Human Papillomavirus and Cervical Cancer

Biomarkers for Improved Prevention Efforts

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Abstract and Introduction

Abstract

While organized screening programs in industrialized countries have significantly reduced cervical cancer incidence, cytology-based screening has several limitations. Equivocal or mildly abnormal Pap tests require costly retesting or diagnostic work-up by colposcopy and biopsy. In low-resource countries, it has been difficult to establish and sustain cytology-based programs. Advances in understanding human papillomavirus biology and the natural history of human papillomavirus-related precancers and cancers have led to the discovery of a range of novel biomarkers in the past decade. In this article, we will discuss the potential role of new biomarkers for primary screening, triage and diagnosis in high-resource countries and their promise for prevention efforts in resource constrained settings.

Cervical Cancer: Incidence & Burden

Invasive cervical cancer (ICC) is a significant cause of cancer-related morbidity and mortality among women worldwide, with substantial geographic variation.[1] Many industrialized countries have achieved significant successes in reducing ICC burden over the past six decades, and with annual incidence rates between 4 and 14 per 100,000, ICC no longer ranks even among the top ten cancers in these settings. The low incidence is achieved through substantial healthcare investments for screening programs and diagnostic workup in these countries. On the other hand, cervical cancer is the leading cancer among women in many resource-constrained settings of the developing world, where incidence and mortality rates are about five- to six-times higher.[1] Rates are highest in sub-Saharan Africa, South-Central Asia and parts of South America, where ICC represents from a sixth up to a fifth of all cancers among women.[1]

Persistent infections with carcinogenic human papillomavirus (HPV) genotypes have long been established as the necessary, but not sufficient, cause of ICC.[2,3] Organized prevention programs in industrialized settings have relied on early detection of HPV-associated dysplastic changes in exfoliated cervical cells ('Pap smear') that reflect underlying precancerous lesions.[4] Cervical cancer screening has been a success owing to the long period, typically extending over many years, carcinogenic HPV infections take to progress to precancerous lesions (cervical intraepithelial neoplasia, grade 3 or CIN3) and ICC, and the availability of relatively safe and effective methods of treatment of cervical precancer. Yet concerns about the substantial cost burden associated with screening, limited accuracy of cytology and complications of unnecessary treatment have prompted research and development of more efficient approaches for cervical cancer prevention. Over the past two decades, substantial improvements in understanding the natural history of HPV-associated cervical carcinogenesis as well as advancements in molecular technologies have led to the availability of novel screening tests that provide alternatives or adjunctive methods to cytology. Prominent among these are the HPV-DNA based screening assays, already widely used as adjunctive methods for primary screening and for triage of equivocal cytology. At the same time, HPV vaccines have been developed and introduced that have high efficacy in preventing HPV infections when administered in HPV-naive populations. There is unanimous agreement that screening efforts have to continue, and screening algorithms have to be made more efficient, since it will take years to decades to see an effect of HPV vaccination on reduction in cancer incidence. In addition,

newer screening approaches are needed to anticipate continuous changes in disease prevalence in populations with increasing vaccination coverage.

Biomarker Principles

In this article, we discuss the current evidence and opportunities for improvement of cervical cancer screening through the use of novel biomarkers. In many countries, Pap cytology is still the primary screening test, either alone or in conjunction with HPV testing (predominantly in the USA).[5–8] In some European countries, a switch to primary HPV screening followed by cytology triage has now been recommended.[9]

New biomarkers may have potential use in primary screening, as triage tests for primary cytology screening, and as triage tests for primary HPV screening. For any biomarker to be useful, the test result has to influence clinical management. Management options include direct referral for treatment, referral to colposcopy to confirm precancer histologically, increased surveillance through more intensive screening or release to routine screening. The management options should be chosen based on an individual's risk of precancer and cancer, indicated by screening test results and other risk indicators such as age.[10,11]

