential diagnosis from non-neoplastic alterations can be facilitated, especially in conjunction with other immunostains, as presented below. Lesion grading can be more accurate, especially concerning aggressive-appearing low-grade lesions, which could easily be upgraded [83]. p16 IHC may also be of use in evaluating cauterized cervical resection margins, since the positive staining pattern of HGSIL is not affected by diathermy in LLETZ biopsies [76]. Awareness of the different patterns of immunoreactivity and its limitations might allow for a most proper use in certain clinicopathological settings. However, significant variability remains in the reported percentage of cases that stain positively for p16, and the need for standardization of sample preparation and evaluation protocols cannot be overemphasized [90].

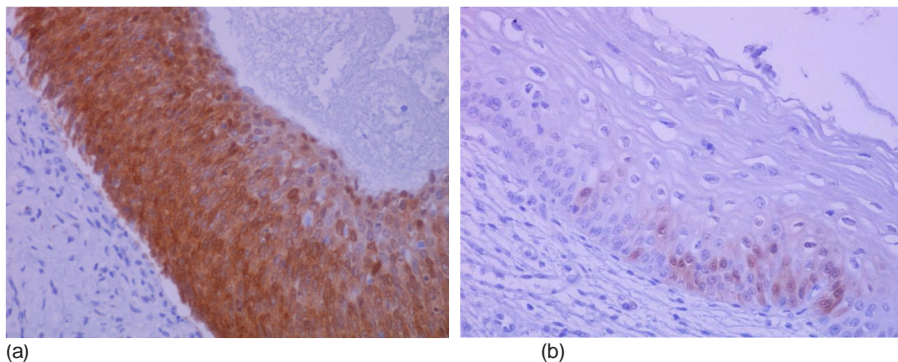


Figure 3. a – b). Diffuse p16 positivity in a HSIL (a) and focal positivity in a LSIL (b).

In the recent consensus recommendations of the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology [31], p16 was considered as the only biomarker with sufficient evidence on which to make recommendations regarding use in squamous lesions of the lower anogenital tract in the context of HPV biology. p16 immunohistochemical evaluation was recommended for the distinction of high grade lesions from their mimics, as well as for diagnosis clarification of a lesion falling in the CIN2 group. However, p16 IHC is not indicated for the distinction of low grade lesions from non-HPV induced alterations or as a routine adjunct to histologic assessment of biopsy specimens with HSIL/ CIN3 or LSIL morphology. An exception to the latter concerns cases interpreted as \leq LSIL that are at high risk for missed high-grade disease, in relation to previous cytology results or HPV-

test [31]. The estimated magnitude of p16 IHC utilization, according to these recommendations, is for <25% of cervical biopsy specimens.

Finally, another aspect of p16 immunostaining is the possibility of correlation with lesion “progression”. It has been suggested that certain phases of a given HR-HPV-associated neoplastic process may have different indices of p16 expression [16]. Although the detailed examination of this subject is not included in the aim of the present text, it should be mentioned that, in an interesting study by Hariri and Oster, 25/26 low-grade lesions with negative p16 staining (concerning diffuse staining) and a minimum follow-up period of five years had a benign or normal outcome, revealing a negative predictive value of p16 in predicting the outcome of CIN 1 cases as high as 96% [80]. In a study including conization specimens with coexisting CIN1 and CIN3 areas, all CIN1 were p16 positive [91], while p16 staining did not predict persistence or clearance of HR-HPV after treatment for CIN in a study by Branca et al. [72]. In a recent report by Ozaki et al. [85] including 99 patients with CIN1 and follow-up data, 26/76 cases in the regression group showed p16 immunopositivity vs 17/23 in the persistence/progression groups.

4.1.2. Cyclins

Cyclins along with cyclin-dependent kinases and their inhibitors are important molecules for the orderly progression of cells through phases of the cell cycle. Their immunohistochemical evaluation has been reported to be of help in the diagnosis of SIL in cervical specimens. Cyclin E is uncommonly expressed in epithelia not infected by HPV and its conspicuous immunopositivity may facilitate the recognition of SIL [16]. In addition, cyclin B1 immunoreactivity above the basal/parabasal cells correlates significantly with HPV detection and could be a marker of HPV presence [21]. Cyclins D and A have been also studied as possible markers of HPV-related lesions.

