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Review

# Advances in Cervical Cancer Control and Future Perspectives

**Magdalena Grce, Mihaela Matovina, Nina Milutin-Gašperov and Ivan Sabol**

Laboratory of Molecular Virology and Bacteriology, Division of Molecular Medicine, »Rudjer Bošković« Institute, Zagreb, Croatia

**A B S T R A C T**

*The knowledge that the persistent infection with high-risk (HR) human papillomavirus (HPV) is the etiological factor in the development of cervical cancer has led to the development of the HPV DNA detection methods as well as the prophylactic vaccine against the most common HR-HPV types, HPV 16 and 18. Despite HPV vaccination, cervical cancer screening will remain the main preventive measure for both vaccinated and non-vaccinated women, but the nature of screening and management of women with cervical disease is being adapted to the new technologies. Although, HPV DNA detection is more sensitive that cytology, its specificity is lower, since most HPV infections are transient. Therefore, other methods are considered to improve the management of women with cervical disease. Typing of HPV DNA and viral load measurements are still used for research purposes only. Detection of viral oncogene E6/E7 transcripts, which is the marker of the productive infection, is a promising tool for follow-up of HPV DNA-positive women. The detection of p16INK4a over-expression, as an indirect test of E6/E7 expression, is used for confirmation of cervical neoplasia. Despite the lack of standardization, the detection of p16INK4a is useful in clinical settings, however its reproducibility in the management of low-grade and borderline cases is low. Future perspectives include the determination of the methylation status of several cellular genes that could predict the progression of the disease.*

***Key words***: *cervical cancer, high-risk (HR) human papillomavirus (HPV), biological markers, Croatia*

## Introduction

The causal relationship between the persistent infection with carcinogenic or high-risk (HR) human papillomavirus (HPV) and cervical cancer is now well established[1,2]. Prophylactic vaccination against the most common causes of cervical cancer, HPV types 16 and 18 accounting for 70–75% of cervical cancer cases, is available since several years ago[3]. However, not all cervical cancer cases could be prevented by vaccination, so screening for cervical cancer remains the main measure of prevention for both vaccinated and non-vaccinated women.

As it takes several decades for cervical changes to develop from atypical, low, mild and high abnormalities to invasive cancer, there is enough time for early detection of precancerous lesions. Therefore, cervical cytology (Papanicolaou or Pap smear) was, and still is, the test of choice for detection of cervical precancerous lesions, since its introduction in the early 1960s[4].

The conventional cytology by Pap smear is easy to perform and it is a relatively low-cost screening test. However, Pap test is highly subjective. The sensitivity and specificity varies between laboratories, from 11 to 99%, and 14 to 97%, respectively[5]. In addition, the need to often repeat the test because of the high prevalence of unsatisfactory and false negative results undermines test cost-effectiveness. Therefore, test accuracy and its cost-effectiveness highly depend on the quality control of its performance[6]. The liquid based cytology (LBC), despite having several advantages, has similar sensitivity and specificity for detecting high-grade cervical intraepithelial neoplasia (CIN2+) compared to conventional cytology[7–9]. LBC is less time consuming, reduces the number of unsatisfactory cervical smears and allows residual sample to be used for additional testing. In addition, LBC can be automated, allowing large number of samples to be processed *per* day.

Nowadays, several tests complementing the Pap test are being evaluated in clinical settings. This review focuses on challenges in cervical cancer early detection and control using new technologies, such as HPV testing

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based either on viral DNA or RNA analysis, detection of the molecular biomarker p16INK4a, and evaluation of DNA methylation status.

## DNA Based HPV Testing

Recognition that cervical cancer is causally linked to HR-HPV types led to the use of HPV DNA testing. HPV DNA tests are based on detection of DNA of at least 13 carcinogenic HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66)[2], either by DNA amplification (polymerase chain reaction-PCR) with type-specific primers or by hybridisation with a cocktail of probes[10]. Those tests are more sensitive, objective, reproducible, and less demanding, in terms of training and quality assurance, compared to cytology. In addition, there are a few commercially available, EMEA (European Agency for the Evaluation of Medical Products) and FDA (Food and Drug Administration of the United States) approved HPV DNA assays, of which the Hybrid Capture 2 (HC2, Qiagen GmbH, Hilden, Germany) is widely used in clinical laboratories. HPV DNA testing with HC2 showed to be very useful for triage of equivocal cytology samples (ASC-US, atypical squamous cells of undetermined significance), and for follow-up of women after treatment for high-grade cervical disease.

