EXPERT REVIEWS

Biomarkers for cervical cancer screening: the role of p16^{INK4a} to highlight transforming HPV infections

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Biomarkers indicating the initiation of neoplastic transformation processes in human papillomavirus (HPV)-infected epithelial cells are moving into the focus of cancer prevention research, particularly for anogenital cancer, including cancer of the uterine cervix. Based on the in-depth understanding of the molecular events leading to neoplastic transformation of HPV-infected human cells, the cyclin-dependent kinase inhibitor p16^{INK4a} turned out to be substantially overexpressed in virtually all HPV-transformed cells. This finding opened novel avenues in diagnostic histopathology to substantially improve the diagnostic accuracy of cervical cancer and its precursor lesions. Furthermore, it provides a novel technical platform to substantially improve the accuracy of cytology-based cancer early-detection programs. Here, we review the molecular background and the current evidence for the clinical utility of the p16^{INK4a} biomarker for HPV-related cancers, and cervical cancer prevention in particular.

Keywords: biomarker • cancer early detection • cancer screening • cervical cancer • diagnostic accuracy • HPV E6 • HPV E7 • human papillomavirus • p16^{INK4a} • viral oncogenes

The global epidemiology of cervical cancer & the impact of screening

Approximately 530,000 women develop cancer of the uterine cervix each year, and approximately 275,000 women die worldwide because of it [201]. After breast and colorectal cancer, cervical cancer is the third most common cancer in women on a global scale. However, in contrast to colon and breast cancer, which are more prevalent in economically more advanced regions, approximately 80% of all cases of invasive cervical cancer are diagnosed in developing countries, whereas only 20% of these cancers occur in developed nations. A closer look into this remarkable epidemiologic pattern reveals that in societies that have introduced and maintain population-wide cervical cancer early-detection and screening programs, the incidence of cervical cancer can be reduced by approximately 70%, whereas societies that are not yet capable of setting up and maintaining such population-wide cervical cancer screening programs or providing appropriate clinical care for those women who test positive for screening tests still suffer from very high incidence and mortality rates [1]. In that regard, cervical cancer was the first example to demonstrate that early detection programs on a population-wide level are indeed capable of substantially reducing cancer-related morbidity and mortality.

The Pap test

The population-wide screening and early-detection programs maintained in most Western countries rely on a simple diagnostic test that became known as the 'Pap test'. Between the mid-1960s and mid-1970s, many countries in Europe and North America adopted this test for population-wide cervical cancer early-detection and screening programs. Although it has never been tested in prospective clinical trials, and without any prior proven evidence for its efficacy, the Pap test soon became the technical platform of population-wide cervical cancer screening initiatives, rapidly leading to a remarkable decline in the cervical cancer incidence and mortality in countries where these programs were initiated [2].

The basis of the Pap test was, and still is, primarily the morphological assessment of

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alterations of the nuclei of basal squamous epithelial cells in samples taken from the outer surface of the uterine cervix. In most cases, neoplastic transformation is the consequence of chromosomal instability that occurs suddenly in distinct somatic cells during a process usually referred to as cancer initiation [3,4]. Chromosomal instability is characterized by multiple numeric and structural aberrations of the chromosomes in the affected cells, leading to substantial shifts in the overall DNA content [5,6]. This process is ascribed as aneuploidy [7]. During the progression of the cervical epithelium towards neoplastic transformation, finally ending in invasive cancer, aneuploidy of the nuclei of the respective cells becomes increasingly prominent by hyperchromatic nuclei, morphological alterations of the membrane of the nucleus, and condensation and abnormal decondensation of distinct chromosomes (coarse chromatin; described in detail in [8]). Therefore, shifts of the DNA content and the process of aneuploidy have been used as the predominant diagnostic criteria in cervical cytopathology that have finally led to the success of Pap test-based screening initiatives.

Particularly in the early phases of the transformation process, these morphological alterations are still discrete and difficult to distinguish from nonspecific morphological alterations of the cells not linked to neoplastic transformation (i.e., metaplasia, inflammation and atrophy of the cervical squamous epithelium). In addition to these ambiguities in the morphologic evaluation, the sensitivity of the Pap test to identify high-grade cervical neoplasia is limited, as early alterations may easily be overlooked by a single observer.

The recently developed Bethesda classification of Pap results aimed to acknowledge this fact by introducing distinct diagnostic categories for those samples that could not be accurately classified [9]. These were referred to as 'atypical squamous cells of undetermined significance (ASC-US)' or 'atypical squamous cells – cannot exclude high-grade cervical squamous intraepithelial lesions'. Approximately 2% of all cytology samples are classified as ASC-US. Of these, approximately 10% have underlying high-grade lesions. These may not be neglected and thus all patients confronted with the diagnosis of ASC-US require careful further clinical work-up to exclude the presence of high-grade cervical squamous intraepithelial lesions. These technical shortcomings of the Pap test are an important burden for the affected patients and an important driver of costs for healthcare providers, and thus underline the strong demand for better biomarkers that may help to overcome the ambiguities of cervical cytopathology [10].

Molecular pathogenesis of cervical cancer & the role of oncogenic human papillomaviruses

Over the past 30 years, the essential causative role of distinct types of human papillomaviruses (HPVs), so-called high-risk (HR)-HPV types, in cervical cancer has been well established [11]. Papillomaviruses are widespread pathogens, infecting the squamous epithelium of the skin and mucosal surfaces. Of the more than 200 characterized HPVs [12], approximately 40 types preferentially infect the epithelium of the genital tract of males and females. Approximately 13 of these 40 were classified as potentially oncogenic and thus referred to as HR-HPV types, since they were found in association with neoplastic diseases. The oncogenic potential of these viruses is quite divergent: HPV types 16, 18 and 45 are clearly very potent oncogenic agents, whereas the oncogenicity of most of the other viruses of this group is less potent [13]. In cross-sectional studies among young adults, HR-HPV infections are found in approximately 15–25% of the 18–30-year-old population, whereas with increasing age, the prevalence of these infections decreases [14].

HR-HPV infections may cause small lesions in the infected squamous epithelium in females and males. These infections and the associated lesions are usually transient and regress after a couple of months [15]. In histology, these acute HPV infections are characterized by 'koilocytes' that occur in the intermediate and superficial cell layers of the infected epithelium and represent the morphological hallmark of squamous epithelia replicating HPVs (Figure 1) [16]. In somewhat less than 10% of infections in the female genital tract, however, the infections persist and eventually progresses to high-grade cervical intraepithelial neoplasia (CIN). Interestingly, these persistent infections preferentially occur in cells with direct anatomical relation to the cervical transformation zone [14].

Various lines of evidence have shown that productive infections apparently occur without significant gender preference in the genital tract of men and women [17]. Productive HPV infections are characterized by a highly regulated gene-expression profile that is strongly adapted to the differentiation status of the squamous cell epithelium of its host cells [17–19]. After the virus has entered the basal cell compartment, only low-level viral gene expression occurs in the basal cells. The expression of low levels of the *E6* and *E7* genes in particular allows the cells to remain in a status compatible with DNA replication and triggers local expansion of the infected cells. With increasing maturation of the squamous cell epithelium in the intermediate squamous epithelial cell layers, expression of the early viral genes becomes more active. Although the host cells in this phase have lost their own capability to replicate, they remain in a state that allows replication of the viral genomes. If the cells finally enter the stage of superficial squamous epithelial cells, the early viral genes are shut off. Interestingly, the expression of late viral genes, including *E4*, *L1* and *L2*, is restricted to terminally differentiated squamous epithelial cells at the surface of the epithelium. The detection of the L1 or E4 proteins is therefore a good surrogate parameter for productive HPV infections. Usually, these infections are transient and do not last longer than 12–24 months [15,20]. It is assumed that during the course of infection, a sufficient number of cytotoxic T cells are triggered that recognize various virus-derived epitopes, which leads to spontaneous resolution of the lesion [17,21,22].