Cyclin B1. It has been reported that E6/E7 oncoproteins of HPV type 18 induced changes in the expression of cell cycle regulatory proteins before immortalization [92]. Significantly increased expression was noted for cyclin B and its transcriptional activation was documented. In 2000, Southern et al. demonstrated increased cyclin B1 expression in HGSILs [93]. In their study cyclin B protein was up-regulated and persisted into the upper epithelial layers in parallel with cyclin A expression in high-grade squamous intraepithelial lesions.

In a study performed in our laboratory, cyclin B1 immunopositivity above the basal/parabasal cells correlated significantly with HPV detection ($p < 0.001$) (Figure 4). In all cases of HSIL immunopositivity for cyclin B1 extended above the basal/parabasal layers, most often involving the superficial layers as well [21]. Furthermore, increased cyclin B1 immunoreactivity was observed in 51/52 low-grade lesions (98.07%), and in seven of 15 biopsies (46.6%) characterized as atypia of unknown significance (AUS). Six of these seven cases tested HPV-positive by PCR.

The main staining pattern observed in low-grade lesions and non-diagnostic atypias in the above study consisted of sporadic cyclin B1 staining in mature squamous polygonal cells often just above the basal layers, with slight differences between flat and elevated lesions. Immu-

nopositivity was seen in 52 of 55 cases with HPV infection detected by PCR, whereas it was seen in only 5 cases without PCR-proven HPV infection. In 4 of the latter cases, however, p16 immunoreactivity was observed, suggesting that HPV could be present though not detected by PCR.

The pattern of immunoreactivity observed in low-grade lesions and AUS cases could be perceived as cytoplasmic accumulation or retention of cyclin B1 in suprabasal squamous cells, which might reflect early events in the inhibition of G2-to-M transition, a well-known phenomenon during HPV infection *in vitro*. The possibility was suggested that these cyclin B1-positive cells could be viewed as a type of “prekoilocytes”, whose eventual progression to koilocytes would depend on several parameters related to the intricacies of HPV infection.

In conclusion, cyclin B1 positivity above the basal/parabasal layers correlates significantly with HPV detection and could be a marker of HPV presence. Thus, it might constitute a helpful finding in difficult to diagnose cases. Immunopositivity in a specimen showing non-diagnostic atypia should prompt reevaluation and/or HPV testing, as it is likely that the case could represent a genuine low-grade intraepithelial lesion. Thus, a search for “pre-koilocytes” with B1 immunostaining might be useful in certain cases.

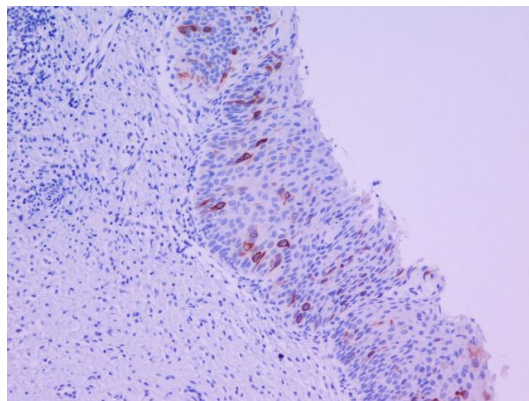


Figure 4. Cyclin B1 immunopositivity above the basal/parabasal cells correlates significantly with HPV detection.

Cyclin E. Cyclin E is another important cell cycle regulator, promoting G1 transition, which has been reported to exhibit increased expression in squamous intraepithelial lesions and invasive cervical carcinomas, although the exact mechanisms are not clear [16].

Moderate to strong immunopositivity for cyclin E was observed in 92.6% and in 91.6% of LSIL and HSIL, respectively, in a study by Keating et al., being positive in 38/41 HR-HPV positive cases. In a group of nondiagnostic squamous atypias cyclin E positivity was associated with HPV positivity [16]. In a study by Bahnassy et al. cyclin E staining increased from CIN1 to invasive carcinoma (from 16.7% to 88.4%, respectively), while gene amplification was detected in 11.1% of CIN1 cases, in 45.5% of CINII, in 55.3% of CINIII, and in 88.4% of carcinoma cases [94].

