Human papillomavirus screening and vaccines for cancer prevention: what is on the horizon?

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This perspective aims to look at how the recently licensed human papillomavirus (HPV) vaccines, based on L1 virus-like particles (VLPs), will impact cervical cancer globally, and what remains to be done in this field. The current VLP vaccines protect against infection from a substantial proportion, but not all, of the oncogenic HPV genotypes, and they have no proven therapeutic benefit for patients with HPV disease. Furthermore, they are too costly for sustained global use, and it is unclear how they may impact current cytologic screening protocols. A number of new technologies show promise in addressing these issues, including molecular screening tools and alternative vaccine strategies.

These are interesting times for clinicians and scientists studying human papillomavirus (HPV). New technologies are driving a second profound shift in the way that HPV-related disease will be managed. The first revolution came with the development of Pap smears and the institution of national cytologic screening and intervention programs. Over the past five decades, this approach has been successful in reducing the incidence of cervical cancer by approximately 80% in the USA, but now comes at a cost of more than US$6 billion per annum. In principle, cervical cancer is a completely preventable disease. Substantial gaps remain in screening programs: cytologic screening, although highly effective, is imperfect, and there are still approximately 5000 deaths from cervical cancer each year in the USA. Furthermore, the benefits of cytologic screening and intervention are not available to most women in low-resource settings. As a consequence, 83% of the global burden of cervical cancer (an estimated 493,000 new cases and 274,000 deaths in 2002) is in developing countries, and cervical cancer remains the second leading cause of cancer death of women worldwide.

This latest paradigm shift has occurred because of the recognition that persistent infection with oncogenic-type HPV is a necessary, although insufficient, cause of cervical cancer. This fact has driven the development of screening, based upon the molecular detection of HPV nucleic acid, and preventive vaccines using L1 virus-like particles (VLPs). Although it is also clear that expression of HPV oncogenes E6 and E7 is required for the maintenance of cancer phenotypes, unfortunately, true antiviral drugs and curative treatments for advanced cervical cancer have not yet been developed. This probably reflects the fact that a lack of enzyme activity intrinsic to E6 and E7 to directly target with small molecules, and inhibitors of E1 helicase, the only enzymic activity of any HPV-encoded proteins, are unlikely to impact high-grade disease or cervical cancer. Therefore, progress has currently been limited to cancer prevention. Here, we will discuss how these new molecular screening technologies might best be implemented. Furthermore, we will address several promising new second-generation vaccine technologies that might be used in cancer prevention: L1 capsomers and polymeric L2 vaccines for the prevention of new infections, as well as treatment of subclinical HPV infections and premalignant lesions using therapeutic vaccine modalities targeting early HPV antigens. These therapeutic vaccines may also prove effective in cervical cancer patients, possibly when combined with other modalities.

Screening for human papillomavirus & disease

Despite measurable success of cytologic screening in many countries, Pap tests suffer from relatively low sensitivity from a single smear, and low specificity when using a referral threshold of atypical squamous cells of undetermined significance (ASCUS). Because of these limitations of Pap tests, screening programs rely on annual testing and follow-up of minor abnormalities, most of which are self-limiting and require no further intervention. The development of new screening algorithms has thus targeted methods that would reduce the number of screening tests performed, as well as methods that would more specifically identify true neoplastic changes rather than mostly transient viral infection. The need for more efficient screening algorithms will become

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more acute with the addition of vaccination to the overall prevention strategy, which will result in a further reduction in the positive predictive value of a single positive test result [1]. The only US FDA-approved HPV DNA test (Hybrid Capture® 2, or hc2, Qiagen Corp., MD, USA) has been shown to be more sensitive for detection of high-grade cervical intraepithelial neoplasia (CIN 2/3) and more specific than Pap testing when used in women over 30 years of age. Current screening recommendations in the USA incorporate the use of HPV DNA tests in two areas:

- Triage of mildly abnormal Pap diagnoses of ASCUS
- Primary screening of women over 30 years of age [2]

The high negative predictive value of HPV DNA testing allows a safe extension of screening intervals from annually to once every 3–5 years [3,4], an algorithm which, if broadly adopted, is expected to result in a reduction in the total number of Pap tests performed per year by nearly 50% [5]. Many studies have recently reported good performance in using HPV DNA testing alone as a primary cervical cancer screening test, eliminating the Pap altogether or using Pap testing as a triage for HPV DNA-positive tests [6].