In persistent infections that occur predominantly at the uterine transformation zone, the above described gene-expression pattern shifts into a state that is now increasingly referred to as 'transforming HPV infection' (Figure 2) [23]. The transforming infection is characterized by substantially enhanced expression of the early viral genes *E6* and *E7* in the basal and parabasal

cells of the infected epithelium. Since both viral proteins interact with critical cellular tumor-suppressor proteins, such as p53 and pRB, the level of these tumor-suppressive proteins becomes reduced owing to continuous enzymatic degradation via the ubiquitin pathway [24]. In addition, both viral oncogenes interfere with multiple additional cellular mechanisms safeguarding the chromosomal stability and hence genetic fidelity of the infected cells [25]. This results in a state of genomic instability, as indicated by increasing aneuploidy, and may finally trigger neoplastic transformation of the respective squamous epithelial cells. It is important to note that these transforming HPV infections are still capable of replicating viral genomes in the intermediate cells (indicated by persisting koilocytosis) and can generate mature HPV particles at the surface of the epithelium, as indicated by the ongoing expression of the viral L1 capsid protein, as long as the cells retain some degree of squamous epithelial differentiation [26–29].

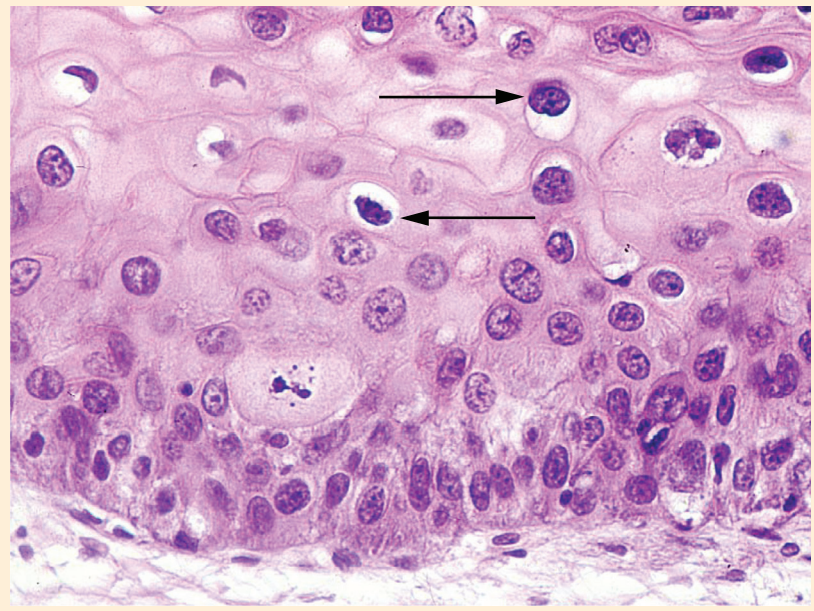

Figure 1. Hematoxylin and eosin-stained section of a low-grade cervical intraepithelial neoplasia lesion of a cervical biopsy. The presence of koilocytes is highlighted by arrows that indicate human papillomavirus replication in the intermediate and superficial cells of the squamous epithelium.

The risk of developing cervical cancer as a consequence of HR-HPV infections is apparently directly related to the degree of genomic destabilization of the respective host cell genome. Thus, the release of the viral oncogenes from their tight control in basal and parabasal cells is the critical event in HPV-related cervical carcinogenesis. This very much resembles cancer initiation events in other cancer entities, such as the primary mutation of the critical tumor-suppressor gene, *APC*, in colorectal cancer [30,31]. It does not imply that all transforming infection events inevitably progress to invasive carcinomas, just as only a minority of colorectal adenomas with *APC* gene mutations will evolve into colorectal cancers. However, compared with the mere productive infection, lesions displaying transforming HPV infections are considered to be at substantially higher risk for neoplastic progression. Based on these considerations and experimental observations, attempts to identify the 'ideal' biomarker for cervical cancer screening currently focus on those markers that allow the identification of HPV-infected cells in the transforming mode of the infection (Figure 2).

Diagnostic implications of the HPV-related pathogenesis of cervical cancer

HPV infections can be easily monitored using simple DNA-based detection methods, ranging from hybridization-based techniques, such as the Qiagen HC2® test, to ultrasensitive PCR-based amplification techniques, such as Roche's Amplicor® test [32]. In clinical terms, the increased sensitivity to detect HPV infections is clearly a substantial advantage when compared with the rather low sensitivity of the Pap test. However, owing to the very high prevalence of transient HPV infections, particularly among younger women, the positive-predictive value of the sensitive amplification-based HPV tests for high-grade disease is substantially lower in comparison with cytology-based screening tests or less sensitive HPV detection methods. Therefore, the clinical utility of any HPV detection assay is a compromise between analytical sensitivity to detect as many HPV infection events as possible and the clinical specificity with regard to predicting HPV-induced cervical disease [33].

The specific detection of transforming HPV infections has repeatedly been attempted by conceptually very different approaches. First, genomic rearrangements of the viral genome, leading to chromosomal integration and disruption of the viral *E2* gene, have been postulated to trigger the enhanced expression of the viral oncogenes (reviewed in [34]). Integration of the viral genomes into the chromosomes of the human host cells was regarded as a key event in cervical carcinogenesis. Consequently, many different assays have been developed to monitor chromosomal integration events. However, extensive research on the true prevalence of integrated viral genomes revealed that many invasive carcinomas arise from HPV-infected squamous epithelial cells in which no integrated viral genome copies were detectable [35]. These studies showed that progression of productive HPV infections to transforming HPV infections and finally invasive cancer cell clones is not necessarily linked to chromosomal integration of the HPV genomes. Since aneuploidy and chromosomal imbalances arise significantly earlier during the preneoplastic progression cascade [4,36,37], it became clear that initiation of HPV-related carcinogenesis substantially precedes genomic integration of HPV genomes [23].

