Minireview

Human Papillomavirus in Cervical Cancer Screening: Important Role as Biomarker

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Abstract

Cervical cytology screening has reduced cervical cancer morbidity and mortality but shows important shortcomings in terms of sensitivity and specificity. Infection with distinct types of human papillomavirus (HPV) is the primary etiologic factor in cervical carcinogenesis. This causal relationship has been exploited for the development of molecular technologies for viral detection to overcome limitations linked to cytologic cervical screening. HPV testing has been suggested for primary screening, triage of equivocal Pap smears or low-grade lesions and follow-up after treatment for cervical intraepithelial neoplasia. Determination of HPV genotype, viral load, integration status and RNA expression could further improve the effectiveness of HPV-based screening and triage strategies. The prospect of prophylactic HPV vaccination stresses the importance of modification of the current cytology-based screening approach. (Cancer Epidemiol Biomarkers Prev 2008;17(4):810–7)

Cervical Cancer Screening

Cervical cancer is the second most common malignancy and cause of cancer-related death in women worldwide (1, 2). Invasive cervical carcinoma is preceded by precursor lesions, which are characterized by disturbances of cellular maturation, stratification, and nuclear atypia (3, 4) and can be classified histologically as cervical intraepithelial neoplasia (CIN) or cytologically as squamous intraepithelial lesions according to the Bethesda terminology (5, 6). Because of this well-defined premalignant phase, cervical cancer is particularly amenable to screening (6). The classic screening method is based on the cytologic evaluation of Papanicolaou (Pap)−stained cervical smears, in which cervical cells are scraped from the transformation zone and transferred to a glass slide (7, 8). During the last 50 years, screening programs based on Papcytology have undoubtedly reduced cervical cancer morbidity and mortality (4, 6, 9-11). In spite of its success, the Paptest is a subjective method with a limited sensitivity of 50% and high susceptibility to intraindividual and interindividual variability (6, 11, 12). Introduction of liquid-based cytology (LBC) has contributed to mitigating the problem of efficiency in processing

samples, but the diagnostic validity in terms of sensitivity and specificity still shows important shortcomings (11, 13-15). Despite the substantial resources spent in cytologic screening and follow-up, cervical cancer still is the 10th most common cause of cancer death in European women (16). Because cervical cancer is the only cancer that is almost completely preventable through regular screening and thus early treatment, improvement and expansion of existing screening strategies and technologies constitutes a main target of the European Council Recommendation on Cancer Screening (17).

Human Papillomavirus in Cervical Carcinogenesis

Over the last decade, astonishing progress has been made in understanding the pathogenesis of cervical cancer. An overwhelming body of evidence shows that infection with distinct types of the human papillomavirus (HPV) is the primary risk factor for the development of cervical cancer and its precursor lesions (2, 17-19). HPVs are small circular double-stranded DNA viruses that belong to the *Papovaviridae* family. The HPV genome is ~8,000 bp in length and encodes eight open reading frames, which are transcribed as polycistronic mRNAs (20). The gene products can be divided into ''early'' (E) and ''late'' (L) proteins, depending on the time of expression during the viral life cycle (Fig. 1; ref. 21). The critical molecules in viral replication are E6 and E7, which functionally inactivate the products of two important tumor suppressor genes, p53 and pRb, respectively. Both oncoproteins induce proliferation, immortalization, and malignant transformation of the infected cells (21, 22).

Of the >100 different HPV types, 40 are known to infect the genital tract (2). These mucosal types are

classified as ''low-risk'' and ''high-risk'' based on their prevalence in cervical cancer and its precursors. Low-risk HPV types, such as 6 and 11, induce benign lesions with minimum risk of progression to malignancy. By contrast, high-risk HPVs (HR-HPV) have higher oncogenic potential (21). The tremendous importance of HPV in cervical carcinogenesis has opened up possibilities for cancer prevention.

