REVIEW

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The importance of immunocytochemistry in the detection of high-grade cervical lesions

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Abstract

Despite the implementation of various screening programs in many countries, cervical cancer continues to be a major health problem. Cervical cytology is the most used screening method, but human papillomavirus (HPV) genotyping, alone or in combination with cytology, has gained ground during the last years. Still, one of the major limitations of HPV-genotyping is the low specificity of HPV as a screening method in young women that are HPV-positive, but with no potential for future disease. Obviously, there is a need for a better screening algorithm. The ideal screening test for cervical high-grade lesions should detect the effect of high-risk (HR)-HPV infection after cell transformation, but not before, and should accurately identify the cases that are more likely to experience disease progression to neoplasia. Solid data regarding the benefit of immunocytochemistry in the evaluation of the patients with modified cervical cytology have been published recently. The use of the dual staining with $p16^{INK4a}$ and Ki-67 could increase specificity of the method for the detection of atypical cells and may perform better in predicting the risk of high-grade dysplasia in the near future.

Keywords: cervical cancer, cervical cytology, HPV, immunocytochemistry.

Introduction

Cervical cancer screening methods have evolved in the recent years, along with the introduction of human papillomavirus (HPV) genotyping [1–14]. Despite the well-documented involvement of the high-risk (HR)-HPV infection in cervical cancer development, genotyping alone lacks in specificity, given that some infections are cleared over time, especially in young women.

The recent advances in immunocytochemistry for the evaluation of patients with modified cervical cytology could have a major impact in the future of cervical cancer screening. We will discuss in the following paragraphs the current issue that is cervical cancer, the trends in cervical cancer screening, the impact of HPV-genotyping and immunocytochemistry in the diagnosis of modified cervical cytology, with a particular accent on dual staining $p16^{INK4a}$/Ki-67 [14–46].

Cervical cancer – the burden of the disease

Cervical cancer continues to be a major health problem. It is considered the seventh most common malignancy worldwide and the third among the female population. Each year, more than 530 000 new cases are diagnosed and more than 275 000 deaths are determined by this disease. Globally speaking, cervical cancer is responsible for 9% of malignancy related mortality, with more than 80% of cases being diagnosed in developing countries. The process of oncogenesis is rather slow and, in most cases, it takes a matter of years for precancerous lesions to progress to invasive cancer. This leaves a wide window of opportunity for the screening programs to detect these patients and refer them for accurate diagnostic and treatment. On the other hand, cervical cancer is considered one of the preventable malignancies, due to the HPV vaccination program. Great interest and advertising was generated by the development of the anti-HPV vaccines. Many countries developed national programs for vaccine implementation in the large population, but the results were far inferior to the ones expected. Therefore, the global incidence of cervical cancer has not decreased over the last years.

Current trends in cervical cancer screening

The most accepted and used screening method for cervical cancer is cervical cytology (Pap smear). The periodicity of performing this investigation and the age at which the first Pap smear should be performed continue to be a subject of debate, with different recommendations across the world. According to the *American College of Obstetricians and Gynecologists* (ACOG) guidelines and the *World Health Organization* (WHO), screening by Pap smears should be performed every three to five years. The current Australian screening program recommends a Pap smear to be performed every two years for women

aged between 18–69 years, but it will be changed later this year, as presented below. The French *High Health Authority* (HAS) recommends a Pap smear every three years after two previous normal Pap smears taken at one year interval, for all women between 25 and 65 years [1].

Though the performance of this method for the identification of high-grade cervical intraepithelial neoplasia (CIN) was investigated by several studies [2, 3], recent data suggest that HPV-genotyping represents a more appropriate method for screening. HPV-genotyping is considered a method with higher sensitivity for the detection of cervical dysplasia. Genotyping can be performed as a 'reflex' test for patients with cytological abnormalities [4]. Several studies suggest that HPV-genotyping could be offered as a replacement for Pap cytology [5, 6].

Patients with cytological abnormalities are referred to colposcopy and if necessary to biopsy. Most patients that are referred to colposcopy on the base of the cytological exam do not actually have high-grade intraepithelial lesions and do not require biopsy. This management strategy is inefficient and results in significant costs due to the inability of current techniques to differentiate between high- and low-risk patients. Data available in literature suggest that a better method with higher specificity for high-grade cervical lesions would be necessary in order to reduce the number of unnecessary colposcopies.

