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Immunohistochemistry in the Diagnosis of Squamous Intraepithelial Lesions of the Uterine Cervix

Evanthia A. Kostopoulou and George Koukoulis
*University of Thessaly
Greece*

1. Introduction

During the last few decades accumulated epidemiological, clinical, and experimental evidence has revealed the important role of human papillomavirus (HPV) in the development of cervical carcinomas, an association almost unique in cancer epidemiology. Several important questions have been answered and a large number of scientific studies have paved the way for the introduction of new and effective vaccines, which will hopefully diminish the incidence of HPV-related carcinomas and precursor lesions in forthcoming years (Crum et al., 2003; zur Hausen, 1977, 2008). However, the exact recognition and proper treatment of clinically important lesions often poses problems both to pathologists and gynecologists.

Morphology remains the gold standard for lesion diagnosis, despite the fact that it can be hampered by inter- and intra-observer variability. Additionally, the contribution of morphology in the field of human papillomavirus research cannot be overemphasized, since cytologic and/or histologic examination allow the recognition of viral cytopathic effects, and, with the aid of immunohistochemical and other *in situ* techniques, may reveal the exact cells, in which some main interactions take place. Thus, the correlation of cellular alterations with new sensitive methods of detection either for human papillomavirus nucleic acids or for HPV-related intracellular interactions might lead both to the identification of different groups of lesions according to their clinical significance, as well as to the correct application of current morphological criteria.

The following chapter will focus on those immunohistochemical methods that can facilitate or confirm the detection of intraepithelial lesions of cervical squamous epithelium (SILs) in biopsy specimens, additionally presenting in brief some data concerning the mechanisms by which these specific cellular targets are related to important actions of HPV oncoproteins. Finally, a short comment concerning the application in diagnosis of methods other than immunohistochemistry has been added.

1.1 HPV in carcinogenesis

In the last three decades a large number of scientific studies have focused on the subject of cervical carcinogenesis. These resulted in the accumulation of data linking several types of

human papillomavirus to the development of cervical cancer (Bosch et al., 2002; Bosch et al., 2006; Crum et al., 1984; zur Hausen, 1977, 2009). The revealed strong association led to the suggestion that HPV is not only the main cause of cervical cancer, but also a necessary cause (Walboomers et al., 1999). Human papillomavirus is associated with more than 99% of all cervical cancer cases. In addition, a significant percentage of vulvar, vaginal, penile, anal and perianal carcinomas are HPV-positive (Fuste et al., 2010; Gross & Pfister, 2004; Insinga et al., 2008; Munoz et al., 2006), often containing HPV 16 DNA (zur Hausen, 2009), 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 region are presented in Table 1.

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 (Bergonzini *et al.*, 2010). Among them, cervical cancer represents a well-studied prototype of a human tumor related to a viral infection.

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 (Munoz et al., 2006; zur Hausen, 2009).

The most common viral types detected in cervical carcinomas include HPV 16, 18, 45, 31, 33, 52, 58, and 35 (Clifford et al., 2003; Munoz et al., 2003). The fraction of squamous cell carcinomas or adenocarcinomas attributable to HPV16 and HPV18, which comprise the two most common types, is 70% and 86%, respectively. The paradox is that, although infection with oncogenic types of HPV is very common, malignancy is a rare outcome. This difference in incidence between infection and cancer development reveals the significance of complex interactions between viral, environmental and host-related factors (Frazer, 2009; Moscicki et al., 2006; Snijders et al., 2006; Whiteside et al., 2008; zur Hausen, 2008). Viral persistence is an important determinant in this sequence of events, while immune status, viral integration into the host DNA, and infection with multiple HPV genotypes have significant roles. These multiple interactions are reflected in the long interval between infection and invasive carcinoma detection, often spanning a period of 15 to 25 years (zur Hausen 2008). Other factors that may modify the risk for HPV DNA-positive women include smoking, the use of oral contraceptives, and previous exposure to other sexually transmitted diseases (Bosch et al., 2006; Collins et al., 2010; Luie et al., 2011; Munoz et al., 2006).

In recent years a large number of scientific studies have resulted in the introduction of effective vaccines, which are expected to diminish the incidence of HPV-related carcinomas of the uterine cervix and other organs (Bogaardts et al., 2011; Frazer, 2009; Stanley, 2010; The FUTURE I/II Study Group, 2010; zur Hausen, 2008). Moreover, they are expected to reduce the incidence of intraepithelial HPV-related lesions. A large number of the latter are caused by non-carcinogenic HPV types and do not constitute precancerous lesions, but still may be

the cause of significant anxiety and distress for the patients. Furthermore, in rare instances they can give rise to life-threatening conditions, like recurrent respiratory papillomatosis.