Applications of HPV Testing

The establishment of HPV as central and necessary cause of cervical cancer was exploited for the development of molecular technologies for viral detection (19, 23) to overcome limitations linked to cytologic cervical screening. HPV DNA testing identifies women at risk for developing cervical neoplasia without the inherent subjectivity of cervical cytologic assessment (11, 24).

Therefore, HPV testing has been suggested for primary screening (16, 25, 26), triage of equivocal Pap smears or low-grade lesions (25, 27, 28), and follow-up after treatment for CIN (25, 29-31).

Primary Screening. Recent independent studies indicate that HPV testing, as a primary screening method, has a higher sensitivity (25-35% higher) and a higher negative predictive value for the detection of preinvasive disease than cytology (11, 32-34). Therefore, in the United States, it was recently concluded to add HPV testing to cytology screening after the age of 30 at an interval of 3 years if both tests are negative (11, 25).

An important drawback of HPV screening, compared with cytologic screening, is its lower specificity (5-10% lower) and low positive predictive value for high-grade CIN due to the high prevalence of transient infections (15, 25). HPV acquisition peaks near the late teens or early 20s and these HPV infections and associated mild lesions almost always clear spontaneously, rendering HPV screening at young age less efficient. Therefore, age plays a tremendous role in the determination of the target population (25, 35).

To date, the applicability of HPV as sole primary screening modality has only been evaluated in cross-sectional comparisons or epidemiologic studies. Large trials comparing this approach to cytology alone are needed to assess the effect of primary HPV screening on
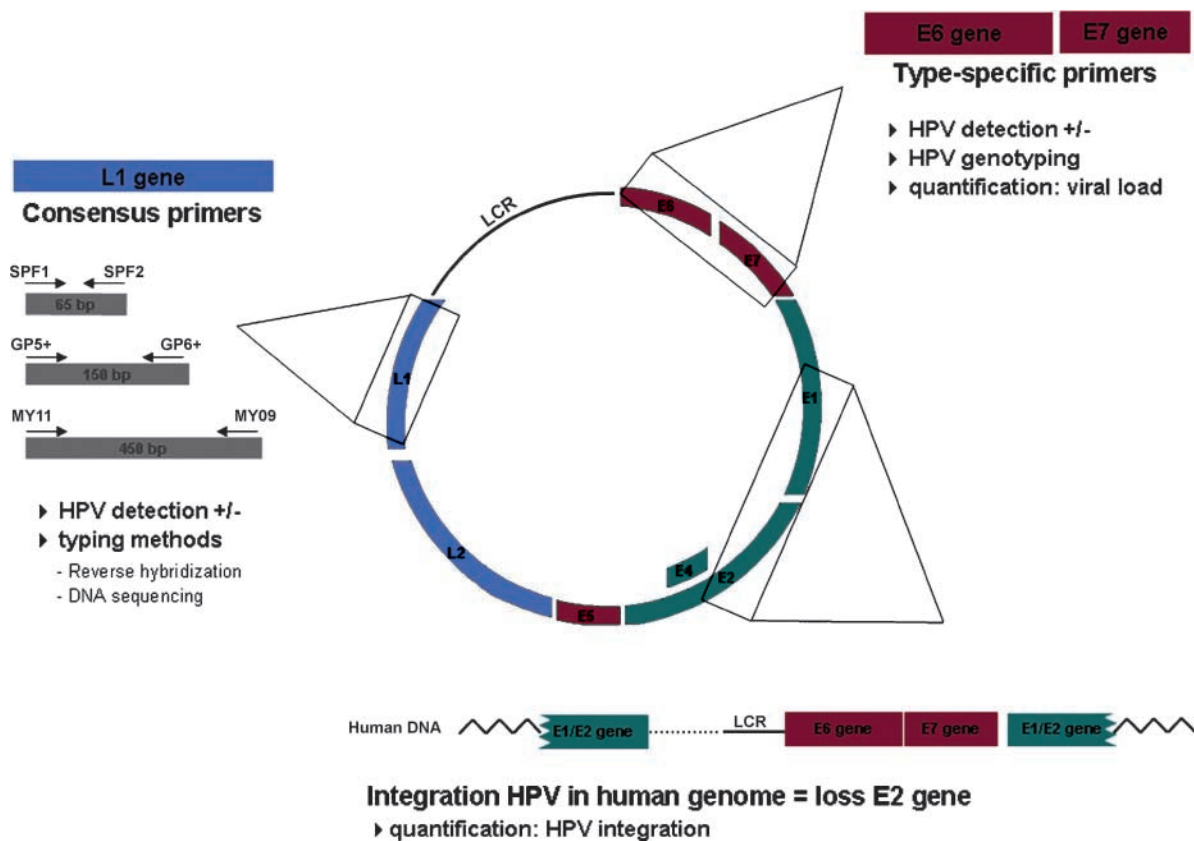
Figure 1. Schematic representation of the HPV genome, highlighting the regions important in PCR-based HPV analysis. L1 region, consensus PCRs, such as SPF, GP5+/6+, and MY09/11, target different conserved regions of the HPV L1 gene, amplifying several genital HPV types in one reaction. Amplicons can be analyzed to discriminate between HPV types by hybridization with type-specific probes or DNA sequencing. E6/E7 region, type-specific PCR assays target specific sequences of viral early genes, usually E6 and E7, and exclusively amplify a single HPV genotype. Application of HPV type-specific PCRs allows immediate discrimination between different HPV types. Quantitative PCR allows simultaneous assessment of HPV presence, genotype, and viral load. E1/E2 region, quantification of the E2 gene allows determination of the HPV integration status.