The impact of HPV genotyping

The most important etiopathogenic factor for the development of high-grade cervical lesions is the persistent infection with HR-HPV. Patients infected with high-risk types, such as type 16 and 18, are less likely to experience spontaneous infection clearance. Several studies also indicate that HR-HPV types and the patient's age are factors that favor infection persistence and disease recurrence, even after loop excision [7, 8]. This places patients with HR-HPV in a risk group that theoretically should be monitored more closely. Many studies advocate in favor of replacing the Pap cytology with HPV-genotyping as the primary screening method. The main argument is represented by the high sensitivity rate of this method. The ATHENA study, the largest study that evaluated the performance of HPV-genotyping compared with liquid based cytology, investigated 47 208 patients and concluded that HPV-testing with separate HPV 16 and HPV 18 detection could be a superior alternative method, more sensitive and more efficient for cervical cancer screening than cytology [9]. Following these results, the HPV-genotyping gained considerable ground as a screening method for cervical cancer.

In the renewed *National Cervical Screening Program* of the *Australian Department of Health*, which will be implemented by December 2017, the Pap cytology will be replaced by HPV-genotyping test. The test will be performed every five years and the target population is represented by women aged between 18 to 69 years. On the other hand, in the USA, the ACOG guidelines still recommend cytology for women less than 30 years old, and co-testing (cytology plus HPV-genotyping), as the primary screening method for women older than 30 years [1].

It is estimated that 10.4% of women with normal cytology are positive for HR-HPV [10]. The clearance rate of infection is very high among young women with a competent immune system, and this explains the low specificity rate of HPV-genotyping test for the detection of high-grade cervical lesions.

One of the major limitations of HPV-genotyping is the fact that there is a very large proportion of women less than 30 years old that are HPV-positive, but with no cervical lesions, that will spontaneously clear the HPV-infection. This significantly lowers the specificity of HPV as a screening method, and could lead to further unnecessary investigations for this category of women, that are HPV-positive, but with no potential for future disease.

Immunocytochemistry importance for diagnostic

Immunostainining, as a method to improve and modulate the management of gynecological malignancies, is not a novelty. Immunohistochemistry has already been included in the assessment protocol of ovarian and breast tumors [11–16]. Solid data regarding the benefit of immunocytochemistry in the evaluation of the patients with modified cervical cytology have been published recently.

The diagnostic value of minichromosome maintenance protein 2 (MCM2) immunocytochemical staining in cervical lesions and its relationship with HPV-infection was evaluated by Zheng (2015). They investigated a group of 187 patients with cytological abnormalities and found that for the detection of cervical lesions MCM2 immuno-cytochemical test was more effective than HPV-type detection [17].

Immunohistochemistry with $p16^{INK4a}$ antibodies has been used as a diagnostic aid in different types of tumors. Diffuse expression of $p16^{INK4a}$ in the cervix can be considered as a surrogate marker of infection with HR-HPV [18].

The use of residual cytological material from ThinPrep® Pap test liquid-based cytology vials for performing the dual staining test $p16^{INK4a}$/Ki-67 seems a very practical idea. Immunohistochemistry on samples from cervical biopsy can be an adjuvant method for an even more accurate diagnostic, but immunocytochemistry could be even more valuable because it could be used as a screening method. The major benefit would be to decide which patients with modified cytology should be referred for further investigations like colposcopy and biopsy.

Immunocytochemical staining can also be used to detect the presence of HPV in tissue sections by using monoclonal antibodies, such as 4C4 clone, IgG1 [19].

Novel immunocytochemical markers

Novel immunocytochemical markers for cervical disease, such as ProEx C, programmed cell death protein-1 (PD-1), discs large 1 (DLG1), E-cadherin –160 C/A have been described, but further studies are required. ProEx C staining has been found to be more sensitive and specific

than HR-HPV testing in patients with atypical squamous cells of undetermined significance (ASCUS) [20–22]. PD-1 and ligand (PD-L1) have been shown to impair local cellular immunity leading to persistence of HPV and progression to cervical cancer. The blockade therapy of these proteins showed promising benefits and can become a future immunotherapy treatment option [23–25]. Human DLG1 tumor suppressor participates in regulating cell polarity and proliferation. Cavatorta *et al.* (2017) evaluated the expression of DLG1 and concluded that that its expression can provide valuable prognostic information, having an important role in the progression of early dysplastic cervical lesions [26]. E-cadherin, a transmembrane glycoprotein with important roles in the maintenance of cervical squamous epithelium integrity, has been proven to represent a valid risk factor for high-grade squamous intraepithelial lesion (HSIL) and *in situ* carcinoma [27].

p16^{INK4a} as marker for cervical lesion

p16^{INK4a} is a tumor-suppressor protein and cyclin-dependent kinase (CDK) inhibitor that blocks CDK4- and CDK6-mediated retinoblastoma protein (pRb) phosphorylation to inhibit E2F-dependent transcription and cell-cycle progression [28].