When assessing screening options, it is important to consider physical and financial harm associated with unnecessary tests and procedures. False-positive test results may cause anxiety, lead to overtreatment of women, increase risks of obstetric complications, and thus increase the downstream costs of a screening program. The goal of cervical cancer screening programs is to prevent cancer, not to treat cervical intraepithelial neoplasia. Currently, a treatment threshold of CIN2 or worse lesions is widely used, despite the fact that a large percentage of CIN2 lesions spontaneously regress.[12] Furthermore, there is increasing evidence that even CIN3 is a heterogeneous group; only about 30–50% of large CIN3s are estimated to invade to cancer over a long time period.[13,14] An important area of cervical cancer biomarker research focuses on the identification of markers for cervical lesions that likely progress to cancer. It is important to note that risk thresholds and available resources can vary substantially between populations and may lead to different screening recommendations based on the optimal trade-offs between benefits and harms.

In the first part of this article, we briefly summarize the evidence on biomarkers that have been widely evaluated and are already in limited use in clinical practice. In the second part, we discuss biomarkers in discovery and validation phases that have the promise to improve screening in the future.

HPV Life Cycle & Natural History & the Basis for Biomarker Selection

The HPV genome consists of a circular double-stranded, 8000 bp long DNA with three regions:

- The upper regulatory region which functions as a transcription and replication control region;
- An 'early' region encoding proteins (E1, E2, E4, E5, E6, E7) for replication, regulation and modification of the host cytoplasm and nucleus;
- A 'late' region encoding the viral capsid proteins (L1, L2).

The prominent areas of research focused on biomarker discovery and validation are conceptually based on events in the HPV life cycle and natural history of HPV-dependent cervical carcinogenesis. While the phylogenetic taxonomy and classification of papillomaviruses continues to be refined, 13 HPV genotypes (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) are considered carcinogenic while some others (HPV types 26, 53, 66, 67, 70, 73, 82) are considered possibly carcinogenic in humans.[15] The molecular mechanisms of how HPV causes cancer have been extensively studied. Two viral oncoproteins, E6 and E7, interfere with key cellular pathways that control cell proliferation and apoptosis. Specifically, E7 disrupts pRb from its binding to E2F and triggers uncontrolled cell cycling. E6 interferes with p53 and abrogates apoptosis, which would normally occur in cells with uncontrolled cell proliferation. E6 and E7

induce substantial chromosomal instability in transformed cells, even at precancerous stages.[15,16] While biomarker discovery continues in multiple directions, current biomarker candidates can be broadly categorized into two groups, viral or cellular markers (Figure 1). The biomarker research pipeline extends from discovery (*in vitro*/preclinical studies) to early stage validation, and then to validation in randomized clinical trials.[17] A tabular representation of the state of availability and status of regulatory approval of commercially marketed biomarkers is presented in Table 1.

Table 1. Major classes of biomarkers being developed and validated for use in cervical cancer prevention research.

Type of biomarker	Test format	Application	Quality of evidence/regulatory approval	Manufacturers and test names
Viral markers				
Detection of carcinogenic HPV DNA (and HPV genotyping)	Signal amplification (e.g., Digene Hybrid Capture-2) Target genome amplification by PCR (e.g., Amplicor®, Linear Array®)	Primary screening Triage of equivocal cytology	Large population-based studies and randomized trials Many tests licensed for use in the USA and Europe, many in final regulatory stages	Qiagen: Digene hc2, careHPV™, QIAensemble™† Roche: Amplicor®, Cobas® 4800†, Linear Array®‡ Cervista® HPV HR† CLART® HPV2‡ Autogenomics: Infiniti® HR-HPV QUAD‡ BioRad: HR-HPV Dx PCR Innogenetics: InnoLiPA™‡ Multimetrix: Multiplex HPV Genotyping Kit‡ Greiner: Papillocheck® HPV-Screening‡ Abbott: RealTime HR HPV®† Not commercialized: GP 5+/6+ EIA‡
Detection of E6/E7 mRNA	Nucleic acid sequence-based amplification Transcription-mediated amplification In situ hybridization	Adjunct to primary HPV-based screening Triage of equivocal or mildly abnormal cytology	Multiple clinical studies published Large population-based studies underway	GenProbe: Aptima® Norchip: PreTect® Proofer‡ BioMerieux: NucliSENS EasyQ® HPV‡ IncellDx: HPV OncoTect®‡
Detection of HPV protein	Immunostaining of histology and cytology slides (L1) ELISA (E6)	Adjunct to primary HPV-based screening Triage of	Some clinical studies published	Cytoimmun: Cytoactiv® ArborVita: AVantage™ HPV E6

