Immunomarkers in Gynecologic Cytology: The Search for the Ideal ‘Biomolecular Papanicolaou Test’

Álvaro P. Pinto^a Martin Degen^b Luisa Lina Villa^c Edmund S. Cibas^a

Departments of ^aPathology and ^bDermatology, Brigham and Women’s Hospital and Harvard Medical School, Boston, Mass., USA; ^cDepartment of Radiology, Cancer Institute of the São Paulo State, University of São Paulo School of Medicine, São Paulo, Brazil

Key Words

Biomarkers • Cervical cytology • HPV • Human papillomavirus • Immunocytochemistry • Papanicolaou

Abstract

Harnessing the knowledge we have gained on the cell cycle disruption caused by human papillomaviruses (HPV) will likely lead to improved screening modalities for cervical cancer and its precursors. An easily applied biomarker that has high specificity and sensitivity would represent an attractive alternative or complement to cytology and HPV testing. To date, a number of promising markers have been investigated. These include p16^{INK4A}, MIB-1, BD-ProEx C, and L1. Newer possibilities involve a variety of gene products associated with aberrations of chromosome 3q, such as telomerase, p63, and PIK3CA, as well the combination of biomarkers such as p16^{INK4A} and MIB-1 in the same assay. Although none of them has yet been incorporated into screening algorithms or found its way into routine practice, their performance characteristics remain a focus of current investigations. This review summarizes what we know and where we hope to go in translating basic pathobiology into clinical practice.

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Introduction

Cancer remains one of the world’s leading causes of death and a major health and economic burden. Worldwide, cervical cancer is the third most commonly diagnosed cancer in women (approximately 530,000 new cases) resulting in 275,000 deaths annually [1]. More than 85% of the new diagnosed cases of cervical cancer occur in developing countries. In countries with an active screening program, widespread cytological screening efforts using conventional Papanicolaou tests have significantly reduced the incidence of and mortality from cervical cancer. In several places, particularly in developed, high-resource countries, conventional Papanicolaou smears have been replaced by liquid-based cytology (LBC) preparation methods. LBC improves the transfer of cells onto slides, yields more homogeneous cell samples, and is better suited for automated screening devices than conventional smears. Despite these improvements, cytological screening continues to be hampered by imperfect sensitivity (SE) and less-than-ideal inter-observer reproducibility, especially for some diagnostic categories. Although LBC allows for ancillary testing from the same sample, in most populations around the world cervical cancer screening is still purely based on traditional cytology.

The discovery of a strong causal association between human papillomavirus (HPV) infection and cervical cancer [2] changed perspectives about cervical cancer prevention by introducing HPV DNA and RNA testing and spurring the development of prophylactic vaccines. HPV molecular tests, however, especially the DNA-based ones, do not discriminate between transient and persistent infection and thus lack sufficient specificity (SP) to act as the stand-alone primary screening modality for significant lesion detection. However, several recently published studies have shown the exquisite SE and moderate to high SP of an HPV RNA test that could be used in routine screening of women >30 years old. At the same time, HPV tests tend to be expensive and thus not readily available to all who might benefit from them. There is a strong demand, therefore, for additional, more sensitive, and specific markers to improve screening. An accurate biomarker would certainly improve standardization and quality control for the diagnosis of cervical cancer.

Immunocytochemical detection of molecular alterations caused by HPV in host cells could potentially be used as an adjunct to cytological screening to improve SE without compromising SP. Considering that cytopathologists are expert morphologists and are used to interpret immunostains, an ideal tool for the detection of high-grade squamous intraepithelial lesions (HSIL) could be an immunomarker applied to a preparation derived from a liquid-based sample. Immunocytochemistry (ICC) is fast, simple, and relatively inexpensive, and provides information linked to cytomorphology. Furthermore, the evaluation of ICC applied to LBC samples could be automated. This review describes the main characteristics, advantages, and limitations of the biomarkers currently available for the detection of HSIL or the likelihood of progression of atypical cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) to HSIL. An attempt was made to include all retrospective and prospective studies that assessed biomarkers by immunostaining of conventional and/or LBC samples. Studies of biomarkers for glandular lesions of the cervix were not included.

What Are the Biomarkers of Cervical Cytology?