In conclusion, cyclin E immunostaining could be used to discriminate reactive from neoplastic epithelium [14] especially in conjunction with other markers, discussed in other parts of the present text. However, it is not useful in the distinction of low-grade from high-grade lesions.

4.1.3. Ki67

Ki-67 is an antigen expressed in the nuclei of proliferating cells during the whole cell cycle, except for the G0 and G1 early phases. MIB-1 is a monoclonal antibody that detects this antigen in paraffin tissue sections. Although positivity is observed under normal conditions in the lower compartments of the multilayered squamous epithelium, staining of the middle and upper layers is indicative of an intraepithelial lesion (Figure -5).

Immunopositivity for Ki-67 increases as a function of increasing lesion grade [16,19,95-98]. HSIL/CIN3 lesions usually show nuclear positivity scattered throughout all epithelial layers, while lower grade lesions show less diffuse immunoreaction. However, immunostains should be interpreted with caution. It is well-known to pathologists that reactive and reparative changes may pose a problem in the examination of cell proliferation, and, in the case of Ki-67, immunostaining may extend through the upper layers of the epithelium. In addition, tangential sectioning may sometimes result in the false impression that positive parabasal cells are more superficially placed, thus leading to a SIL diagnosis [99].

It should be noted that Ki-67 immunohistochemical stain may be especially helpful in differentiating atrophic epithelial changes from high-grade lesions [14]. It can also be used in the evaluation of cauterized margins, which often pose diagnostic problems [20]. In addition, Ki-67 immunostaining can be used as an adjunct to other markers. In one study of metaplastic cervical epithelia, addition of Ki67 positivity in >50% of lesional cells to p16 “band” positivity increased specificity (from 92.5 to 96%) and positive predictive value (from 85.7 to 91.7%) for the diagnosis of high-grade CIN [100]. Dual IHC stain with evaluation of colocalization of antibodies represents an interesting approach to this theme [101].

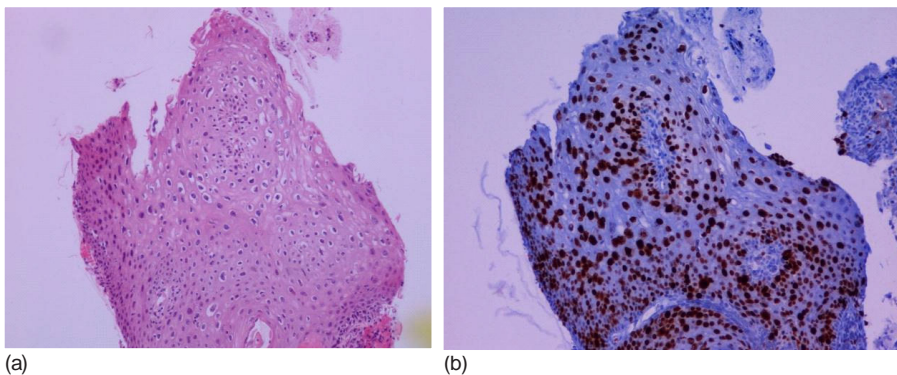


Figure 5. Ki-67 positivity in a low-grade SIL.

4.1.4. *ProEx C*

ProEx C is a recently developed biomarker reagent that targets two proteins having a significant role in the regulation of DNA replication during S-phase: the minichromosome maintenance protein 2 (MCM2) and DNA topoisomerase IIa (TOP2A). TOP2A is a nuclear enzyme that regulates the enzymatic unlinking of DNA strands during chromosome replication. MCM2 functions also during DNA replication by loading the pre-replication complex onto DNA and unwinding the latter through helicase activity to permit synthesis. These proteins are overexpressed in aberrant S-phase induction and have been shown to be overexpressed in CINs and cervical carcinomas [98,99,102,103]. ProEx C appears to be efficient in distinguishing reactive epithelial changes from squamous lesions. The stain can be used alone or in conjunction with p16.