		equivocal or mildly abnormal cytology		
Cellular markers				
$p16^{ink4a}$ (also with addition of Ki-67)	Immunostaining of histology and cytology slides ELISA	Primary screening Triage of equivocal or mildly abnormal cytology	Multiple clinical studies published Large population-based studies underway	mtm Laboratories: CINtec® and CINtec® PLUS
MCM2 and TOP2A	Immunostaining of histology and cytology slides	Primary screening Triage of equivocal or mildly abnormal cytology	Some clinical studies published	Becton Dickinson: ProEx™C

[†]Results include partial HPV genotyping.

[‡]**Genotyping assay.**

HPV: Human papillomavirus; MCM2: Minichromosome maintenance protein 2; TOP2A: Topoisomerase IIA.

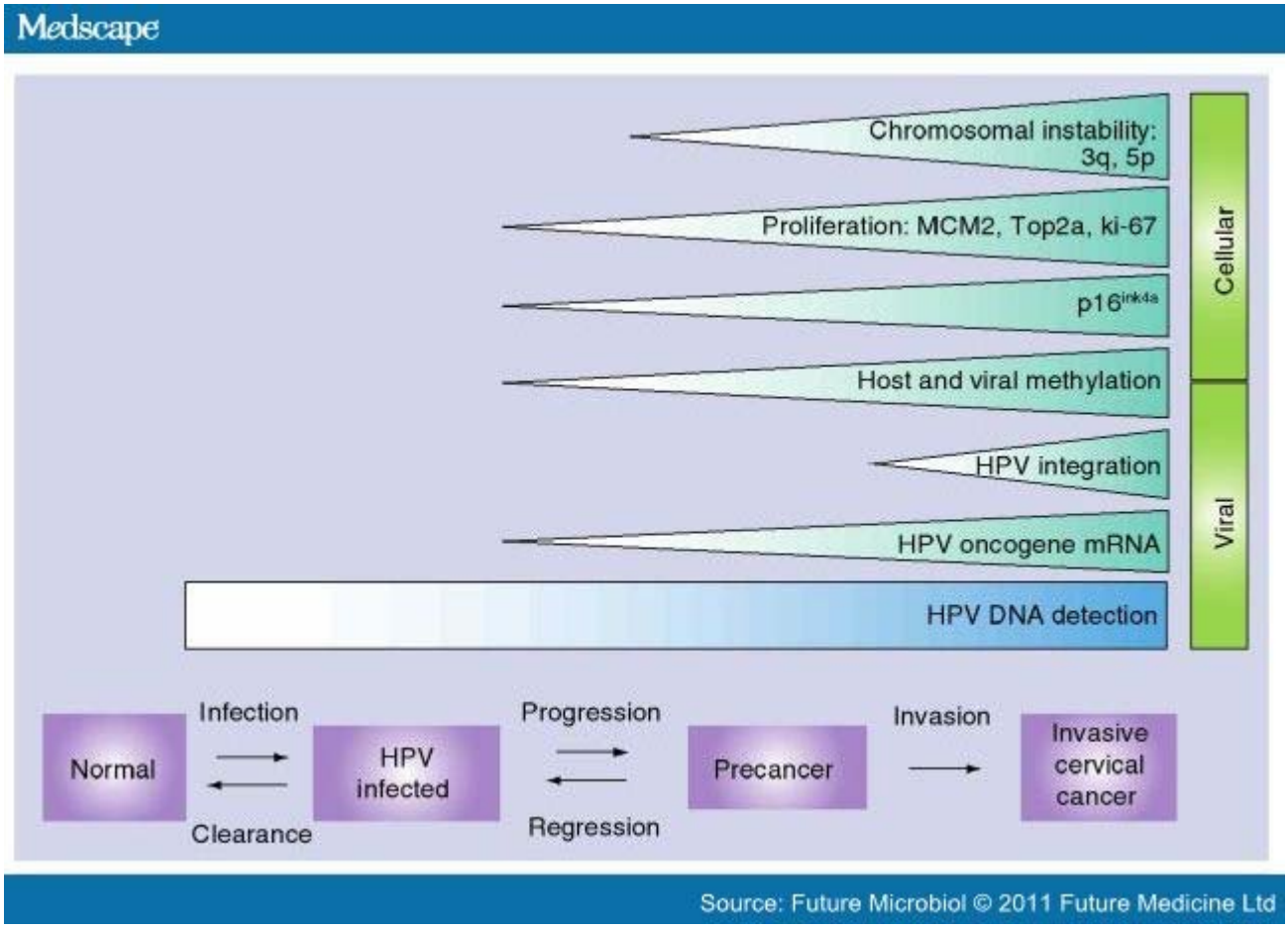

Figure 1. Human papillomavirus natural history and cellular and viral biomarkers used in cervical cancer screening. HPV infection happens shortly after sexual initiation. Most infections clear spontaneously, but a few carcinogenic HPV infections may persist and initiate oncogenic changes in epithelial cells at the cervical transformation zone. In a small fraction of cases, these persistent abnormalities may progress to invasive cervical cancer in the absence of early detection and treatment. Viral and cellular biomarkers indicating key steps of the functional progression model (HPV infection, precancer and invasive cancer) have been discovered, with some currently in early discovery stages, while others have already been commercialized.

HPV: Human papillomavirus.

The Limitations of Cervical Cytology

Cytology was introduced in the early 1950s as a primary screening method as part of annual preventive examinations, even though it was never subject to evaluation for effectiveness in randomized trials. The declining incidence rates in settings in Northern America, Europe, and Australia have provided widely accepted proof of the effectiveness of cytology-based screening.[1,18] Screening with cytology has become a well-established component of standard preventative care in most industrialized settings.[19,20] For example, more than 80% of women surveyed in a US study reported receiving Pap smears in the past 3 years.[18] In fact, over half of incident cases of ICC in the US continue to occur in women who have never or rarely been screened.