cancer incidence and mortality (35). In Europe, several randomized controlled trials are being conducted to establish the performance of HPV testing as a primary cervical cancer screening test. The main postulated outcome of these trials is a reduction in the cumulative incidence of high-grade CIN 3 to 5 years after screening among baseline HPV-negative compared with baseline cytology-negative women. Until the results are published in 2008, the Pap smear continues to be the standard screen test in the European Union (11, 16, 25). The approach of using HPV as the sole primary screening modality has several advantages: HPV assays provide an automated, objective, and highly sensitive test; the need for cytology would be reduced, improving its quality; unnecessary triage of equivocal and low-grade lesions would be avoided; and the screening interval could be safely prolonged, improving cost-efficiency and convenience of screening (35).

Triage of Equivocal Pap smears. Low-grade squamous intraepithelial lesion (LSIL) and atypical squamous cells of undetermined significance (ASCUS) represent the largest fraction of abnormalities in cervical cancer screening and comprise the most histologically confirmed high-grade abnormalities. HPV testing was proposed to achieve cost-effective management of these diagnostic categories (36, 37). The ASCUS-LSIL Triage Study has investigated in a prospective, randomized fashion the optimal management of LSIL and ASCUS by three strategies: immediate colposcopy, HPV triage, and repeated cytology. HPV triage seemed to be at least as sensitive as immediate colposcopy in the detection of high-grade CIN, whereas the number of women referred for colposcopy was halved (38). Therefore, HPV triage emerges as the best strategy for management of women with ASCUS. As other studies strengthened the effectiveness of this approach (28), the use of HPV detection in triage of ASCUS has been introduced into many international guidelines.

On the other hand, because cytologic interpretation of LSIL is fairly reproducible and the majority of LSIL cases (>80%) are HPV positive, the use of HPV testing for the management of LSIL is not cost-effective, as the majority of women would still be referred for colposcopy (27). As yet, it is still unclear whether determination of HPV genotype or viral load would be useful in triage of LSIL.

Follow-up after Treatment. Women treated for CIN should be followed up regularly to monitor their outcome. HPV testing was suggested to predict residual or recurrent CIN in women treated for high-grade cervical lesions. Current data show that HPV testing indicates residual disease more quickly, with higher sensitivity and similar specificity than follow-up cytology or histological assessment of section margins. A negative HPV test allows shortening of the posttreatment surveillance period, but more long-term data are necessary to present detailed evidence-based follow-up algorithms (29-31).