The staining pattern of ProEx C in histologic sections is evaluated in a way reminiscent of MIB-1, since only nuclear positivity above the basal one-third layer is considered positive [99]. ProEx C has been reported to stain reactive epithelial cell nuclei less and LSIL nuclei more than MIB-1 [99]. Strong positive staining for ProEx C involving the lower and upper halves of the epithelium was observed in 92% of high-grade squamous intraepithelial lesions in a study by Badr et al. [102]. Condylomas and CIN I showed greater variability in patterns of staining, with immunopositivity extending into the upper half of the epithelium in 48% of cases. According to the authors, the stain can be applied to confirm the diagnosis of HSIL and to triage cases of atypical squamous metaplasia. According to Shi et al. [103], ProEx C is a better marker than p16 for the detection of LSILs, showing positivity in 94% of the cases in a series of 34 LSILs.

One study by Pinto et al. [98] examined cases with the differential diagnosis of HSIL vs reactive epithelial changes. ProEx C showed 87% sensitivity and 71% specificity for SIL in biopsy material. The authors reported a larger number of cells stained by ProEx C in comparison to MIB-1 in both HSIL and LSIL cases. In addition, the combination of p16 and ProEx C predicted more NoSIL (including normal, reactive, and/or atrophic epithelia) than p16 and MiB-1 (61% vs 43%). These observations suggested that ProEx C could be more useful in the distinction of reactive epithelial changes from SILs than MiB-1.

Sanati et al. reported a sensitivity and specificity of 89% and 100%, respectively, for ProExC immunostain in distinguishing high-grade squamous intraepithelial lesions from squamous metaplasia, while positive and negative predictive values were 100% and 82%, respectively [104]. In a recent study by Guo et al., diffuse positivity for ProExC significantly increased from benign cervix/CIN 1 to CIN 2 or 3/carcinoma, while the highest specificity for CIN 2+ and CIN3+ (100% and 93%, respectively) was achieved when immunostaining was positive for both ProExC and p16, suggesting that it is advantageous to use these two markers together in order to distinguish high-grade lesions from their mimics [105]. The use of the same two markers, p16 and ProExC, as a first step, followed by Ki-67 immunostaining in discordant cases, has been suggested as cost saving strategy by Walts and Bose [106]. According to these authors, performing the two above stains initially and adding Ki-67 only when p16 and ProExC yield discordant results provided the same diagnostic accuracy while reducing the cost, since only one third of the cases required performance of the third stain.

4.1.5. L1 capsid protein

HPV L1 capsid protein is the major structural protein of human papillomavirus. In the last few years L1 immunoreactivity has been examined repeatedly in cytologic material. Nuclear positivity is mainly observed in productive lesions and is gradually lost in high grade lesions and carcinomas.

It has been suggested that combined L1/p16 IHC may be helpful for clinical management, especially in cases in which the grade of the lesion is difficult to assess [91]. In a study by Negri et al. L1 was expressed in 34.85% of CIN1 cases, including 12/38 (31.56%) cases with coexisting CIN3 in conization specimens and 11 of 28 (39.29%) biopsy specimens from women with cytologically proven regression [91]. In the latter group a high staining score was often observed. The authors suggested that combining p16 and L1 immunostaining might allow for a distinction between different risk patterns of LSIL.

In a study by Galgano et al. immunohistochemical staining of HPV L1 was negatively associated with the increasing severity of their consensus diagnosis ($p_{\text{trend}} < 0.001$) and decreased with increasing intensity of p16INK4a ($p_{\text{trend}} < 0.001$) and Ki-67 ($p_{\text{trend}} < 0.001$) [107]. Positivity for L1 was observed in 32%, 32.2%, and 16.5% of CIN1, CIN2, and CIN3/AIS, respectively, and was also observed in 3.3% of negative specimens. It was negatively associated with having a CIN2+ or CIN3+ diagnosis (OR=0.62, and 0.18, respectively). The authors reported that L1 IHC detection was neither sensitive nor specific for any group of cervical neoplasia in biopsy material and this was attributed to the complexity of the temporal evolution of the HPV virion production which may be quite transient. It is interesting that L1 positive cases with a negative consensus diagnosis in this study commonly had at least 1 reviewer diagnosis of CIN1, revealing once again the difficulties in the distinction of SIL vs negative for SIL.