[21] Although the clinical sensitivity of a single Pap smear is quite modest (60–70%),[22,23] the success of cytology-based screening is achieved by frequently repeated Pap testing, causing substantial cost burdens on the healthcare system.[24,25] Multiple efforts have focused on improving the accuracy and cost effectiveness of cytology-based screening protocols.[26] The introduction of liquid-based cytology has decreased the proportion of inadequate slides, and has permitted reflex testing for other molecular markers.[27,28] Yet false-negative rates associated with cytology continue to be substantial, primarily since cytological detection still relies on visual identification and subjective interpretation of morphologic changes induced by carcinogenic HPV.[27] About 10–15% of women with equivocal (atypical squamous cell of undetermined significance [ASC-US])[29] and mildly abnormal (low grade squamous intraepithelial lesions [LSIL])[30] results have an underlying CIN3. Since ASC-US and LSIL represent significant proportions of cytological results, further workup is required to make management decisions.[29] It is expected that cytological screening will be especially challenged in HPV-vaccinated populations, as the reduction of CIN2+ prevalence will be much higher than the reduction of low-grade abnormalities, further decreasing the signal-to-noise ratio of cytology testing.[31,32] Finally, most promising efforts to curb rising healthcare costs rely on prolonging screening intervals through improvements in negative predictive value of the screening test, a weakness of low-sensitivity cytology screening.[32]

The Role of HPV DNA Testing in Cervical Cancer Screening

Testing for HPV DNA, the necessary cause of virtually all ICC, provides a biologically salient approach for screening.[33,34] The detection of HPV in cervical scrapings was one of the first, and to date is the most widely evaluated, alternative to cytology.[26,35] Current HPV detection technologies are focused on hybridization with signal amplification of HPV DNA (e.g., Digene Hybrid Capture® 2 (hc2) (by Qiagen);[36] Cervista® HPV HR (by Hologic)[37]) or genomic amplification using PCR (e.g., Amplicor® HPV Test and Cobas® HPV Test (by Roche)[38,39]), with most results reported as aggregate presence or absence of carcinogenic HPV types.