Another main result of human papilloma virus research was the introduction in clinical practice of new diagnostic techniques (Cuzick et al., 2006; Gravitt et al., 2008; Poljak & Kocjan, 2010; Snijders et al., 2010). These allow for a more precise evaluation of the following: a) the presence of HPV in biologic specimens and the viral type present, b) the viral load, and c) the presence of an HPV-associated lesion demanding further therapeutic measures in cytological or biopsy material.

Finally, an important aspect of human papilloma virus research is the fact that the complex interactions between HPV oncoproteins and their multiple cellular targets offer to investigators the opportunity to study important cellular pathways related to the carcinogenic process in general.

1.2 Interactions between HPV oncoproteins and cellular pathways

High-risk mucosal HPVs encode three transforming proteins: E5, E6 and E7. Their multiple biological activities have been extensively studied in the last few decades; however, several aspects remain to be elucidated (McLaughlin-Drubin M & Münger K, 2009a).

HPV E5 is able to transform mouse fibroblasts and keratinocytes in culture (Straight et al., 1993). It is believed to contribute to early stages of carcinogenesis and works in concert with E6 and E7 (Talbert-Slagle & DiMaio, 2009; Hu et al., 2009). The latter proteins 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.

One of the main actions of HPV E7 proteins is the interaction 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 HR- HPV E7 (McLaughlin-Drubin M & Münger K, 2009a; Munger et al., 1991). 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 (McLaughlin-Drubin M & Münger K, 2009b; Zerbass et al., 1995). It also results in retaining differentiating keratinocytes in a DNA synthesis competent state.

High-risk HPV E6 proteins target p53 for proteasomal degradation through association with the cellular ubiquitin ligase E6AP (McLaughlin-Drubin M & Münger K, 2009b; Scheffner et al., 1990). Low-risk HPV E6 proteins can also associate with E6AP; however, high-risk HPV proteins target p53 for ubiquitination.

Furthermore, HR-HPV E6 and E7 proteins cooperate to generate mitotic defects and aneuploidy through induction of supernumerary centrosomes and multipolar mitoses in epithelial cells (Duensing et al., 2000), 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 (Crum et al. 1984).

Finally, integration of HPV genome into host chromosomes is an important event in cervical carcinogenesis (Hopman et al., 2006; Pett & Coleman, 2007), which 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.

2. Immunohistochemical stains in the diagnosis of Squamous Intraepithelial Lesions (SIL)

Clinical management of preinvasive cervical disease consists of confirmation of SIL diagnosis by histopathological examination, followed by treatment or careful follow-up of certain lesions, according to the current guidelines. Histopathological diagnosis of CIN is based on well-defined criteria. However, in certain cases distinguishing both low- and high-grade lesions from their mimics may pose problems (Crum & Rose, 2006; Kostopoulou et al., 2001; Kurman et al., 1992), even to experienced gynecologic pathologists. The distinction of florid reactive changes, immature metaplastic patterns, and atrophic changes from HPV-induced alterations may cause difficulties. 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 (Bollmann et al., 2005; Cho et al., 2005; Guillaud et al., 2005; Prasad et al., 1994; Salvia et al., 2004; Scheurer et al., 2007). However, studies attempting to correlate HPV presence and replication to certain cytohistologic alterations are becoming less frequent and/or fruitful.

In recent years molecular studies have revealed several markers that might be of utility in the diagnosis of squamous intraepithelial lesions, 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. The immunohistochemical stains that are currently in use in several laboratories worldwide, as well as some new promising markers are presented in the following text. 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.

2.1 p16

One extensively studied marker is p16 INK4A (hereafter referred to as p16), a cyclin-dependent kinase inhibitor. p16 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 (Lukas et al., 1995; Ohtani et al., 2004; Quelle et al., 1995; Serrano, 1997; Sano et al., 1998).

The expression of p16 is altered in several human tumors by deletions, mutations, or methylation (Cohen & Geradts, 1997; Nakashima et al., 1999; O'Neill & McCluggage, 2006; Ruas & Peters 1998) and has also been altered in cervical carcinoma cases. However, increased expression 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 (Lukas et al., 1995; Giarre et al., 2001; McLaughlin-Drubin & Munger, 2009b). Correlation has been reported between HR-HPV oncogene expression and high scores of p16 positivity (Andersson et al., 2006), and enhancement of p16 RNA level has been observed *in vitro* after immortalization by high-risk HPV types (Nakao et al., 1997). 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 (reviewed by Kostopoulou et al., 2011) and its possible correlation with HR-HPV types and/or lesion "progression". Indeed, p16 is one of the best studied markers in gynaecologic pathology. However, percentages of immunohistochemical 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, and the reported percentages of p16 immunopositivity are presented, together with the criteria and the antibodies used by the authors. Importantly, 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. It should be also noted that some authors interpret focal positivity as a false-positive reaction. Positivity in the studies presented below varied from 5.6% to 100% for low-grade lesions and from 45.2% to 100% for high-grade lesions (Table 2). The percentage of immunopositivity observed in non-neoplastic epithelia also varied between 0% and 32.7%.