The integration of high-risk (HR) HPV into the host genome, resulting in continuous expression of the viral oncoproteins E6 and E7, the viral load, and immunological and hormonal factors all play an important role in

the deregulation of the cell cycle that occurs in cervical neoplasia. The ideal molecular marker, therefore, is not necessarily a surrogate marker for the presence of HR-HPV. Rather, it would reflect the disruption of the cell cycle derived from the summation of all forces that act on the cell during carcinogenesis. Most of the currently available molecular markers are targets of the E2F transcription factor family. They include the molecules required for cell-cycle progression (e.g., cyclin E), DNA replication (e.g., minichromosome maintenance proteins – MCMs), DNA synthesis (e.g., PCNA – proliferating cell nuclear antigen), and cell cycle control (e.g., p16^{INK4A} and p21/WAF1) [3]. Some of these biomarkers, including cyclin E, p21/WAF1, and PCNA, have been barely or never tested in cytological samples and therefore will not be discussed further here. The focus of this review is on those markers that already have been tested for cervical pre-cancerous lesions in cytology. Some of them have been the focus of research on the cell cycle and its deregulation by HPV and can be translated into potential clinical benefits [3].

Figure 1 illustrates the molecular regulation and deregulation of the cell cycle in normal and HPV-infected epithelium, respectively. The retinoblastoma gene product (pRb) is a key regulator of cell cycle progression. In resting cells, unphosphorylated pRb binds and inactivates several transcription factors, including members of the E2F family, thereby blocking a cell's transition into S (synthesis)-phase. Upon mitogen stimulation, the cyclin D1/CDK4/6 complex is activated and hyper-phosphorylates and inactivates pRb resulting in the release of active E2F in the epithelial basal layer. As a consequence, E2F-responsive genes are de-repressed and genes required for S-phase entry, such as cyclin E, PCNA, Ki-67, MCMs, TOP2A (DNA topoisomerase 2- α), p14^{ARF}, and p16^{INK4A} are activated. An important function of p16^{INK4A} involves a feedback mechanism that regulates levels of the cyclin D1/CDK4/6 complex, and thus controls levels of the S-phase progression genes. p14^{ARF} regulates p53 levels through activity of the ubiquitin ligase murine double minute (MDM2), which maintains p53 at low levels preventing induction of cell cycle arrest and/or apoptosis. Cyclin E forms a complex with CDK2, which also phosphorylates pRb, further promoting the G₁-S transition. The cyclin/CDK complexes are inactivated by p21/WAF1, which is tightly controlled by the tumor suppressor p53 (fig. 1a).

In HPV-infected epithelium (fig. 1b), cell cycle progression is no longer dependent on external stimuli, but is instead stimulated by the HPV oncogene E7 in basal as

Fig. 1. Simplified illustration showing biomarkers of cervical neoplasia and their effects on the cell cycle. **a** Normal epithelium: external growth factors activate the cyclin D1/CDK4/6 complex, which in turn hyperphosphorylates pRb. E2F is released and activates genes for S-phase progression. Among these genes are p16^{INK4A} and p14^{ARF}. A p16^{INK4A}-induced feedback mechanism inactivates cyclin D1/CDK4/6 complexes and inactivates E2F. p14^{ARF} controls p53 levels in the cell and activates p21, which is a

CDK inhibitor. **b** HPV-infected epithelium: HPV oncogene E7 binds to and inactivates pRb releasing active E2F in basal as well as para-basal cell layers. There, E2F induces S-phase genes. Importantly, the p16^{INK4A} feedback loop is bypassed leading to prolonged E2F activity. p14^{ARF} over-expression results in inactivation of MDM2, which can no longer regulate p53 levels. HPV oncogene E6 binds p53 and targets it for degradation resulting in the suppression of p53 target genes.

well as supra-basal cell layers. E7 binds and targets pRb for degradation, leading to the displacement of active E2F and consequently to the activation of S-phase genes. Furthermore, the feedback mechanism that normally results from p16^{INK4A}-induced inhibition of the cyclin D1/CDK4/6 complex is bypassed. E7 activity induces p14^{ARF} expression, which inactivates MDM2 and, therefore, p53 is stabilized. To avoid p53-induced cell cycle

arrest and/or apoptosis, the HPV oncogene E6 binds p53 and targets it for degradation, resulting in loss of expression of p53-responsive genes (i.e., p21/WAF1).

Figure 2 illustrates that gynecologic cytology biomarkers have been the focus of research for more than a decade. These studies have shown that positive staining for markers like p16^{INK4A}, MIB-1, and BD-ProEx CTM is highly correlated with the presence of HSIL. They also

Fig. 2. Number of studies published per year on p16^{INK4a}, MIB-1, BD-ProEx C, and capsid protein L1.

illustrate how basic research into the cell cycle and the effects of HPV on cell cycle control can be decoded into clinical gain [3].

What Are the Main Goals for Immunomarkers?