The primary benefit of using HPV testing is the high sensitivity and high negative predictive value, since the absence of carcinogenic HPV indicates an extremely low risk of CIN3/ICC for 5–10 years, thereby allowing for safe prolonging of screening intervals.[32] The role of HPV DNA testing as a solitary primary screening test (to replace cytology) or as an adjunct to cytological screening has been evaluated in large randomized trials over the past decade.[40–46] Results show overwhelming evidence that HPV DNA testing

has a higher sensitivity in comparison with cytology for detection of CIN3.[26,47,48] Yet its utility is constrained by its limitation of lower specificity than cytology, since the majority of HPV infections are transient and would not progress to cervical dysplasia.[49,50] The high prevalence of benign and self-limiting HPV infections, and the low prevalence of cervical cancer precursors (let alone ICC), in the second and third decades of life further limit the use of HPV DNA testing for these age groups. Hence, the use HPV DNA testing in primary screening is currently primarily focused on women 30 years or older.[9] At any age, however, a single negative HPV DNA test indicates a very low risk of precancer over the next 5–10 years and allows clinicians to extend screening intervals safely.[51]

Since the FDA approval of Digene hc2 as a test for triage of ASC-US cytology in 2000, its use has increased steadily in the USA.[29,52] In the ALTS trial, it was found that while HPV testing was deemed to have utility in distinguishing women with ASC-US who were at risk for precancer, it was limited in its discriminating capacity for mildly abnormal (LSIL) cytology given the high background prevalence of carcinogenic HPV in this population.[53] The availability of genotype-specific information for HPV could potentially provide additional risk stratification in HPV-positive women. This may be of particular relevance in the detection of HPV types 16 and 18, since HPV 16-associated lesions are more likely to be persistent and have higher carcinogenicity than other HPV types,[54,55] and since HPV 18 is more associated with lesions within the endocervical canal that are frequently missed by cytology.[56] Indeed, some newer HPV DNA detection assays are able to provide type-specific information for HPV 16/18[39,57–61] (Table 1). A typical application is HPV16/18 genotyping in HPV-positive, cytology-negative women. Positivity for HPV16/18 may warrant earlier referral to colposcopy because of the higher risk associated with these types. However, it remains to be determined in clinical studies and cost-effectiveness analyses whether HPV genotyping provides sufficient risk stratification in a screening population.

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				HR HPV®† Not commercialized: GP 5+/6+ EIA‡
Detection of E6/E7 mRNA	Nucleic acid sequence-based amplification Transcription-mediated amplification In situ hybridization	Adjunct to primary HPV-based screening Triage of equivocal or mildly abnormal cytology	Multiple clinical studies published Large population-based studies underway	GenProbe: Aptima® Norchip: PreTect® Proofer‡ BioMerieux: NucliSENS EasyQ® HPV‡ IncellDx: HPV OncoTect®‡
Detection of HPV protein	Immunostaining of histology and cytology slides (L1) ELISA (E6)	Adjunct to primary HPV-based screening Triage of equivocal or mildly abnormal cytology	Some clinical studies published	Cytoimmun: Cytoactiv® ArborVita: AVantage™ HPV E6
Cellular markers				
$p16^{ink4a}$ (also with addition of Ki-67)	Immunostaining of histology and cytology slides ELISA	Primary screening Triage of equivocal or mildly abnormal cytology	Multiple clinical studies published Large population-based studies underway	mtm Laboratories: CINtec® and CINtec® PLUS
MCM2 and TOP2A	Immunostaining of histology and cytology slides	Primary screening Triage of equivocal or mildly abnormal cytology	Some clinical studies published	Becton Dickinson: ProEx™C

†Results include partial HPV genotyping.