Several studies and meta-analyses have demonstrated that HPV DNA testing is highly effective for the triage of women having ASC-US. It is more sensitive and equally specific as cytology in identifying women with underlying clinically relevant cervical disease (CIN)[11,12]. Cuzick et al.[12] determined that the HC2 assay for triage of women with ASC-US has an overall sensitivity of 93.1% (95% CI: 91.1–95.1%) and 95.5% (95% CI: 92.7–98.2%), and specificity of 62.3% (95% CI: 57.6–67.1%) and 60.5% (52.9–68.2%) for detecting CIN2+ and CIN3+, respectively. For triage of women with LSIL (low-grade squamous intraepithelial lesions) the sensitivity of HC2 was also very high, 97.2% (95% CI: 95.6–98.8%) for detecting CIN2+ and 97.1% (95% CI: 94.0–100%) for detecting CIN3+ but the specificity was very low, 30.6% (95% CI: 22.7–38.6%) for detecting CIN2+ and 26.1% (95% CI: 15.1–37.1%) for detecting CIN3+. However, for women aged 35 or more, the HPV positivity rate was much lower than for younger women and the potential value of HPV DNA testing as an adjunct to cytology in this group was substantially better than for younger women[12,13].

Comparison of the accuracy of HPV DNA testing for the triage of ASC-US or worse with cytology showed that the sensitivity of HC2 was on average 14% higher than repeat cytology for detection of CIN2+ (ratio: 1.14; 95% CI: 1.08–1.20) and has similar specificity (ratio: 0.99; 95% CI: 0.88–1.10)[12]. The Croatian study, Pajtler et al.[14] using PCR method to estimate the predictive value of HPV testing for disease prognosis, emphasises the need to carefully evaluate the value of HPV testing in a routine practice.

Studies concerning post-treatment follow-up, although heterogeneous, indicate that HPV DNA testing performed better than follow-up by cytology to predict success or failure of treatment. It has significantly higher sensitivity and its specificity is not significantly lower compared to cytology[10]. Based on these convincing data, application of HPV DNA testing has been recommended in the European Guidelines on Quality Control in Cervical Cancer Screening[15] and has been introduced into routine practice in many countries, including Croatia[9,14,16].

HPV DNA testing for primary screening, with cytology reserved for triage of HPV positive women, has been evaluated in several randomised controlled studies as a sole test or in combination with cytology[17]. All these studies suggest that HPV DNA testing is more sensitive than cytology in primary cervical screening in women over the age of 30. HPV DNA testing was substantially more sensitive in detecting CIN2+ lesions than conventional cytology (96% *vs*. 53%) but was less specific (91% *vs*. 96%). However, the specificity of both tests increased with age. The sensitivity of HPV DNA testing was uniformly high at all ages, whereas the sensitivity of cytology was substantially better in women over the age of 50 than in younger women (79% *vs*. 60%). The biggest advantage of HPV DNA testing in primary screening is its negative-predictive value (NPV) that guarantees safe extension of the interval for re-screening of HPV negative women to 5 or more years[18].

In the Proposal for the programme of early detection of cervical cancer in Croatia submitted in 2004 to the Ministry of Health and Social Welfare[19], HPV testing as primary screening was suggested to be implemented after the first round of the programme. Actually, the priority is still to establish the nation-wide cervical screening programme based on conventional cytology, which is a prerogative for subsequent piloting of HPV testing as primary screening in Croatia.