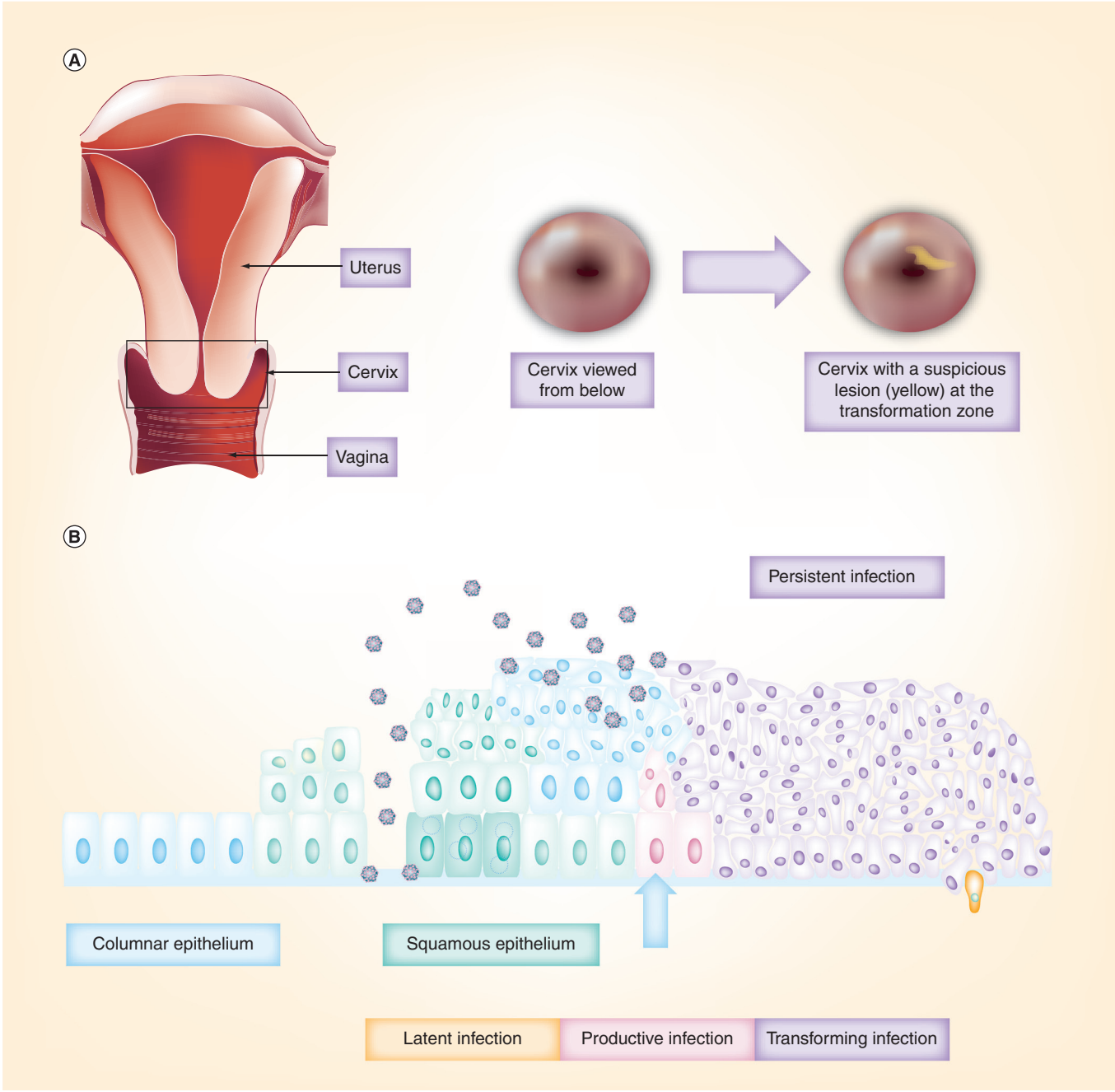

Figure 2. The course of human papillomavirus infections at the transformation zone of the uterine cervix. (A) Transforming human papillomavirus (HPV) infections may become clinically visible as whitish lesions that lose the capacity to store iodine and thus remain whitish upon iodine application. They are almost exclusively observed in direct anatomical relation to the transformation zone of the uterine cervix. **(B)** Schematic representation of the three phases of a HPV infection. Minor lacerations of the squamous epithelium permit the virus to come into contact with its natural host cell at the bottom of the squamous epithelium. Upon viral uptake, transport to the nucleus and release of the circular episomal viral genome occur, and genetic activity of the virus appears to be blocked (latent infection, dark turquoise cells). In individual infected cells, some gene expression may suddenly occur. This allows for some limited growth stimulation and limited local expansion (light turquoise cells to the left of the arrow). Once these cells start to differentiate, the full program of viral DNA replication and synthesis of mature virions in the intermediate and superficial squamous epithelial cells (turquoise basal cells, blue intermediate and superficial squamous epithelial cells) is initiated. This stage of a HPV infection is referred to as a productive HPV infection. In some of the HPV-infected basal and parabasal squamous epithelial cells, the intracellular control of the HPV *E6* and *E7* genes is lost (indicated by the blue arrow). The latter is characterized by high-level expression of the *E6* and *E7* genes (purple cells) and strong diffuse overexpression of $p16^{INK4a}$ (see Figure 4B & D). For further details, please refer to the text of this review.
Adapted with permission from [23].

Some approaches focus on the detection of E6/E7 mRNA transcripts [38]. Based on the amplification of the respective viral oncogene transcripts, it was hoped that the detection of the viral mRNAs may indicate the transforming infection mode. Two assays using this concept have been commercially developed. One, the NucliSENSEasyQ™ assay, is distributed by BioMerieux and allows the detection of the E6/E7 transcripts in a total of five HPV genotypes (16, 18, 31, 33 and 45). The other, GenProbe's Aptima™ assay, allows the detection of 14 different HPV genotypes. The clinical evaluation and comparison of both assays revealed that the NucliSENSEasyQ assay displayed very high specificity, but only low sensitivity, to detect high-grade precancerous lesions, whereas the clinical evaluation of GenProbe's Aptima assay showed exactly the opposite, with very good sensitivity but only marginally improved specificity compared with HPV-DNA testing assays [39]. This may be partly due to the more comprehensive number of HPV types included in the Aptima assay compared with the NucliSENSEasyQ assay and does not necessarily relate to other test-specific differences. A recent report showed that the transition of productive to transforming HPV 16 infections may go along with shifts of the HPV 16 transcriptome, and that the combined detection in four HPV-derived transcripts may allow assessment of the progression stage of HPV 16-induced lesions [40].

Other attempts have been made to detect enhanced expression of the viral *E6* and *E7* gene products in either biopsy tissue sections or cytology samples by using immunohistochemistry and immunocytology [41,42]. However, so far there is no firm proof that these approaches will yield clinical utility and will be able to satisfy the diagnostic demands for routine applications. In part, this may be due to the comparably short half-life of both viral oncogene products [43] and also due to the lack of sufficiently specific monoclonal antibodies. Work is ongoing to improve the detection of viral oncogene products in clinical samples using alternative detection tools [44]; however, although preliminary data yield some promise, the current evidence does not yet allow us to conclude whether this will be a feasible approach in the clinical screening routine.

Proliferation-associated cellular protein biomarkers in cervical cancer screening

Alternative strategies have taken a more indirect approach. They focused on cellular proteins that are indirectly induced by the activity of the viral oncoproteins in proliferating basal and parabasal cells in HPV-induced lesions displaying the transforming mode of HPV infection. A number of cellular proteins have been investigated with regard to their potential utility as markers for cervical preneoplastic lesions. Almost all of these marker proteins are directly or indirectly involved in regulation of the cell cycle.

Gene-expression profiling data comparing mRNA patterns expressed in normal and HPV-transformed cervical epithelial cells revealed numerous candidate genes that might be suitable as biomarkers to identify transformed lesions in histology and cytology [45]. Promising candidates for detecting (pre-)malignancy include minichromosome maintenance (MCM) proteins 2–7 [46]. As members of the DNA prereplication complex, they are essential for DNA replication in all eukaryotic cells [47]. All MCMs are selectively expressed in normal epithelial proliferative compartments, but not in differentiating cells. However, a hallmark of preneoplastic lesions is continuous proliferation and thus widespread expression of MCM proteins, which can be detected by immunohistochemistry in either tissue sections or in abnormal cells in cytology specimens [47,48]. The enhanced expression of MCMs has been used to improve the detection of high-grade lesions and squamous cell carcinoma in cervical smears and also other HPV-induced preneoplastic lesions at other anatomical sites [46,48–52]. MCM proteins reflect the active proliferating state of the cells, but cannot differentiate between proliferation due to normal tissue reconstitution, inflammation, metaplasia or other benign repair conditions, or indeed due to abnormal cell cycle regulation as it occurs in HPV-induced neoplasia. Topoisomerase-α (TOP2A) is a nuclear enzyme that regulates DNA topology during chromosome replication. Among other aspects, it appears to be a valuable marker in predicting the responsiveness of breast cancers to chemotherapy regimens including anthracyclins [53]. A combination of antibodies against the MCM-2 and TOP2A proteins has been included in the ProExC™ test kit distributed by Becton, Dickinson and Company. This assay seems to improve the sensitivity and specificity of conventional cytology, in particular, if it is used as an adjunct test to triage women with HR-HPV infections [54].

