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MOLECULAR MARKERS OF EARLY CERVICAL NEOPLASIA

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Abstract

Pure morphological distinction of high-grade squamous intraepithelial lesions (HSILs) from their mimics can be challenging. Diagnosis can be difficult with nonconventional HSILs associated with a metaplastic phenotype, squamous intraepithelial lesions (SILs) that defy precise classification such as “eosinophilic dysplasias”, and those that overlap with columnar neoplasms, including stratified variants of adenocarcinoma in situ (“SMILE”). Gene expression and protein profiling have identified biomarkers with the potential to decrease diagnostic variability and increase specificity of histological and cytological analysis. Among the ones clinically useful for HSIL detection are p16^{INK4A} and MIB-1 which complement each other, differentiating SIL from normal/atrophic (MIB-1 low) or reactive/immature metaplastic (p16^{INK4A} scattered) epithelium. Additional markers, including ProExTMC, have been proposed but their added value is yet to be established. In the final analysis, biomarkers are most helpful for distinguishing benign immature or atrophic proliferations from HSIL. The distinction of LSIL from HSIL must be made on the hematoxylin and eosin-stained section and should be made with care, given the potential consequences of a diagnosis of CIN2 or CIN3.

Keywords

biomarker; immunohistochemistry; p16^{INK4A}; ProExTMC; MIB-1; cervical biopsy; liquid based cytology; cervix neoplasia; cervical intraepithelial neoplasia (CIN); squamous intraepithelial lesion (SIL); HPV

It is well established that most cervical cancers develop from precursor lesions associated with high-risk human papillomaviruses (HR-HPV). These lesions have been classically termed dysplasia and are more currently designated as cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SIL). SILs are classified as low and high grade according to their putative potential to progress to carcinoma if untreated. While low grade squamous intraepithelial lesions (LSIL) rarely are followed by a diagnosis of malignancy, high grade

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squamous intraepithelial lesions (HSIL) are considered the true precursor of cervical squamous cell carcinomas (SCC). The importance of the accurate diagnosis of the latter is crucial to both optimal care and to interrupt the natural history of this disease.¹ A histopathological diagnosis is routinely obtained on biopsies or loop electrical excision procedure (LEEP) specimens and is the basis for the proper care of patients with SIL previously detected by cytology or a combination of cytology and molecular tests such as hybrid capture HPV DNA detection. At this point, high specificity is the desired goal in order to prevent false-positive results that may lead to patient psychological stress, unnecessary conization or hysterectomy, and increased medical costs.

Variants, differential diagnosis and inter-observer variability of SIL

The histological features of nonconventional variants and the most common differential diagnosis or “mimics” of SIL have been described previously in a recent article by Nucci². Important elements of that review will be summarized here and will serve as basis for the subsequent description of the molecular markers applications. Our main focus will be on HSIL, the most clinically relevant lesion for SCC prevention and the one for which over-diagnosis can result in unnecessary surgery.

One of the most common sources for inaccurate interpretation of SIL is atrophy, characterized by altered maturation, nuclear crowding, and increased nuclear/cytoplasmic ratio (Figure 1). While severe atrophy may be confused with HSIL, milder disturbances in maturation may mimic LSIL, with pseudo-koilocytosis in the lower end of this spectrum. Also, superficial vacuolated cells of reactive mature or partially atrophic epithelium may mimic koilocytes (Figure 2). A critical issue is the separation of HSIL from LSIL, as gauging the degree of atypia that exceeds the limits of an LSIL diagnosis is not always easy and over-diagnosis can have important therapeutic implications². Under the current guidelines, however, the distinction between reactive epithelium and LSIL is not so critical in that the diagnosis of the latter should not trigger an ablative procedure if there is no concern for HSIL on clinical or cytologic grounds. Thus, the practicing pathologist is discouraged from expending undue energy to verify a subtle LSIL or ordering excessive immunostains to resolve mild atypias (Figure 2). On the other hand, the distinction between an immature reactive or metaplastic proliferation and HSIL is important and sometimes, difficult (see below).

