Immunoprevention of human papillomavirus-associated malignancies

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Abstract (185/200) words

Persistent infection by one of fifteen high risk human papillomavirus (hrHPV) types is a necessary but not sufficient cause of 5% of all human cancers. This provides a remarkable opportunity for cancer prevention via immunization. Since Harald zur Hausen’s pioneering identification of hrHPV types 16 and 18, found in ~50% and ~20% of cervical cancers respectively, two prophylactic HPV vaccines containing virus-like particles (VLP) of each genotype have been widely licensed. These vaccines are beginning to impact infection and HPV-associated neoplasia rates after immunization campaigns in adolescents. Here we review recent progress and opportunities to better prevent HPV-associated cancers, including: broadening immune-protection to cover all hrHPV types, reducing the cost of HPV vaccines especially for developing countries that have the highest rates of cervical cancer, and immune-based treatment of established HPV infections. Screening based upon George Papanicolaou’s cervical cytology testing, and more recently detection of hrHPV DNA/RNA, followed by ablative treatment of high grade cervical intraepithelial neoplasia (CIN2/3) have substantially reduced cervical cancer rates, and we examine their interplay with immune-based modalities for the prevention and eventual elimination of cervical cancer and other HPV-related malignancies.
Introduction

Cervical cancer remains the third leading cause of cancer in women worldwide and virtually all cases can be attributed to infection via one of fifteen high risk HPVs (hrHPV) (1). Importantly, significant fractions of other anogenital malignancies (vaginal, vulval, anal and penile) as well as oropharyngeal and oral cancers (2) are also caused by HPV infection, predominantly by the HPV16 genotype. Infections with hrHPV and their associated neoplasia remain highly prevalent and although there are effective screening strategies available for detection of HPV infection at the cervix, importantly, HPV screening strategies are not routinely applied to other anatomic sites.

Since 16-18% of cancer cases globally are caused by infections annually (3) and preventive vaccination against infectious agents ranks among the most cost-effective of medical interventions, the concept of ‘cancer immuno-prevention’ offers enormous promise to improve human health. Indeed, this promise is being realized with the introduction of preventive vaccines against Hepatitis B (HBV) vaccine and Human Papillomavirus (HPV) vaccines, and complements both cervical cytology screening efforts and chemoprevention via drug treatments for Helicobacter pylori, Hepatitis C, and HIV (1). While prevention of liver cancers linked to HBV via vaccination has already been demonstrated (4-6), it is too early to see the impact of HPV vaccination on cancer incidence although HPV disease rates are clearly dropping (7).

Unfortunately, HPV vaccination uptake has been slow in some developed and many developing countries. The current high cost of the vaccine and need for administration to adolescents have hindered widespread introduction. Motivation for uptake among the general population has also been complicated by perceived concerns for safety, potential for increased promiscuity, lack of efficacy and need, but with continued
scientific reporting of the benefits and safety of HPV vaccination, it is hopeful that these concerns will be resolved and implementation will improve.

In this review, we will discuss the potential of immunization for both prevention and treatment of HPV disease as primary and secondary cancer prevention strategies. Due to space limitations, it is not possible to cover these areas comprehensively and our intent is only to provide a concise summary of the most recent advances of over a century of progress (Figure 1) that address immune-prevention of HPV malignancies and how they could interface with current cervical cancer prevention efforts.

Etiology of HPV and the early successes of HPV vaccination

Over 100 different HPV genotypes have been fully sequenced and can be generally divided into cutaneous and mucosal types. While most infections are benign, those caused by a subset (approximately 15) of the mucosal HPV types can progress to malignancy and are considered ‘high risk’ (hrHPV). The hrHPV are the primary etiologic agent of almost all cervical cancers (8-12). HPV16 (13) and HPV18 (14) are the most studied HR types as they cause ~70% of all cervical cancers (15). More recently, the link between mucosal HPV and cancers has been expanded to certain subsets of anal, vaginal, vulval, penile and oropharyngeal cancers (9). HPV16 is also the cause of approximately 90% of HPV-associated cancers at these non-cervical sites (9). The currently licensed HPV vaccines, Gardasil® (Merck & Co) and Cervarix® (GSK), are based on the major capsid protein L1 which has self-assembled into non-infectious virus-like particles (VLP) (16, 17) and both target HPV16 and HPV18. While the mucosal hrHPV types have received the most attention, the ‘low risk’ mucosal HPV types also produce disease with considerable morbidity, including recalcitrant anogenital warts, and life-threatening laryngeal papillomas (e.g. HPV6 and 11). Gardasil® also prevents
infection and disease associated with the two most prevalent types in benign genital warts (90%), HPV6 and HPV11.