Simple addition of HPV DNA testing as currently available may therefore reduce the total number of screens performed for most women over their lifetime. However, it still suffers from a relatively low specificity and detection, and follow-up of transient infections will remain a significant problem. Certain genetic events are associated with progression and high-grade disease. These include genomic integration and alterations in the methylation of HPV episomes, upregulation of viral oncogene expression and gain/loss of particular chromosomal regions. Thus, combining HPV testing with PCR approaches to detect HPV integration, quantitative alterations in the viral transcriptional program or aberrant methylation of the HPV genome, or fluorescent *in situ* hybridization to identify aberrant chromosomal rearrangements, such as 3q gain, show promise to improve the specificity of this approach [7]. Protein biomarkers may also be useful, for example p16, in improving triage after HPV testing [8].

Importantly, there are several improvements in technology that could further increase the use of HPV testing globally, where one of the most important barriers to successful screening is the reticence of the community to participate in screening programs. Algorithms that allow for mailing self-collected vaginal (or penile) swab samples to a central laboratory are likely to reduce both physician costs and inconvenience to the patient, thereby increasing compliance. In rural areas and in the developing world, the use of rapid PCR reactions could allow point-of-care detection and typing of HPV without the need for shipment to a central laboratory, and potentially provide the option for single-visit screening and treatment.

Advances in the cervical cancer screening protocols will continue to be focused on improving sensitivity with fewer tests and increasing specificity to reduce the number of unnecessary follow-up procedures. Most importantly, cervical cancer screening will continue to be a necessary part of comprehensive cervical cancer prevention strategies, even with the introduction of highly efficacious vaccines against HPV 16 and 18, as a total of 30% of invasive cancers are caused by nonvaccine-targeted gentoypes.

Preventive vaccine strategies

The new HPV VLP vaccines are now being utilized in a significant fraction of the US population. It is clear that the most cost-effective approach is to vaccinate women prior to the initiation of sexual activity, as the VLP vaccines are preventive, not therapeutic, and HPV infections are currently so widespread in the population [9,10]. Recent studies indicate that vaccination of women at mid-life is still effective in preventing new HPV infections, but it is questionable whether this approach will reach an acceptable cost:benefit ratio from a public-health perspective. It is known that, in addition to high-grade CIN and adenocarcinoma *in situ*, the precursors of cervical cancer, the HPV vaccine prevents vulval intraepithelial neoplasia (VIN) and vaginal intraepithelial neoplasia (VaIN), the precursors of vulval and vaginal cancer, as well as most benign genital warts in women [11]. Studies addressing protection of men from benign genital warts are ongoing. It is likely that HPV vaccination will also protect against anal intraepithelial neoplasia, the precursor of anal cancer. Protection against oral challenge with HPV is also likely, and this should be studied for potential to prevent HPV-related head and neck cancers [12].

A full course of the current HPV vaccine costs US$360, making it the most expensive preventive vaccine. This is troubling because those most in need of this vaccine are the poor, who generally do not have access to cytologic screening programs. There are efforts from the manufacturers

to supply the vaccine at cost, but this is likely still too high for long-term global access to these important vaccines. A more sustainable approach may be local manufacture of a low-cost alternative. HPV L1 capsomers produced in *Escherichia coli* are receiving attention in this regard [13,14]. Expression in bacteria provides a very high yield with simplified purification, and L1 capsomers are very stable. This stability may facilitate long-term storage and potentially abrogate the need for a cold chain, which can be problematic for delivery to remote and poor areas. Furthermore, HPV 16 L1 capsomers are being manufactured for early-phase clinical testing by Shantha Biotech (Hyderabad, India) for Drs R Garcea (University of Colorado, CO, USA) and R Schlegel (Georgetown University, Washington, DC, USA). Shantha Biotech is an Indian company specialized in low-cost vaccine manufacture, and supplies the WHO.