The application of immunocytochemically detected biomarkers ('immunomarkers') to cervical cancer screening could increase the accuracy of HSIL detection in cervical screening. Moreover, an immunomarker might also identify which cases of ASC-US and LSIL will progress to HSIL and invasive disease. Table 1 summarizes the published data on immunomarkers for these two distinct applications. Most of the studies (23 on p16^{INK4A}, 11 on MIB-1, 5 on ProEx C, 2 on L1, 2 on p63/p73, 1 on the catalytic subunit- α of the phosphatidylinositol 3-kinase gene, PIK3CA, and 1 on telomerase) tested cases representing all the cytological diagnostic categories regarding squamous cells: negative for intraepithelial lesion or malignancy (NILM), ASC-US, atypical squamous cells, cannot rule out HSIL (ASC-H), LSIL, HSIL, and squamous cell carcinoma. Other studies focused on single diagnostic categories, either NILM (2 on p16^{INK4A}), ASC-H (1 on p16^{INK4A}), LSIL (1 on L1), HSIL (3 on p16^{INK4A} and 1 on p63), or carcinoma (2 on p16^{INK4A} and 1 on MIB-1). Other studies investigated the applicability to two or more categories, such as NILM/ASC-US (1 on p16^{INK4A} and 1 on MIB-1), NILM/HSIL (1 on MIB-

1), ASC-US/ASC-H (1 on p16^{INK4A}), ASC-US/LSIL (4 on p16^{INK4A}, 2 on MIB-1, and 1 on the dual-staining p16^{INK4A}/MIB-1), and ASC/LSIL/HSIL (3 on p16^{INK4A} and 1 on MIB-1).

Study conclusions relating to the effectiveness of biomarkers can be roughly categorized as 'positive' or 'negative' (table 1). The parameters measured include SE, SP, positive predictive value (PPV), negative predictive value, accuracy, inter-observer agreement, and/or positive correlation patterns with biopsy results or the presence of HR-HPV [4]. Biomarkers were tested alone, in comparison with each other, or in comparison with other biomolecular assays (e.g., hybrid capture, HPV E6/E7 mRNA detection, or enzyme-linked immunosorbent assay – ELISA). Of note, the authors sometimes drew conclusions that were only indirectly related to the biomarker's effectiveness. For example, one group of investigators combined and tested a modified Papanicolaou counter stain to be used with the immunomarker [5]. In some cases, a biomarker proved to be more useful for one diagnostic category than another [6]. It has also been shown that immunomarker expression can be successfully quantified by computer-assisted analysis [7]. These additional findings are designated 'other conclusions' in table 1. Studies that came to negative conclusions are described below in the discussion of each biomarker. In the studies that investigated or compared more than one immunomarker (e.g., p16^{INK4A}, MIB-1, and ProEx C), information about each marker was retrieved.

Table 1. Published studies on biomarkers applied to cervical cytology preparations

Biomarker	Studies n	Year of first study	Type of preparation			Application		Effectiveness conclusion ^a		Other	
			C	LBC	CB	D	P	pos.	neg.	conc. ^b	assay ^c
p16 ^{INK4A}	33	2002	1	28	5	32	–	26	4	5	3
MIB-1	14	2002	3	8	3	13	1	8	5	–	–
p16/MIB-1	1	2011	–	1	–	1	–	1	–	–	–
ProEx C	5	2006	–	4	1	5	–	4	–	–	–
L1	5	2003	1	4	1	1	4	5	–	–	–
p63/p73	2	2007	–	2	–	2	1	1	–	–	–
PIK3CA	1	2006	–	1	–	1	–	1	–	–	–
TRT-H239	1	2004	–	1	–	1	–	–	1	–	–

C = Conventional Papanicolaou test; CB = cell block; D = diagnosis or detection of HSIL; P = progression of lesser-grade lesions to HSIL.

^a Number of studies with conclusions related to the performance of the marker for detecting HSIL.

^b Number of studies with conclusions (conc.) other than detection of HSIL.

^c Number of studies that evaluated the immunomarkers in a different way than applied to cytology slides.

p16^{INK4A}

The protein p16^{INK4A}, derived from the host p16^{INK4A}/CDKN2A tumor suppressor gene, has been identified as a biomarker for transforming HPV infection and therefore can be used as a surrogate marker of HR-HPV infection. In the absence of HPV, p16^{INK4A} blocks the activity of the cyclin-dependent kinases CDK4/6, resulting in greater binding of pRb to the transcription factor E2F, thus down-regulating progression through the G₁-S transition checkpoint of the cell cycle (fig. 1a). Unbound E2F also acts in a negative feedback loop with pRb. When HR-HPV infects the host cell, the viral oncoprotein E7 binds and inactivates pRb, thereby releasing E2F, which promotes cell cycle progression, a molecular switch that is usually activated by CDK4/6. Thus, the p16^{INK4A}-induced feedback loop is lost, and p16^{INK4A} is over-expressed (fig. 1b). As a result, the protein accumulates in the nucleus and cytoplasm of affected cells and can be detected by ICC, usually with a brown reaction product. In cytological samples, p16^{INK4A}-positive cells show brown cytoplasm with a darker brown nucleus.