Although the classic spectrum of changes related to LSIL and HSIL are well described in most text books, the transformation zone has a high degree of morphologic plasticity and high risk (HR-HPV) and low risk (LR-HPV) HPVs can infect different cell types, including columnar, reserve, mature, intermediate, and immature squamous cells. These nonconventional SIL variants are described as follows:

1) SIL with immature metaplastic phenotype

Presumably, this subset of precursors results from the infection of immature metaplastic cells by HR-HPV; they can be subdivided into 3 patterns:

i) *HSIL akin to atypical immature metaplasia (AIM)* - Immature and often uniform metaplastic cells with relatively high nuclear density and hyperchromasia in all epithelial layers including the upper ones (figure 3). This is associated with a lack of normal cytoplasmic maturation with discrete cell borders on the surface, as well as a lack of surface columnar cells or mucin droplets, features that are usually seen in immature squamous metaplasia. The origin of this variant is the squamous immature cell. ii) *Eosinophilic dysplasia (ED)* – Expansile proliferation of cells that lack normal maturation, have abundant eosinophilic cytoplasm with distinct cell membranes, a modest increase in nuclear cytoplasmic ratio and focal nuclear dysplastic features with mild nuclear enlargement, hyperchromasia, irregular nuclear membrane contour,

and distinct nucleoli. ED has been proposed as a variant of HSIL; however, the degree of atypia is variable and it is likely that lesions falling in this spectrum could be classified in either the low or high grade category (Figure 4). Currently, we classify problematic lesions as “SIL in metaplasia, possibly low (or high) grade” and allow the clinician to incorporate other considerations (age, size of lesion, cytology etc) into the management scheme. The cell origin of this SIL variant appears to range from cells with well to intermediate squamous differentiation. iii) *SIL occurring in the setting of columnar differentiation change* – These can be seen associated with microglandular change or in some cases, as mild reserve cell expansions with mild atypia, and prominent columnar cell differentiation, the latter manifested by mucin droplets. Atypia is usually mild. These lesions can be classified as SIL of uncertain grade but if the changes are modest, we classify them as LSIL (Figure 5).

2) Stratified variants of adenocarcinoma in situ (so-called stratified mucin-producing intraepithelial lesion (“SMILE”))

This is best classified as a variant of adenocarcinoma in situ (AIS), with a phenotype that strongly resembles HSIL because of its stratification. Another histological key feature is the discrete mucin production in all layers of the epithelium, in contrast to the reserve cell expansions where the mucin production is distinctly superficial. It may coexist with an invasive carcinoma with an adenosquamous pattern, and limited studies suggest that SMILEs may constitute a risk factor for invasion (Figure 6)

3) Immature condyloma (squamous papilloma or papillary immature metaplasia)

This is a variant of LSIL that can be misclassified as HSIL because of its immature appearance, but is associated with LR-HPV. It is composed of a slender, filiform papillae lined by squamous epithelium only showing slight nuclear crowding and mild superficial koilocytotic atypia mimicking immature metaplasia (Figure 7). Unlike mature condylomas that exfoliate cells with conspicuous cytopathic effect (koilocytosis), immature condyloma is usually preceded by a negative or undetermined atypical cytological result, and so, is less likely to be identified as a SIL or as non-specific atypia (ASCUS) by cytology.

Although histological assessment has been classically considered as the “gold standard”, a significant amount of intra and inter-observer variability for SIL diagnoses has been well documented¹. This is mainly due to the SIL mimics and the SIL morphological variations described above; the former a common finding in routine biopsies and the latter a feature inherent to the pathogenesis of SILs. In general, the high end of the diagnostic spectrum of HSIL, cervical intraepithelial neoplasia grade 3 – CIN3, achieves the highest index of histopathological diagnostic agreement. However, in our personal experience, the level of agreement can be highly related to the individuals involved in the comparison (i.e. number of years of training and/or experience). High inter-observer variability for SIL has been observed also in liquid-based cytology¹. Surrogate biomarkers have been shown to decrease diagnostic variability and increase specificity of histological and cytological analysis on a routine basis.

