

Rapid Communication

Clinical utility of HPV genotyping

Chris J. Meijer^a, Peter J. Snijders^a, Philip E. Castle^{b,*}

^a Department of Pathology, Unit of Molecular Pathology, Vrije Universiteit Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

^b Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD, USA

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Introduction and HPV testing

Cervical cancer screening is presently done by finding abnormal cells in cervical smears (i.e., cervical cytology or Pap smears). However, cervical cytology is not full-proof. Cervical cytology is insensitive for the detection of cancer and precancer [1], requiring many rounds of screening to achieve programmatic effectiveness.

It is now recognized that virtually all cervical cancers, both of the squamous and of the adenocarcinoma histologic types, are causally related to cervical infections by 14 oncogenic human papillomavirus (HPV) genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) [2,3]. With the advent of methods that detect nearly all oncogenic HPV types in aggregate, the question has risen whether testing for oncogenic HPV can improve more reliably the detection of cervical carcinomas and their precursors (cervical intraepithelial neoplasia grade 2 [CIN2] and 3 [CIN3]). There is now compelling evidence that testing for oncogenic HPV is more sensitive and has a higher negative predictive value (NPV) for CIN2 or worse (CIN2+) compared to cervical cytology [4–6] at the cost of a small decrease in specificity and positive predictive value compared to cytology. HPV testing also implies detecting an endpoint in cervical carcinogenesis that is earlier than the development of cervical abnormalities; this translates into a longer safety margin in following up women who are HPV-negative. The sensitivity and NPV of HPV testing are so high that few cancers and CIN2+ lesions are missed. Therefore, a negative test result provides years of reassurance. Because HPV infections are so common in young women after sexual debut and precancerous lesions so rare, the use of HPV DNA testing should be delayed until 10–15 years after the average age of sexual debut in any given population. For example, in the U.S., HPV testing as an adjunct to cytology in primary screening is

not recommended until age 30 and older [7]. Importantly, in the absence of CIN2+ lesions, the older a woman is who tests positive for oncogenic HPV, the increasing likelihood she will have a persistent infection [8], which strongly predicts progression to CIN2+.

Many large randomized controlled trials are in progress to determine the most efficient and cost-effective algorithm for detection of CIN2+ in primary screening [9,10]. In a couple of years, these trials will conclude and the likely conclusion is that HPV testing will be shown to be a superior primary screening test to cytology.

The evidence is already compelling that testing for oncogenic HPV is the most sensitive and cost-effective in the follow-up of women treated for CIN2+ [11] and in the triage of women with equivocal cytology (atypical squamous cells [ASC] in the U.S. and borderline or mild dyskaryosis [BMD] in Europe) [12–14]. It has been established that testing for oncogenic HPV should be used in the monitoring of women treated for CIN3 either alone or in combination with cytology since HPV testing is not only more sensitive for CIN lesions but is less influenced by the tissue repair reaction, which often yields problems in cytological evaluation of cervical smears in the early months after treatment. Moreover, there is a 5–10% risk of disease recurrence, which is a much higher incidence of disease than in the general population, and therefore the most sensitive test should be employed during follow-up. A negative oncogenic HPV test implies immediate reassurance, and women can be returned to routine screening. Triage of women with ASC or BMD for colposcopic evaluation of the cervix has been shown to reduce the number of follow-up smears without the cost of an increase in colposcopic referrals and is more cost-effective than evaluation of these women by cytological follow-up at 6 and 18 months.

A remark of caution should be made about the sensitivity of the oncogenic HPV test. For clinical purposes, it is important not to use the most sensitive oncogenic HPV tests. Based on the data collected from several studies, we recently proposed

* Corresponding author. Fax: +1 3014020916.

E-mail address: castlep@mail.nih.gov (P.E. Castle).