Among the candidate immunomarkers for cervical pre-cancer lesion detection, p16^{INK4A} is one of the most promising and the most studied (fig. 2). Several studies have tested it in either LBC or cell block preparations (table 1), and the majority have demonstrated the effectiveness of p16^{INK4A} for improving the cytological detection of HSIL. When compared against other adjunctive tests (Qiagen Hybrid Capture-2, Roche Amplicor HPV test,

and several others) in a population with previous abnormal cytology, p16^{INK4A} showed lower SE but good SP (lower only than PreTect HPV-proofer) and the highest PPV [8]. Another study [9] demonstrated that p16^{INK4A} ICC can significantly improve SP and PPV of colposcopy in the management of women with ASC-US cytology. Although studies vary in their methodological approach, and conclusions are usually drawn from cases pre-selected for colposcopy, they clearly demonstrate (1) a strong correlation between positive p16^{INK4A} cytological staining and the severity of the histological lesion [10–12]; (2) the potential to increase the accuracy of cytological examination [12–17], and (3) the potential for efficient triage of ASC-US [9, 18–20], ASC-H [6, 21], and LSIL [18–20] cases.

Only 4 of 33 studies came to negative conclusions about the effectiveness of p16^{INK4A} as a marker of cervical cytology. Benevolo et al. [22] compared p16^{INK4A} with Hybrid Capture 2 (HC2; Qiagen, Valencia, Calif., USA) and cytological examination in 471 Thin Prep-processed (Cytoc, Boxborough, Mass., USA) cases and found HC2 to be the most sensitive, but cytological examination to have the highest SP and PPV of the 3 methods. It is possible that p16^{INK4A} might have performed better had the authors used a morphology-based score to classify their p16^{INK4A} interpretations. The other 3 studies with negative conclusions for p16^{INK4A} compared this marker with ProEx C and PIK3CA, and the results are discussed below under the headings of those markers.

A systematic review and meta-analysis of all studies that evaluated the use of p16^{INK4A} immunostaining in cytology and histology [23] found positivity rates of 12, 45, and 89% for NILM, ASC-US/LSIL, and HSIL cases, respectively. A similarly good correlation between the frequency of p16^{INK4A} expression and the severity of cellular abnormalities was found in the histological studies [24]. Despite such good correlations, the authors concluded that there was insufficient evidence for the routine application of p16^{INK4A} to clinical practice. Limitations in the interpretation of the p16^{INK4A} stain include: (1) the lack of standardized criteria in cytology for the evaluation of staining intensity and nuclear versus cytoplasmic staining; (2) the absence of consensus for threshold values of positivity; (3) the presence of sporadic p16^{INK4A} immunoreactivity in normal, atrophic, and metaplastic squamous cells, as well as endocervical cells, inflammatory cells, and bacteria [15]. Scoring systems like the one proposed by Wentzensen et al. [25] to assess morphological criteria such as nuclear size and nuclear-cytoplasmic ratio may increase the SP for HSIL detection. According to some authors [26], a scoring system could simply rely on the well-established cytomorphological criteria for HSIL.

Finally, 3 studies evaluated p16^{INK4A} in a different way than by ICC (table 1). Two tested the performance of an ELISA for p16^{INK4A} (mtm laboratories, Heidelberg, Germany), the first being a proof-of-concept study with 319 cases [27], and the second a larger study [28] on 1,781 women by the same research group. This second study used an enhanced version of the p16^{INK4A} ELISA and showed promise in screening when combined with HC2, with comparable SE (91.8%) and higher SP (86.0%) compared to p16^{INK4A} ICC (table 1). When tested by itself, however, SE of p16^{INK4A} ELISA was very low (50.9%), still retaining a very good SP (90.4%). The third study evaluated p16^{INK4A} over-expression by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis [29]. They found it not to be appropriate for cervical screening purposes. p16^{INK4A} expression measured by RT-PCR did not correlate with the cytological diagnosis or HPV status because the biomarker transcripts of the dysplastic cells were diluted by the RNA of the normal cells and their over-expression could not be detected by RT-PCR.

MIB-1

Ki-67 is an antigen that identifies proliferating cells and is expressed in all phases of the cell cycle (G₁, S, G₂/M). MIB-1 is a monoclonal antibody that detects this antigen

in the nuclei of fixed cells or tissues embedded in paraffin. HPV infection activates host cell cycle progression with increased cell cycle kinetics, and this phenomenon is reflected in an increased MIB-1 staining index. With MIB-1 immunostaining, positive cells typically show dark brown staining limited to the nucleus. MIB-1 tends to stain mainly basal and para-basal cells, but intermediate and superficial cells can also be stained by this antibody.