An interesting approach to low-cost and large-scale production of L1 is the use of recombinant plant technology. L1 in crude extracts of transgenic tobacco plants was able to induce similar levels of neutralizing antibodies in vaccinated mice as compared with L1 VLPs prepared in insect cells [15]. Several studies suggest that L1 VLPs may be administered without injection, for example, via oral or nasal routes [16–19]. Thus, potentially, ingestion of crude preparations of L1 generated in plants or yeast might provide a simple, low-cost vaccine.

Another Indian company, Dr Reddy's Laboratories Ltd (Hyderabad, India), is examining recombinant *Salmonella enterica* serovar Typhi Ty21a expressing HPV 16 L1 as a live vaccine, and clinical trials are planned in the near future in a collaboration with Drs D Nardelli-Haefligger (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland) and J Schiller (National Cancer Institute, MD, USA) [20]. This approach has several potential advantages: it combines two important vaccines targeting typhoid and cervical cancer; it can be delivered without needles (which add significantly to the cost and fear of injected vaccines); and it does not require purification technologies. However, the safety of live vectored vaccines is a potential issue.

One important characteristic of all L1-based vaccines is that they induce type-restricted protection: while they are highly effective against the types used to develop the VLPs, they are only partially effective against the most related HPV types, and of less value against more distantly related types [21,22]. The current L1 VLP vaccines target only two of the at least 15 oncogenic HPV types. This suggests that more highly multivalent vaccines may be required, resulting in increased complexity of manufacture and cost. However, the two oncogenic types targeted by current L1 VLP vaccines are by far the most important: HPV 16 is responsible for approximately 50% of all cervical cancers, and HPV 18 for approximately 20%. Furthermore, they appear to offer at least partial protection against some other oncogenic types, notably HPV 31 and 45, which are relatively common in cervical cancer and are closely related to HPV 16 and 18, respectively. This limits the gains from expanding the valency of the current HPV vaccines, but it may become more important if cross-type protection wanes more rapidly than homologous type protection.

Despite these caveats, a broadly effective HPV vaccine would be highly advantageous [22]. Eradication of cervical cancer would likely require such a cross-protective vaccine. Indeed, patients are frequently infected with multiple different HPV types. Importantly, without broad protection, cytologic screening programs must remain in some form. This greatly reduces the cost:benefit ratio of both HPV vaccination and screening. A broadly protective HPV vaccine that provides life-long immunity could potentially eliminate the need for cytologic screening of the cervix. Finally, there are theoretical concerns that a niche created by elimination of the two most common oncogenic types through vaccination would be rapidly filled by other HPV types. This is not likely, given the lack of evidence for competition, and the niche would be more likely filled by the many benign types if this actually occurs. Another possible benefit for an alternative and preferably broadly protective vaccine would be commercial competition to drive down the cost of HPV vaccination overall.

Vaccination with the minor capsid protein L2 shows promise for inducing broad protection [23]. L2 vaccines, like L1 VLPs, are protective by inducing neutralizing antibodies [24,25]. However, whereas the antibodies induced by L1 VLP are high titer and exhibit type-restricted neutralization, L2 induces broadly cross-neutralizing antibodies at a lower titer. The low-titer response to L2 as compared with L1 VLP reflects its lack of structure and raises concerns regarding efficacy, particularly over the long term. This lack of structure may be used to an advantage for L2, because L2 polypeptides derived from different HPV types can be concatenated into a single fusion protein. Such a polymeric L2 polypeptide

appears to be particularly effective in inducing broadly cross-neutralizing antibodies. Since even concatenated L2 vaccines remain less immunogenic than L1 VLP, they will likely require a potent adjuvant or additional boosters to maintain immunity in the long term. R Roden (Johns Hopkins University, MD, USA), J Schiller and D Lowy (National Cancer Institute, MD, USA) are developing a polymeric L2 fusion protein in collaboration with Shantha Biotechnics for early-phase clinical testing.