‡Genotyping assay.

HPV: Human papillomavirus; MCM2: Minichromosome maintenance protein 2; TOP2A: Topoisomerase IIA.

In the USA, cytology and HPV DNA co-testing are being widely used. In Canada and many European settings, a strategy with primary screening by HPV testing followed by cytology triage of HPV DNA positives ('sequential' or 'two-stage' testing) was proposed[32] and has been evaluated in multiple randomized clinical trials.[62] This strategy takes advantage of the high negative predictive value of HPV DNA testing and maximizes sensitivity, while reserving cytology for those who have higher likelihood of dysplastic lesions. The reliance on cytology, with subjective interpretation and substantial inter-observer variability, along with potential for sampling/collection errors, however, remains a challenge.

Novel Biomarkers in Cervical Cancer Prevention

Given limitations in use of both cytology and HPV DNA based approaches as standalone tests for screening, the focus of cervical cancer prevention research has been on development and validation of new disease-specific biomarkers of HPV-associated transformation.[63–66] The underlying biological basis and utility of some prominent biomarkers is discussed below and their functional relevance in relation with various stages of cervical carcinogenesis is schematically presented in Figure 1.

E6/E7 mRNA Detection

The progression from a transient to a transforming HPV infection is characterized by a strong increase of HPV *E6/E7* mRNA and protein expression.[67] Multiple studies have evaluated the role of detection of mRNA transcripts in cervical scrapings to identify cervical precancers.[68–76] At least two commercial platforms are currently available: PreTect® Proofer (Norchip [marketed as NucliSENS EasyQ® by BioMerieux in some European markets]) and APTIMA® (GenProbe) (Table 1). In a recent meta-analysis by Burger and colleagues,[77] 11 studies that evaluated HPV E6/E7-based mRNA detection against HPV DNA testing for detection of CIN2+ reference standard were summarized. Given the considerable heterogeneity, pooling of data was not possible. A 'best evidence synthesis' for E6/E7 mRNA HPV testing accuracy was provided, that reflected a sensitivity ranging between 0.41 to 0.86 for the PreTect Proofer/NucliSENS Easy Q assays while a higher range – from 0.90 to 0.95 – for the APTIMA assay. The specificity ranged from 0.63 to 0.97 and from 0.42 to 0.61 for the PreTect Proofer/NucliSENS EasyQ and APTIMA assays, respectively. The considerable difference in sensitivity (and specificity) between PreTect Proofer/EasyQ and APTIMA may in part be explained by the difference in type coverage: The former tests detect only five types (HPV16, 18, 31, 33, 45), while the latter covers 14 types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68).

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†Results include partial HPV genotyping.