Randomized controlled trials for both Gardasil® (FUTURE trials) (18-22) and Cervarix® (PATRICIA and the Costa Rica HPV vaccine trial, CVT) (23-25) examined three immunizations in young women. The vaccines demonstrated high immunogenicity, excellent safety profiles and showed efficacy in preventing incident vaccine-related HPV infection as well as incident persistent infection related to vaccine HPV types. Several countries have adopted national HPV immunization programs which have begun to bear fruit. For example, in 2007 Australia was one of the first countries to adopt such a campaign with Gardasil® and a significant decline (<1% of women versus 10.5% prior to introduction of vaccines in 2006) in genital wart diagnoses in women (26, 27) as well as reduced cervical abnormalities in teenage girls (28) has been reported. Significant declines in the reporting of genital warts in men were also observed although this finding was attributed to herd-immunity rather than direct vaccination (29).

**Improving access to current vaccines**

When the HPV vaccine was first introduced, the duration of immunity was also unknown. Therefore pre-teens were considered the optimal population for immunization given the importance of vaccination prior to sexual debut (7). The targeting of 9-26 year old patients, especially young adolescents however has complicated vaccination because it is infants that traditionally receive the majority of vaccinations and hence, compliance towards 3 doses has been an outstanding issue. In light of this, it is reasonable to consider exploring the safety and immunogenicity of HPV vaccines in infants. Alternatively, co-administration of current HPV vaccines with other childhood combination vaccines against multiple infectious agents should also be considered since
this potentially reduces the costs of vaccine administration. Indeed, some HPV co-
immunization studies thus far have shown that antibody responses from either vaccine
to be non-inferior to when administered alone (30-33).

The cost of the two commercial vaccines has also limited vaccine uptake, especially in
the developing world where >80% of cervical cancer cases occur. The vaccines were
introduced at $120 dose, i.e. $360 total, although recent GAVI pricing of $4.50/dose for
developing countries has been negotiated and is hopefully sustainable (34). On a
national scale, the costs of HPV vaccination provide a significant barrier to mass
vaccination programs especially as they are born in addition to ongoing cytologic
screening programs. It is clearly important to understand how completion of an HPV
vaccination regimen impacts a patient’s need for cytologic and/or HPV screening.
Presently, because the vaccines target only 2/15 oncogenic types, cervical cytology
screening continues. However, since HPV vaccination reduces the incidence of high
grade CIN, the predictive value and cost-effectiveness of cytologic screening will drop in
this population. This issue might potentially be addressed by implementing screening via
HPV testing and increasing the screening interval.

Several studies are examining whether fewer doses (2 doses versus 3) can be
administered with acceptable protective efficacy and duration (35-41). Although more
independent studies are required to ensure sustained efficacy and examine cross-
protection of related types, the data thus far are supportive of a two dose regimen. Such
studies have also been examined by health authorities such as the WHO SAGE
(Strategic Advisory Group of Experts on immunization) and the overall conclusions were
that a 2-dose prime-boost schedule within an interval of 6 months is non-inferior to the
standard 3-dose schedule (42). However it is important to note that there were some
findings suggesting poorer clinical efficacy with 2 doses which were attributed to a failure in controlling the intervals between the prime and booster immunization. WHO SAGE recommends adolescents within 9-13 years range for the 2-dose regimen and that the interval between the prime and booster shot needs ≤6 months or else a third boost will be required. As a result of this, there is now considerable interest in the efficacy of a single dose vaccination. Remarkably, in one recent study, a subset of patients that received only one dose of Cervarix® still exhibited detectable antibody responses after four years and no evidence of breakthrough infection (43). While these findings must be interpreted cautiously, they suggest that a trial to specifically address the efficacy of a single dose may be warranted.