In a recent study by Gatta et al. L1 was expressed in the nuclei of superficial cells of dysplastic epithelia, often with characteristics of koilocytes [108]. L1 positivity was observed in 8/32 CIN1 biopsies (25%) and in 1/10 CIN2 cases (10%), but it was not observed in CIN3 and carcinoma cases examined. Their only case which showed a punctate signal with catalyzed signal-amplified colorimetric DNA in situ hybridization, suggestive of viral integration, belonged to the L1-negative/p16-positive group.

4.1.6. Other markers

In the above paragraphs some of the most important markers used in the histopathologic diagnosis of SIL have been presented. Ki67 and p16 have been used for several years in many laboratories worldwide, while ProEx C and L1 have only been in use for the last few years. However, several other markers have been tested in cervical biopsies for their potential utility in diagnosis. It should be noted that in recent years development of high-throughput technologies, as gene expression profiling, has increased the potential for biomarker discovery. However, although some of these markers showed promising results, in most cases they did not present any specific advantages in comparison to the already existing biomarkers from a diagnostic point of view.

4.2. Immunohistochemical stains in the diagnosis of glandular intraepithelial lesions

Several entities have to be distinguished from AIS, including cervical endometriosis, tubal metaplasia, reparative changes, Arias-Stella reaction, atypia due to irradiation, atypical forms of microglandular hyperplasia, as well as other viral infections, specifically Cytomegalovirus and Herpesvirus. Biomarkers can be of help in these cases.

4.2.1. p16

Already discussed in the paragraphs concerning squamous intraepithelial lesions, p16 has also emerged as a potentially useful marker in the evaluation of glandular cervical alterations [20, 109-111]. Most AIS lesions show diffuse nuclear and/or cytoplasmic immunopositivity (Figure 6). Microglandular hyperplasia and reactive lesions are usually negative for p16. Positivity can be observed in tubal metaplasia; however, it is commonly focal/weak.

p16 was diffusely and strongly expressed (3+) in 29/29 AIS in a study by Negri et al [110], while patchy positivity was observed in tubal metaplasias, endometriosis and endometrial samples. Likewise, all 19 AIS cases showed diffuse nuclear and cytoplasmic reactivity in a study by Little and Stewart [111], while rare single cells were positive in normal endocervical epithelium. Staining was focal in most cases of tubo-endometrioid metaplasia in this study; however, eight of these latter cases included glands that were diffusely p16 positive and/or showed Ki-67 labeling in >25% of cells.

These results emphasize the importance of using panels of antibodies in some of these problematic cases in conjunction with careful morphologic examination.

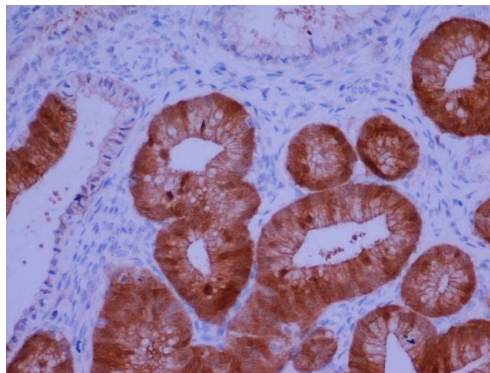


Figure 6. p16 immunopositivity in AIS. Compare with adjacent normal glands. Abrupt transition is focally observed between normal and neoplastic epithelium.

4.2.2. Ki-67

Nuclear positivity for Ki-67 is usually observed in >30% of cells in adenocarcinoma in situ, and often in the majority of the cells. In the contrary, only a small percentage of cells (<10%) stain

positively in tubal metaplasia. In practice, it is not usually necessary to undertake a count of positive cells, as there are typically only scattered positive nuclei in tubal metaplasia, while the majority of nuclei are positive in AIS [20].

All cases of adenocarcinoma in situ showed markedly increased Ki-67 labeling together with diffuse nuclear and cytoplasmic reactivity for p16 in the study by Little and Stewart [111], and typically the positive cells were sharply demarcated from the adjacent normal, unstained endocervical epithelium.

However, AIS may occasionally exhibit a low proliferation index [110]. Additionally, some benign lesions, like endometriosis, may show a high proliferation index. Thus, a combination of different markers is more useful than isolated stains in the evaluation of glandular intraepithelial alterations.