A major drawback of these proliferation marker-based assays is the expression of marker proteins in normal proliferating cells that do not allow the unequivocal distinction between transformed and otherwise proliferating cells [55]. The use of MCM- or TOP2A-based test kits thus still requires the morphological distinction of abnormal from normal nuclei of cells based on the conventional cytological criteria [50]. The simple staining of potentially abnormal cells apparently improves the sensitivity if compared with the conventional Pap test [50,54].

The cyclin-dependent kinase inhibitor p16INK4a & its role in future cervical cancer screening

Gene products that are overexpressed in HPV-transformed cells as a direct consequence of the expression of the transforming viral oncoproteins E6 and E7 may represent a better alternative to proliferation-associated proteins. The $p16^{INK4a}$ protein acts as a cyclin-dependent kinase inhibitor in normal cells and is an integral component of normal cell cycle control (Figure 3A) [56]. $p16^{INK4a}$ inhibits phosphorylation of the cyclin D-dependent kinase 4 and 6 complex, which in turn hyperphosphorylates the pRB gene product [57–60]. CDK4/cyclin D-induced hyperphosphorylation of pRB results in the inactivation of pRB and the release of bound E2F transcription factors. Increased free E2F subsequently activates S-phase progression genes, thereby overcoming the G1/S-phase restriction point and pushing the cell into the S-phase of the cell cycle [61]. The increased levels of $p16^{INK4a}$ result in less active phosphorylation of CDK4/cyclin D, such that the latter complex is less capable of phosphorylating pRB and therefore less free E2F accumulates in the nuclei of the respective

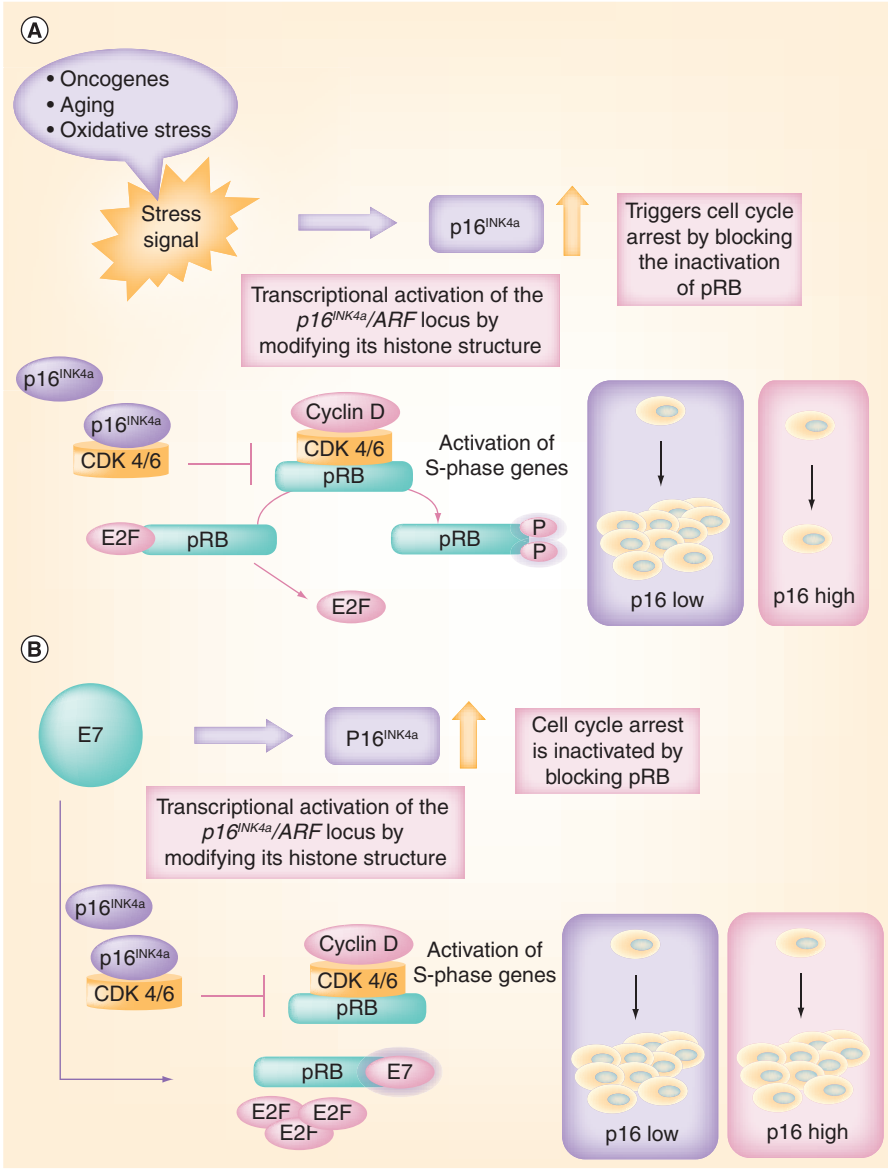

Figure 3. Schematic representation of the p16^{INK4a}–cyclin D–pRB pathway by various stress symbols and a schematic representation of the submerging of this pathway by the human papillomavirus E7 protein. (A) Schematic representation of p16^{INK4a}-mediated response mechanisms towards cellular stress signals. Genomic stress induces enhanced p16^{INK4a} protein expression by modifying the histone structure of the *p16^{INK4a}/ARF* locus. This triggers immediate cell cycle arrest, since p16^{INK4a} inhibits the cyclin D–cyclin-dependent kinase complex that hyperphosphorylates the pRB protein into its inactive state. Since the hypophosphorylated pRB binds more E2F, less free E2F is available to trigger further cell cycle progression into the S-phase, thus causing cell cycle arrest, thereby preventing further cell cycle progression at the G1/S-phase transition. Inducers of this process may be any genomic damaging events, aging, reactive oxygen species signals or activated oncogenes, such as expression of *kras*. **(B)** Effects of human papillomavirus E7 on *p16^{INK4a}* gene expression. The enhanced expression of the high-risk human papillomavirus E7 protein triggers a similar 'oncogenic stress stimulus' as the mutant *kras* gene. This again results in modification of the histone architecture of the *p16^{INK4a}/ARF* locus and hence enhanced expression of p16^{INK4a}. However, at the same time, E7 inactivates the cell cycle-arresting activity of pRB. Thus, the final part of the p16^{INK4a}-mediated cell cycle arrest mechanisms is inactivated and, as a result, the cells continue to proliferate, despite very high levels of p16^{INK4a}.
(A) This figure is based on data and mechanisms reviewed in [56–61].
(B) Data taken from [71].

cells. The biological consequence is cell cycle arrest and induction of senescence [62]. Interestingly, cell cycle arrest in cells expressing high levels of p16^{INK4a} appears to be irreversible, thus providing robust protection against genomic damage induced by genomic stress, such as oncogene activation, aging or other conditions that may result in the activation of the *p16^{INK4a}* locus [63]. p16^{INK4a} is expressed at higher levels in cells upon genotoxic damage during aging, in cells displaying cellular maturation and differentiation disturbances, or in cells with activating oncogene mutations [58]. The exact mechanisms leading to the induction of p16^{INK4a} expression are not yet fully understood. Various cellular stress inducers trigger demethylation of the histone K27 marks and thus may change the histone architecture of the p16^{INK4a} locus. This in turn no longer allows polycomb repressor complexes to bind and suppress the expression of the *p16^{INK4a}* gene that is subsequently expressed at substantially enhanced levels [64–66]. An early report by Khleif *et al.* suggested that accumulating E2F activity in cycling cells may induce an autoregulatory pathway, resulting in upregulation of the p16^{INK4a} gene product [67]. Using immortalization experiments of human cervical epithelial cells transfected by HPV genomes *in vitro*, Pater and colleagues demonstrated that immortalization of these cells by HPV oncongenes results in enhanced p16^{INK4a} expression [68]. Based on these experimental findings, we speculate that enhanced expression of the papillomavirus oncogenes may also lead to substantial overexpression of p16^{INK4a} in cells that overexpress the HPV E7 gene product in the course of transforming HPV infections. A series of studies in the late 1990s convincingly confirmed the hypothesis that overexpression of p16^{INK4a} might serve as a highly valuable biomarker for the clinical detection of high-grade HPV-induced lesions [69,70].