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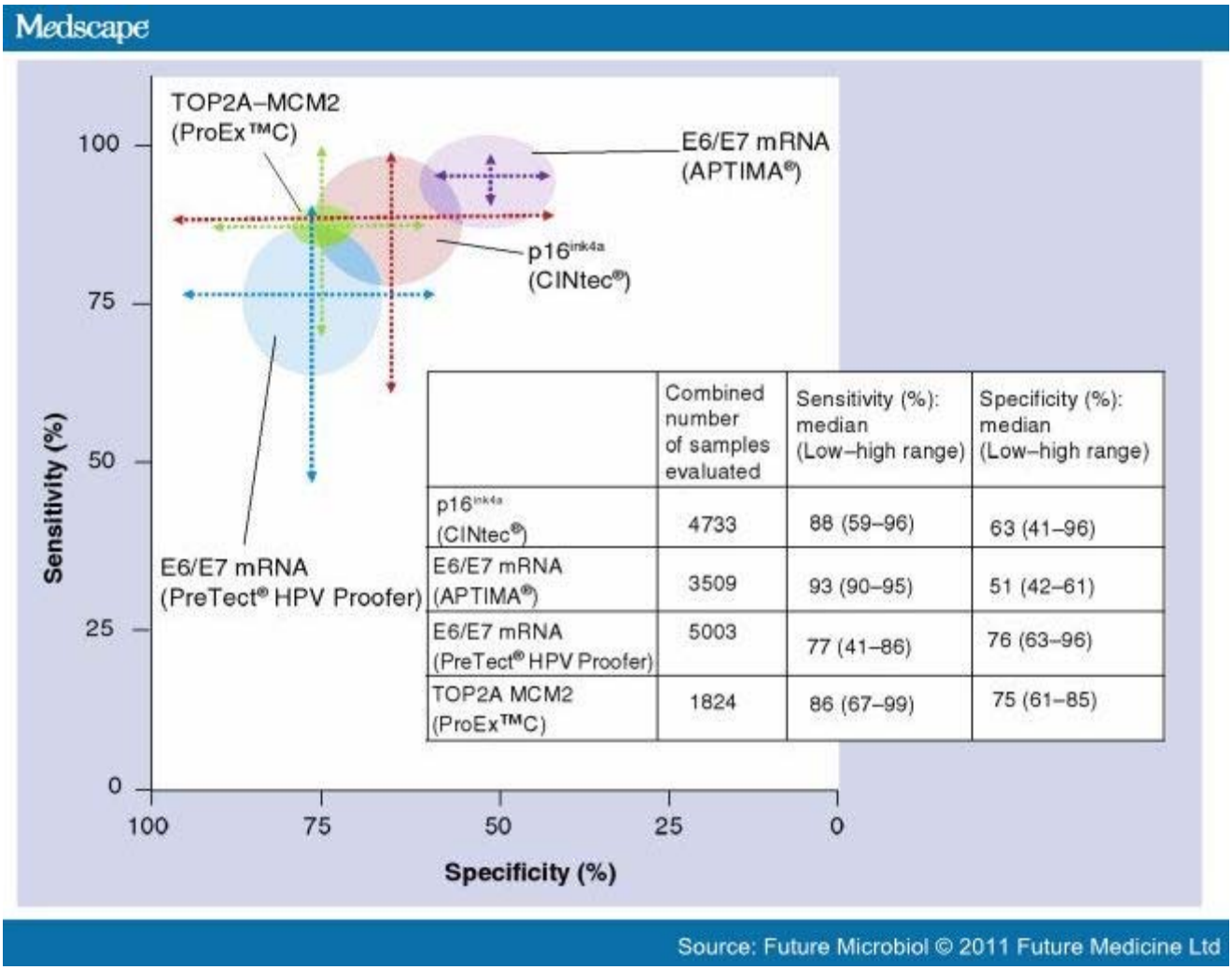
HPV: Human papillomavirus; MCM2: Minichromosome maintenance protein 2; TOP2A: Topoisomerase IIA.

$p16^{ink4a}$

The biomarker most widely evaluated is $p16^{ink4a}$, a cyclin-dependent kinase inhibitor that is markedly overexpressed in cancerous and precancerous cervical tissue. $p16^{ink4a}$ is a cellular correlate of the increased expression of the viral oncoprotein E7 that disrupts a key cell cycle regulator, pRb, in transforming HPV infections. The disturbance of the Rb pathway leads to a compensatory overexpression of $p16^{ink4a}$ through a negative feedback loop.[64] The resultant overexpression and cellular accumulation of $p16^{ink4a}$ is a specific marker of cervical precancerous lesions and can be measured through immunocytochemical staining of histology and cytology slides and using ELISA assays.[78]

A commercially available CE-marked assay (CINtec®, mtm Laboratories) has been widely validated. Liquid-based cytology systems such as ThinPrep®, SurePath™, CYTO-screen system® and others have been used in these studies. p16^{ink4a} has been evaluated as a standalone test and as an adjunct to cytology[79–84] or HPV testing.[80,85,86]The role of p16^{ink4a} based detection in screening and triage has been reviewed in previous articles.[63,87] These reviews noted substantial heterogeneity in methods used for defining p16^{ink4a} positivity in the cytology application, including quantitative and morphologic approaches. The sensitivity has ranged between 0.59 and 0.96 and the specificity has ranged between 0.41 and 0.96 for the detection of CIN2+ lesions in clinical studies, reflecting the heterogeneity in test interpretation and analyzed populations (Figure 2). Recently, a dual immunostain of p16^{ink4a} with Ki-67 (CINtec® PLUS) has been introduced that is supposed to substantially simplify and standardize the evaluation of stained slides.[80,84]