In a recent study (Kostopoulou et al., 2011) 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 mainly in the lower epithelial layers, and cases with occasional positive cells, dispersed or in small groups, commonly above the parabasal layer. Diffuse positivity (Figure 1) in the horizontal plane involved either all epithelial layers, or 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%). In LSIL 41/55 cases (74.5%) showed some type of positivity, most commonly focal/sporadic (Figures 2 and 3). Interestingly, three out of eight LSILs showing diffuse immunoreactivity were characterized by markedly increased nuclear dimensions in the upper epithelial layers in comparison to other lesions characterized as low-grade. Another interesting finding of the above study was the different HPV type distribution 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 (Kostopoulou et al., 2011). The percentage of high-risk or probable high-risk HPV associated LSILs positive for p16 was 71.4% (25/35). This was not significantly different from immunopositivity observed in low-risk HPV associated lesions. Moreover, study of the pertinent literature revealed that a significant percentage of LSILs testing positive for HR-HPV by PCR or HC2 does not exhibit any p16 immunopositivity. The percentage of p16 positivity reported for HR-HPV positive LSILs varied from 32.4% to 94.4% (Ishikawa et al. 2006; Kalof et al., 2005; Kostopoulou et al., 2011).

Reference	Number of SILs examined	LSIL positivity	HSIL positivity	Non-neoplastic epithelia	Evaluation of staining	Antibody used in the study
Agoff et al., 2003	269	56.6%	84.5%	11.5%	N and C ≥5% cells	E6H4 (MTM)
Branca et al., 2004	137	35%	81.2%	0%	N and/or C	Polyclonal (Abcam)
Negri et al., 2004	127	74.7%	100%	ND	N and C ≥5% cells in lower third	CINtec p16 Histology Kit (DakoCytomation)
Volgareva et al., 2004	113	37.2%	45.2%	3.2%	N and/or C	E6H4 (MTM)
Wang et al., 2004	113	72%	94.7%	32.7%	Any reactivity	E6H4 (MTM)
Dray et al., 2005	104	74.1%	96.1%	7.0%	N and/or C	JC8 (Biocare Medical)
Murphy et al., 2005	117	100%	98.7%	0%	N or C	p16 (PharMingen)
Ishikawa et al., 2006	141	24.5%	87.5%	0%	Moderate and strong	E6H4 (MTM)
Focchi et al., 2007	153	90.9%	100%	7.9%	C and N ≥5% cells	Ab7 16PO7 (Neomarkers)
Hariri & Oster, 2007	140	71.4%	100%	6%	Continuous basal and parabasal	p16 Histology Kit (Dako)
Van Niekerk et al., 2007	184	57.1%	96.9%	22.9%	N and C ≥5% cells in each layer	E6H4 (DakoCytomation)
Godoy et al., 2008	115	50%	96.2%	0%	C and N	CINtec p16 Kit (Dako)
Dijkstra et al., 2010	406	5.6%	96.7%	ND	Diffuse, >1/3 of epithelium	Ab-4, 16P04 (Lab Vision)
Tan et al., 2010	129	26.7%	79.7%	0%	N and C ≥5% cells	p16 (NeoMarkers)

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

^a Only studies including more than 100 cases of squamous intraepithelial lesions in histopathologic specimens and published in the last ten years are presented.

Table 2. p16 immunopositivity in low- and high- grade squamous intraepithelial lesions reported in the literature^a

The results of the above studies point towards the use of p16 immunostain in conjunction with histopathologic evaluation. Addition of a consecutive p16-stained slide to the HE-stained slides has been shown to improve significantly interobserver agreement for both punch and cone biopsies (Bergeron et al., 2010; Dijkstra et al., 2010; Horn et al., 2008), and to help in the identification of occult lesions (Ordi et al., 2008). The differential diagnosis from non-neoplastic alterations can be facilitated, especially in conjunction with other immunostains, as presented below. Moreover, lesion grading can be faster, especially

concerning aggressive-appearing low-grade lesions, which otherwise might be upgraded (Dijkstra et al., 2010). Awareness of the different patterns of immunoreactivity 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 several unresolved technical issues remain, underlining the need for standardization of sample preparation and evaluation protocols (Mulvany et al., 2008; Tsoumpou et al., 2009).