A recent study by McLaughlin-Drubin and colleagues suggests that the enhanced expression of p16^{INK4a} in HPV-transformed cells may also be related to the activation of a distinct histone demethylase (KDM6B) induced by the oncogenic stress conferred by expression of the *E7* oncogene (Figure 3B) [71]. As the E7 protein in the same

cell also inactivates the essential pRB-mediated control functions of the cell cycle, E7-expressing cells may instead continue to proliferate, despite their exceedingly high p16^{INK4a} levels (Figure 3B). Taken together, these observations further corroborate the idea that overexpression of p16^{INK4a} in cells transformed by HPVs may represent a specific biomarker for HPV-mediated transformation, specifically highlighting those cells that have lost the normal control of the viral oncogenes.

To evaluate the diagnostic implications of this concept, overexpression of p16^{INK4a} was first investigated in tissue sections obtained from HPV-induced cervical lesions. In the first series of studies, sections of biopsies of cervical lesions, including CIN grades 1, 2 and 3 (CIN1, 2 and 3), as well as several cervical cancer biopsies, were stained with monoclonal antibodies raised against p16^{INK4a}-derived epitopes. Two initial studies first analyzed the expression of p16^{INK4a} by using formalin-fixed paraffin-embedded biopsies or tissue sections. These early reports demonstrated that all high-grade cervical lesions, defined as CIN2+, displayed strong and diffuse staining of the basal and parabasal cells. In CIN1, approximately 50% of lesions revealed similar diffuse staining of the basal and parabasal cells, whereas the other half did not display this staining pattern [69,70]. These data were rapidly confirmed by a series of further studies in the following years (reviewed in [72]).

The use of p16^{INK4a} in histopathology

In most countries maintaining population-wide cervical cancer screening programs, Pap cytology is being used to identify women with potentially abnormal lesions who are then referred to colposcopy and eventually biopsy. Small punch biopsies taken during colposcopy are fixed in formalin, embedded in paraffin, and sections stained with hematoxylin and eosin (H&E) are then investigated by a pathologist. The pathologist's diagnosis is usually established according to morphological criteria outlined by the WHO classification system for cervical pathology [73]. Clinical decision-making processes primarily rely on the histopathologic diagnosis established through the morphological interpretation of H&E-stained tissue sections by the pathologists. For cervical cancer screening, this is of particular importance since in most countries, women who are diagnosed as having a CIN2 lesion or higher are recommended to undergo a loop electrosurgical excision procedure and surgical removal of the dysplastic epithelium.

A precise histopathologic diagnosis is of great importance in this context, since low-grade lesions have the potential to spontaneously regress [13], whereas ablation therapy may have a potentially negative impact on the reproductive outcome of women [74]. Several studies in the past have shown that the reproducibility and interobserver agreement of the pathologists' diagnoses are far from being perfect [10,71,75–80]. The degree of interobserver variability is usually expressed as a κ-coefficient, which determines the level of agreement among observers typically corrected by chance. The κ-coefficient for H&E-stained sections of cervical lesions is usually in the range of 0.45–0.50 [10], which is regarded as only moderate agreement. Particularly in the early lesions, the clear assessment of the nature and progression status of the respective lesions is sometimes very difficult. In the more advanced lesions, the distinction of truly abnormal cells from squamous epithelial metaplasia or atrophic lesions is sometimes difficult and results in a substantial number of women receiving ablative therapy even though their lesions would never have progressed to invasive cancer. This overtreatment is worth particular consideration in cervical cancer screening programs, as it may cause various obstetric side effects, such as as premature delivery, sometimes with grave and lifelong substantial disabilities in the affected children (reviewed in [74]). A more precise technology allowing for better reproducibility in diagnostic assessment may contribute substantially to fewer side effects of screening programs, better care for the patients and thus, in the long run, presumably also to a substantial reduction of the costs associated with cervical cancer screening programs.

Given the fact that overexpression of p16^{INK4a} as a consequence of the enhanced expression of the *E7* oncogene in HPV-infected basal cells highlights the initiation of the carcinogenic cascade, eventually leading to cervical cancer, the use of monoclonal antibodies directed against p16^{INK4a} should allow much better discrimination of true lesions from their histopathologic mimics, such as immature metaplasia and other squamous epithelial changes (Figure 4). In the first study to test this hypothesis, there was significantly better agreement in the interpretation of p16 expression (κ-value: 0.899 [95% CI: 0.84–0.99] [81], which is regarded as almost perfect agreement) compared with H&E-stained slides, particularly for high-grade CIN from κ-values of 0.566 to 0.749. These data have essentially been confirmed by a series of subsequent reports uniformly demonstrating that the conjunctive use of H&E and p16^{INK4a} immunohistochemistry substantially improves the reproducibility of the histopathology diagnosis of cervical precancerous lesions [10,82–87].

It is important to note that under certain circumstances, enhanced expression of p16^{INK4a} may also be observed in cervical epithelial lesions not related to HPV infection or neoplastic transformation (Figure 5). Several lines of evidence suggest that the enhanced expression of p16^{INK4a} in these cells is part of the physiological stress response mechanism that should prevent further proliferation of cells that may have acquired oxidative stress signals, and triggers irreversible cell cycle arrest, as discussed earlier (Figure 3A). This pattern of p16^{INK4a} expression in nontransformed epithelia is always focal and not usually observed in the basal and parabasal cell layers, whereas in HPV-transformed lesions, the p16^{INK4a}-expression pattern is always diffuse and most prominent in the basal and parabasal cell layers. In line with this notion are the focal p16^{INK4a} staining patterns most frequently observed in metaplastic or atrophic squamous epithelial lesions, particularly in older women. However, focal staining patterns are never found in epithelia with p16^{INK4a}-expressing cells retaining the capacity to proliferate, as can be demonstrated by the combined use of two antibodies, one against p16^{INK4a} and one against Ki67 [88]. Owing to the typical staining patterns of p16^{INK4a} in the basal and parabasal cell compartments, there are usually no difficulties in distinguishing lesions displaying diffuse p16^{INK4a} overexpression, indicating neoplastic transformation, compared with those that display the focal staining pattern as a reactive response, indicating disturbances of the normal squamous epithelial differentiation pattern.