The first studies on MIB-1 were published in 1995 and 1997 (fig. 2), with promising results. Since then, several studies followed, but MIB-1 has never been tested by itself in a large cohort (table 2). When tested for HSIL detection against other markers like p16^{INK4A} (table 3) and ProEx C, MIB-1 generally shows lower SE and SP [14, 30–34]. MIB-1 quantification has also been tested as a possible predictor of lesion progression in histological [24] and cytological [7] specimens. In cytology, when compared to other proliferation markers, MIB-1 was less selective and less informative for the progression of LSIL [7]. MIB-1 complements p16^{INK4A} in the analysis of histological sections by differentiating SIL from atrophic epithelium with atypia [24]. Its complementary role to p16^{INK4A} in cytology has also been tested [16, 30, 33, 35, 36] and is discussed below.

BD-ProEx C

MCM 2 functions during DNA replication by loading the pre-replication complex onto DNA and by unwinding DNA through helicase activity to permit DNA synthesis. 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 over-expressed when the S-phase cell cycle induction is aberrant. A cocktail of two monoclonal antibodies that targets the expression of these two proteins was tested for the first time for the detection of cervical HSIL in cytological samples in 2006 [37]. BD-ProEx C then became commercially available for application in histological and cytological specimens [38].

Biomarkers with an exclusively nuclear distribution are easier to detect than those producing cytoplasmic staining, as the latter can be difficult to discriminate from non-specific antibody binding. BD-ProEx C staining, like MIB-1, is exclusively nuclear, and its histological distribution in cervical intraepithelial neoplasias (CINs) is very similar to that of MIB-1. In cytological specimens, again like MIB-1, ProEx C occasionally stains normal glandular cells and tubal metaplasia [39]. Also, ProEx C and MIB-1 stain random nuclei of para-basal cells in atrophic epithelium. This immunoreactivity of atrophic

Table 2. SE, SP, PPV, and negative predictive value (NPV) of biomarkers tested individually or combined

First author	Cases n	Category tested	SE %	SP %	PPV %	NPV %	Gold standard
p16^{INK4A}							
Nasioutziki [12], 2011	226	all	95.0	92.0	71.0		H (biopsy)
Reuschenbach [66], 2010	275	all	86.6	74.8			H + p16 IHC
Szarewski [8], 2008	953	all	83.0	68.7	52.3		H (biopsy + LEEP)
Benevolo [22], 2008							H
Monsonogo [9], 2007	248	all	88.2	61.9	14.6	98.6	H (LEEP)
Bergeron [20], 2006	210	all	96.0	83.0			H
		ASC-US	95.0	84.0			
		LSIL	100	81.0			
Samarawardana [17], 2010	403	all	81.7	78.1	41.2		H (biopsy)
Denton [18], 2010 ^a	810	ASC-US	92.6	63.2–71.1			H (biopsy)
		LSIL	92.2	37.3–53.3			
MIB-1							
Dunton [67], 1997	124	all	89	65	60	91?	H (biopsy)
		ASC-US/LSIL	96	67	49	98	H (biopsy/curettage)
Zeng [68], 2002	49						
p16^{INK4A}/MIB-1 dual stain							
Schmidt [36], 2011 ^a	776	ASC-US	92.2	80.6			H
		LSIL	94.2	68.0			
ProEx C (MCM2 plus TOP2A)							
Tambouret [40], 2008	624	all	81	82			H (biopsy)
		(+ cytology)	92	84			
Depuydt [39], 2011	3,126	all	78.3	91.4	12.7		H (biopsy)

H = Histology; IHC = immunohistochemistry; LEEP = loop electrosurgical excision procedure. Dunton (1997) used air-dried smears. All other studies were done on LBC samples.

^a From the European CINtec Cytology Study Group.

Table 3. The diagnostic accuracy of various biomarkers analyzed by receiver operating characteristic (ROC) curves

Study	No. of cases	Category tested	Accuracy %	Other markers accuracy, %		Gold standard
p16 ^{INK4A} *	291	ASC-US	76.0			HPV DNA testing by PCR
		LSIL	79.0			
		ASC-H/HSIL	88.0			
		HSIL	95.0			
MIB-1*	147	ASC-US	68.0			
		LSIL	72.0			
		HSIL	86.0			
PIK3CA	74	CIN3	90.0	p16 = 82.0	MIB-1 = 83.0	H (biopsy or conization)

* From the same group – Sahebali et al. [14, 34].

cell nuclei is a pitfall in the cytological distinction between HSIL and atrophy.

From 2006 until today, a few studies have tested the role of BD-ProEx C in cervical cancer screening (table 1). When combined with any level of cytological atypia, BD-ProEx C has SE of 92% and SP of 84% for the detection of HSIL [40]. In another study, when different cervical cancer screening strategies were investigated [39], primary HR-HPV PCR screening followed by BD-ProEx C was determined to be the best screening strategy for CIN2+ detection with the highest SE (76%) and PPV (41.7%), and a high SP (98.3%). The cocktail BD-ProEx C was the first successful attempt to use a combination of more than one antibody for cervical cancer screening enhancement.