The positive reaction for p16^{INK4a} in cycling cells is a direct indicator of HPV-E7 overexpression, which is a marker of cell cycle alteration. Immunohistochemical analysis has demonstrated that diffuse staining for p16^{INK4a} is present in almost all cases of high-grade cervical lesions and, on the other hand, it is rarely detected in normal epithelium or low-grade lesions. The value of p16^{INK4a} as a complementary marker of high-grade intraepithelial lesions of the uterine cervix was investigated by Dray *et al.* (2005). Immunostaining for p16^{INK4a} was performed on 189 samples obtained after punch biopsy of the cervix, and a strong correlation between p16^{INK4a} expression and the presence of HSILs on Hematoxylin–Eosin sections was observed [29].

However, the expression of p16^{INK4a} can be rarely observed in normal tissue with no malignant potential. Focal and occasionally diffuse expression of p16^{INK4a} can be observed in benign endocervical columnar cells, squamous metaplasia and in endometriosis lesions [30]. This represents the major limitation for the use of p16^{INK4a} alone as a marker for the detection of high-grade lesions.

Several studies investigated the specificity of p16^{INK4a} cytology-based testing, and all of them found it substantially higher than the specificity of HPV-testing [31–34]. Nieh *et al.* (2005) investigated the correlation between HR-HPV viral load and p16^{INK4a} expression in Pap smears categorized as ASCUS. Follow-up biopsies were performed to establish the severity of the lesions. They found a significant association between p16^{INK4a} expression in ASCUS-categorized smears, with the presence of squamous intraepithelial lesions (SILs) on follow-up biopsies and positive HR-HPV viral loads. They concluded that p16^{INK4a} immunostaining on smears seems to be a more accurate method than HR-HPV viral load for the detection of intraepithelial lesions among patients categorized as having ASCUS on Pap smears [35].

According to most studies, p16^{INK4a} immunoperoxidase shows greater specificity than sensitivity for squamous lesions and can serve as an adjuvant method for the assessment of cases with modified cytology [36].

The role of Ki-67

Ki-67 is considered a proliferation marker that is confined to the parabasal cell layer of normal stratified squamous mucosa of the cervix. The overexpression of Ki-67 in the stratified squamous epithelium in cervical dysplasia correlates with the extent of disordered maturation [37].

Ki-67 is elevated in HPV-infected mature squamous epithelia and can be used to distinguish between low-grade squamous intraepithelial lesions (LSILs) and HSILs [38, 39].

On the other hand, Ki-67 may be positive in HPV-negative squamous metaplasia or heeling epithelium. In conclusion, the expression of this marker in immature squamous epithelium cannot be considered specific for HPV-infection.

The need for better screening in cervical cancer – p16^{INK4a}/Ki-67 dual staining

The use of the dual staining with p16^{INK4a} and Ki-67 could increase specificity of the method for the detection of atypical cells. Ki-67 can be detected exclusively in the nucleus of proliferating cells, whereas cells in G0 phase do not express it [40, 41]. Because the overexpression of p16^{INK4a} is strongly associated with the impairment of the cell-cycle control mechanism, the expression of both p16^{INK4a} and Ki-67 in the same cell should not be encountered in normal conditions. In conclusion, the concomitant expression of p16^{INK4a} and Ki-67 in the same cell may serve as an indicator of deregulation of cell cycle control [42].

Schmidt *et al.* [43] conducted, in 2011, one of the first major studies investigating the performance of dual-staining protocol, p16^{INK4a}/Ki-67 expression in cervical cytology samples, for identifying high-grade CIN in women with ASCUS and LSIL on Pap smear. The study involved 776 patients and the residual samples of liquid-based samples from cytology tests were used. They found that dual-staining cytology provided sensitivity rates that were comparable to the rates previously reported for HPV-testing and p16^{INK4a} single-staining cytology, but with improved specificity [43].

Kisser & Zechmeister-Koss (2014) [44] performed a review of literature aiming to assed the accuracy of p16^{INK4a}/Ki-67 dual staining for the triage of patients with ASCUS and LSIL. They found five studies which reported a sensitivity of p16^{INK4a}/Ki-67 testing ranging from 0.86% to 0.98% and specificity ranging from 0.43% to 0.68%, in patients with LSIL, and a sensitivity ranging from 0.64% to 0.92% and a specificity ranging from 0.53% to 0.81%, in patients with ASCUS. Their survey concluded that p16^{INK4a}/Ki-67 testing cannot be recommended for the triage of women with ASCUS or LSIL cytology, due to insufficient high-quality evidence and that more data is needed to prove the benefit of the introduction of the p16^{INK4a}/Ki-67 test in the management protocol of patients with cytological abnormalities [44].