**Protection against more genotypes**

The current HPV vaccines are not approved for protection against the non-vaccine hrHPV types that can also cause cervical cancer and indeed are currently responsible for ~30% of all cases (44). Further analysis from both pre-clinical and the HPV vaccine trials showed cross-protection against hrHPV types that are not directly targeted by the current HPV vaccines was observed but was generally partial, limited to a few genotypes and is of unclear duration (45-47). Therefore, it remains an important goal to extend protection to all hrHPV types without substantially driving up cost of immunization.

Broad protection is especially important for immune compromised individuals who suffer more disease associated with HPV types beyond those targeted by the current vaccines. HIV+ patients have much higher rates of multi-type infections and an increased risk for HPV-associated cancers (e.g. anal cancer in men) (48) despite the introduction of HAART therapy. Linkage studies have also shown a 2-22 fold increase incidence of cervical cancer in HIV+ women compared to HIV- women (49). While B cell responses
are somewhat compromised in HIV+ patients and solid organ transplant recipients (SOTR), vaccination studies in other infectious diseases show they are still capable of generating effective neutralizing antibody responses (50-52). In light of this, several trials are ongoing to evaluate if this is also true for the current HPV vaccines (reviewed in (52)). In one recently completed trial (53), all HIV-positive women (HAART naïve) were seropositive for HPV16 and 18 antibodies following vaccination with the bi-valent vaccine although titers were overall lower than the healthy control group. The safety profile was also consistent with clinical experience with healthy women. It is worth emphasizing however that certain meta-analysis and population studies indicate that HPV16 infection is under represented in HIV+ patients, suggesting these populations acquire more distinct HPV genotypes or they experience more multi-type infections. While this further complicates current HPV vaccine uptake as well as policy making decision since not all types are targeted by the current HPV vaccines, the findings also support the efforts to make a more broadly protective HPV vaccine.

An interesting avenue for exploration is targeting of the *epidermodysplasia verruciformis* (EV)-associated ~30 cutaneous types which have been proposed as a co-factor along with UV radiation in the development of non-melanoma skin cancers (NMSC) (54, 55). EV is a rare inherited condition that predisposes patients to widespread cutaneous warts and squamous cell carcinoma predominantly caused by HPV5 and HPV8. Both HIV and SOTRs have a higher risk of developing warts, keratotic skin lesions (e.g. actinic keratosis) and NMSC in association with EV type HPV infections (56, 57). However, the etiologic link to NMSC remains controversial and there are concerns over the timing of infection which appears to occur throughout the lifespan. Recent studies in the mouse system nevertheless are encouraging and vaccination offers an approach to test for an etiologic role for EV type HPV in NMSC in these populations. Taken together, it is now
clear that the field of HPV malignancy prevention via vaccination needs to address more types of HPV (i.e. not just HPV16 and 18).

**Second generation HPV vaccines**

The need for broader protection against cancer-related HPV has spurred the efforts of many laboratories to develop next generation vaccine candidates. Merck & Co, the manufacturers of Gardasil, has created a nonavalent vaccine (V503, targeting the 7 most common oncogenic HPV types in cervical cancer and genital wart types HPV6 and 11) which is now in advanced phase 3 clinical trials (NCT00543543, NCT00943722 and NCT01651949). Preliminary results in a 3-dose vaccine regimen report that the immune responses (with respect to HPV6, 11, 16 and 18) from V503 are non-inferior to Gardasil (58). Additionally, the nonavalent vaccine prevents approximately 97% of high-grade cervical, vulvar and vaginal diseases caused by 5 additional oncogenic types (59). However, the cost of manufacturing this vaccine (and hence it's subsequent pricing) is not expected to be cheaper than the current vaccines and this price will likely remain as a limitation towards global implementation.