HPV Analysis: Possibilities and Methods

HPV Detection. The two methodologies most widely used for HPV DNA detection are PCR and the Hybrid Capture II system (HC2, Digene Corp.). HC2 is a nucleic acid hybridization assay for the qualitative detection of DNA of 13 carcinogenic (probe A) and 5 benign HPV types (probe B) in cervical specimens. It is the only HPV test which has been Food and Drug Administration (FDA)−approved for ASCUS triage and cervical cancer screening in combination with cytology after the age of 30 and is used in clinical settings worldwide as a robust and reproducible screening assay (14, 39). However, this test shows a number of disadvantages, such as the inability to identify specific HPV genotypes and the risk of cross-hybridization of additional HPV types with the probe mix (40, 41). Moreover, its applicability in routine screening is hampered by cost implications, clinical effectiveness of repeated testing in follow-up, and its lower sensitivity compared with PCR (12, 23).

There are two relevant approaches for detection of HPV DNA by PCR: consensus PCR and type-specific PCR. The most widely used PCR assays use consensus primers, such as GP5+/6+, MY09/11, and SPF, which target a highly conserved region of the HPV L1 gene, amplifying numerous genital HPV types in one reaction (Fig. 1; refs. 42, 43). The GP5+/6+ PCR set consists of two primers that detect a broad range of HPVs at lowered annealing temperature (23). MY09/11 PCR, on the other hand, is synthesized with several degenerate nucleotides in each primer, generating a mixture of 25 primers that are capable of amplifying a wide spectrum of HPV types (44, 45). Although the MY09/11 and GP5+/6+ systems yield a nearly identical prevalence of HPV in a set of clinical samples, the MY09/11 primers detect twice as many multiple infections (46).

PGMY09/11 primers were designed to improve MY09/11 sensitivity across the type spectrum with increased detection of multiple infections and improved reproducibility and specificity (42, 47). Surprisingly, one report shows similar analytic sensitivities for MY09/11 and PGMY09/11, with better detection of several important HPV types, including HPV16, by MY09/11. As such, caution should be exerted and reproducibility should be monitored when comparing performances of different primer systems (48).

SPF primers are technically analogue to PGMY09/11, as they also include inosine, which matches with any nucleotide and allows PCR at optimum annealing temperature, leading to higher HPV detection rates than those of MY09/11 (49, 50).

Because type-specific PCRs often target specific sequences of viral early genes and exclusively amplify a single HPV genotype, multiple PCRs must be done to detect the presence of HPV DNA in one sample (Fig. 1; ref. 23). Recently, a comparison between MY09/11 consensus PCR and type-specific PCRs showed that consensus PCR frequently missed clinically important HPV infections. The MY09/11 false negativity could be the result of poor sensitivity, mismatch of MY09/11 primers or disruption of L1 target by HPV integration, or DNA degradation. Furthermore, MY09/11 PCR lacked specificity for oncogenic HPVs. When type-specific PCRs are combined with fluorescent probes for real-time detection, multiplexing of several primers allows high-throughput, type-specific HPV detection with excellent cost-effectiveness and turnaround times (51).

A commercial PCR-based assay for HPV detection in cervical scrape specimens is the Roche Amplicor HPV

test, which involves a pool of primers amplifying the same 13 HR-HPV types as included in HC2 assay (52, 53).

HPV Typing. HPV types differ in their ability to induce cervical carcinogenesis. HPV16 and HPV18 are the most prevalent types in invasive cervical cancer (54). Therefore, HPV genotyping approaches could be more appropriate for the identification of individuals at risk of disease than a presence/absence test (55).

After consensus PCR amplification, HPV types can be discriminated by reverse hybridization with type-specific probes, but also DNA sequencing can be done (Fig. 1). However, the latter technique is inappropriate for genotyping of multiple infections, undermining its clinical applicability (56). The most frequently used reverse hybridization technology is the line probe or line blot assay, comprising multiple probes immobilized as parallel lines on a membrane strip. The commercial Roche Linear Array HPV genotyping test, which was developed based on the PGMY09/11 PCR in combination with a line blot assay, has become a convenient tool in epidemiologic studies for the detection and typing of HPV DNA (57).