4.2.3. *bcl-2*

Bcl-2 is a member of a large family of proteins, some inhibiting and others favoring apoptosis.

Lesions of adenocarcinoma in situ with significant apoptosis show negative or focally positive bcl-2 immunostains [109]. In the contrary, tubal metaplasia and endometriosis typically exhibit diffuse cytoplasmic positivity, reminiscent of normal fallopian tube epithelium and proliferative endometrium [20]. However, normal endocervical glands are also negative. Consequently, immunostaining for bcl-2 can comprise part of a panel of antibodies, including p16 and Ki-67 [20,112]. It can also be used in the evaluation of cauterized margins [20], as already discussed for p16 and Ki-67 in squamous lesions.

4.2.4. *Other markers*

Except for the above discussed markers, several other immunostains have been reported to be of help in the diagnosis of glandular lesions. Vimentin can be useful in distinguishing AIS from endometriosis and tuboendometrial metaplasia, since the latter two entities exhibit cytoplasmic positivity. They also show positivity for estrogen receptor, while AIS is negative or focally positive [20]. One additional marker is carcinoembryonic antigen (CEA), which is observed cytoplasmically in a significant percentage of AIS cases, whereas normal endocervical epithelium shows only luminal or no staining.

Interestingly, complete negativity for cyclin D1 was commonly observed in AIS in a study by Little and Stewart [111], in contrast to tuboendometrial metaplasia and normal endocervical epithelia, although staining was typically focal in the latter. Microglandular hyperplasia and mesonephric duct elements were also cyclin D1 positive although relatively few cases were examined in that study.

It should be also noted that the intestinal type of AIS has been reported to show CK7 positivity and CK20 negativity or extremely focal positivity, in spite of the presence of morphological intestinal differentiation in a few cases examined, as reported by McCluggage [20].

Finally, immunohistochemical stains for cytomegalovirus and herpesvirus can be of use for confirmation of these infections, although they are not usually likely to be confused with AIS [34].

5. In Situ Hybridization (ISH)

Detection of HPV nucleic acids is performed by methods that can be broadly subdivided into: a) methods based on target amplification, and b) those based on signal amplification [113]. In addition to several existing liquid phase techniques, in situ hybridization (ISH) methods have been developed for the detection of nucleic acids in cytological and histological specimens. Efforts at improving ISH performance have focused both on amplifying nucleic acid targets before hybridization or on amplifying signals afterwards (e.g. by using in situ PCR, or tyramide signal amplification). Both fluorescent detection and coloured substrate deposition followed by bright-field microscopy can be used, and can be combined with tyramide signal amplification. In addition, ISH assays can be automated along the same lines as immunohistochemistry.

Issues concerning sensitivity of ISH techniques in comparison to PCR have been repeatedly raised. However, these techniques are becoming increasingly sensitive [114,115]. One main contribution of ISH to HPV research is the fact that it permits concurrent morphological evaluation of the cells examined, especially in the case of histological specimens, which is a significant advantage in comparison to liquid phase techniques. In addition, the signal patterns observed in HPV in situ hybridization have been reported to be associated with the physical status of viral DNA in the cell, that is episomal or integrated. Specifically, the punctate pattern of positivity has been linked to viral forms integrated in the host genome [86,116-118].

In a study by Kalof et al. punctate signals were detected in 17/17 (100%) CIN 2/3 lesions, but in only 13.6% of high-risk HPV-positive CIN 1 lesions [86]. In cytology material Ho et al. reported a punctate pattern in 8.7% of CIN1 lesions vs 34.0% of CIN3 lesions [119].

ISH and PCR had fair to good agreement in detecting HPV DNA across CIN categories in a study by Guo et al.; however, ISH detected significantly fewer HPV-positive cases in carcinomas than PCR did, probably as a result of lower copy numbers of episomal HPV DNA in the latter [120]. In addition, although the pure punctate pattern of HPV indicated a high level of viral integration, in cases with mixed signal patterns the level of HPV integration could not be accurately determined, probably due to a variation in the percentage of the two patterns in these cases.