Unfortunately, neither L1 nor L2 vaccines show clear evidence for therapeutic activity against pre-existing lesions [10,23]. One Phase I study suggested that vaccination with HPV 6 L1 VLP can enhance clearance of genital warts versus historic controls, but this observation needs to be followed up with contemporaneous controls [26]. The absence of clear therapeutic activity upon vaccination with the late proteins may reflect the absence of their expression in the basal epithelial cells that harbor the virus. Consequently, therapeutic HPV vaccines typically target one or more of the early viral antigens. The failure of HPV VLP vaccines to treat existing HPV lesions suggests that they will be of no benefit to those with existing infections and disease, and that the impact of preventive vaccination upon rates of cervical cancer will not be fully apparent for at least a couple of decades. Therefore, therapeutic vaccines are still urgently needed despite the licensure of the HPV VLP vaccines.

Therapeutic vaccine strategies for human papillomavirus disease

In the USA, CIN 2/3 is common, with an estimated age-adjusted incidence ranging from 30 to 60 per 100,000 women [27]. If left undetected and/or untreated, a subset of CIN 2/3 lesions will progress to invasive squamous cell carcinomas. Conversely, complete histologic regression of established CIN 2/3 does occur spontaneously in up to 30% of women [28,29]. Since conventional histopathological assessment of biopsy tissue at the time of diagnosis does not predict regression, all CIN 2/3 lesions are treated by either surgical resection or ablation. Surgical resection of cervical dysplasias has been associated with subsequent risk of preterm delivery in several large studies [30–32]. The development of immunotherapeutic strategies would prevent morbidity, even in high-resource settings. Moreover, because a subset of CIN 2/3 lesions does indeed regress, this patient population is a potentially informative cohort for which to develop therapeutics.

A large body of studies in animal models of human cancers demonstrates that therapeutic vaccines can in fact benefit the host. In animal models, therapeutic vaccination in the setting of established disease has resulted in complete tumor rejection, reduction of tumor burden in metastatic disease, and improved survival. It is also clear that efficacy is greater in the setting of minimal residual disease than in the setting of advanced disease [33].

The expression of two HPV oncoproteins, E6 and E7, is functionally required for the initiation and persistence of CIN 2/3, the lesion that is the immediate precursor to invasive disease. E6 and E7 present compelling targets for immunotherapies because they are functionally required for disease persistence, and are non-'self' proteins expressed in CIN 2/3 lesions and invasive disease, but not in normal tissue.

To date, a number of groups has tested peptide, vaccinia-based, DNA-based and protein vaccines, in patients with a wide spectrum of preinvasive HPV disease [34–41]. In general, while it does appear feasible to elicit HPV-specific T-cell responses in patients with established premalignant HPV disease, none of these clinical trials has demonstrated a strong correlation between measured T-cell responses in the peripheral blood and disease outcome (**Table 1**).

This lack of correlation may be attributable to several factors, not the least of which is that these lesions occur in the clinical setting of immune evasion, manifested as a chronic, mucosally sequestered viral infection. Although non-self, the initial presentation of HPV antigens occurs in an immunologic environment that must tolerate a spectrum of commensal organisms, and so, on balance, the 'default' setting is one of temperance, or relative immune privilege [42], not necessarily in an antigen-specific manner. Although locally mediated immune privilege is likely, systemic immunologic antigen-specific anergy would not be necessary for the persistence of dysplastic lesions. The mechanisms of immunologic homeostasis in the lower genital tract are incompletely understood, and many lessons may be learned from researchers studying HIV transmission [43,44]. It may be that immune responses generated by constructs tested to date have not been of sufficient magnitude; current efforts include the evaluation of heterologous prime-boost regimens, as well as the use of vectors that have been shown in other human disease settings to be more immunogenic than peptide, protein or DNA vaccine approaches.

Table 1. Human papillomavirus type 16 E7-targeted clinical investigations.