HPV, cell cycle deregulation and biomarkers

In the HPV infected squamous cell, E6 and E7 viral oncoproteins bind host regulatory proteins leading to degradation of p53 protein and inactivation of retinoblastoma (Rb) protein, two tumor suppressor gene products. These interactions are the essence of the HR-HPV mediated carcinogenesis and ultimately lead to deregulation of the cell cycle manifested by abnormal expression of cell cycle-associated proteins, such as p16. Abnormal expression of the p16 protein can then be used as an immunohistochemical marker of SIL. Based on this, biomarkers targeting key molecular pathways in early and late cervical carcinogenesis have been tested in large-scale prospective cohort studies conducted during the past 3 decades. Goals of previous

and ongoing studies are to get new insights into the HPV induced carcinogenesis and to disclose additional markers that will distinguish productive from transforming HPV infections and predict disease outcome³. In relation to the latter, despite efforts made in the field, the discovery of an ideal marker or set of markers that will predict progression of HSIL to a more advanced disease still waits. Some of the most studied markers classified by their main role in the initial stages (i.e., SIL) of cervical carcinogenesis include: 1) cell cycle regulation markers - p53, p21, Bax, pRb, p16^{INK4A}, p27, MCM2 and TOPII α (ProExTM C), cyclin D1, cyclin A, cyclin E; 2) cell proliferation markers - proliferating cell nuclear antigen (PCNA), c-myc, Ki-67 (MIB-1); 3) apoptosis markers - telomerase, survivin; 4) transcription markers - Skn-1, Oct-1, AP-2, NFkappaB; 5) cell signaling pathway markers - ERK, JNK, p38 kinases – subgroups of mitogen activated protein kinase (MAPK) signaling cascade; and 6) markers of productive HPV infection - HPV L1 capsid protein.

Gene expression and protein profiling have identified a number of differentially expressed genes and proteins in premalignant and malignant cervical disease. Although several of these biomarkers have been studied and are suitable for histopathological sections⁴ or liquid-based preparations⁵, just a few had been proven to be clinically useful for HSIL detection⁶. Most of them are based on protein detection by immunohistochemistry (IHC) and are useful to identify whether or not a cervical pre-cancer disease is present^{4,7}, but not to assign a grade for SIL. Commercial available assays are: 1) the cyclin-dependent kinase inhibitor p16^{INK4A} (CINtecTM); and 2) the DNA replication markers minichromosome maintenance protein 2 (MCM2) plus the DNA topoisomerase II α (TOP IIA), in conjunction called ProExCTM (BD Diagnostics). Both products are available for detection in histological and cytological specimens. Among the noncommercial biomarker assays are: 1) the proliferation marker Ki67 (MIB-1); and 2) chromosomal amplification of 3q, which can result in increased telomerase activity. The latter is an interesting marker based on in situ hybridization (ISH) for detection of HSIL and invasive squamous carcinomas.

A very recent tissue microarray study found that the expression of p16^{INK4A}, MIB-1, and ProExCTM in combination was the most sensitive for determining the severity of dysplasia among other biomarkers⁸. These three immunohistochemical markers are currently considered to be the most clinically useful for SIL detection. Their roles in the cell cycle and their staining patterns in SIL, SIL variants, and SIL mimics, as well their clinical application will be described below.

Practice points

- The need of an accurate diagnosis to base management, and the high inter-observer variation observed in SIL diagnoses justifies the use of immunohistochemical biomarkers in histological sections.
- p16^{INK4A}, MIB-1 and ProEx C are clinically useful for SIL detection

p16^{INK4A}

p16^{INK4A}, the protein derived from the *p16^{INK4A}CDKN2A* tumor suppressor gene, has been identified as a biomarker for transforming HPV infections. Physiologically, p16^{INK4A} blocks the activity of cyclin-dependent kinases CDK4/6, acts in a negative feedback loop with another tumor suppressor, Rb protein, and down regulates progression through the G1-S transition checkpoint of the cell cycle, so that cell proliferation is held in check⁹. When HR-HPV infects the host cell, the viral oncoprotein E7 binds and disrupts Rb from its binding to E2F transcription factor and thereby promotes cell cycle progression, a molecular switch that is usually activated by CDK4/6. The feedback loop is lost and p16^{INK4A} is overexpressed. As a

result, the protein is accumulated in the nucleus and cytoplasm of the affected cells and can be detected by IHC.