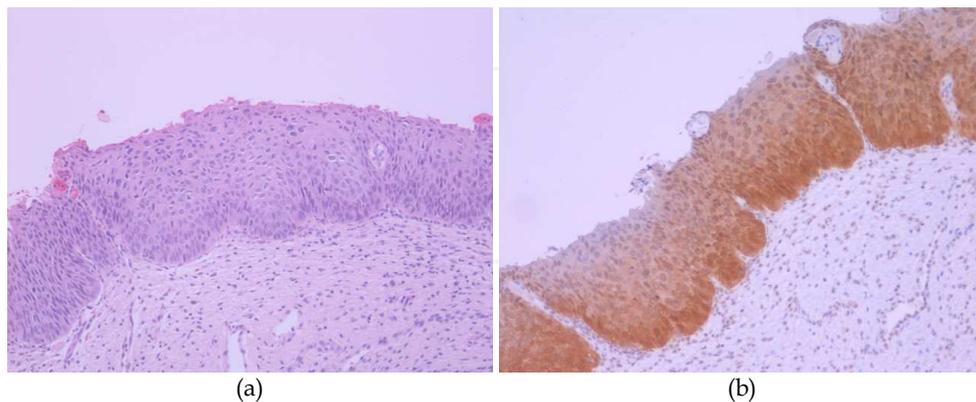


Fig. 1. (a,b). High-grade squamous intraepithelial lesion (HSIL-CIN2): (a) Hematoxylin and eosin staining, (b) p16 immunostain showing diffuse positivity.

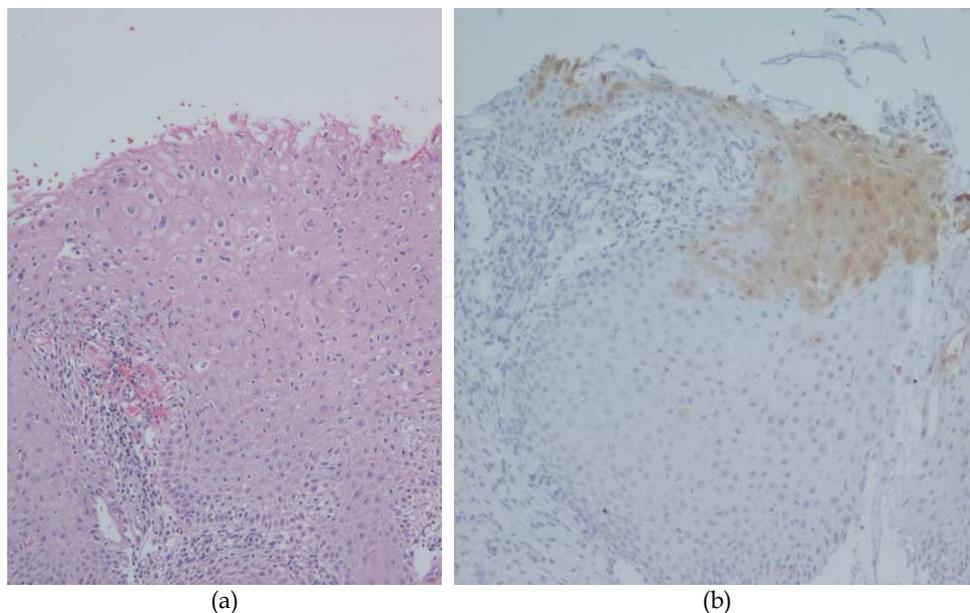


Fig. 2. (a,b). Low-grade squamous intraepithelial lesion: (a) Hematoxylin and eosin staining, (b) p16 immunostain showing focal positivity.

It is of note that: (a) in several studies, especially with increasing number of cases, there often appears a small group of HSILs that do not show any immunoreactivity, and (b) a significant percentage of LSILs associated with HR-HPV, as detected by PCR or HC2, does not exhibit p16 immunopositivity (Kostopoulou et al., 2011). 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. However, p16 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 (Dray et al., 2005).

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 (Keating et al., 2001). 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 (2007) 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%. In a study including conization specimens with coexisting CIN1 and CIN3 areas, all CIN1 were p16 positive (Negri et al., 2008), while p16 staining did not predict persistence or clearance of HR-HPV after treatment for CIN in a study by Branca et al. (2004).