SPF PCR served to develop the commercial INNO-LiPA HPV assay, which is capable of genotyping 25 different HPV types simultaneously and has proved to be sensitive, specific, simple, and rapid in the assessment of HPV (52, 58).

Although these assays are capable of typing a relatively large spectrum of HPV genotypes, they cannot be automated or deployed in a high-throughput platform. Therefore, improved genotyping methods have recently been developed, such as the Luminex xMAP system in which microspheres coated with HPV type-specific probes provide a rapid and cost-effective method to simultaneously detect 26 different HPV genotypes (59).

Also the application of HPV type-specific PCRs allows immediate discrimination between different HPV types in a high-throughput clinical setting (23, 43).

HPV Viral Load. The amount of HR-HPV DNA in a cervical sample, i.e., the viral load, has been suggested as a variable to distinguish HPV infections of clinical relevance. A high HPV load could be considered as a type-dependent risk marker for high-grade cervical lesions or carcinoma (60-63). However, the initial optimism regarding its clinical value was tempered by inconsistencies in the association between viral load and duration of infection, HPV clearance, and subsequent risk of acquisition or progression of disease (2).

The amount of HPV DNA can be determined by quantitative real-time PCR, and a multiplex format allows simultaneous assessment of HPV presence, genotype, and viral load (Fig. 1; refs. 51, 64, 65). Most studies using quantitative HPV PCR methods show a substantial overlap of viral load values among women with and without high-grade CIN, especially in the range of high viral loads. This approach precludes cutoff values for high-grade CIN on the basis of high viral loads, as such limiting the clinical applicability of viral load analysis (35). A recent publication concludes that high viral load is associated with prevalent cervical cancer precursors for most HR-HPV genotypes, but only HPV16 load predicts the development of incident disease (66).

HPV Integration. In cervical cells, HPV can occur in episomal form, integrated form, or both. Viral integration in the human genome often happens in the viral E1 or E2 region and can result in the loss of negative feedback control of oncogene expression by the regulatory E2 protein. Moreover, HPV integration increases the stability of integrant-derived E6 and E7 transcripts (67).

The physical state of the virus can be determined by the failure to amplify full-length E2 using PCR, but also by using more comprehensive Southern blot hybridization. Real-time PCR assays, which simultaneously measure E2 and E6 copy numbers, have recently been developed to determine the integration status (Fig. 1). However, unavoidable technical limitations related to the abundance of episomal forms, low viral load, or the length of the E2 amplicon should be considered when interpreting integration studies (2, 68). For now, it remains largely unclear whether the measurement of HPV integration status could be a useful biomarker for progressive disease. Several studies suggest that identification of integrated viral forms could support HPV-based screening and triage strategies (2, 69-75).

HPV RNA Detection. In cervical carcinogenesis, expression of viral oncogenes is a prerequisite for progression toward malignancy and maintenance of the cancerous phenotype, with E6 and E7 as the main arbitrators (21, 22). Therefore, detection of RNA transcripts of genes involved in oncogenesis enables differentiation between asymptomatic HPV infections and infections associated with high-risk lesions and cervical carcinoma and could therefore be considered as a better risk factor than mere DNA detection (12, 35, 55, 76). RNA as a potential target for routine clinical diagnostics may improve sensitivity, reproducibility, and specificity compared with DNA. As fixation of cells can interfere with RNA quality, it would be favorable that fixatives used for routine collection and processing of cervical specimens maintain RNA integrity (77, 78).