A recent paper presented a method for selecting antibodies, describing selection and characterization of four antibodies to MCM6 and MCM7 for use in ICC with cervical samples. The achieved goal of the two finally selected antibodies, the anti-MCM6 (9D4.3) and anti-MCM7 (2E6.7) clones, was to stain HSIL cells with minimal background staining of normal cells [41]. Further studies to evaluate clinical performance of these antibodies are required.

L1

L1 is primarily the name of the major capsid protein of HPVs. L1 is also the name of an antibody against a protein of the HPV16 capsid that is expressed in the early productive phase of the viral life cycle and is progressively lost during cervical carcinogenesis [42]. Therefore, this antibody has an inverse pattern of distribution along the spectrum of evolution of SIL when compared to the other biomarkers discussed here. L1 stains predominantly the nucleus, but cytoplasmic staining is also observed, similar to p16^{INK4A}.

A recent study using LBC samples reports the highest L1 staining in LSIL cases, which reflects productive HPV infection [43]. L1-negative cases tend to progress to HSIL, while L1-positive cases do not [44]. Furthermore, the combination of L1 and p16^{INK4A} antibodies in LBC samples [42, 45] and cell blocks [30] has been proposed for prognostic prediction of LSIL. Finally, a prospective study published this year demonstrated a linear association of L1 expression with the progression or regression of LSIL in patients infected with HPV [46]. These preliminary results are compelling and warrant further investigation.

Chromosome 3 and Its Related Biomarkers

There is a high prevalence of chromosome 3 alterations in squamous cell carcinomas [47]. Alterations in the short arm of chromosome 3 are a common feature early during the development and progression of squamous cell carcinomas of the head and neck, lung, and, to a lesser extent, uterine cervix [48]. '3p loss' may be the loss of the whole short arm or breakage at 3p14.2, the site of the most common chromosomal fragile site and the *FHIT* gene, a tumor suppressor gene which spans it [49]. Reduced expression or complete loss of bis(5'-adenosyl)-triphosphatase, also known as fragile histidine triad protein (FHIT) expression, was found to be a useful prognostic marker for HSIL, with increased probability of progression to invasive cervical carcinoma (CC) [50]. So far, this marker has not been tested in cytological samples. However, the most characteristic chromosomal abnormality identified in invasive CCs is gain of chromosome arm 3q, detected in approximately 70% of CCs [47, 51]. Comparative genomic hybridization (CGH) studies in cytological samples [52–54] have commonly identified a 3q gain, or more specifically a gain at the segment between chromosome band 3q24/25 and band 3q28, which was associated with HSIL. These observations have led to the hypothesis that this genetic aberration might play a pivotal role in the transition from pre-invasive lesions to invasive CCs [54]. The last 3 biomarkers discussed here are related to the putative oncogenes located at this chromosomal region (3q); they are often over-expressed due to this chromosomal gain.

The human telomerase RNA gene (*hTERC*) and PIK3CA gene are located in the chromosome segment 3q26, while *p63* is located at 3q28. None of these genes and their products have been investigated and studied as extensively as p16^{INK4A} and the other markers previously mentioned. Nevertheless, the application of interphase fluorescence in situ hybridization to routine LBC preparations for 3q26 gain detection has recently received attention. The similarity of the method to that of Uro-Vysion, which is already utilized in some laboratories for the analysis of urine specimens, may work in its favor. It has been proposed that cases with 3q26 gain identify a subset of LSILs with more aggressive biologic behavior. Such cases could be referred to colposcopy, and 3q-negative LSIL patients be followed conservatively [55, 56]. Prospective clinical trials and cost-effectiveness studies are yet to be performed. In parallel, the biomarkers located around this area should also be further explored, as well other candidate oncogenes at 3q24–28.

Telomerase

Telomerase, the product of hTERC, is involved in the maintenance of the length and stability of the chromosomal ends through multiple cellular divisions [55]. Gene amplification and consequent protein over-expression of telomerase results in stable chromosomal ends in cells, extension of the time to senescence and apoptosis, and contribution to cell immortalization. A large number of studies have confirmed that the detection of hTERC amplification at chromosome 3 by fluorescence in situ hybridization has great potential as a tool to detect equivocal or LSIL cytological cases that will progress to HSIL [55–57]. The detection of telomerase by ICC, however, does not seem to be as promising, as concluded by Cheung et al. [58]. They found that telomerase expression did not distinguish normal from neoplastic cells in the 60 LBC samples they examined. Furthermore, the authors showed that ICC for telomerase stains both nuclei and cytoplasm, as well as some mature and metaplastic squamous cells, and benign endocervical and endometrial cells, with high background staining.