Positive p16^{INK4A} immunostaining is expressed as a diffuse, strong, cytoplasmic and nuclear staining pattern in squamous and glandular (i.e., adenocarcinoma in situ, ACIS) lesions associated with high-risk HPV infection, especially HSILs and ACIS. However, two thirds of LSIL will also stain strongly, the exceptions being exophytic and immature condylomas (Figure 8). These low grade lesions which are associated with low risk HPV infections tend to exhibit a focal and scattered weak blush of p16^{INK4A} rather than the diffuse strong granular staining pattern typical for HSIL.

The role of diffuse p16^{INK4A} immunostaining as a predictor of disease progression has also been explored in an effort to identify those LSILs that need more intensive follow-up¹⁰. A recent study¹¹ proposed the combination of p16^{INK4A} with L1 IHC for the estimation of the biological progression risk of LSIL. L1 is an antibody against a protein of the HPV capsid that is expressed in the early productive phase of cervical carcinogenesis and is progressively lost in the subsequent phases, when p16^{INK4A} gets over-expressed. Moreover, a variety of lesions unassociated with HPV which show focal or patchy p16^{INK4A} staining have also been evaluated. These lesions include squamous metaplasia, tubal metaplasia, endometrioid and serous adenocarcinoma, the later showing in fact a diffuse and strong staining pattern. Therefore, the context in which p16^{INK4A} is applied and also the distribution and intensity of its staining pattern must be considered before using this stain to confirm the presence of an HPV-associated lesion.

Another variable that must be appreciated in the context of predicting outcome of such studies is the reproducibility of LSIL diagnosis. We have shown previously that while 10% of LSILs may be followed by an outcome diagnosis of HSIL, the actual figure is less than half of this following pathologic re-review. In other words, progression can be an artifact of misclassifying an LSIL as HSIL on outcome biopsy. In our experience, when misclassified cases are excluded, the risk of progression is very low, calling into question the real value of p16 immunohistochemistry in predicting behavior of LSIL. Moreover, we have found that 40% of CIN2 lesions (consensus diagnosis) will regress in 6 months. Additionally, Syrjanen³ showed that p16 staining in CIN2 did not predict outcome.

In the case of immature atypias, p16^{INK4A} may be more helpful, simply because the differential diagnosis includes HSIL. Atypical immature metaplastic lesions that are p16^{INK4A} positive have been shown to have a higher incidence of subsequent HSIL and are therefore better classified as HSIL with an immature metaplastic appearance¹² (Figure 9). Likewise, negatively staining lesions can be dismissed as reactive atypias (Figure 10). Still others, particularly inflammatory lesions of decidedly uncertain significance, can also stain strongly for p16, and their classification remains problematic (Figure 11).

Among all markers, p16^{INK4A} remains to be the most reliable for HSIL detection in daily practice. In general, the proportion of cervical smears and histological sections overexpressing this marker has been shown to increase with the severity of the cytological/histological abnormalities. In CIN3, the higher end of the spectrum of HSIL, p16^{INK4A} immunostaining had shown 100% sensitivity and 95% specificity¹³. A recent systematic review and meta-analysis of all studies that evaluated the use of p16^{INK4A} in cervical cytological and histological specimens have confirmed this correlation between marker and degree of morphological abnormality¹⁴. Nevertheless, it was not able to formulate any recommendation regarding the applications of p16^{INK4A} in clinical practice. According to the authors, the main reason for this was that the majority of the studies utilizing p16^{INK4A} have focused on the marker/abnormality correlation. Another reason was the discrepancies in sample interpretations,

reducing reproducibility and interpretation of the data across different investigations. To date, just a small number of studies have addressed questions regarding p16^{INK4A}'s role in primary cervical cancer screening, in the triage of equivocal low-grade smears versus HPV-DNA tests, and in the progression risk of LSIL or cervical intraepithelial neoplasia grade 2 (CIN2).