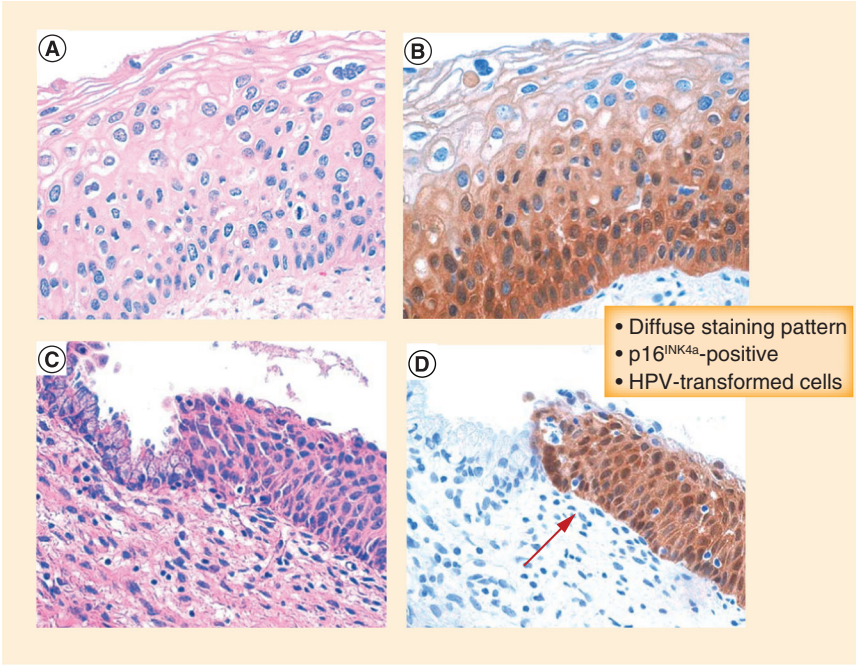

Figure 4. Typical diffuse p16^{INK4a}-positive basal and parabasal cells. (A) Hematoxylin and eosin-stained section of a cervical intraepithelial neoplasia grade 2+ lesion. **(B)** A consecutive section of the same lesion as in **(A)**, stained with the E6H4 monoclonal antibody against p16^{INK4a} purchased from Roche mtm laboratories AG. **(C)** Hematoxylin and eosin-stained section of a putative cervical intraepithelial neoplasia grade 2+ lesion adjacent to the cervical transformation zone. **(D)** A consecutive section of the same lesion as in **(C)**, stained with the E6H4 monoclonal antibody against p16^{INK4a}. The arrow indicates the diffuse staining of basal and parabasal cells. Note the adjacent unstained columnar cells on the left as a further part of the cervical transformation zone.
HPV: Human papillomavirus.
Reproduced with permission from [82].

The improved diagnostic accuracy contributed by p16^{INK4a} immunohistochemistry is further corroborated by an interesting study published by Zhang and colleagues [89]. These authors used p16^{INK4a} to refine the histologic gold standard of a large cervical cancer screening study comparing cytology, HPV testing and visual inspection with acetic acid. Here, only CIN2/3 histology results that exhibited diffuse p16^{INK4a} staining were considered 'real' disease. Using this refined disease definition, the sensitivity of HPV-DNA testing and Pap cytology was substantially improved. This suggested that the p16^{INK4a}-negative cases were mimics of high-grade dysplasia rather than true abnormal lesions.

The use of p16^{INK4a} in cytopathology

The distinct staining features of p16^{INK4a} in cervical biopsies suggests that the use of monoclonal antibodies against p16^{INK4a} might also be able to improve cytological cervical cancer screening techniques. The first published reports using this approach trace back to early work by Bibbo and colleagues, who showed that in principle p16^{INK4a} immunocytochemistry can also be used to identify HPV-transformed cells in cervical cytology samples [90,91].These early reports were soon corroborated by a series of further studies showing that cervical epithelial cells can indeed be stained on conventional as well as liquid-based cytology specimens [92–98]. Although the technical standards to stain and evaluate the staining patterns differed substantially in individual reports, these data clearly suggested that using a p16^{INK4a}-based immunocytochemistry approach offers great potential to improve the drawbacks of conventional Pap cytology. This novel immunocytochemistry approach for the evaluation of cervical cytology samples was subsequently extensively used in additional studies. In particular, for the triage of women with positive HPV test results, but for whom cytology scored as negative for intraepithelial lesion or malignancy [92,99], or those with ASC-US and low-grade squamous intraepithelial lesion (LSIL) cytology results, p16^{INK4a} immunocytochemistry yielded great potential [20,100–102].

The fact that some metaplastic or atrophic squamous epithelial cells of the intermediate and superficial cell layers could also express p16^{INK4a}, even if they were not infected or transformed by HPV, became an apparent drawback, since in cytology the special architecture of the tissue is lost and cannot be further used as a criterion to distinguish the two types of p16^{INK4a}-expressing cells by the expression pattern within the epithelium. Bibbo *et al.* [91], and later also Sahebali *et al.* [103], therefore proposed simply counting the number of p16^{INK4a}-positive cells on cytology slides and defining a certain threshold number of p16^{INK4a}-positive cells per 1000 epithelial cells on the slides to determine whether the sample should be called negative or positive for being suspicious for high-grade cervical lesions.

An alternative qualitative approach, applying morphological cytology criteria as outlined earlier, was used in further work to identify cervical cytology samples from women with high-grade cervical lesions [104]. Here, slides were screened for p16^{INK4a}-stained cells and then in a second step interpreted according to the degree of their nuclear abnormalities. To facilitate this process and to incorporate digital criteria into the evaluation algorithm as far as possible, a binary score was proposed by Wentzensen *et al.*, which allowed the classification of any p16^{INK4a}-stained cells in cytology samples [105]. Using this system to triage ASC-US and LSIL cases revealed that the sensitivity of cytology could be substantially improved into the range of the readily available HPV tests; however, with substantially improved specificity compared with the latter [106]. These data were confirmed by subsequent studies and revealed significantly improved receiver–operator characteristics in comparison with the HPV-DNA or RNA-based assays [39].

However, the morphological interpretation of p16^{INK4a}-stained cells in cytology samples still remained somewhat subjective. To further overcome these limitations, it became evident that combining two antibodies against two markers that theoretically

should be exclusively expressed in either HPV-transformed cells or those undergoing metaplastic changes would provide a useful technical solution. In cells expressing p16^{INK4a} in response to aging (atrophy), metaplasia or other conditions, the downstream p16^{INK4}–CDK4–pRB pathway (Figure 3A) should remain intact and in any case trigger immediate and presumably irreversible cell cycle arrest. Hence, these cells should never concomitantly express proliferation-associated markers such as Ki-67. In HPV-transformed cells, however, the downstream p16^{INK4}–CDK4–pRB pathway is inactivated by the inactivation of pRB through the HPV E7 protein. Consequently, cells that overexpress p16^{INK4a} as a response to viral oncogenes should be able to concomitantly express Ki-67 (Figure 3B). This hypothesis was proven by a detailed analysis of the Ki-67 and p16^{INK4a} staining patterns in a series of cervical biopsy samples encompassing metaplastic as well as neoplastic lesions (Figure 6). These data convincingly demonstrate that concomitant expression of p16^{INK4a} and Ki-67 in the same cell is only observed in epithelia displaying a p16^{INK4a}-expression pattern associated with transformation by the HPV oncogenes *E6* and *E7* [88].