p63/p73

p63 and p73 are homologues of the p53 gene. Multiple isoforms of the proteins of both genes exist. The antibodies anti-p63 4A4 (against isoforms TAp63 and Δ Np63 of p63) and antibody TAp73 (against isoform TAp73 α of p73) were tested recently in a series of 529 LBC samples [59]. The authors concluded that immunoreactivity for TAp73 and p634A4, together with morphological assessment, could detect HSIL and cervical cancer. More importantly, p634A4 immunoreactivity in women with ASC-US and a high TAp73 index in women with LSIL correlated with a higher rate of progression to HSIL or above. It is important to highlight that these results were obtained by incorporating morphological criteria into the immunostain evaluation using a scoring system. Similar to p16^{INK4A}, p63 stains the normal immature squamous cells present in atrophic samples, and morphological evaluation is thus needed in order to minimize false-positive results. These and other isoforms of p63 and p73 have potential and should be further explored.

PIK3CA

PIK3CA is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. Redon et al. [48] identified this gene as the most likely oncogene to be over-expressed as a consequence of the 3q gain in head and neck squamous cell carcinomas. PIK3CA is an important component of the lipid signaling pathway. The subsequent ac-

tivation of the downstream serine/threonine protein kinase has been related to carcinogenesis in ovarian cancer, squamous cell carcinoma of the head and neck, and uterine cervix. In 2006, Goto et al. [32] tested PIK3CA as a carcinogenesis-related marker for squamous intraepithelial lesions in LBC for the first time. They found Ki-67 and PIK3CA might be useful as adjunct tools to HPV-DNA testing (table 3). Ki-67 could reflect the potential of cell growth while PIK3CA could be a carcinogenesis-related marker in early precancerous lesions. This study differs from most of the biomarker studies reviewed here because the histological endpoint used was CIN3 and not CIN2+.

Have the Main Questions about Biomarkers Been Answered?

Let us revisit the two main goals for the markers reviewed here.

ASC-US/LSIL Progression to HSIL

In the United States of America, HR-HPV-DNA tests like HC2 are currently recommended for the management of women with an ASC-US Papanicolaou result. The most carefully studied biomarker for the triage of women with equivocal cytological results is p16^{INK4A}. The studies that compared the accuracy of p16^{INK4A} with HC2 (Qiagen) for an underlying CIN2+ in women with ASC-US [60, 61] or LSIL [61, 62] were revisited in the p16^{INK4A} meta-analysis [23]. Although p16^{INK4A} in general showed lower or equal SE but higher SP compared to HC2, the authors concluded that the evidence was not enough to affirm that the immunomarker could outperform the viral testing in these diagnostic categories. Similar results were described in studies using histological sections immunostained with p16^{INK4A} [24]. Regarding the other markers, only MIB-1 has been proposed in the past to have prognostic value. Biomarkers located on chromosome 3q have not been extensively studied in this regard, but may have potential.

Promising data using the Cytoactive® HPV L1 test supports the notion that cells that lack L1 capsid protein may escape immunological recognition allowing the progression to L1-negative LSIL to HSIL [42, 44, 63].

HSIL Detection

The studies reviewed here assessed the role of these biomarkers for HSIL detection, but none analyzed them in the context of primary screening for cervical cancer. In all studies, selection bias, also known as sample distor-

tion, could not be excluded. Most of the studies included only samples from patients referred to colposcopy and that have histological results available. Despite this obvious limitation, SE, SP, and accuracy were calculated by many of the studies (table 2). A consensus on test performance for primary screening recommends that SE and SP of any new primary screening test exceed 90% [64]. Isolated markers tested in studies with a large number of cases have achieved values between 68 and 83%, respectively. The cocktail of two antibodies ProEx C achieved SP of 91%, but with an SE <80%. Other studies that combined two or more antibodies are still rare and will be discussed below.