MIB-1

Ki67 is an antigen that identifies proliferating cells and is expressed in all parts of the cell cycle (G1, S, G2/M). MIB-1 is a monoclonal antibody that detects this antigen in the cell's nuclei in paraffin tissue sections. HPV infection activates host cell cycle progression with increased cell cycle kinetics and this phenomenon is reflected in an increased MIB-1 staining index.

Positive MIB-1 staining in parabasal cells of the squamous epithelium is found under normal conditions. Distinction of a SIL from nonneoplastic epithelium is based on the presence of MIB-1 positive cells in the middle and upper thirds of the epithelium⁹. HSIL (i.e., CIN3 lesions) usually shows diffuse nuclear positivity scattered throughout all layers of the epithelium (Figure 9), whereas the distribution of MIB-1 staining in LSIL can be less diffuse with only small clusters of squamous cells staining in the upper two-thirds of the epithelium (Figure 8); moderate/high grade lesions (i.e., HSIL/CIN2) may show variable numbers of MIB-1 immunoreactive cells in the middle and upper thirds of the epithelium.

Pitfalls include over-interpretation of positive MIB-1 staining in the presence of inflammation in reactive epithelium (Figure 11), and in intraepithelial lymphocytes that will also stain positive for MIB-1. Also, tangential sectioning sometimes may result in the impression that MIB-1-positive parabasal cells are superficially placed, leading to confusion with a SIL. Finally, interpretation of MIB-1 stained slides may be more difficult in small samples and when less than a full thickness epithelium is present.

Atypical immature metaplastic proliferations, when diffusely positive for MIB-1, are usually HSIL, provided there is also diffusely strong p16 immunoreactivity¹². Stratified adenocarcinomas *in situ* should show diffuse positivity for MIB-1 throughout the entire thickness of the lesion, including the mucin-producing cells¹⁵. Immature condylomas display variable staining for MIB-1 with a low proliferation index in the mid and upper epithelial layers, somewhat less than that seen in LSIL and mature condylomas, and much less than HSILs and papillary carcinomas.

The role of MIB-1 quantitation in providing information about the progression risk of LSIL and CIN 2 has been discussed in several studies¹⁶ but remains a work in progress.

p16^{INK4A} and MIB-1

The recognition and combined application of p16^{INK4A} and MIB-1 in daily practice were previously described in a review article by Nucci and Crum in 2007.² This review indicated p16^{INK4A} as the most useful marker for distinguishing immature metaplasia from HSIL, and Ki67 for distinguishing atrophic changes from HSIL, pointing out their complementary role in routine anatomic pathology practice.

Follow-up studies have since reported that HPV-associated immature metaplastic proliferations showing p16^{INK4A} and MIB-1 over-expression subsequently recur as a bona fide HSIL in many instances. Also, it has been hypothesized that immature metaplastic proliferations that are HPV and p16^{INK4A} positive but hypo-proliferative with MIB-1 may be early or regressing lesions. Most eosinophilic dysplasias are diffusely and strongly positive for p16^{INK4A}, and hyper-proliferative with MIB-1. AIM - atypical immature metaplasia and ED - eosinophilic dysplasia are not terms commonly used in surgical pathology reports. Therefore,

lesions with those morphological features that diffusely express p16 and are hyper-proliferative with MIB-1 should be classified as HSIL on a routine basis.

ProEx™ C

The minichromosome maintenance protein 2 (MCM2) functions during DNA replication by loading the pre-replication complex onto DNA and by unwinding DNA through helicase activity to permit DNA synthesis. DNA topoisomerase II α (TOP2A) is responsible for the enzymatic unlinking of DNA strands during replication. These two proteins play a significant role in the regulation of DNA replication during S-phase. They are both overexpressed when the S-phase cell cycle induction is aberrant. Both, MCM2 and TOP2A were recently identified by gene expression studies. ProEx™ C is a cocktail of two monoclonal antibodies that targets the expression of these two proteins. This assay recently became commercially available for application in histological and cytological specimens.