Currently, one RNA-based HPV assay is commercially available, the PreTect HPV Proofer (Norchip AS; ref. 79). This assay incorporates nucleic acid sequence-based amplification (NASBA) of E6/E7 mRNA transcripts before type-specific detection for HPV16, HPV18, HPV31, HPV33, and HPV45 (ref. 23; Fig. 2). It has been suggested for triaging HPV DNA-positive women and women with equivocal cytology (73, 80, 81). Compared with HPV DNA detection, the presence of E6/E7 mRNA transcripts was less sensitive, but more specific for the detection of disease and follow-up (55). Application of NASBA technology is not restricted to the use of the PreTect HPV proofer kit. In-house primers and molecular beacons can be developed to detect HPV types of interest. As NASBA is a sensitive and fast technique with commercially available basic reagents, it is well suitable for application in a routine screening setting. In principle, RNA detection can be applied as a primary screening test, but this has never been evaluated. Sensitivity is limited when only five HR-HPV types are detected. A broad spectrum mRNA test (15 types), Aptima (GenProbe), is currently under development (35). Preliminary evaluation of the prototype assay showed that HR-HPV E6/E7 mRNA detection, compared with HR-HPV DNA detection, improved the association of positive test results with cervical precancer

and cancer by reducing the number of tests positive in women without precancer without reducing clinical sensitivity for cervical precancer and cancer (82).

High RNA quality is required for the application of real-time reverse transcriptase PCR to evaluate E6/E7 mRNA expression levels (83). The biological importance of the extent of oncogene expression in cervical carcinogenesis suggests that quantification of E6 and E7 transcription may be useful as a prognostic tool to identify women at increased risk of developing cervical cancer.