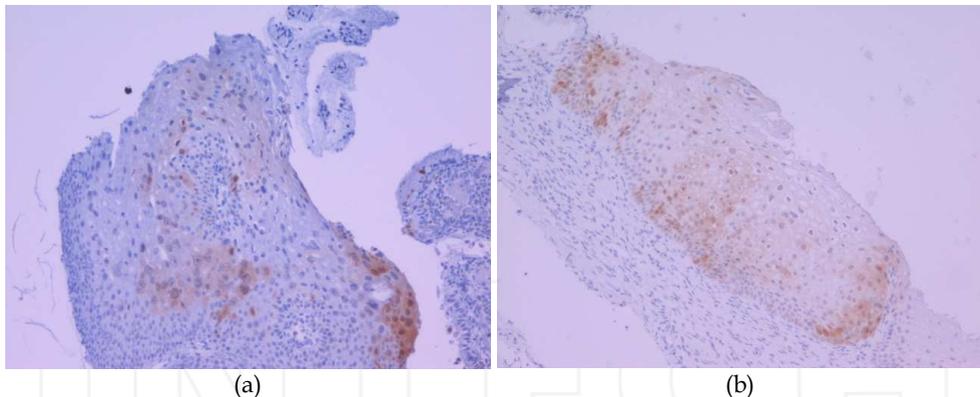


Fig. 3. (a,b). Common patterns of p16 positivity in low-grade lesions

2.2 Cyclins

Cyclins have been reported to be of help in the evaluation of cervical biopsies. Cyclin E is uncommonly expressed in epithelia not infected by HPV and its conspicuous immunopositivity may facilitate the recognition of SIL (Keating et al., 2001). In addition, cyclin B1 immunoreactivity above the basal/parabasal cells correlates significantly with HPV detection and could be a marker of HPV presence (Kostopoulou et al., 2008a). Cyclins D and A have been also studied as possible markers of HPV-related lesions.

2.2.1 Cyclin B1

It has been reported that E6/E7 oncoproteins of HPV type 18 induced changes in the expression of cell cycle regulatory proteins very early and before immortalization (Pei, 1996). 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. 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 immunostaining above the basal/parabasal layers was observed in all cases of HSIL (100%), most often involving the superficial layers as well (Kostopoulou et al., 2008a). Furthermore, increased cyclin B1 immunopositivity was observed in 51/52 low-grade lesions (98.07%) (Figure 4), 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.

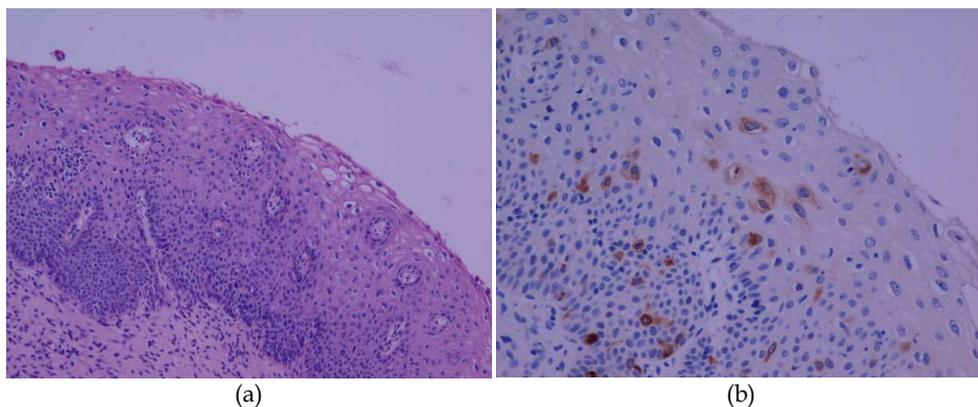


Fig. 4. (a,b). Low grade squamous intraepithelial lesion: (a) Hematoxylin and eosin staining, (b) Cyclin B1 immunostain, showing sporadic positivity in mature squamous polygonal cells above the basal layers.

The essential feature of the staining pattern observed in low-grade lesions and AUS cases 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. This pattern of immunoreactivity 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 immunopositivity was detected, 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. Several mechanisms could be related to this reaction (Kostopoulou et al., 2008a), while this pattern 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.

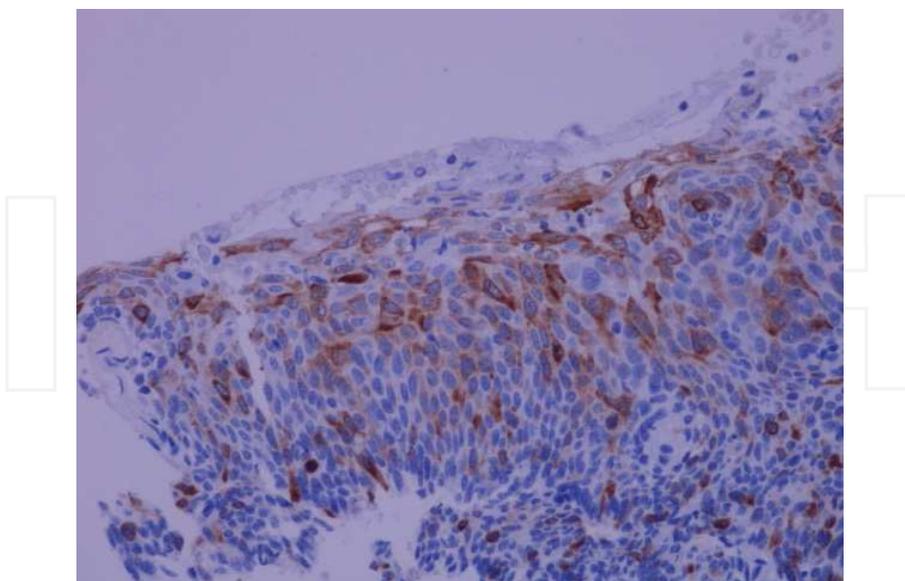


Fig. 5. Cyclin B1 positivity in an HSIL.