	Combined number of samples evaluated	Sensitivity (%): median (Low–high range)	Specificity (%): median (Low–high range)
p16^{ink4a} (CINtec®)	4733	88 (59–96)	63 (41–96)
E6/E7 mRNA (APTIMA®)	3509	93 (90–95)	51 (42–61)
E6/E7 mRNA (PreTect® HPV Proofer)	5003	77 (41–86)	76 (63–96)
TOP2A MCM2 (ProEx™C)	1824	86 (67–99)	75 (61–85)

Figure 2. Graphical representation of the range of estimates of sensitivity and specificity of studies evaluating commercially available biomarkers at the cervical intraepithelial neoplasia grade 2+ threshold. The sensitivity ranges are plotted along the y-axis and specificity along the x-axis. The median values of the range of estimates of sensitivity and specificity are used as the points of convergence of the sensitivity and specificity range lines. The circles around each graph reflect the combined total number of samples used in the studies that were summarized. This graphical representation does not weigh the studies by sample size, and the median value does not reflect a computed summary/pooled measure. The purpose of this graph is to visualize the wide range of performance estimates reported in studies evaluating these biomarkers. The heterogeneity is related to various factors, including but not limited to differences in targeted populations, differences in clinical end points and heterogeneity of biomarker performance.

Markers of Aberrant S-phase Induction

The cell cycle activation mediated by HPV oncogenes in transforming infections is characterized by aberrant S-phase induction. An assay detecting two proteins indicating aberrant S-phase induction, topoisomerase IIA (TOP2A) and minichromosome maintenance protein 2 (MCM2) is commercially available (ProEx™ C by Becton Dickinson).[88]Few clinical studies with limited sample size have shown that it has a sensitivity ranging between 0.67 and 0.99 and specificity ranging between 0.61 and 0.85 (Figure 2).[89–94]

Other Biomarkers Undergoing Clinical Validation

Other cellular makers such as CK13 and CK14,[95] MCM5 and CDC6,[96] Survivin[97] and CEA[98] have also been evaluated in various stages of development. Most are marked by nonuniformity in determination of end points and limited sample sizes. Other viral markers such as HPV L1 capsid protein[99–101] and E6 oncoprotein detection[102,103]have been evaluated in a limited number of small studies, but more evidence is needed to determine their utility.

Biomarkers for Low-resource Settings

In the context of resource-constrained settings, the failure to establish and sustain cytology-based screening has necessitated research on operationally simple and less resource-intensive approaches for cancer prevention and control.[104] Visual methods such as visual inspection with acetic acid and visual inspection with Lugol's Iodine provide immediate *in vivo* detection of visually apparent precancerous cervical lesions and the potential to link screening results and same-visit treatment by cryotherapy (or appropriate referral for cryotherapy-ineligible lesions). While visual inspection with acetic acid/visual inspection with Lugol's Iodine have been extensively evaluated[105,106]and have high operational feasibility in the hands of nonphysician health providers, they miss anywhere between 20 and 50% of true disease due to variations in definitions of disease positivity, inherent subjectivity in test results, and challenges in quality assurance and control.[105,107] There is a huge need for utilizing novel biologically-based approaches in resource-constrained settings of the developing world for improving access and accuracy of screening.[108] careHPV™ is a new assay developed by Qiagen that is a low-cost adaptation of the Digene hc2 assay and can be performed rapidly (<2 h) without access to running water or electricity, an ideal solution for operation in field settings.[109] This assay has been shown to have performance characteristics approaching those of hc2,[109] and in

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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