Since then, more data that advocates in favor of the dual staining as a screening method was published. Posatti-Resende *et al.* (2015) [45] compared the accuracy of double staining for $p16^{INK4a}$/Ki-67 and the molecular test for HR-HPV to identify high-grade CIN in women with ASCUS and LSIL on Pap cytology. Their study included 201 patients and found a sensitivity of 87.5% and a specificity of 79.5% for the HPV-test and, respectively, 62.5% and 93.1% for $p16^{INK4a}$/Ki-67 for diagnosing CIN2/CIN3 in patients with ASCUS. Among women with LSIL, the sensitivity and specificity in the diagnosis of CIN2/CIN3 were 87% and 34.7% for the HPV-test and 69.6% and 75.3% for the dual staining. Interestingly, this study showed different performance rates for patients less than and over the age of 30 years. In younger women, less than 30 years, with ASCUS and LSIL, $p16^{INK4a}$/Ki-67 showed greater accuracy in identifying high-risk lesions. The specificity for the dual staining method was 85.7% compared to 66.7% for the HPV-test in patients with ASCUS and 66.7% compared to 6.9% for HPV-test in women with LSIL. In the group of patients over 30 years, both methods had similar results [45].

These intriguing results can be explained by the high rate of HPV-infections among young women, infections that will eventually be cleared spontaneously and will not produce cervical lesions.

Similar results were reported by Vrdoljak-Mozetič *et al.* (2015) [46] who conducted a prospective study including 155 patients and found that the sensitivity of $p16^{INK4a}$/Ki-67 dual staining was comparable to that of the HR-HPV-test, but the specificity was substantially higher for the detection of high-grade cervical lesions among patients with LSIL on Pap smear [46].

The place of immunocytochemistry in the screening algorithm is the near future

Cervical cancer remains a leading cause of morbidity and mortality worldwide. Obviously, there is a need for a better screening algorithm. The ideal screening test for cervical high-grade lesions should detect the effect of HR-HPV-infection after cell transformation, but not before, and should accurately identify the cases that are more likely to experience disease progression to neoplasia.

Cervical cancer screening guidelines were issued in most countries, with detailed management algorithms, that usually consist in cytology, testing for HR-HPV-infection, colposcopy and invasive procedures (such as cervical biopsies and endocervical curettage). As a response to the overwhelming data that demonstrated the importance of persistent HPV-infection and the development of cervical lesions, many studies investigated the value of HPV-testing as a screening method for cervical cancer [7, 8].

The results were very promising in terms of sensitivity of the HPV-testing, and the idea of using the HPV-test as a primary screening method instead of the classic cytology test gained ground. The strength of this test consists in its high sensitivity. Virtually all cases with lesions that have the potential to progress to neoplasia will be detected as positive for HPV-infection. On the other hand, the weakness of the method is represented by the low specificity, especially in young patients. Because most women are infected at some point in their lives with HPV, but the majority of the infections are cleared by their own immune system, the HPV-test will detect many patients that are positive for HPV, but will not develop cervical lesions. This aspect is even more common in females younger than 30 years. Practically speaking, using the HPV-test as the primary screening method means that many healthy women will be referred for further unnecessary investigations and exposed to the stress of having a potentially suspect lesion. On the other hand, the use of the cytology alone can leave some patients with high-grade lesions undiagnosed. Hence, here is a window of opportunity for use of immunocyto-chemistry. The $p16^{INK4a}$/Ki-67 dual staining performs better then $p16^{INK4a}$ detection alone, and has a better specificity for high-risk lesions then HPV-genotyping. The dual staining $p16^{INK4a}$/Ki-67 has lower sensitivity compared to HPV-testing, and most likely will never be proposed as a primary screening method. However, it could be very useful for the triage of patients with cytological abnormalities, such as ASCUS and LSIL, or patients with HR-HPV prior colposcopy. Especially in patients younger than 30 years, immunocytochemistry could be advantageous for risk stratification. Because the samples from liquid-based cytology can be used and there is no need for another visit to the doctor, the method could be proposed as a reflex test if abnormal cytology is detected. The use of this method could significantly decrease the number of unnecessary colposcopies and biopsies.

Considering the major etiopathogenic role of the HPV-infection in the molecular pathogenesis of cervical cancer, HPV-testing may be superior for stratifying the long-term risk for cervical malignancies, but the $p16^{INK4a}$/Ki-67 dual-staining cytology, that mark the viral E6/E7 oncoprotein-mediated inactivation of tumor suppressor proteins and subsequent cell-cycle deregulation, may perform better in predicting the risk of high-grade dysplasia in the near future [31].

Conclusions

There is an obvious need for a better screening algorithm for cervical cancer. The ideal screening test for cervical high-grade lesions should detect the effect of HR-HPV-infection after cell transformation, but not before, and should accurately identify the cases that are more likely to experience disease progression to neoplasia. Solid data regarding the benefit of immuno-cytochemistry in the evaluation of the patients with modified cervical cytology have been published recently. Therefore, the use of the dual staining with $p16^{INK4a}$ and Ki-67 could increase the specificity of the method for the detection of atypical cells and may perform better in predicting the risk of high-grade dysplasia in the near future.

Conflict of interests

The authors declare no conflict of interests.

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