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.

2.2.2 Cyclin E

Cyclin E, another important cell cycle regulator, which promotes G1 transition, has been reported to exhibit increased expression in squamous intraepithelial lesions and invasive cervical carcinomas, although the exact mechanisms are not clear (Keating et al., 2001).

In a study by Keating et al., (2001) moderate to strong immunopositivity for cyclin E was observed in 92.6% and in 91.6% of low-grade and high-grade intraepithelial lesions, respectively, being positive in 38/41 HR-HPV positive cases. Furthermore, in a group of nondiagnostic squamous atypias cyclin E positivity was associated with HPV positivity.

In a study by Bahnassy et al. (2007), cyclin E staining increased from CIN1 to invasive carcinoma (16.7% to 88.4%, respectively), while gene amplification was detected in 11.1% of CIN1 cases and in 88.4% of carcinoma cases.

In conclusion, although cyclin E staining is not useful in the distinction of low-grade from high-grade lesions, it could be used to discriminate reactive from neoplastic epithelium (Crum & Rose, 2006), especially in conjunction with other markers, as discussed in other

parts of the present text. As is the case with the other immunostains examined in this text, standardization of staining and evaluation protocols are important for the appropriate application of these markers in certain diagnostic dilemmas.

2.3 Other proliferation/cell cycle markers

2.3.1 Ki-67

Ki-67, an antigen expressed in the nuclei of proliferating cells, has also been studied as an indicator of CIN. Ki-67 is expressed in the nucleus during the whole cell cycle, except for the G0 and G1 early phases. 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 6).

Immunopositivity for Ki-67 increases as a function of increasing lesion grade (Arafa et al., 2008; Conesa-Zamora et al., 2009; Carreras et al., 2007; Keating et al., 2001; Mimica et al., 2010; Pinto et al., 2008), but immunostains should be interpreted with caution, since reactive and inflammatory lesions may result in increased epithelial proliferation. It is well-known to pathologists that reactive and reparative changes may pose a problem in the examination of proliferation markers and in the case of Ki-67 immunostaining positive nuclei may extend through most of the epithelium. However, Ki-67 immunostaining can be used as an adjunct to other markers, as already discussed.

It should be noted that Ki-67 immunohistochemical stain may be especially helpful in differentiating atrophic epithelial changes from high-grade lesions (Crum & Rose, 2006).

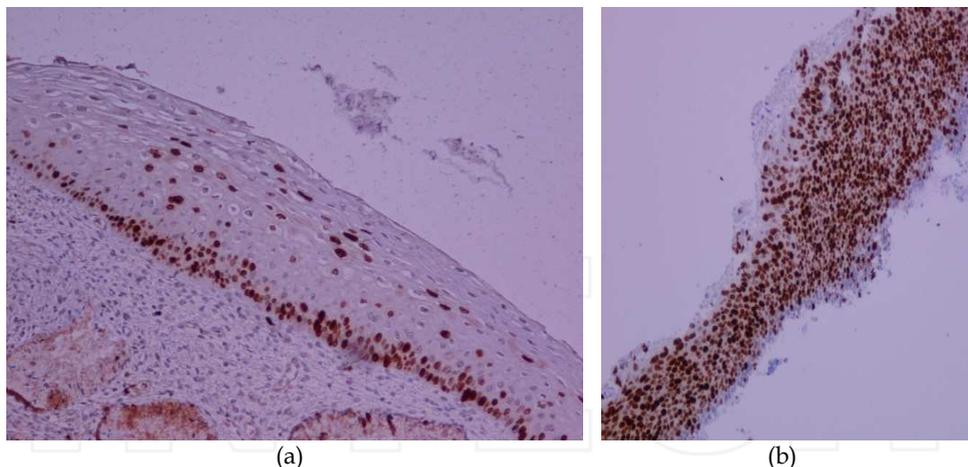


Fig. 6. (a,b). Ki-67positivity in (a) a low- and (b) a high-grade intraepithelial lesion.