In light of this, several groups have attempted to simplify the manufacturing process with alternative L1-VLP eukaryotic expression systems such as tobacco plants, yeast or modified insect cells that secrete the VLPs, which together with local low cost manufacturing of a generic product may drive down costs (Reviewed in (60)). Delivery of L1 VLP via live recombinant vectors such a *S. typhi*, measles virus or adenovirus also have great potential because this potentially offers simpler manufacturing, no need for needles/syringes and potentially fewer doses, but enthusiasm is tempered by potential safety concerns with a live vaccine and pre-existing immunity to the vector. In bacterial production systems VLPs are not readily formed; rather the expression of L1 proteins
results in VLP sub-units known as pentamers or capsomeres (61, 62). The neutralizing epitopes of VLP are preserved in capsomeres, and vaccination with capsomeres also induces strong neutralizing antibody responses in animals although lower than for L1-VLP (63). However, the use of an appropriate adjuvant with capsomeres can achieve neutralizing titers similar to VLPs (64). Vaccination with L1-expression vectors is another potential approach but efficient in vivo delivery and avoiding interference remain significant hurdles.

Given the importance of broad immunity covering all hrHPV, several research groups including ours have investigated the HPV minor capsid protein L2 as a candidate antigen for second generation HPV vaccines. Studies with L2 have shown that the N-terminus contains several highly conserved protective epitopes and is required for several events during the infectious life cycle (Reviewed in (65)). Importantly, L2 vaccination induces antibodies that can cross neutralize a large diverse range of HPV genotypes both in vitro and in vivo (66-69). Moreover because L2’s epitopes are linear, they can be readily expressed in E. coli. Unfortunately, co-expression of L1/L2 VLPs results in an L1-response only suggesting L2 is immune-subdominant in the context of a HPV virion (70, 71). To further complicate matters, vaccination of L2 alone although broadly neutralizing is still not as immunogenic as the L1-VLPs even with the use of a potent adjuvant. To boost immunogenicity, several groups have attempted several methods including the concatenation of multiple L2 epitopes (72), using scaffolds (73) and alternative display methods of L2 on VLP platforms of papillomavirus (74, 75) or other viruses (76). While some of these strategies have yielded higher immune responses, the overall responses were still below those of the current VLP, and it is not known if immune titers will be as long lasting as the current vaccine. The apparently weak response to L2 may reflect in part the use of an in vitro neutralization assay with poor sensitivity to L2-specific
protective antibodies. Nevertheless, it appears that low titers of neutralizing serum antibody are sufficient to confer robust protection in pre-clinical animal models, suggesting that it may not be necessary to achieve similar titers as L1 VLP for durable immunity.

**Therapeutic HPV vaccines and new immunologic considerations**

Since HPV is a very common sexually transmitted infection, there remains an urgent need for a therapy to effectively treat existing chronic HPV infections and disease (Figure 2). No therapeutic activity has been demonstrated for the licensed HPV vaccines likely reflecting the absence of detectable L1 expression in HPV-transformed tumor cells or basal keratinocytes that harbor the infection. HPV viral oncoproteins E6 and E7 are required for the induction and maintenance of cellular transformation(77), and are consistently and specifically co-expressed in all infected cells including HPV-associated cancers(78, 79). While the targeting of other viral antigens like E1, E2 and E5 with vaccination is effective for therapy in animal models of disease, it is likely that tumor cells could escape immune responses by loss or down regulation of E1, E2 and E5 expression, but this is not possible for E6 or E7. Therefore, most therapeutic HPV vaccines being tested clinically target HPV E6 and/or E7.

It is clear from natural history studies that most immune competent persons eventually clear HPV infections and low grade intraepithelial lesions, such that the virus becomes undetectable and the cervical histopathology returns to normal (Figure 2). In contrast, the rate of spontaneous clearance is much lower in immune compromised patients, suggesting that infection elicits a delayed but eventually effective anti-HPV immune response in the majority of patients with an intact immune system (80, 81). In fact, a subset of high grade intraepithelial lesions does undergo complete regression (80),
which is presumably immunologically mediated. While immunotherapies should aim to enhance such anti-viral immunity in those unable to clear HPV naturally, to date, no algorithms exist that can distinguish persons at risk for either high grade dysplasias or invasive disease. Therefore, the standard of care for high grade intraepithelial neoplasia is resection. In a landmark therapeutic clinical trial by Kenter et al (82) wherein HPV16+ VIN3 patients were vaccinated with synthetic overlapping long peptides (SLP vaccines) covering HPV-16 E6 and E7, 9 out of 19 patients (47%) exhibited complete regression of the disease and HPV16 specific T-cell responses were detected. Although the regression rate was less than 50%, these findings suggest that it is possible to treat HPV-specific disease and induce complete regression via a vaccine-induced T-cell response.