Higher sensitivity of HPV DNA testing over cytology offers several advantages, including, most importantly, the potential of reducing cervical cancer rates, while simultaneously reducing the number of lifetime screens and increasing the efficiency of cervical screening particularly as the proportion of HPV vaccinated women increases in the population. Other HPV DNA based techniques, such as real-time PCR for assessing the viral load and/or the physical state of the viral genome, and HPV DNA typing of most common HR HPV types are also valuable biological markers of disease progression. Currently, these tests are reserved for research purposes only. In case of viral load measurement, there are no validated tests available for clinical use and, therefore, the true clinical relevance of the viral load measurement has not been properly evaluated. HPV DNA types can be determined by either type-specific PCR, hybridisation of the consensus-PCR amplicon with specific probes or restriction fragment length polymorphism of the consensus-PCR amplicon[10,20]. In addition, there are commercially available HPV DNA typing methods, of which the INNO-LiPA HPV Genotyping assay (Innogenetics

Group, Gent, Belgium) and Roche LBA (Molecular Systems, Pleasanton, California), both designed for the identification of all 13 HR HPV types and more [few probable HR and many low-risk (LR) types], are the most widely used[21]. The commercial tests are useful for epidemiological studies, but not as a clinical tests, as they can be confusing for both the clinicians and women. A recent study on HPV frequency in Croatia, showed that five most commonly found HPV types in cervical cancer, HPV 16, 18, 31, 33 and 45, were also the most frequent types found in high grade cervical lesion, which confirms clinical relevance of HPV DNA typing[22,23].

## RNA Based HPV Testing

While the sensitivity of HPV DNA testing is very high for detecting high-grade squamous intraepithelial lesions (HSIL), its specificity is lower than cytology (discussed in the previous Chapter). As most HPV infections are in fact transient and regress spontaneously[2], this low specificity of DNA testing means that many women will needlessly be referred to colposcopy and treatment. Therefore, to make HPV DNA testing more efficient, different biological markers are being evaluated to identify the lesions that will progress. One very promising marker is the presence of the viral oncogene transcripts E6 and E7.

The role of HPV E6 and E7 oncogenes in cell immortalization and transformation is reviewed in detail by Munger and Howely[24]. Briefly, E6 and E7 proteins disrupt normal cell cycle and cell death control. During the viral life cycle transcription of oncogenes is tightly regulated and the gene expression is low[25]. In addition, the transcription of E6 and E7 oncogenes is ongoing only in the deep layers of the epithelium, specifically in histological low-grade lesions (CIN1)[26]. Even though those lesions can be HPV DNA positive, they remain HPV mRNA negative. However, in CIN2+ lesions, the regulation of transcription is gone and a large amount of E6/E7 transcripts can be found throughout the epithelium and can be detected in the samples taken for analysis[26,27].

The PreTect HPV-Proofer assay (NorChip/BioMérieux Inc., Klokkarstua, Hurum, Norway) enables the detection and identification of E6/E7 transcripts of HPV types 16, 18, 31, 33 and 45[28], while APTIMA® HPV assay (Gen-Probe Inc., San Diego, CA, USA) allows the detection of HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68, but not the identification of each transcript[29]. The number of HPV types covered by the respective commercially available assays represents an issue to be considered. However, HPV RNA testing is a promising tool for triage of HPV DNA-positive women, who, if they are also RNA-positive, are at greater risk of disease progression.

In a recent large comparative study[30], several DNA and RNA based methods were assessed for the detection of CIN2+. Non-genotyping DNA tests in that study had sensitivity, specificity and positive predictive value (PPV) close to 99+%, 25% and 35%, respectively. RNA based methods in the same study had similar sensitivity but increased specificity and PPV. For the PreTect HPV-Proofer assay, sensitivity, specificity and PPV values were 73.6% (95% CI: 67.8–78.8), 73.1% (95% CI: 69.5–76.4) and 52.0% (95% CI: 46.8–57.2), respectively. The APTIMA assay had a sensitivity of 95.2% (95% CI: 92.0– 97.4) and again both specificity and PPV were higher than DNA test values, 42.2% (95% CI: 38.4–46.0) and 39.9% (95% CI: 36.2–43.8), respectively[30].