2.3.2 Aberrant S-phase

Two relatively new biomarkers include the minichromosome maintenance protein 2 (MCM2) and DNA topoisomerase IIa (TOP2A) (Pinto et al., 2008). These two proteins have a significant role in the regulation of DNA replication during S-phase. They are overexpressed

when S-phase induction is aberrant and have been shown to be overexpressed in CINs and cervical carcinomas (Badr et al., 2008; Pinto et al., 2008; Shi et al., 2007). 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. ProEx C (Tambouret et al., 2008) is a recently developed immunohistochemical assay that targets these two proteins and appears to be efficient in distinguishing reactive epithelial changes from squamous lesions, alone or in conjunction with p16.

According to Shi et al. (2007), ProEXC is a better marker than p16 for the detection of LSILs, showing positivity in 94% of the cases in a series of 34 LSILs. In a study by Badr et al. (2008) 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. 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.

Pinto et al. (2008) included in their study 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, providing a lower false positive rate relative to the latter.

In a study by Sanati et al. (2010) sensitivity, specificity, positive and negative predictive value of ProExC in distinguishing high-grade squamous intraepithelial lesion from squamous metaplasia were 89%, 100%, 100%, and 82%, respectively. In a recent study by Guo et al., (2011) 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.

Walts and Bose (2009) suggested as cost saving strategy the use of two markers initially, p16 and ProExC, followed by Ki-67 immunostaining in discordant cases. According to the above 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.

2.4 Other markers and applications

In the present text an effort has been made to cover the immunohistochemical markers, which are currently most useful from a diagnostic point of view, and have been evaluated in several studies and laboratories.

In addition to the above biomarkers, which are in use in many pathology departments worldwide, a large number of other markers have been examined for their potential utility in the diagnosis and/or prognosis of cervical precursor lesions and in resolving problematic

cases (Galgano et al., 2010; Khan et al., 2008; Kostopoulou et al., 2008b). The results of these studies have been described in detail in the pertinent literature. In addition, image analysis methods have been used in an attempt to bring more objectivity to the interpretation of biopsy specimens. Furthermore, although the detection of SILs in cytology material and the evaluation of screening strategies are beyond the scope of the present text, it should be mentioned that the contribution of the above markers is important in this context, as presented in brief in the following (Carozzi et al., 2008; Depuydt et al., 2011; Tsoumpou et al., 2009).

2.4.1 L1 capsid protein

One recently studied marker, which has been examined repeatedly in cytologic material, is L1. Nuclear positivity for HPV L1 capsid protein, the major structural protein of human papillomavirus, is mainly observed in productive lesions and is gradually lost in high grade lesions and carcinomas.

It has been suggested that combined L1/p16 immunostaining may be helpful for clinical management, especially in cases in which the grade of the lesion is difficult to assess (Negri et al., 2008).

In a study by Galgano et al. (2010), this protein, which should be highly correlated with a productive viral infection, was neither sensitive nor specific for any group of cervical neoplasia in biopsy material. 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 had commonly at least 1 reviewer diagnosis of CIN1, revealing once again the difficulties in the distinction of SIL *vs* negative for SIL and the importance of a panel of immunostains in this specific context.

2.4.2 In situ hybridization techniques

Detection of papillomavirus nucleic acids is currently performed by methods that can be broadly subdivided into methods based on target amplification and those based on signal amplification (Snijders et al., 2010). In addition to several existing liquid phase techniques, in situ hybridization (ISH) methods have been developed for cytological and histological specimens. Both fluorescent detection and coloured substrate deposition followed by bright-field microscopy can be used, and can be combined with tyramide signal amplification. ISH assays can also be automated along the same lines as immunohistochemistry. Finally, except for HPV nucleic acids, other applications of in situ hybridization include the detection of amplification of the gene coding for the telomerase RNA component (TERC) at 3q26 (Hopman et al., 2006; Zheng et al., 2010).

Issues concerning sensitivity of the above techniques in comparison to PCR have been repeatedly raised. However, ISH techniques are becoming increasingly sensitive and can now detect low copy numbers of HPV DNA (Kelesidis et al., 2011; Montag et al., 2011). In addition, their important contribution to HPV research is the fact that they allow concurrent morphological evaluation of the areas examined, mainly in the case of histological specimens. Furthermore, the signal patterns observed in HPV ISH have been reported to be associated with the physical status of viral DNA in the cells examined, that is episomal or integrated. Specifically, the punctate pattern of positivity has been linked to the presence of

integrated viral forms in the host genome (Cooper et al., 1991; Evans et al., 2002; Hopman et al., 2005).

In a study by Guo et al. (2008) ISH and PCR had fair to good agreement in detecting HPV DNA across CIN categories, but ISH detected significantly fewer HPV-positive cases in carcinomas than PCR did, probably as a result of lower copy numbers of episomal as compared to integrated HPV DNA in the latter. In addition, although the pure punctate pattern of HPV indicated a high level of viral integration, the level of HPV integration could not be accurately determined in cases with mixed signal patterns, probably due to a variation in the percentage of the two patterns in these cases. Recently, Ho et al. (2011) reported a punctate pattern in 8.7% of CIN1 lesions *vs* 34.0% of CIN3 lesions in cytology material, while Alameda et al. (2011) reported a correlation of the punctate pattern with lesion persistence in cytology specimens.