The ProEx™ C signal is intense, diffuse, and comprises full epithelium thickness in HSIL, yet presents with a variable pattern of staining in LSIL, and is usually negative in reparative or reactive immature squamous metaplasias. The staining pattern of ProEx™ C in histologic sections is similar to MIB-1 in that only nuclear positivity above the basal one-third layer is considered positive. Nevertheless, staining for MIB-1 and ProEx™ C in HSIL is discordant in about 35% of cases¹⁷. In our experience, ProEx™ C stains normal reactive epithelial cell nuclei less, and LSIL nuclei greater, than MIB-1 (Figure 12). Nevertheless, ProEx™ C is comparable to p16^{INK4A} and MIB-1 in the detection of HSIL in tissue sections^{18,19}. Recently, we tested this marker in a small challenging series of HSILs and benign mimics⁷. ProEx™ C appeared to provide an equivalent level of sensitivity and a higher level of specificity for HSIL alone or in conjunction with p16^{INK4A}. However, p16^{INK4A} allows for easier interpretation of SIL due to the strong diffuse immunostaining of SILs containing high risk HPVs, including both LSILs and HSILs.

Regarding ProEx™ C application in cytology, a recent study found higher sensitivity (98.8%) and specificity (82.4%) for detecting HSIL against Hybrid Capture II HPV DNA testing HR-HPV status (71.1% and 64.7% respectively) in 100 specimens with a diagnosis of atypical squamous cells - cannot exclude HSIL (ASC-H)²⁰. Another study on liquid-based cervical cytological samples²¹ suggested that ProEx™ C can be more selective and informative for the progression of low-grade (CIN1) and moderate-grade (CIN2) lesions than MIB-1. The authors also proposed an analytical threshold of ProEx™ C - positive nuclear area (3%) for dividing squamous lesions into low and high risk of progression, in order to define patients who need differentiated clinical management.

Concerning its use on HSIL variants, two studies^{8,19} related a variable ProEx™ C staining pattern in AIM, similar to what is seen in LSIL or condyloma. The specific application of ProEx™ C on HSIL variants other than AIM has never been addressed.

Despite these preliminary findings, data on the clinical utility of the ProEx™ C assay in histological^{18,19,7} or cytological²² specimens are still limited, and its potential role as a marker for SIL is far from being confirmed and completely understood.

p16^{INK4a} and ProEx C

The combination of p16^{INK4a} and ProEx™ C may result in a high diagnostic value for the detection of HSIL and LSIL in cervical biopsies according to one study²³. This study found p16^{INK4a} to be more sensitive and specific for identifying HSIL, and ProEx™ C a better marker for the detection of LSIL. However, it is the role of ProEx™ C as a detector of HSIL cells with aberrant S-phase induction that has been stressed and demonstrated by most

publications^{8,19,22,20,7}. In our hands⁷, the combination of p16^{INK4A} and ProExTM C predicted more non-SILs than p16^{INK4A} combined with MIB-1. This suggests that ProExTM C may be more efficient (lower false positive rate for NoSIL) than MIB-1 as complimentary surrogate marker in distinguishing HSIL from benign mimics. A recent study (24) was designed to evaluate the efficacy of these three stains alone and in combinations to determine the most useful strategy in the diagnosis of SIL in histological sections and provide directions in cases with discordant staining patterns. The best cost/beneficial combination was found by using p16^{INK4A} and ProExTM C initially, followed by Ki-67 only when the first markers reveal discordant results.

Practice points

- p16^{INK4A} is currently the most reliable immunohistochemical marker for HSIL detection.
- p16^{INK4A} and MIB-1 complement each other by differentiating SIL respectively from reactive or atrophic epithelium with atypia.
- ProEx C is comparable to MIB-1 as a complement to p16^{INK4A} in clinical settings.