that the amount of oncogenic HPV DNA in a cervical scrape might be an important parameter to distinguish oncogenic HPV infections that are clinically irrelevant [15]. That viral load is potentially an important clinical determinant can already be concluded from studies involving different testing methods for oncogenic HPV displaying different analytical sensitivities for detecting oncogenic HPV DNA. For example, we recently compared the clinical performance of the GP5+/6+ PCR and SPF10/LIPA assays in a nested case–control study of women with normal cytology participating in a population-based screening program [16]. Among case women (i.e., women who developed lesions CIN3+ within a median follow-up time of 2.2 years), GP5+/6+ PCR and SPF10/LIPA were 88% positive (sensitivity) and did not differ significantly. By comparison, among control women (i.e., women with lesions \leq CIN1 diagnosed within a median follow-up time of 7.0 years), GP5+/6+ PCR was much less likely to test positive (7% vs. 32%) than SPF10/LIPA. As a consequence, the specificity and positive predictive value of the GP5+/6+ PCR for CIN3+ were markedly higher than of the SPF10/LIPA assay (93% versus 68% and 67% versus 29%, respectively). The clinical consequences of such very analytically sensitive tests are that many women testing positive will be unnecessarily sent for colposcopic evaluation. Thus, an ultra-analytically sensitive test does not imply better clinical utility because it will yield poorer specificity. The clinical performance of oncogenic HPV tests for CIN3+ should therefore be verified in controlled trials before their use in clinical settings to avoid too many follow-up visits.

Although HPV DNA testing may ultimately be shown to be a more accurate screening test than cytology, and therefore supplant it as the primary screening test, its lower specificity will result in an increase in referral of women for unnecessary follow-up and potentially treatment and therefore added costs. While the added costs are more than offset by the lengthening of screening intervals for HPV-negative women, the inconvenience of unneeded follow-up and the potential for unnecessary treatment, which has important reproductive negative consequences [17], cannot be ignored. To increase specificity while maintaining the sensitivity of oncogenic HPV DNA testing, two approaches might be considered. First, cytology could be used as the secondary test [5]. Second, and the focus of this review, is to use HPV genotyping.

What types of HPV should be part of any test?

Presently 14 types have been characterized as oncogenic and 3 (i.e., HPV 26, 73, and 82) have been characterized as possibly oncogenic [2,3]. The prevalence of HPV is dependent of the geographical region, but HPV 16 is by far the most prevalent and oncogenic HPV type [18]. Typically HPV18, 45, 31 and 33 are the next most prevalent types but the order varies between geographical areas. In other areas, such as Asia, HPV58 and HPV52 are the next most common after HPV16 and HPV18 [19]. Which types should be included in a pooled test for oncogenic HPV and the added value of including rare (probably) oncogenic types in the oncogenic

HPV test is a matter of debate. Of note, oncogenic HPV tests that have been clinically validated in large population-based studies (i.e., Hybrid Capture 2 and perhaps GP5+/6+ PCR-EIA, which detect 13 and 14 oncogenic HPV types, respectively, in aggregate) have shown good clinical sensitivity and specificity for CIN2+ in population-based screening studies. Adding extra (probably) oncogenic HPV types to these assays with a very low prevalence in cervical cancer but with a high prevalence in women without disease may negatively influence the specificity of oncogenic HPV test for CIN3+ significantly [20]. As a result, excessive numbers of women would be referred to colposcopy without significant increases in detection of CIN3+. Thus, decreasing the number of oncogenic HPV types in these tests does not seem an option but increasing the number of oncogenic HPV types with other oncogenic HPV types will result in only incremental increases in clinical sensitivity with concomitant larger decreases in clinical specificity.

Type-specific HPV testing

Several studies have now shown that women with an HPV16 infection have a significantly greater risk for developing CIN3+ compared to other oncogenic types [21,22]. Likewise, HPV18-positive women with normal cytology have an increased risk not only for CIN3+ [22] but also for adenocarcinoma and its precursor lesions [23,24] compared to other oncogenic types. Comparing the prevalence of oncogenic HPV types in adenocarcinomas and squamous cell carcinomas and their precursors with that of women with normal cytology in one study, HPV16 has a preferential risk for both SCC and adenocarcinoma whereas HPV18 has a preferential risk for adenocarcinoma [23]. Large, rigorous case-series consistently find HPV16 the most prevalent and HPV18 the second most prevalent genotypes in SCC [2]; HPV18 the most prevalent and HPV16 the second most prevalent genotypes in adenocarcinomas [24], which are more often missed by cytology screening than SCC.

In toto, there is compelling evidence that HPV16 and HPV18 are the most oncogenic types and might warrant separate detection performed either as a triage for an oncogenic HPV-positive test or concurrently with the pooled oncogenic HPV test. The question is whether there are other types that warrant separate detection. For any risk marker or biomarker, the strength of association as measured by odds ratios (and relative risks in cohort studies) must be ~ 25 or greater to have clinical utility [25]. Intuitively, the criteria for choosing any type for separate detection should be both high positive predictive value (PPV) (absolute risk) and etiologic fraction (i.e., the attributable proportion of cervical lesions assigned to a given genotype as cause). Low PPV or etiologic fraction for any type would make testing for that type limited in its clinical utility or cost-ineffective, respectively. A third criterion must be the likelihood of any type to progress to cancer [26]. Since the ultimate goal is to prevent cancer, not detect CIN2+, those types that cause CIN2+ but are rarely associated with invasive lesions may not be good candidates for separate detection

although they might be included in the pooled test to maintain a highly sensitive screening test. Those types that cause CIN2 and CIN3 but never cause cancer should not be added to any test.