Study	Study type	Patient population	Vaccine	Schedule	Results	Toxicities	Ref.
Borysiewicz *et al.* (1996)	Phase I/II	Late-stage cervical cancer	Vaccinia-HPV 16/18 E6/E7	Single dose	HPV-specific CTL detected in 1/3 evaluable patients	No serious systemic side effects; no short- or medium-term complications	[34]
Kaufmann *et al.* (2002)	Phase I/II	Stage Ib or IIa cervical cancer	Vaccinia-HPV 16/18 E6/E7	Weeks 0, 4	4/29 CTL responses, 8/29 serologic responses	Mild-to-moderate local toxicity	[48]
Steller *et al.* (1998)	Phase I	Refractory cervical or vaginal cancer	E786–93 lipopeptide	Weeks 0, 3, 6, 9	E786–93-specific CTLs in 4/10 evaluable patients before vaccination, in 5/7 evaluable after two vaccinations, in 2/3 evaluable after four vaccinations	Transient grade I	[35]
Ressing *et al.* (2000)	Phase I/II	Terminal, refractory cervical cancer	HPV 16 E7 HLA-A*201 peptides	Weeks 0, 4, 8,12	Peptide-specific CTLs with PADRE help in 4/19 patients	Transient grade I	[36]
Muderspach *et al.* (2000)	Phase I	CIN2/3 or VIN2/3	HPV 16 E712–20 in patients 1–10, plus E786–93 in patients 11–18	Weeks 0, 4, 8, 12	CTL response in 8/16 patients	Transient grade I and II toxicities	[37]
Klencke *et al.* (2002)	Phase I	High-grade anal dysplasia (AIN 2/3)	DNA-HPV 16 E783–95 on microspheres	Weeks 0, 3, 6, 9	10/12 patients E7-specific $CD8^+$ T cells by ELISPOT	Transient grade I/II toxicities	[38]
Sheets *et al.* (2003)	Phase I	CIN 2/3	DNA-HPV 16 E7 HLA-A*201 epitopes on microparticles	Weeks 0, 3, 6	11/15 patients peptide-specific CTL	Transient grade I	[39]

AIN: Anal intraepithelial neoplasia; CIN: Cervical intraepithelial neoplasia; CTL: Cytotoxic T lymphocyte; ELISPOT: Enzyme-linked immunosorbent spot; FIGO: International Federation of Gynecology and Obstetrics; HPV: Human papillomavirus; PADRE: Pan-DR epitope; VIN: Vulval intraepithelial neoplasia.

Table 1. Human papillomavirus type 16 E7-targeted clinical investigations (cont.).

Study	Study type	Patient population	Vaccine	Schedule	Results	Toxicities	Ref.
Baldwin *et al.* (2003)	Phase II	High grade HPV + VIN	Vaccinia-HPV 16/18 E6/E7	Single dose	Increased anti-vaccinia Ig titer in 11/12 patients. In 6/10 patients with evaluable material, vaccine induced increase in specific T-cell response	Mild vaccine-site reactions	[46]
Davidson *et al.* (2003)	Phase II	HPV16 + VIN 3	Vaccinia-HPV 16/18 E6/E7	Single dose	Vaccine induced increase in anti-vaccinia Ig response in 17/18 patients. Increased HPV-specific cellular response following immunization in 14/17 patients	No unexpected vaccine-related adverse effects	[47]
Garcia *et al.* (2004)	Phase II	CIN 2/3	ZYC101a plasmid-encoding fragments of HPV16/18 E6/E7 in microparticles	Weeks 0, 3, 6	No correlation between HPV-specific cellular response and regression	Mild vaccine-site reactions	[41]
Einstein *et al.* (2007)	Phase II	CIN 3	*Mycobacterium bovis* BCG Hsp70–HPV 16 E7 fusion protein	Weeks 0, 4, 8	HPV specific cellular response not reported	Grade 1 local reaction to vaccine	[40]

AIN: Anal intraepithelial neoplasia; CIN: Cervical intraepithelial neoplasia; CTL: Cytotoxic T lymphocyte; ELISPOT: Enzyme-linked immunosorbent spot; FIGO: International Federation of Gynecology and Obstetrics; HPV: Human papillomavirus; PADRE: Pan-DR epitope; VIN: Vulval intraepithelial neoplasia.