Unfortunately, monitoring of E6/E7-specific cellular immune responses has proven more complex than for the antibody responses to VLP vaccines ((83-87) and reviewed in (88) and (89)). To date, trials using a plethora of therapeutic vaccine platforms have elicited E6- or E7- specific cellular immune responses in the peripheral blood that were weak and often did not infiltrate the tumor regions. More importantly, even if HPV antigen-specific T-cell responses were detected in the peripheral blood, these responses did not always correlate with clinical response. Recently, Maldonado et al (90) suggest that T-cell responses are sequestered in the lesion micro-environment, at the site of antigen. In patients with HPV16+ CIN2/3 primed twice with a DNA vaccine and boosted with a recombinant vaccinia eight weeks prior to a standard therapeutic resection, they found that in subjects that had residual disease, clonally expanded, proliferating effector immune responses that were organized in lymphoid aggregates were localized in lesional mucosa. Intraepithelial CD8+ infiltrates were increased compared to pre-vaccination, and these infiltrates were associated with histologic features of apoptosis in
dysplastic epithelial cells. Vaccinated subjects who had shared HLA alleles had shared T cell receptors (TCRs) in tissue T cells. The frequencies of these TCRs were variable in the peripheral blood, suggesting that the tissue responses were the result of a process of selection, as opposed to transudate. These observations raise the question of whether earlier vaccine studies in which HPV-specific T-cell responses in the blood were very weak or not detectable may have elicited local responses that were not measured.

In the heterologous prime-boost vaccination study, within-subject comparisons of tissue samples obtained before and after vaccination suggested that previous studies may have been, in effect, censoring histologic endpoints. Because pre-invasive HPV lesions are clinically indolent, and directly accessible, they present an opportunity to better understand mechanisms of disease clearance, and tissue-localized obstacles to clearance. Based on earlier studies of tissue predictors of clinical outcomes in unvaccinated CIN2/3 lesions that demonstrated down-regulated expression of adhesion molecules in the neovasculature associated with persistent lesions (91), clinical testing of peripheral vaccination with the heterologous prime-boost regimen, in concert with direct manipulation of the lesion microenvironment with topical TLR agonist is ongoing (NCT00788164). Clearly there is much to be learnt about the mechanisms of targeting vaccination responses to the relevant site (92), and that it is beneficial to monitor cellular immune responses systemically and at the site of infection (93).

The negative influence of the local tumor microenvironment on clinical responses to therapeutic vaccination is also becoming more recognized. Much work is currently focused on using different strategies to alter the local microenvironment to enhance immune-surveillance against tumors (Reviewed in (94) and (95)). For example, the local application of the topical immune modulator imiquimod can alter the local microenvironment via activation of innate (TLR7) signaling and foster an effective
immune response. Such local inflammatory responses likely enhance targeting to the relevant site and overcome local immune suppressive responses. Indeed, topical imiquimod treatment is partially effective against genital warts, cervical and vulvar neoplasia (96, 97). However, as the HPV disease enlarges and progresses, the effects of imiquimod become limited (98). This situation may require additional and systemic treatments to overcome more profound immune suppression in cancer such as combination therapy of HPV therapeutic vaccination with either chemotherapeutic agents or with low doses of radiation. Indeed, in pre-clinical models, conventional cytotoxic therapies either prime or enhance pre-existing or HPV therapeutic vaccination induced anti-tumor specific immune responses (99-101), but additional work to determine the optimal combinations and timing of administration is needed.