According to Kong et al. (2007), 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 polymerase chain reaction. In a more recent study by Kelesidis et al. (2011), ISH exhibited a sensitivity of 89.5% for the detection of CIN2+ lesions, while PCR showed sensitivity of 94.7% for these lesions. A percentage of ISH-positive cases was not detected by PCR (performed on liquid-based sample media), emphasizing the technical problems and limitations of the techniques.

Voss et al. (2009) compared a fluorescence in situ hybridization (FISH) HR-HPV assay to Hybrid Capture 2 (HC2) and polymerase chain reaction (PCR) for the detection of HR-HPV subtypes in cervical cytology specimens. 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.

It is apparent from the above results that the applications of HPV ISH are partly dependent on the sensitivity of the assay and its sufficiency to carry a high negative predictive value (Crum & Rose 2006). This is especially important if clinical decisions are based on a negative result. However, ISH represents a useful tool for ancillary molecular HPV testing in cervical specimens, and may be important in certain clinicopathologic situations.

2.4.3 Applications in cytology

The preceding text focused mainly on the application of immunohistochemistry in SIL diagnosis in histopathology specimens. However, several of the above markers have been applied in cytopathology material, as presented in brief in the following paragraphs. The introduction of liquid-based techniques, which has been one of the most important advances in this field, has facilitated relevant applications.

The most studied marker in cytology is p16. Positivity has been observed in 10%-86% of LSIL and in 42%-100% of HSIL, as reviewed by Tsoumpou et al. (2009). The lack of general consensus regarding threshold values for p16 positivity is especially important in cervical cytology specimens. Several authors have used both quantitative and qualitative criteria, evaluating the number of positive cells as well as cell morphology, recognizing the fact that p16 overexpression may be often detected in nondysplastic cells. In the contrary, other investigators used only quantitative criteria.

It has been suggested that p16 immunocytochemical testing can be used as a reflex test in conjunction with liquid-based cytology following a cytologic result of ASC-US or LSIL, or be used on destained conventional or liquid-based cytology specimens (Denton et al., 2010). p16 in conjunction with Ki-67 provide high sensitivity for the detection of CIN2+ lesions (Schmidt et al., 2011; Yu et al., 2010).

The prognostic utility of L1 immunocytochemistry, especially in association with p16 in cytology, has been reported by several authors (Griesser et al., 2004; Sarmadi et al., 2011; Yoshida et al., 2008).

The use of HPV in situ hybridization has already been discussed in the previous section. It is of note that, in a recent study, prior knowledge of HPV status resulted in significantly higher detection rate of CIN2+ in cytology specimens compared to screening blinded to HPV status, with limited loss of specificity (Benoy et al., 2011). This raises several important questions, although more research is needed to study the significance of this type of knowledge provided prior to cytological reading.

3. Conclusion

Although histopathology remains the “gold standard” for the diagnosis of SIL, both low- and high-grade, certain biomarkers have emerged as helpful adjuncts. Their combined use may assist in the histopathologic classification of preinvasive lesions and facilitate the distinction from non-HPV induced alterations. It is clear from the above that the diagnosis of a squamous intraepithelial 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 morphological evaluation, the latter comprising the most important part of the diagnostic procedure. Standardization of protocols and familiarity with the patterns of immunostaining, especially in nonneoplastic cervical tissue, are important requirements for the proper use of the above markers. Awareness of the strengths and limitations of each particular technique cannot be overemphasized. In addition, the performance of several markers and methods in the detection of lesions related to HPV types other than those addressed by the current vaccines remains to be carefully evaluated.

4. References

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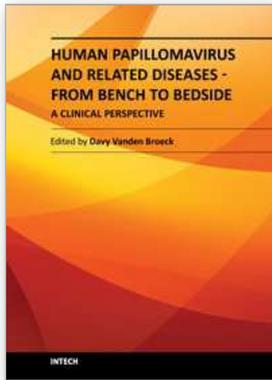
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Cervical cancer is the second most prevalent cancer among women worldwide, and infection with Human Papilloma Virus (HPV) has been identified as the causal agent for this condition. The natural history of cervical cancer is characterized by slow disease progression, rendering the condition, in essence, preventable and even treatable when diagnosed in early stages. Pap smear and the recently introduced prophylactic vaccines are the most prominent prevention options, but despite the availability of these primary and secondary screening tools, the global burden of disease is unfortunately still very high. This book will focus on the clinical aspects of HPV and related disease, highlighting the latest developments in this field.

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821