Markers Tested in Series and Combination

Several studies have tested more than one biomarker on the same sample. The complementary role of p16^{INK4A} and MIB-1 on LBC and cell block preparations was explored by Yu et al. [30], Risse et al. [35], Sahebali et al. [16], and Longatto et al. [33]. The first two studies found that cell blocks ('cytology') immunostained for p16^{INK4A} and MIB-1 in combination were complementary to LBC, improving diagnostic accuracy for HSIL [30] and squamous cell carcinoma [30, 35] and allowing for an unequivocal and refined diagnosis. Yu et al. [30] also called attention to the possibility of combining p16^{INK4A} with HPV L1 for prediction of progression of cervical lesions. The third study (Sahebali et al. [16]) examined 500 LBC slides and found that MIB-1 occasionally stained normal proliferating cells, but that these cells were negative for p16^{INK4A}. On the other hand, immunoreactivity for p16^{INK4A} was seen in squamous metaplasia but not with MIB-1. These authors also stained 199 consecutive negative and 'equivocal' (ASC-US) LBC samples that tested positive for HR-HPV types by HC2 or PCR. The authors found the combination of p16^{INK4A} and MIB-1 useful for the management of women with equivocal cytology. Evidence from these studies resulted in the development of an ICC dual-stain protocol, which simultaneously detects p16^{INK4A} and Ki-67 expression in cervical cytology samples [36, 65]. The p16^{INK4A}/Ki-67 dual stain was tested on ASC-US/LSIL cases for HSIL identification, and immunostain positivity was scored independently of the interpretation of cytomorphological abnormalities. A previous study from the same group evaluated the same cases for p16^{INK4A} using a refined score to classify cases as positive that included abnormal cytomorphology plus p16^{INK4A} immunoreactivity. These two studies are in-

cluded in table 2. While p16^{INK4A}/Ki-67 dual staining had equivalent SE to p16^{INK4A} alone, SP was substantially improved. According to the authors, the explanation for this gain in SP comes from the mutual exclusion of the simultaneous co-expression of these two proteins in the same cervical cell under normal physiological conditions, since Ki-67 (a proliferation-associated antigen) and p16^{INK4A} (cell-cycle dependent kinase inhibitor with a tumor suppressor function) have antagonistic effects in cells with intact cell cycle control. A potential advantage of the p16^{INK4A}/Ki-67 stain could be to minimize or eliminate the need for cytomorphology interpretation, which contributes to inter-observer variability. This improvement may also facilitate the development of computer-assisted slide reading approaches in the future.

Fewer studies have compared biomarkers against each other. Only two studies compared BD-ProEx C with p16^{INK4A} [31] and MIB-1 [7, 31] in 79 and 76 cytological samples, respectively. Halloush et al. [31] found BD-ProEx C to be comparable to p16^{INK4A} and MIB-1 (each as a single stain), with the advantage that the background is cleaner and the findings are easier to interpret than p16^{INK4A} in cell blocks derived from LBC, since, like MIB-1, it only stains nuclei. Beccati et al. [7] found the BD-ProEx C to be more selective and more informative for progression of LSIL than MIB-1 in LBC. Unfortunately, BD-ProEx C, like MIB-1, occasionally and perplexingly stains some benign para-basal cells from atrophic epithelium, which may lead to a false-positive interpretation. Finally, in a study of 74 cases by Goto et al. [32] PIK3CA was shown to be the best method to detect CIN3 as compared to p16^{INK4A} and MIB-1. Diagnostic accuracies of the three markers were determined by receiver-operating characteristic curves, based on the fraction of the immunopositive cells independently counted in 3 high power fields by two pathologists. As mentioned earlier, the use of histological CIN3 and not CIN2+ as the endpoint in this study represents a potential pitfall, since the ultimate goal of cervical cancer screening is to detect HSIL or CIN2+ and not CIN3+.

Future Perspectives for Cervical Screening

Despite the recent introduction of HPV vaccines, screening will need to continue for cervical cancer prevention. It is very likely that recent advances in our understanding of the effects of HPV on cell cycle control will one day be translated into clinical practice in the form of a superior screening modality.

A consensus on test performance for primary screening recommends that SE and SP of any new ancillary primary screening test exceed 90% [64]. Furthermore, a future ideal screening test would not just improve the detection of HSIL but also identify patients with lesser abnormalities that are more likely to progress to HSIL. As described above, immunomarkers have the potential to improve SE and SP, and therefore significant effort has been put into testing their application to clinical practice. Examined individually, the biomarkers reviewed here do not achieve this stringent recommendation, especially if attention is focused on the studies with the larger case numbers (table 2).

With respect to population screening programs, a fundamental point to be considered is the cost-benefit ratio of the test. Molecular HPV tests, in general, are more expensive and technically challenging than immunostains. This makes it attractive to consider future screening mo-

dalities that include one or more of the biomarkers described above. General markers of cell proliferation like MIB-1, MCMs or ProEx C, and surrogate markers of HR-HPV infection like p16^{INK4A} have shown promising results as reflected by the relative high volume of the literature reviewed here. Other potential candidates need to be tested until we find an ideal combination. Following the example of the p16^{INK4A}/MIB-1 dual staining combination and the MCM2/TOP2A combination (ProEx C), combinations are often superior to any single marker and should be tested. A critical determinant for the success of future investigations will be the standardization of sample preparation and interpretation. Furthermore, before reaching the point of routine application, this ideal 'biomolecular' or 'immune-enhanced Papanicolaou test' needs to be evaluated in large prospective clinical trials with appropriate colposcopic, histological, and clinical endpoints as well as adequate follow-up.

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