The types next in importance after HPV16 and HPV18 appear to vary regionally. One study found that, among women with normal cytology, HPV33-positive women were at an increased in women with CIN2+ [27]. Another study [28] found HPV58 to be the most risky type after HPV16 and HPV18. In other geographic locations, different types appear to be the next most important after HPV16 and HPV18 [19,29].

Because it seems unlikely that validated tests with adjunctive testing for specific genotypes will develop to meet regional needs, one solution is to genotype for all oncogenic HPV types. Currently, there is no FDA-approved test for HPV genotyping. In addition, there are practical considerations, as described below, which might limit the utility of full genotyping. Perhaps the most rational design for clinical utility is to include separate, validated HPV16 and HPV18 detection with the pooled oncogenic HPV test.

Clinical management based on genotyping

The goal of detecting an additional biomarker, whether the biomarker is type-specific detection of HPV genotypes or another biomarker, is to further stratify women according to risk of precancer and cancer (CIN3+). Intuitively, clinical decisions are then made according to the risk state; women at the greatest risk being managed the most aggressively while others less so. Thus, clinical resources are focused on the management of oncogenic women and lower-risk women receive less clinical attention, resulting in more cost efficiency and less over-treatment. One concern, particularly in the U.S., is that this stratification will lead to more aggressive management of the oncogenic women without concomitant less aggressive management of women in lower risk strata. In this scenario, there would be little or no added benefit to women while increasing costs and over-treatment that is now recognized to have reproductive consequences [17].

The question of risk stratification raises an important theoretical question: what is the minimum risk for CIN3+ that warrants colposcopic evaluation? Historically, prior to the advent of HPV triage and in clinical settings that have not adopted HPV triage, women with equivocal smears (atypical squamous cells of undetermined significance [ASCUS]) were sent to colposcopy at the discretion of the clinician in the U.S. In Europe, there was a greater tendency to wait for repeated non-normal cytology before referral to colposcopy. Current U.S. guidelines suggest the HPV triage of ASCUS and that oncogenic HPV-positive ASCUS, LSIL, or worse cytology warrants colposcopic evaluation. From ALTS, the 2-year risk of CIN3+ for this group of women was $\geq 15\%$. Among the HPV16-negative, oncogenic HPV-positive ASCUS, and HPV16-negative LSIL, the risk is 8–10%, similar to the risk of ASCUS unqualified by HPV triage.

Where co-testing with cytology and HPV exists in general screening, the clinical dilemma is appropriate management of

women who are oncogenic HPV-positive and cytologically negative. Several studies have now demonstrated that cytologically negative women who test HPV16 or HPV18-positive are at a greater risk of prevalent or incipient CIN3+ than women who have LSIL cytology. It is therefore rationale based on historical standards of care to send women to colposcopy for HPV16 or HPV18 positivity even if cytology is negative. By comparison, women who are oncogenic HPV-positive, HPV16- and HPV18-negative, and cytologically negative were at a relatively low risk of CIN3+. Thus, it seems plausible to retest women who are positive for the other oncogenic HPV types (i.e., HPV16- and HPV18-negative) and cytologically negative in a year or two, depending on the acceptable risk, to allow for transient infections by the weaker oncogenic HPV types to clear. Women would then be sent to colposcopy for evidence of type-specific oncogenic HPV persistence; in the absence of complete genotyping for the oncogenic HPV types, repeat oncogenic HPV-positive over 1–2 years may be a conservative surrogate for type-specific HPV persistence in cytologically negative, 30 years and older women. As screening programs begin to rely on testing for oncogenic HPV as the primary screening method, it seems likely that similar decision tree will be used if HPV genotyping is used either to triage a positive screening test for oncogenic HPV or as the primary screening test: women who test positive for the highest risk HPV types (HPV16 and 18) may warrant immediate colposcopy or more intensive follow-up (6–12 month follow-up) because of their elevated risk of CIN3+ whereas oncogenic HPV+ women who test negative for these types might be followed up less intensively (1–2 year follow-up) to reduce referral to colposcopy. Women in the latter group would be sent to colposcopy as the result of a repeat oncogenic HPV-positive test as evidence of oncogenic HPV persistence.