A study of Cuschieri et al.[31] indicated that mRNA of E6/E7 might also be a marker of persistence since 67% of RNA-positive, and only 27% of RNA-negative samples persisted for 2 years. Several issues need to be addressed before clinical application of HPV RNA testing. The RNA stability represents a major problem for the accuracy of HPV RNA testing. Small scale studies have shown that samples are well preserved in LBC medium even for several weeks, but RNAse contamination and RNA degradation could be a problem[32,33].

## Detection of p16INK4a

HPV oncoproteins E6 and E7 act by deactivating tumour suppressor genes p53 and pRB, respectively. It has been proposed that inactivation of pRB in cells infected with HR-HPV could lead to over-expression of cyclin-dependent kinase inhibitor p16INK4a, which is negatively regulated by pRb. This hypothesis has been confirmed for HPV positive cervical cancer cell lines that showed strong over-expression of p16[34]. Those findings promoted the possibility of using immunostaining in the detection of p16INK4a as an additional test for distinguishing between normal and neoplastic cervical epithelia.

Von Knebel Doeberitz group conducted a study on histological samples, and tested several different antibodies for detection of p16INK4a. Their results supported the hypothesis of over-expression of p16INK4a in neoplastic cervical lesions. They also confirmed that over-expression of p16INK4a is a good marker for cells expressing viral E6/E7 oncoproteins[35]. The results of the study by von Knebel Doeberitz group were the basis for the development of a screening test for immunochemical detection of p16INK4a over-expression, CINtec® p16INK4a Histology kit (mtm laboratories AG, Heidelberg, Germany).

Klaes et al.[36] have confirmed that the detection of over-expression of p16INK4a can improve the interobserver agreement in the histological diagnosis of cervical lesions in tissue sections. Immunostaining for p16INK4a proved to be particularly helpful in distinguishing between CIN1 and CIN2+ lesions. They also found lack of p16INK4a immunostaining in CIN1 lesions infected with LR-HPV, compared to CIN1 lesions infected with HR-HPV[36]. Results of this study showed that immunostaining for p16INK4a also helped in focusing pathologist's attention to small clusters of dysplastic cells, and, therefore, improved diagnosis in cases where the number of dysplastic cells was low[36].

Kanao et al.[37] conducted a study to determine the levels of mRNA for p16INK4a and the alternative transcript

from the same gene locus, p14ARF in cervical cancer tissues positive or negative for HPV infection to evaluate possible correlation between p16INK4a/p14ARF over-expression and HPV infection. This study showed over-expression of p16INK4a/p14ARF only in HPV-positive cancer tissues, and suggested that HPV-negative cancers might have the opposite mode of inactivation of p53 and pRb, that involves inactivation of p16INK4a/p14ARF gene. However, the study did not include sufficient number of HPV-negative cervical cancer samples to draw final conclusions[37].

In the study by Wang et al.[38], patients with CIN1 lesions were followed-up for 5 to 7 years. The over-expression of p16INK4a could not be correlated with HPV status, and was detected even in normal and inflammatory epithelium, although in lower percentage of cases (12.5% in normal epithelium, 75% in CIN and 75% in SCC). However, they have noticed that cases positive for p16INK4a developed cervical cancer in the shorter time than those without over-expression of p16INK4a.

Wentzensen et al.[39] investigated the methods for improving the use of p16INK4a over-expression in LBC screening, since it has been noticed that non-dysplastic cells can also show increased expression of p16INK4a. They tried to define morphologic criteria of the nuclei, to enable scoring of p16INK4a-positive cervical cells, to identify patients with HSIL.

Carrozi et al.[40] tested the use of detection of p16INK4a over-expression in conjunction to HPV testing. They found p16INK4a over-expression in 88% (95% CI 80–94) of HPV-positive specimens that were histologically confirmed CIN2 or worse. Therefore, they concluded that p16INK4a staining is a useful tool to improve the specificity of HPV DNA testing, since it is strongly associated with the CIN2+ lesions. In addition, the performance of p16INK4a staining might be improved by using higher cut-offs or nuclear scoring criteria in younger women, where HPV infections are often transient[40].