According to Kong et al., in cases of atypical squamous metaplasia, p16 reactivity (focal strong and diffuse strong) was significantly more sensitive than ISH in correlating with the presence of human papillomavirus as detected by PCR [121]. Voss et al. compared a fluorescence in situ hybridization (FISH) HR-HPV assay to Hybrid Capture 2 (HC2) and PCR for the detection of HR-HPV subtypes in cervical cytology specimens [122]. FISH was concordant with HC2 and PCR in 85% and 82% of the specimens, respectively, while HC2 and PCR were concordant in 84% of the specimens. In a more recent study by Kelesidis et al., ISH exhibited a sensitivity of 89.5% for the detection of CIN2+ lesions, while PCR showed sensitivity of 94.7% for these lesions. Importantly, a percentage of ISH-positive cases was not detected by polymerase chain reaction (performed on liquid-based sample media), emphasizing the technical problems and limitations of the techniques [114].

As is apparent from the above results, the applications of HPV DNA ISH are partly dependent on the sensitivity of the assay and its sufficiency to carry a high negative predictive value [14]. This is especially important if clinical decisions are based on a negative result. However, ISH represents a useful tool for ancillary molecular HPV testing when concurrent morphological evaluation of the area examined is necessary.

Except for HPV nucleic acids, there are also other applications of in situ hybridization techniques in cervical specimens, including the detection of amplification of the gene coding for the telomerase RNA component (TERC) at 3q26 [56,123,124]. TERC amplification has been reported to increase with severity of dysplasia and it has been suggested that this might serve as a marker in the distinction between low- and high-grade lesions.

Except for DNA ISH, in situ RNA detection shows promising results in certain applications. RNA markers have emerged as an important group of biomarkers after the widespread use of genome-wide gene expression profiling techniques. New sensitive RNA in situ hybridization methods can detect more than one target simultaneously, can be applied on formalin-fixed paraffin-embedded tissue, and they allow for simultaneous evaluation of morphology [125]. As expected, preanalytical variables related to fixation can affect biomarker measurements and should be considered in the application of these techniques.

Both cellular and viral proteins related to lesion prognosis, previously examined at the mRNA level with PCR techniques, might prove to be important markers for RNA ISH applications. Recently, transcriptional analysis of HPV16 genes by in situ hybridization in histological sections of cervical dysplasia revealed transcription patterns that bring into question some of the current beliefs on the mechanism of HPV-16 infection in the progression to cervical cancer [126]. The detection of HPV E6/E7 mRNA expression appears also promising in the case of multiple infections [127].

In addition, the polymerase chain reaction has been used for target amplification before hybridization, with promising results. *In situ* PCR combines increased sensitivity with the anatomical localization provided by in-situ hybridization, and allows the examination of the specific genetic material at a specific cellular level. Although the method is subject to both false positive and negative outcomes, it allows correlation of viral DNA localization with relevant target proteins, thus providing important information concerning the development of cervical neoplasia [128,129], which cannot be obtained by polymerase chain reaction-based methods alone.

6. Other techniques

Finally, it should be noted that new techniques continue to emerge in the field of cervical lesion detection. Of particular interest in the context of biopsy processing is the development of a water-soluble protein-saving biopsy processing method, which is followed by analysis of proteins of the supernatant samples. This method resulted in the identification of proteins that discriminate between grades of cervical neoplasia, while preserving the tissue for conventional microscopic analysis [130,131].

7. Conclusions

In the above text important ancillary techniques currently in use in several laboratories worldwide for the evaluation of cervical biopsies have been presented, along with their main applications in diagnosis. Suggested panels of antibodies for specific diagnostic dilemmas have been discussed. Furthermore, the significance of certain negative stains has been presented. It should be noted that histopathologic examination remains the “gold standard” for the diagnosis of low- and high-grade SIL and AIS; however, certain biomarkers have emerged as helpful adjuncts, assisting in a more precise diagnosis of cervical precursor lesions.

It is obvious from the above presented data that the diagnosis of a lesion in a diagnostically challenging case cannot at present be based solely on any particular marker, but rather on a combination of markers with careful morphologic evaluation. Important requirements for the proper use of the presented markers include standardization of protocols and familiarity with the patterns of immunostaining. Finally, an important issue, not specifically analyzed, which may emerge in the next few years and merits further study, is the exact performance of these markers in the detection of lesions related to uncommon HPV types, other than those addressed by the current vaccines.

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