Another reason for the lack of correlation between measures of HPV-specific response measured in peripheral blood with lesion outcome may be a relative lack of homing of effector cells to the site of lesions. Topical imiquimod, a TLR-7 agonist that elicits a local proinflammatory microenvironment, is used to treat external genital HPV disease, but is not approved for use for either vaginal or cervical disease. Epithelium exposed to topical imiquimod is subsequently infiltrated with inflammatory effector cells, and both dendritic cell maturation and migration are enhanced. Ultimately, a combination of enhanced HPV-specific adaptive immunity with a local immunologic milieu that allows access to lesions is a strategy that is likely to improve clinical outcomes. As a fraction of CIN 2/3 lesions do in fact regress, and the lesions are accessible, this cohort presents an opportunity to determine immunologic parameters of a 'successful' immune response, as well as variables associated with 'failure', very early in the neoplastic process. From an immediate practical standpoint, identification of variables that are associated with regression would allow a subset of patients to avoid surgery.

Finally, most patients who present with invasive disease in the USA have early, stage IB disease. As Stage 1B cervical cancer is clinically staged during surgery, the outcome for these patients is heterogenous; for example, 47% of Stage IB cervical cancer patients who receive standard surgical care will eventually have a recurrence and require adjuvant therapy, whereas surgery alone is effective in the remainder. Current histopathologic criteria, aside from lymph node involvement at the time of resection, do not reliably predict in which patient disease will recur. The identification of patients at risk for recurrence would permit better stratification of care. A growing body of evidence suggests that the immunological microenvironment within the primary tumor plays a critical role in disease progression [45]. Therefore, study of tissues from stage IB cervical cancer patients may lead to the identification of better predictors of disease outcome, for example, measures of the immunologic signature within the primary lesion might predict the likelihood of recurrence and/or the potential for efficacious treatment with a therapeutic HPV vaccine.

Conclusion

Our understanding that persistent oncogenic HPV infection is a necessary cause of cervical cancer clearly indicates that this disease can eventually be eradicated. We have important tools in the fight against cervical cancer; screening by HPV testing and cytology for high-grade CIN, and the ability to essentially cure the patient by ablation of these precursor lesions, and, most recently, HPV L1 VLP vaccines to prevent most oncogenic HPV infections. However, a large fraction of women worldwide do not have access to these valuable cancer-prevention tools. Furthermore, we currently lack virus-specific therapies that are needed to treat cervical cancer patients and HPV infections. As the HPV oncogenes lack intrinsic enzymic activity and thus are difficult to target with small-molecule drugs, immunotherapy presently shows the greatest promise to help those afflicted with HPV disease.

Future perspective

There are many promising approaches to improve the predictive value of screening for premalignant cervical disease. Improved accuracy and lower costs for screening will be particularly significant upon the widespread introduction of the current HPV vaccines, as the incidence of disease is likely to plummet and the proportion of false positives will thus increase. Implementation of rapid molecular testing will provide on-site HPV quantification and typing for immediate treatment. Self-sampling is likely to improve coverage and reduce costs. Testing for characteristic genetic changes may provide prediction of lesions likely to progress.

Much work needs to be done to facilitate the global introduction of preventive HPV vaccines. The protection can be expanded to cover all oncogenic types by increasing the valency of the current HPV vaccines, or possibly by introducing polymeric L2 vaccines. Efforts to drive down the cost of vaccination, reduce the number of doses and potentially provide needleless immunization and life-long protection remain critical. Incorporation into other vaccine regimens could potentially further extend access.

The study of cancer immunology, particularly mechanisms of immune suppression and evasion, has made great strides recently that will likely facilitate our efforts to develop immunotherapies for HPV-related disease. Current vaccine technologies are able to trigger systemic T-cell responses to HPV oncogenes, but these are often ineffective against disease. The combination of vaccination with new approaches to alter the tumor microenvironment to facilitate

cellular immune clearance of infected cells has the potential to improve the efficacy of immunotherapy. These studies are likely to have important implications for immunotherapy of other cancer types.

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