Exploitation of HPV Consequences

HPV infection and its consequences are associated with changes in expression levels and/or function of host genes (6). Improved understanding of the molecular pathways of cervical carcinogenesis led to the discovery of clinically useful biomarkers. This translational research approach identified markers that reflect deregulation of the cell cycle in cervical neoplasia, such as p16, Ki-67, MCM proteins, and cyclin E (6, 84, 85). However, many of these biomarkers are only indicative of the presence of aberrant S-phase induction and lack specificity for cervical malignancy (6). Alternative candidate markers can emerge from an approach focused on proteomic analysis of cervical cancer samples. Proteomics is widely accepted as a powerful tool in the development of molecular diagnosis and the identification of disease biomarkers in the postgenomic era (86-89). Proteomic profiling of altered proteins may provide new
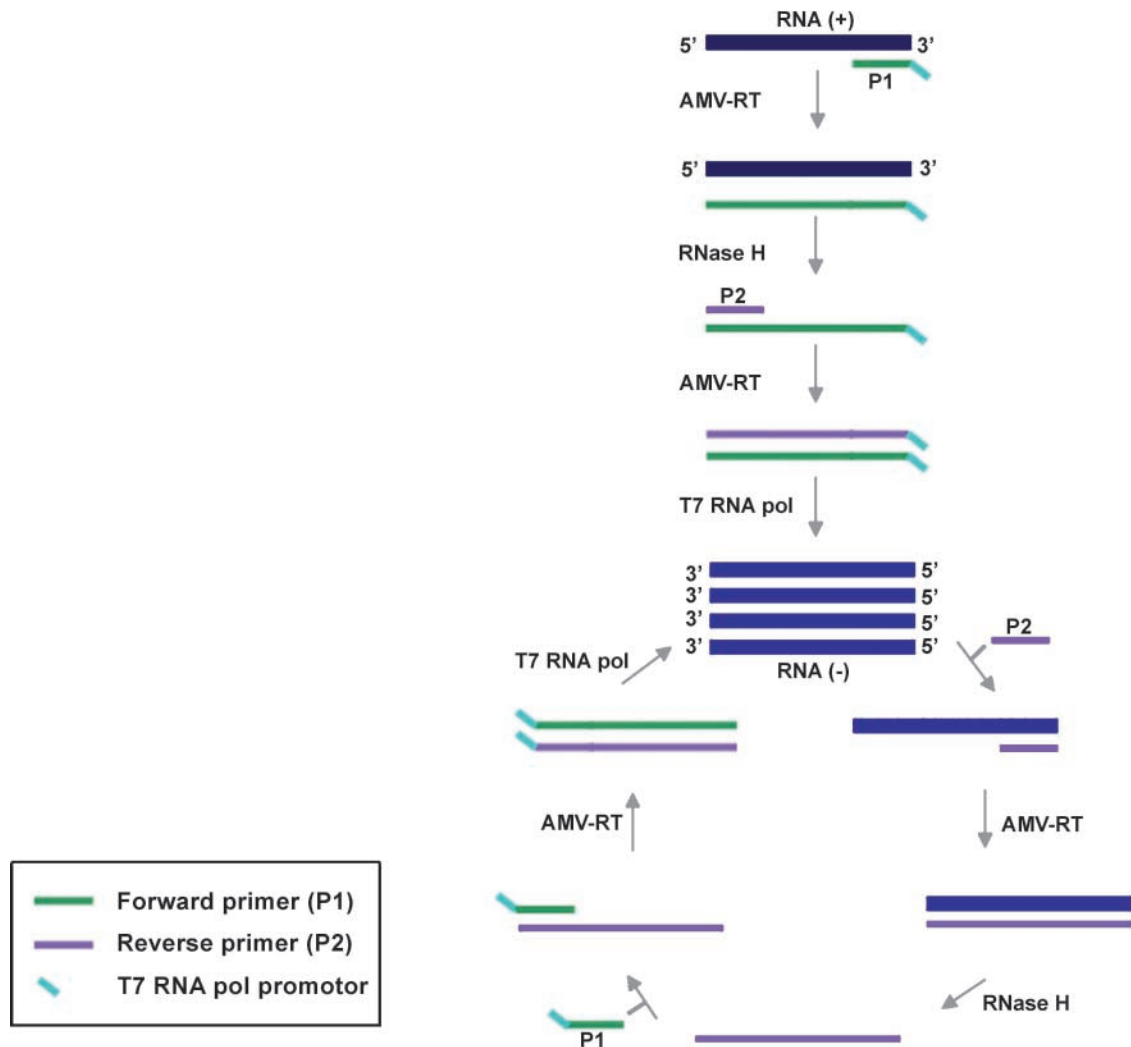
Figure 2. NASBA amplification reaction. NASBA is a sensitive transcription-based amplification method that specifically targets RNA and has been applied for the detection of viral genomes, viroids, rRNAs, and mRNAs. The technology relies on three enzymes: avian myeloblastosis virus reverse transcriptase (*AMV-RT*), RNase H, and T7 RNA polymerase, which uses the T7 promotor at the 5′ end of the forward primer. In NASBA, only nucleic acids which are single-stranded in the primer binding regions function as a template. Because the reaction is isothermal (41°C), specific amplification of single-stranded RNA in the presence of double-stranded DNA is possible, as long as DNA denaturation is prevented in the sample preparation procedure. This makes the method useful for specific mRNA detection in a background of genomic DNA, even for intronless genes. The resulting single-stranded RNA amplicons can be easily detected by hybridization with sequence-specific probes, such as molecular beacons.

insights into cervical carcinogenesis and yield new biomarkers, serving as molecular signposts to detect early cancer and people at risk for developing disease (90).

HPV Vaccination: Implications for Screening

The strong relationship between HPV and cervical cancer has also opened up the possibility of primary prevention by the development of prophylactic vaccines against HR-HPV infections. HPV vaccines are based on virus-like particles (VLP) assembled from recombinant HPV capsid proteins L1 and L2 (91). To date, two effective prophylactic VLP L1 vaccines, which are capable of inducing virion neutralizing antibodies, have been developed (37, 92-98). Cervarix is a bivalent HPV16/18 vaccine developed by GlaxoSmithKline (GlaxoSmithKline Biologicals), and the FDA−approved vaccine Gardasil is a quadrivalent HPV16/18/6/11 vaccine developed by Merck (Merck and Co. Inc.). These four HPV types cause the vast majority of anogenital disease (99, 100). HPV16 and HPV18 account for 62% to 77% of all cervical cancers, depending on the geographic region (54). HPV6 and HPV11 infections are responsible for over 90% of the low-risk HPV-associated disease (100). Ongoing clinical trials are currently investigating the long-term efficacy of both bivalent and quadrivalent vaccines (92).