Several factors also limit the action of HPV-specific cytotoxic T cells on infected cells. Immune suppressive cells such as the T-regulatory cells (T-regs) or Tumor-associated macrophages (TAMs) (102, 103) are also important contributors immune-suppression locally in the tumor-microenvironment. Studies in cervical cancer also indicate a central role for CD4+ T-regulatory cells in immune evasion (86, 103). Currently, there is also much interest in using monoclonal antibody-based systemic therapies that target co-inhibitory receptors such as CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) or PD-1 (Programmed Cell-Death protein 1) on CTLs. Recent studies show the use of anti-CTLA-4 antibodies (e.g ipilimumab or tremelimumab) in clinical trials in several cancers achieved complete clinical responses in some cases associated with the reactivation and infiltration of CTLs into the tumor bed. These findings in turn suggest the potential use of CTLA-4 inhibition in combination with HPV therapeutic vaccination to treat HPV+ cancer.
A second potential target is the recent discovery of PD-1, another inhibitory member of the CD28/CTLA-4 family of co-receptors which is expressed not only in T-cells but other immune cells such as B-cells, macrophages and even NK-cells. Importantly, PD-1 ligands have been shown to be more highly expressed in tonsillar crypts as well as in both HPV-associated head and neck squamous cell cancer (HPV-HNSCC) tumor-associated macrophages and tumor cells. Concurrently, it was found that the majority of the CD8+ tumor-infiltrating CTLs had high expression of PD-1. Taken together, this provides evidence for the PD-1/PD-L1 pathway in maintaining an immune-suppressive tumor microenvironment and provides a rationale for blocking this PD-1/PD-L1 pathway to improve the immune response against HPV-HNSCC (104, 105). Surprisingly, however, most cervical cancers are PD-L1 negative (106), implying differences in the immune microenvironment of cervical cancer and HPV-HNSCC.

End points

In HPV vaccine studies, protection against or clearance of high grade CIN (CIN2/3) is typically a primary end point since it is the recognized precursor lesion of cervical cancer (Figure 2). However, because of the diagnostic variability for CIN2, many therapeutic studies now focus on CIN3 only to test clinical activity. Therapeutic effects of a vaccine are often delayed because of the time needed to develop an effective immune response as opposed to the more direct action of a small molecule. Therefore it is critical in such studies to follow the end points over a sufficient period to capture the effects of vaccination. However, the safety of a delay in treatment of CIN2/3 with LEEP must be carefully considered because of the risk, albeit low over a limited period, of progression. Indeed, a follow-up of 19 weeks has been safely used, and delays of 9 months for LEEP treatment are taken in routine clinical practice for newly pregnant women with CIN2/3. Care must also be taken when interpreting an effective vaccine response since CIN2/3
patients exhibit significant rates of spontaneous regression (~25%) and inflammation triggered by biopsy may influence responses (80). An alternative disease upon which to test HPV therapeutic vaccines is VIN2/3 given its lower regression rate (~1.2%) (107, 108), although this is much less prevalent than CIN2/3. Indeed the low rate of spontaneous regression was used to support the absence of a control group in the landmark HPV16 E6/E7 SLP clinical trial (82).

Similarly, much thought is needed in deciding the end points as well as what constitutes ‘efficacy’ of current prophylactic vaccines. These regulatory and policy-making decisions profoundly affect vaccine development and uptake ((109), Reviewed in (110)). The use of CIN2/3 as the endpoint for the qualification of second generation preventive HPV vaccines will greatly increase trial sizes over the use of persistent HPV DNA detection as an endpoint, and may impede their development and drive up cost of bio-similar vaccines. This issue is particularly important for vaccines intended to prevent HPV+ head and neck cancer because there is currently no precursor lesion and screening protocol defined, and it is neither feasible nor ethical to use cancer as an endpoint. However, robust protocols exist for detection of persistent oral hrHPV infection and warrant serious consideration as an endpoint.

The use of immunologic endpoints such as L1 VLP ELISA or in vitro neutralization titers in serum might also be considered for non-inferiority studies of biosimilar L1 VLP vaccines, although the correlate of protection has yet to be properly defined. Fortunately there has been minimal, if any evidence of breakthrough infection by vaccine types in appropriately immunized patients. However, this renders the determination of a minimal neutralizing titer associated with protection very difficult, although one possibility is to use the infection by non-vaccine types for which protection is partial and compare serum
cross-neutralizing titers in these patients with those who are not infected. It will also be important to understand the role of memory B cells and the recall response in long term protection (i.e. is there sufficient time for the inoculum to elicit a rapid and local antibody response if the local protective antibody level has waned below that required for sterilizing immunity), as this has also been suggested as an important factor. The measurement of relevant immune correlates for therapeutic HPV vaccines is much more controversial especially given the greater technical complexity of the assays, the diversity of effector cells, the importance of targeting of the anti-viral responses to the lesion site and the potential of an immune suppressive local environment. These issues suggest the importance of monitoring the response locally, which creates significant technical hurdles over measurement of systemic immune responses in blood.