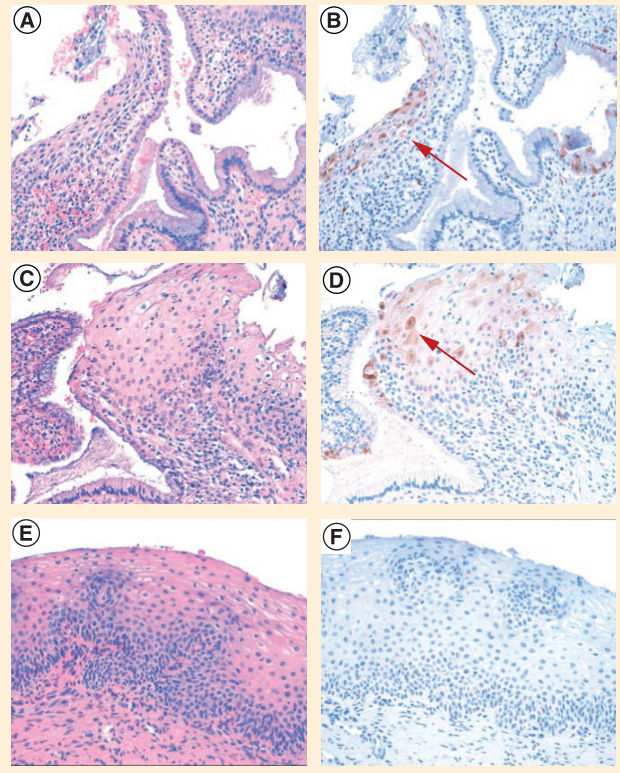

Figure 5. Examples of focal p16^{INK4a}-positive lesions. (A) Lesion negative for dysplasia (hematoxylin and eosin [H&E] stained; ×100). **(B)** Same lesion as in **(A)** (p16 stained; ×100). The arrow indicates focal p16^{INK4a} staining of intermediate and superficial squamous epithelial cells. **(C)** Cervical intraepithelial neoplasia grade 1 (H&E stained; ×100). **(D)** Same lesion as in **(C)** (p16 stained; ×100). The arrow indicates focal p16^{INK4a} staining of intermediate and superficial squamous epithelial cells. **(E,F)** A study sample showing no immunoreactivity within the squamous epithelium. **(E)** Lesion negative for dysplasia (H&E stained; ×100). **(F)** Same lesion as in **(E)** (p16 stained; ×100). Cases with a focal staining pattern and cases showing no immunoreactivity are categorized as negative for CINtec® p16^{INK4a} immunostaining. **(A–D)** Focal staining pattern – that is, staining of isolated cells or small cell clusters, particularly not of the basal and parabasal cells. Reproduced with permission from [82].

In recent studies, this combined use of two antibodies against p16^{INK4a} and Ki-67 in a one-step staining approach (p16^{INK4a}/Ki-67 dual stain, CINtec plus™ [Roche mtm laboratories AG]) was used to triage women who tested positive for HR-HPV infections but had negative cytology test results. In this study, women ≥30 years of age who tested Pap negative but were positive for HPV were included in the analysis. p16^{INK4a}/Ki-67 dual-stained cytology was performed from residual cellular material available from the liquid-based cytology vial collected during the initial Pap/HPV cotesting screening visit. p16^{INK4a}/Ki-67 dual-stained cytology tested positive at baseline in 108 out of 425 (25.4%) Pap-negative/HPV-positive cases. Sensitivity of dual-stain testing for the detection of biopsy-confirmed CIN2+ during preliminary follow-up within the group of Pap-negative/HPV-positive women was 91.9% for CIN2+ (34 out of 37 cases) and 96.4% for CIN3+ (27 out of 28 cases). Specificity was 82.1% for CIN2+ and 76.9% for CIN3+ on biopsy. This study therefore shows that triaging Pap-negative/HPV-positive women with p16^{INK4a}/Ki-67 dual-stained cytology indeed identifies women with a high probability of underlying CIN2+ and may efficiently complement HPV-based screening programs to prevent cervical cancer [107]. Precisely the same approach was further used for the triage of patients with ASC-US and LSIL cytology test results [102]. The objective of this latter study was to analyze the diagnostic performance of the dual-stain approach for identifying high-grade CIN2+ in women with Pap cytology results categorized as ASC-US or LSIL in residual material of a study where p16^{INK4a} performance was evaluated [100]. Liquid-based cytology residual material was evaluable from 776 ASC-US or LSIL cases and subjected to p16^{INK4a}/Ki-67 dual staining. The presence of one or more double-immunoreactive cell(s) (Figure 7) was regarded as a positive test outcome, irrespective of morphology. Test results were correlated to histology follow-up. The sensitivity of the p16^{INK4a}/Ki-67 dual stain for biopsy-confirmed CIN2+ was 92.2% for the ASC-US cases and 94.2% for the LSIL cases, while specificity rates were 80.6% for ASC-US and 68.0% for LSIL. Importantly, similar sensitivity/specificity profiles were obtained for groups of women aged <30 years versus women aged ≥30 years. Hence, the dual-stain

cytology test showed comparable sensitivity to, but significantly higher specificity than, HPV testing.

The results show that the p16^{INK4a}/Ki-67 dual stain provided a comparably high sensitivity for the detection of underlying CIN2+ in women with ASC-US or LSIL Pap cytology results following HPV testing or the previous p16^{INK4a} single-stain cytology test. However, the specificity of this morphology-independent interpretation of p16^{INK4a}/Ki-67 dual-stain tests was further improved compared with the earlier p16^{INK4a} single-stain cytology test in the same cohort and, of course, in comparison to HPV testing.

In addition to these exciting data, the p16^{INK4a}/Ki-67 dual-stained cytology approach was used in a prospective study to identify women with CIN2+ lesions from a primary screening cohort. Again, the accuracy was compared with HPV testing and Pap cytology. A total of 27,349 women 18–65 years of age and attending routine cervical cancer screening were prospectively enrolled in five European countries. Women were referred to colposcopy if they had cytology results of ASC-US or worse, positive p16^{INK4a}/Ki-67 dual-stained cytology results and/or positive HR-HPV test results (as well as women aged 30 years and older, if only the HPV test was positive). The p16^{INK4a}/Ki-67 dual stain was substantially more sensitive than Pap cytology (classified as ASC-US or worse; 93.3 vs 67.7% in women less than 30 years of age and 87.8 vs 64.9% in women more than 30 years of age) for detecting CIN2+, with no difference in specificity (92.3 vs 92.8% in women less than 30 years of age and 96.3% for both tests in women more than 30 years of age). In women more than 30 years of age, HPV testing in this screening cohort was slightly more sensitive than the p16^{INK4a}/Ki-67 dual stain (95.6 vs 87.8%), but significantly less specific (93.1 vs 96.3%) [108]. Taken together, these new data from this very large cohort clearly show that there is a role for the combined use of antibodies against p16^{INK4a} and Ki-67 as an unequivocal parameter for a primary screening test to identify women with CIN2+ lesions who require detailed colposcopic workup. The gain of sensitivity compared with Pap testing is comparable to primary HPV testing in women older than 30 years of age; however, the gain of specificity compared with HPV-DNA testing strongly argues in favor of the dual-stain test. In women less than 30 years of age in whom HPV-DNA testing is not recommended, the dual p16^{INK4a}/Ki-67 staining performance in comparison to Pap testing will reduce psychological distress for patients with transient HPV infections. Thus, this approach could be applied as a single test in the population 25–65 years of age and simplify and reduce the costs associated with population-wide cervical cancer screening programs with the HPV testing approach.

Conclusion

The evidence-based delineation of proteomic biomarkers by the molecular dissection of critical pathways leading to cellular transformation in cervical carcinogenesis makes it possible to develop clinically useful assays that carry the potential to rapidly overcome the substantial drawbacks of established cervical cancer screening technologies. It is therefore highly likely that these new concepts will soon enter clinical practice for cervical cancer screening around the world.