The establishment of vaccination programs and reasonable levels of coverage do not imply that cervical screening programs can be discontinued (15, 92, 101). One reason is that the primary target population consists of 9-year-old to 13-year-old females and the ''catch-up'' vaccination of older women will likely be achieved at much lower coverage rates. Moreover, vaccination will not protect against all oncogenic HPV types, although some cross-protection against other HR-HPV types will be achieved by vaccinating against HPV16 and HPV18. Inevitably, screening and prevention strategies should be adapted to one another. Cervical cytology screening programs will require modification to attain cost-effective cervical cancer control and surveillance of vaccinated populations. They could assume a new role in monitoring the long-term effectiveness of vaccination and the changes in the natural history of HPV malignancy (11, 15, 102).

HPV-Based Approaches for Cancer Prevention in Developing Countries

Cervical cancer shows the greatest burden in developing countries, which suffer with considerable barriers to set up cytology-based screening programs (35, 103). After all, high-quality cytology requires trained personnel and specialized equipment, and Pap-based algorithms entail multiple visits. Although HPV vaccines may eventually provide the best possible solution for prevention of cervical cancer in these countries, innovative HPV-based approaches might enable the establishment of a feasible and effective screening policy. However, HPV testing mostly requires high-technology laboratory-based molecular analyses, involving high costs. Currently, several initiatives aim at the development of rapid, simple, accurate and affordable HPV tests. One test, based on Digene's HC2, allows testing for oncogenic HPVs in 96 samples in <2 h. Another assay by Arbor Vita Corpora-

tion targets HR-HPV E6 and yields results in 20 min. Because these tests offer rapid results, women could ultimately be screened and treated during the same visit. Because the need for a good sample is less imperative for HPV testing than for cytology, the possibility of self-sampling has been explored. Optimistic results concerning the diagnostic accuracy of self-collected vaginal specimens and cost effectiveness indicate that this strategy might be valuable for the improvement of population coverage (35).

Conclusion

The organization of an effective public health prevention program is complex. The scientific knowledge about cervical cancer and HPV, which has accumulated over the last decades, has opened the possibility to improve existing prevention strategies and screening practices. HPV testing could reduce incidence and mortality from cervical cancer. Sensitive molecular testing techniques can bypass the limitations of cervical cytology screening and can offer women greater protection against cervical cancer at lower cost (11). Ongoing randomized control trials of primary HPV screening will yield the degree of evidence necessary for public health policymakers to make informed decisions about the future of cervical cancer screening programs (15). Especially in an era of HPV vaccination, it makes public health sense to develop a screening system based on HPV monitoring. One of the most neglected aspects of the potential effect of prophylactic HPV vaccines is the evaluation of existing screening practices to permit synergy between primary and secondary prevention efforts (104). Therefore, the biggest issue that should be debated upon the screening community is not whether to incorporate HPV testing into screening programs, but how to incorporate it (11, 14).

In our opinion, HPV detection, with a higher sensitivity and negative predictive value for the detection of preinvasive disease than cytology, is without a doubt the preferred primary test in a routine screening setting. The more specific test, cytologic reflex testing, should be done to triage HPV-positive samples and guide the clinical response. Both tests can be done on the same LBC sample. In the early phase of adopting HPV primary screening, both HPV detection and cytology could be done simultaneously, mainly to increase the confidence of cytopathologists/cytotechnologists in HPV screening. Clinicians should properly educate their patients about the effect of their HPV positivity, an aspect which is overlooked when commercial interests of vaccine-producing companies seem to surpass the importance of patients' tranquillity of mind.

Beyond the appropriate introduction of HPV testing, an improvement of population health can be expected from an increased coverage of the target population and quality control of the different steps in the screening process. In Belgium, the 3-year Pap screening coverage in women 25 to 64 years old currently amounts to only 59%, whereas many of the women are overscreened (105). These data underline the importance of implementing cancer screening programs with a call/recall system, appropriate quality control at all levels, with effective diagnostic, treatment, and after-care service following evidence-based guidelines.

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