The need for broad protection is becoming increasingly clear in light of differing prevalence of certain key hrHPV in different populations, notably HPV52 and 58, although HPV16 and 18 are the dominant types in cervical cancer worldwide. While there is not clear evidence for competition between types and most mathematical modeling studies suggest genotype replacement is unlikely, one study has recently reported an increase in the prevalence of non-vaccine HPV types after vaccination (111). As there is insufficient data currently to further substantiate such findings (112), long term follow-ups of vaccinated populations will be required to answer such questions. However, the development of highly multivalent L1 VLP or other L2-based second generation HPV vaccines will further reduce concern for such issues.

**Screening for HPV-associated malignancies in the era of HPV vaccination**

In considering the impact of HPV vaccination, it is critical to address how it is best integrated with Pap screening. Vaccination with L1 VLP will not render cervical
screening redundant in the near term as it has no therapeutic effect. In addition, screening is not recommended for women >26 years (based on the assumption that they have had an active sexual history), and vaccination is recommended for 9-26 year olds, i.e., many older women have not benefitted from HPV vaccination. Furthermore, because of the limited hrHPV type specificity of the licensed vaccines, screening will still detect disease associated with non-vaccine types. However, these screening programs must be re-evaluated since the predictive value and cost-effectiveness of screening will be significantly lower in vaccinated women (113) (Reviewed in (114)), and dramatically so with the advent of the nonavalent vaccine.

To begin to address such issues, it has been proposed that primary screening be done via HPV DNA testing first followed by Pap cytology triage (Reviewed in (115), (116)). These HPV DNA detection assays are more sensitive for disease as compared to PAP screening; however, in terms of determining true disease, the assays are at least 10% less specific than the Pap smear(117). Nonetheless, cohort studies have subsequently shown that there is actually minimal over-diagnosis when HPV DNA testing is used as the sole modality with cytology reserved for triage of HPV-positive women with increased screening intervals (e.g. once every 3 years)(118, 119). Indeed, the US FDA has recently approved the Roche cobas HPV test methodology as the first HPV DNA test for primary cervical cancer screening (there are current 4 FDA-approved assays but only 1 is approved for primary screening). The cobas® test specifically identifies HPV 16 and HPV 18, while concurrently detecting 12 other types of high-risk HPVs. It will be up to professional medical societies and organizations to determine how this HPV DNA test will be incorporated into current screening protocols. Logically, DNA testing combined with cytology will probably the best regimen for developed countries with robust healthcare infrastructure, and might be used to trigger treatment with a therapeutic HPV
vaccine. Many resource-limited developing countries have adopted visual inspection by acetic acid (VIA)-only programs as it is a cheaper and immediate point-of-care approach compared to delayed HPV DNA/Pap testing in a central laboratory, although clearly less predictive and potentially problematic to combine with an immunotherapy without knowledge of the hrHPV genotype (assuming a type-specific immunotherapy).

In contrast to the cervix, measures to detect and screen for HPV-associated oropharynx cancers have been lacking due to a lack of well-defined precursor lesion (Reviewed in (120)). While primary prevention via vaccination should prove promising in the long term (121), there is currently no secondary prevention option. Recently, a strong association between HPV16 E6 serum antibodies and HPV-associated oropharynx cancers was reported (122). Furthermore, these HPV16 E6 antibodies were present in a notable proportion of patients with HPV-associated oropharyngeal cancers for >10 years before diagnosis, but this approach currently lacks sufficient predictive value alone. HPV DNA testing in oral cavity specimens (123) is a particularly promising approach for screening, although more work is needed to understand the predictive value of this bio-marker testing strategy for HPV+ HNSCC and its precursors. HPV malignancies of the oropharynx are predominantly due to a single genotype, HPV16, and thus an immunotherapeutic approach focused on HPV16 T-cell based vaccines could be the primary goal for immunotherapeutic control of these infections, particularly in those who have seroconverted to HPV16 E6 positivity.