Final remarks

Pathologists have three important decisions to make when evaluating cervical biopsies, which are to confirm SIL, distinguish LSIL from HSIL (CIN2 or greater) and exclude malignancy. Among the biomarkers described in this review, p16^{INK4A}, a surrogate marker of HPV infection, and MIB-1, a marker of cell proliferation, are currently the most useful in daily practice. When used concurrently, these two biomarkers can help the practitioner pathologist to identify whether or not a SIL is present, differentiating it from normal, atrophic, reactive or immature metaplastic epithelium. ProExTM C appears to be a reasonable alternative to MIB-1 in this complementary role with p16^{INK4A}^{7,24}. A recent study suggested a clearer separation of adenocarcinomas *in situ* from tubal metaplasia with ProExTM C, since this reagent does not stain the latter whereas p16 staining of either tubal metaplasia, endometrium (or endocervix) can be focally intense. This may be one of the more useful applications of ProExTM C in the cervix.²⁵

Recently, p16^{INK4A} has been shown to increase the specificity of HPV Hybrid Capture II test in a subset of a large randomized controlled trial²⁶. The validity of p16^{INK4A}, as well the recently introduced ProExTM C as ancillary tools in a “biomolecular Pap test” has to be further tested, optimized, and automated in multicenter trials.

Research directions

- The search for novel biomarkers with independent prognostic value in cervical cancer or SIL.
- The role of p16^{INK4A} and ProExTM C as potential “biomolecular Pap tests”.

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Figure 1. Atrophic squamous epithelium (A) H&E, (B) corresponding negative p16 immunostain (photos courtesy of E. Pirog).

Figure 2. Mild squamous atypias can occur in both reproductive age and menopausal women. Immunostains are not required in the majority of cases given the lack of impact on patient management.

Figure 3. HSIL with a metaplastic phenotype. The cells are more uniform than more classic HSILs; however the nuclear heterogeneity, crowding and lack of surface maturation characterize this lesion.

Figure 4.

SIL occurring in a more mature metaplastic background (compared to Figure 3). Nuclei tend to be well spaced, often with mild chromatin abnormalities (A) H&E, (B). Mib-1 staining is positive (C), p16 stain is diffusely positive due to the presence of high risk HPV types. We would nonetheless classify this as SIL in metaplasia, favor LSIL.

Figure 5. SIL with columnar differentiation, favor LSIL. This is another interesting SIL. Note that on H&E, the nuclei are enlarged but display minimal chromatin abnormalities and conspicuous

cytoplasmic maturation (A) H&E, (B) Mib-1 staining is low and the criterion of positivity (expansion of positive cells into the superficial 2/3 of the epithelial thickness) cannot be assessed in this case (C). Note however the intense p16 staining of all the cells, including columnar and reserve cells.

Figure 6. Stratified mucin-producing intra-epithelial lesion (SMILE; also known as stratified adenocarcinoma in situ). Like the SIL in Figure 5, this lesion displays columnar differentiation. Note however, the much higher nuclear density and nuclear atypia.

Figure 7.

Immature condyloma (LSIL). This lesion is a consequence of infection of immature metaplastic epithelium by HPV types 6 or 11. Characteristic features are villiform architecture, uniform nuclei with small nucleoli and uniform nuclear spacing. In such lesions the Mib-1 index is usually low to moderate and the p16 stain is either negative or patchy in distribution.

Figure 8. LSIL. (A) H&E, showing koilocytosis, (B) both diffuse basal and patchy (left) staining for p16, (C) positive Mib-1 staining (D). another LSIL showing weak p16 staining;

Figure 9.

HSIL with a metaplastic phenotype (A) H&E, (B) positive Mib-1 staining; (C) strong and diffuse staining for p16.

Figure 10. Reactive epithelial changes. A) H&E, B) negative p16 staining, C) positive Mib-1 staining; Mib-1 stain positivity in this case reflects lack of epithelial maturation. The case illustrates higher specificity of p16 in classification of immature inflammatory lesions.

Figure 11.

SIL. Immunostaining of a markedly inflamed atypical squamous epithelium (A) H&E (B) positive staining for p16. The intense staining is consistent with SIL; however, the lesion is difficult to grade.

Figure 12.

Comparison of ProEx-C and Mib-1 staining in reactive epithelium (A&B) and LSIL (C&D). Note the staining in reactive epithelium is higher with Mib-1 (A) than ProEx-C (B). In contrast the Mib-1 index in LSIL (C) is lower than ProEx-C (D).