Guidelines for colposcopic evaluation must be balanced with the recognition that recent improvements in cervical cancer screening have not been matched by concomitant advances in colposcopic evaluation and diagnosis of women with abnormal screening results. Of concern, recent data have demonstrated that the sensitivity of colposcopically directed biopsy to detect underlying precancer is only $\sim 70\%$ [30]. Thus, despite its historical status as the diagnostic reference, colposcopically directed biopsy must now be considered the technically weak link in the overall cervical cancer prevention program. Taking more biopsies, based on appearances or using a systematic, 4-quadrant approach, may improve the performance of colposcopy. However, extensive validation of these approaches is lacking.

Limitations in colposcopy raise an important but controversial topic. If the reproducibility and sensitivity of HPV testing for detection of precancer/cancer prove to be superior to the cytology/colposcopy combination, are we willing to treat women surgically based on (virologic) risk status? In general, the specificity and positive predictive value of HPV testing are mediocre. But there are notable exceptions; for example, even in the absence of histologically confirmed precancer, cytologically abnormal women with prevalent HPV16 infection are at very

high absolute risk of having a missed, small precancerous lesion (i.e., cervical intraepithelial neoplasia grade 3). Long-term persistence for HPV16 also carries very high absolute risk of cervical precancer and cancer [31]. Cervical cancer prevention is achieved by removing the entire zone of cancer susceptibility, the cervical squamo-columnar transformation zone. In practice, clinicians do not treat individual precancerous lesions; they treat the transformation zone for the risk of cancer. It is conceivable that treatment might eventually be triggered by validated, virologic, or molecular markers of risk, even when the “lesion” is colposcopically invisible.

Advantages and disadvantages of full genotyping

While it stands to reason that some degree of genotyping can usefully guide the aggressiveness of clinical management, the exact form is uncertain. The potential utility of genotyping must be weighed against added cost to the patients, added complexity for clinical laboratories, and the increased burden for clinicians to interpret these additional data. The adoption of partial or full genotyping will depend on several critical factors: (1) test performance (clinical sensitivity and specificity; reliability) leading to FDA approval; (2) automation and high throughput for practical use in clinical laboratories; (3) algorithms for clinical interpretations of viral patterns; and (4) acceptance of a virologic (vs. histopathologic) model of cervical carcinogenesis by clinicians.

One advantage of genotyping is the ability to increase programmatic performance of screening using HPV by also identifying which HPV-positive women have persistent oncogenic HPV. Detection of short-term HPV persistence may increase programmatic specificity of screening based on HPV [32]. If full genotyping is not introduced into screening, one important question is how often do women who repeatedly test positive for oncogenic HPV (with or without separate detection of HPV16 and HPV18) have a persistent oncogenic HPV infection.

Genotyping tests

Currently, there are no genotyping tests that are FDA-approved. Thus, the data demonstrating the potential utility of genotyping, based on research assays, have outdistanced the availability of suitable commercial and validated tests. In its absence, clinical laboratories will be tempted to develop their own in-house assay based on DNA amplification (PCR) technologies. The use of these “home-brews” or analyte-specific reagents (ASRs) warrants caution and should be strongly discouraged because they typically lack sufficient validation of clinical performance and reliability. Assays must demonstrate clinical performance for detection of rigorous endpoints, such as CIN3+. Clinical performance does not simply translate to good sensitivity but must also include good positive predictive value. Thus, an ultra-analytically sensitive screening test will only result in over-referral without added gains in detection of CIN3+. Consequently, candidate typing assays should be compatible with current clinically validated

tests that detect oncogenic HPV in aggregate in terms of sensitivity and specificity. Second, the test must be reliable. This includes intra-batch, inter-batch, and inter-operator reproducibility and consistency in performance over time (e.g., sensitivity and PPV for CIN3+). For example, HC2 has shown good reliability both for test reproducibility by multiple testing laboratories [33,34] as well as consistent clinical performance for detection of CIN2+ throughout Europe and North America [6].

Careful validation is often expensive, but there are no short cuts. Simple correlation studies of one assay versus another validated assay using convenience specimens do not equate to clinical performance. The FDA licensure typically requires demonstrated performance for the detection of CIN2+. For a test that is predicted to be 90% sensitive for CIN2+, a sample size of 134 cases of CIN2+ is needed to achieve a 95% confidence interval of 85%–95%; in a high-risk population with a 2% risk of CIN2+, this translates to a population sample size of 6700. For reproducibility, a few repeated tests within a batch cannot determine if there is assay drift over time, which can only be assessed with careful quality control measures. Without validation and the precautions of quality control and assurance, genotyping has the capability of causing more harm than benefit.