Concluding remarks

The finding that hrHPV infection is necessary although insufficient for the development of cervical cancer has driven tremendous advances in cancer immunoprevention, including two licensed prophylactic vaccines (Figure 1). We anticipate further significant
advances in broadening protection to all hrHPV, lowering the number of doses and cost of HPV vaccination and eliciting therapeutic immunity. Such developments in immunotherapy of HPV will complement major advances in screening, notably HPV DNA, RNA and possibly oncoprotein testing as a first line screening modality in the cervix and also possibly at non-cervical sites. The advent of such opportunities will require significant changes in health policies for best implementation and realization of their potential to eliminate HPV-related cancer and drive down costs so that all may benefit.

FIGURE LEGENDS

Figure 1. A summary of milestones over a century of human papillomavirus research and implementation of measures to prevent cervical cancer.

Figure 2. A diagram summarizing the stepwise progression from HPV infection of the cervical transformation zone to cervical cancer and the opportunities for intervention.

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George Papanicolaou develops the "Pap smear".

HPV DNA and virus particles (Koilicytotic atypia) found in abnormal cervical smears.

Active HPV genes (E6/E7) identified and transforming properties described.

IARC declares HPV16 and HPV18 as group 1 carcinogens (e.g., oncogenic) for cancers of the cervix.

A HPV16 VLP vaccine tested in humans demonstrates safety and high immunogenicity.

FDA approves HPV vaccines for young women and subsequently boys.

Phase III studies of 2- and 4-valent HPV L1-VLP vaccines show high safety, immunogenicity and efficacy against vaccine-related HPV types.

Testing of 9-valent L1 VLP vaccine.

1920: Pap smear introduced into clinics for cervical cancer screening.

1940: Harald Zur Hausen discovers HPV16 and HPV18 DNA in cervical cancers.

1970: Discovery of Virus-like Particle (VLP) formation by L1 capsid protein.


1990: Hybrid Capture 2 HPV test as an adjunct to Pap testing.

2000: FDA approves the Hybrid Capture 2 HPV test as an adjunct to Pap testing.

2010: FDA approves the use of an HPV Test as a primary screening procedure prompting re-evaluation of clinical algorithms.

2014: Imiquimod used to treat VIN patients.


Legend:

- **1920**: Pap smear introduced into clinics for cervical cancer screening.
- **1940**: Harald Zur Hausen discovers HPV16 and HPV18 DNA in cervical cancers.
- **1970**: Discovery of Virus-like Particle (VLP) formation by L1 capsid protein.
- **1980**: Oral HPV infection strongly associated with oropharyngeal cancers.
- **1990**: Hybrid Capture 2 HPV test as an adjunct to Pap testing.
- **2000**: FDA approves the Hybrid Capture 2 HPV test as an adjunct to Pap testing.
- **2010**: FDA approves the use of an HPV Test as a primary screening procedure prompting re-evaluation of clinical algorithms.
- **2014**: Imiquimod used to treat VIN patients.
- **2014**: HPV therapeutic vaccine trial using overlapping HPV16+ E6/E7 peptides induces a T-cell response and associated lesion regression in VIN patients.
Fig. 2

Primary Immuno-Prevention: Prophylactic HPV vaccination

Secondary Prevention: HPV/cytologic Screening

Conventional therapy: Surgery/Radiation/Chemotherapy

Neutralizing antibodies

Treatment of high grade CIN by LEEP

Normal Cervical Transformation Zone

HPV Infection

Progression of productive lesion CIN1 (left) to cervical cancer precursor, CIN2/3 (right)

Invasion

Metastasis

Invasive HPV+ Cancer

Immune modulators e.g. Imiquimod

Secondary Immuno-prevention

Immunotherapy

Therapeutic vaccines that induce HPV-specific T cells
Cancer Prevention Research

Immunoprevention of human papillomavirus-associated malignancies


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