However, a recent meta-analysis that included 61 different studies showed that proven usefulness of the detection of p16INK4a over-expression is undermined by the lack of standardized methodology[41]. This problem is more pronounced in p16INK4a cytology, since the histological criteria for p16INK4a positivity are well defined. Average proportion of p16-positive smears in cytological samples was 12% for normal, 45% for ASC-US, 45% LSIL and 89% for HSIL, and only 2% for normal, 38% CIN1, 68% CIN2, and 82% for CIN3 in histological samples. The results of different studies were also very heterogeneous, especially in the cytological samples graded as ASC-US (10–100% of p16 positive) and LSIL (10–86% of p16 positive). The same problem was observed for histological samples graded CIN1, where over--expression of p16 varied from 0–100% in different studies. The lack of reproducibility, that was especially high in the assessment of low-grade lesions, invoked the conclusion that there is still not enough data to give recommendations about the best use of p16INK4a detection in clinical practice[41].

Only a few studies evaluating the use of p16INK4a as the adjunct to cytology was conducted in Croatia, so far[9,42,43]. The authors of these studies all found that p16INK4a was a useful adjunct to cytology. The results obtained so far call for continuing efforts to try to determine the best use of p16INK4a as a marker of the progression of cervical lesions, since there are still too many discrepancies emerging from different studies to be able to use the method as a reliable test of disease progression, especially in low grade and borderline samples.

## Future Perspectives: Evaluation of DNA Methylation Status

The identification of specific biomarkers of early malignant progression would be useful to improve the selection of women with the increased risk of cervical disease. Therefore, the appearance of epigenetic biomarkers in relation to the stages of cervical carcinogenesis is considered as a promising diagnostic tool[44].

It is well established that DNA methylation is a frequent epigenetic event in many human cancers[45,46]. Over the last decade, a growing number of studies evaluating methylation status of host genes in cervical tissue have been published[45–50]. A large number of cancer-related genes are being recognized that harbour dense methylation of cytosine in normally unmethylated CpG-rich sequences, called CpG islands, located within the 5' gene promoter regions[51]. Most of candidate biomarkers for methylation analyzed in cervical tissues were previously observed in other types of cancer[50].

Nowadays, methylation assays are still being evaluated. Although the majority of the studies uses methylation specific PCR (MSP) followed by Methylight, other studies use quantitative MSP protocols, bisulfite sequencing, and other methods (reviewed by Wentzensen et al., 2009)[50]. Briefly, the MSP amplifies genomic DNA that is modified by previous treatment with sodium bisulfite, which converts all of the unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. Modified DNA is the template for PCR amplification with specific primers for the methylated and unmethylated forms of the gene[49].

In the last decade, numerous studies analyzed methylation status of various tumour suppressor genes (p16, RARβ, FHIT, GSTP1, MGMT, hMLH1, MAL, DAPK, IGSF4, CDH1 and PAX1) and found that methylation was a frequent event in CIN2+ lesions and tumour samples, very low in CIN1 samples and negative in control samples from healthy women, suggesting that aberrant methylation may indicate increased risk for cancer development[44,47–49]. In 2009, Wentzensen et al. conducted a systematic research on 51 published studies on the methylation status of 68 genes in cervical tissues[50]. Among 15 genes analyzed in detail in those studies, 7 genes (CDH1, FHIT, TERT, CDH13, MGMT, TIMP3 and HIC1) showed very heterogeneous methylation frequencies in cervical cancer. Only three markers, DAPK1, CADM1

and RARβ, showed elevated methylation in cervical cancers consistently across those studies.

There are no methylation markers that could be used in cervical cancer screening, currently, however, tremendous efforts to identify epigenetic biomarkers of progression of cervical disease are under way. In addition, the methods for the evaluation of DNA methylation status are still time consuming and cumbersome, and to be applicable in clinical practice they have to be well defined and standardized. Therefore, early detection of cervical cancer requires large scale well-powered epidemiologic studies designed to properly identify, and then validate methylation markers candidates.