To increase the efficiency of clinical validation, there may be utility in choosing clinical sites where cytology and HC2 testing are already being done routinely. Because of the high sensitivity of co-testing by cytology and HC2 for CIN2+, a stratified random sampling approach could be used to reduce the number of patients enrolled: all women positive for either cytology (\geq ASC) or HC2+ and random sample of the double negatives would be enrolled. Such an approach would reduce the testing in a population of women who are at very low risk of CIN2+, i.e., those who tested negative by cytology and HC2, while still allowing clinical specificity of a new test to be determined. This approach, if accepted by the FDA, may permit more companies to seek FDA approval rather than attempting to circumvent approval by using an ASR approach.

Vaccinated populations

HPV vaccines composed of self-assembled L1 virus-like particles (VLP) have shown great promise for the prevention of HPV16 and HPV18 infections [35,36]. Assuming that there will be widespread vaccination in high-resource populations and that vaccination will have sufficient duration to prevent most HPV16 and HPV18 infections, we can anticipate a direct impact on both oncogenic HPV and cytology screening tests. Specifically, it is plausible to expect that both tests will have reduced positive predictive value, but owing to intrinsic properties of cytology, the latter will suffer more because its sensitivity and specificity are likely to decrease in settings with very low lesion prevalence [37]. In these populations, it may be necessary to use more specific measures of risk such as oncogenic HPV persistence or eventually, if fully validated, more specific markers of transformation such as p16^{INK4a} [38] or oncogenic E6/E7 expression [39].

Developing countries

The clinical utility of HPV genotyping is to make current screening more programmatically efficient by better allocating resources according to risk. In developing countries, where more than 80% of cervical cancer incidence and mortality occur and where screening programs are either ineffective or non-existent, it is unclear whether HPV genotyping can be made available and would be useful. In regions where a once or twice in a lifetime screen-and-treatment approach is applied, maximizing sensitivity may be more important than stratifying women according to risk. Currently, a low-cost oncogenic HPV DNA test is under development via an initiative by PATH (People for Appropriate Technology in Health, Seattle, WA) and will soon be ready for evaluation. However, no similar HPV genotyping test is currently being developed. Thus, like the currently developed HPV16 and HPV18 L1 VLP vaccines, it is uncertain if or when HPV genotyping technology will be available to underserved populations.

Summary

In summary, cervical cancer screening is now entering a new era in which we will increasingly rely on measuring the causal viral infection, oncogenic HPV, rather than the pleomorphic cellular changes caused by the infection. As successive cohorts of women receive HPV vaccination, periodic screening with HPV tests will provide a useful means to monitor the duration of protection in the population. As we move from cytology-based screening to HPV-based screening, genotyping may prove useful in stratifying HPV+ women according to risk of prevalent or incipient precancer and cancer to determine the appropriate clinical management strategy. However, to achieve benefit to patients, the addition of HPV genotyping to cervical cancer screening must not be abused by excessive referrals to colposcopy and over-treatment, which can be exacerbated by the use of poorly validated tests.

Appendix A

The Working Group members participating in the preparation of this document are:

Raymond J. Apple, Roche Molecular Systems, Alameda, CA, USA; Philip E. Castle, National Cancer Institute, Bethesda, MD, USA; Gary M. Clifford, IARC/WHO, Lyon, France; Silvia Franceschi, IARC/WHO, Lyon, France; Eduardo L. Franco, McGill University, Montreal, Canada; Sue J. Goldie, Harvard University, Boston, MA, USA; Susanne Krüger-Kjær, Danish Cancer Society, Copenhagen, Denmark; Shalini Kulasingam, Duke University, Durham, NC, USA; James Linder, University of Nebraska Medical Center, Omaha, NE, USA; Chris J. Meijer, VU University Medical Center, Amsterdam, The Netherlands; Joseph Monsonego, Institut Alfred Fournier, Paris, France; Nubia Munoz, Instituto Nacional de Cancerologia, Bogota, Colombia.

Disclosure:

Dr. J. Linder is an employee of Cytyc Corporation (Marlborough, MA, USA);

Dr. R. Apple is an employee of Roche Molecular Systems (Alameda, CA, USA).

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