Expert commentary

The identification of p16^{INK4a} as a specific marker for neoplastic transformation of cervical squamous epithelial cells and, in

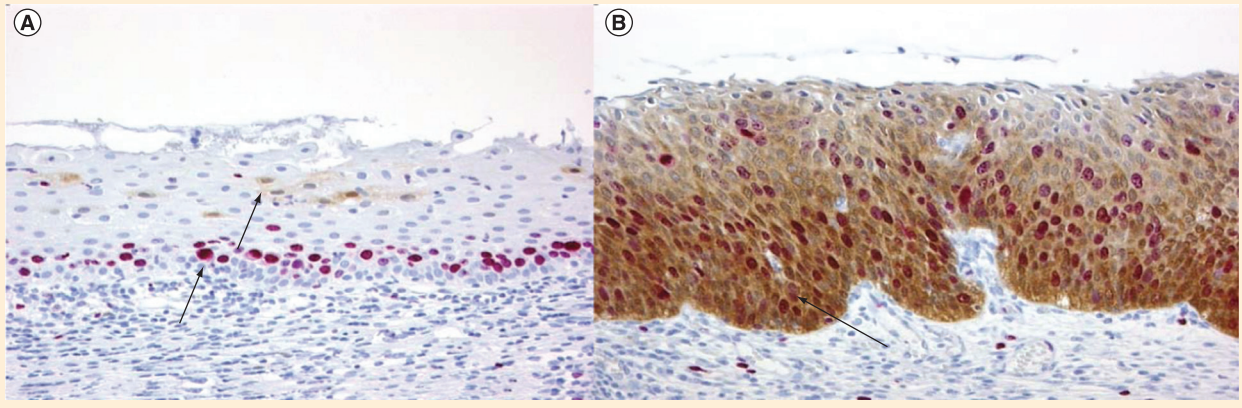

Figure 6. Combined detection of p16^{INK4a} and Ki-67 allows for the unambiguous identification of human papillomavirus-transformed cells. (A) Atrophic cervical epithelium with a few p16^{INK4}-positive cells (focal staining pattern) in the intermediate cell compartment. This section was also stained with an antibody against the Ki-67 antigen to highlight the proliferating cell compartment in the basal cell layer. The upper arrow marks the focally stained cells in the intermediate and superficial cell layers. The lower arrow marks the Ki-67-stained cells in the parabasal cell layer, which in this case are p16^{INK4} negative. **(B)** Cervical intraepithelial neoplasia grade 3 lesion (human papillomavirus 16-positive) stained for both p16^{INK4} and Ki-67. In the human papillomavirus-transformed lesion, costaining with both antibodies is observed as an unequivocal sign of cellular transformation. The arrow indicates the double-stained transformed cells that display strong reactivity for both markers in the same cells.
Reproduced with permission from [109].

particular, its combination with Ki-67 as marker for proliferating cells, allows us, for the first time, to unequivocally identify even the earliest steps of neoplastic transformation in HPV-infected epithelial cells. Based on the underlying molecular mechanisms, these markers indicate the initiation of the destabilizing genomic events that trigger neoplastic transformation. Clinical trials have convincingly demonstrated that the routine use of these markers significantly improves the reliability of the histopathology of cervical lesions. This results in better patient care, the avoidance of unnecessary surgical interventions and, at the same time, improved sensitivity to identify patients who indeed require surgical intervention. The application of these markers to identify transformed cells in cytology specimens allows substantially improved sensitivity and specificity of these diagnostic approaches in comparison with either conventional Pap stains or the use of HPV tests. This will help to improve the quality and reliability of cervical cancer early-detection and screening programs. Finally, this biomarker-based technical improvement will predictably help to reduce the costs of cervical cancer screening programs to identify women who require further medical interventions, and will lead to substantial relief from anxiety and fear for those who are now confronted with only equivocal test results.

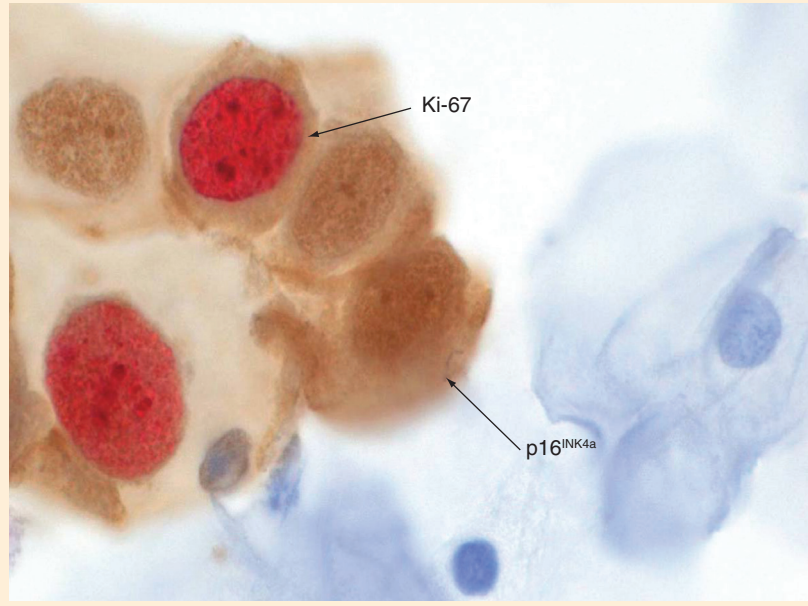

Figure 7. Example of human papillomavirus-transformed cells coexpressing p16INK4 (brown stain) and Ki-67 (red stain) in the same cell in a liquid-based cervical cytology sample. The detection of a single of these double-stained cells strongly suggests the presence of high-grade lesions (cervical intraepithelial neoplasia grade 2 or higher). The cells were stained using the CINtec® Plus kit developed by Roche mtm laboratories AG.

Five-year view

Based on the scientific and clinical data presented in this review, we anticipate the routine use of p16^{INK4a} as an essential marker to either diagnose or to rule out HPV-induced cervical high-grade lesions in the forthcoming years. It is highly likely that novel nomenclatures of cervical lesions will rely either on the presence or absence of p16^{INK4a} overexpression. Hence, p16^{INK4a} will soon become the guiding marker to rule in or to rule out transformation of squamous epithelial cells as a consequence of HPV infections.

The use of the double staining for p16^{INK4a} and Ki-67 is on its way to becoming an established tool in the triage of women with normal cytology, but with positive HPV tests, as well as for those who have either ASC-US or LSIL cytology reports. Based on the existing data, we may further speculate that within the next few years, the routine use of p16^{INK4a}/Ki-67 as even the primary screening test may become the most reliable parameter to predict the presence of lesions that require further diagnostic workup. If this assumption is substantiated by further large sets of clinical data, we can expect that this may also be a technical approach that could be used in developing countries. If so, it will be an important step forward in the global fight against cervical cancer.

Financial & competing interests disclosure

D Schmidt and C Bergeron have been temporary clinical advisors and speakers for mtm Laboratories, and M von Knebel Doeberitz was a member of the supervisory board of mtm Laboratories. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The molecular dissection of the mechanisms by which human papillomavirus (HPV) infections contribute to cervical carcinogenesis allowed the identification of p16^{INK4a} as an ideal biomarker to identify HPV-infected and -transformed epithelial cells.
- Overexpression of p16^{INK4a} allows just HPV-infected, but not yet transformed, epithelial cells to be distinguished from those that have already become transformed by the activation of the viral HPV oncogenes *E6* and *E7* in the basal and parabasal cells of the infected epithelium.
- The clinical application of this novel biomarker-based concept in either histology or cervical cytology revealed convincing data strongly supporting the theoretical concept, as well as its clinical translation to diagnostic cervical histopathology and cytopathology.

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