## Conclusion

Cytology screening complemented by HPV DNA and/or RNA testing, and immunohistochemistry of p16INK4a represents a powerful tool for management of women with cervical precancerous lesions. Figure 1 summarizes the possible clinical application of the new methods, described in this review, for screening of the target population of women and monitoring those with abnormal findings for early detection of precancerous lesions. Detection of HR-HPV DNA is already widely used in clinical practice. However, p16INK4a over-expression, and most other DNA and RNA based methods require further standardisation and extensive research. This is particularly true for DNA methylation analysis, since several methylation biomarkers were identified but none was adapted and evaluated for clinical application, yet.

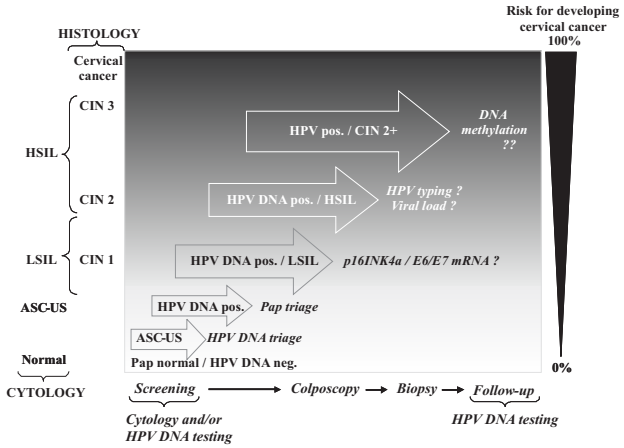


*Fig. 1. Possible clinical application of the new methods for screening and monitoring of cervical precancer and cancer.*

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*M. Grce*

*Laboratory of Molecular Virology and Bacteriology, Division of Molecular Medicine, »Ruđer Bošković« Institute, Bijenička cesta 54, 10002 Zagreb, Croatia*
*e-mail: grce@irb.hr*

## NOVE SPOZNAJE U KONTROLI RAKA VRATA MATERNICE I BUDUĆE PERSPEKTIVE

## S A Ž E T A K

Saznanje da je trajna infekcija visokorizičnim tipovima HPV-a uzrok nastanka raka vrata maternice dovelo je do razvoja testova zasnovanih na detekciji DNA HPV-a te profilaktičkog cjepiva protiv dvaju najučestalijih visokorizičnih tipova, HPV 16 i 18. Usprkos primjeni cjepiva, probir za rak vrata maternice ostaje glavni način prevencije i za žene koje su primile cjepivo i za one koje nisu cijepljene, ali je način probira i tretman žena kod kojih su nađene promjene epitela prilagođen novim tehnologijama. Iako je detekcija DNA HPV-a puno osjetljivija od citologije, njena specifičnost je manja, jer su mnoge infekcije HPV-om prolazne. Zbog toga se razmatraju druge metode koje bi poboljšale praćenje oboljelih žena. Tipizacija HPV-a i određivanje količine virusa za sad se koriste samo u istraživanjima i nemaju kliničku primjenu. Metoda detekcije transkripata virusnih onkogena E6 i E7, biljega produktivne infekcije, čini se obećavajućom za praćenje žena koje su pozitivne na prisustvo DNA HPV-a. Metoda koja se već koristi u kliničkom okruženju, premda nije standardizirana, je detekcija povećane ekspresije p16INK4a, kao indirektni pokazatelj ekspresije virusnih onkogena. Tom metodom se potvrđuje prisustvo promjena cervikalnog epitela u citološkim i histološkim uzorcima. Usprkos nedostatku standardizacije, metoda se pokazala korisnom kao dopuna klasičnom citološkom i histološkom pregledu uzoraka, ali je njena reproducibilnost kod žena sa promjenama epitela niskog stupnja i graničnim promjenama niska. Metode koje se razmatraju i možda će se koristiti u budućnosti uključuju i određivanje metilacijskog statusa nekoliko staničnih gena, iz čega bi se moglo predvidjeti kako će bolest napredovati, ali one su još uvijek u fazi istraživanja.