

Immunoprevention of Human Papillomavirus-Associated Malignancies

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Abstract

Persistent infection by one of 15 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 approximately 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 affect 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. *Cancer Prev Res*; 8(2); 95–104. ©2014 AACR.

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 15 high-risk HPVs (hrHPV; ref. 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.

Because 16% to 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 immunoprevention" 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). Although 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 discuss the potential of immunization for both prevention and treatment of HPV disease as primary and secondary cancer prevention strategies. Because of 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 (Fig. 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

More than 100 different HPV genotypes have been fully sequenced and can be generally divided into cutaneous and mucosal types. Although 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 approximately 70% of all cervical cancers (15). More recently, the link between mucosal HPV and cancers has been expanded to

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doi: 10.1158/1940-6207.CAPR-14-0311

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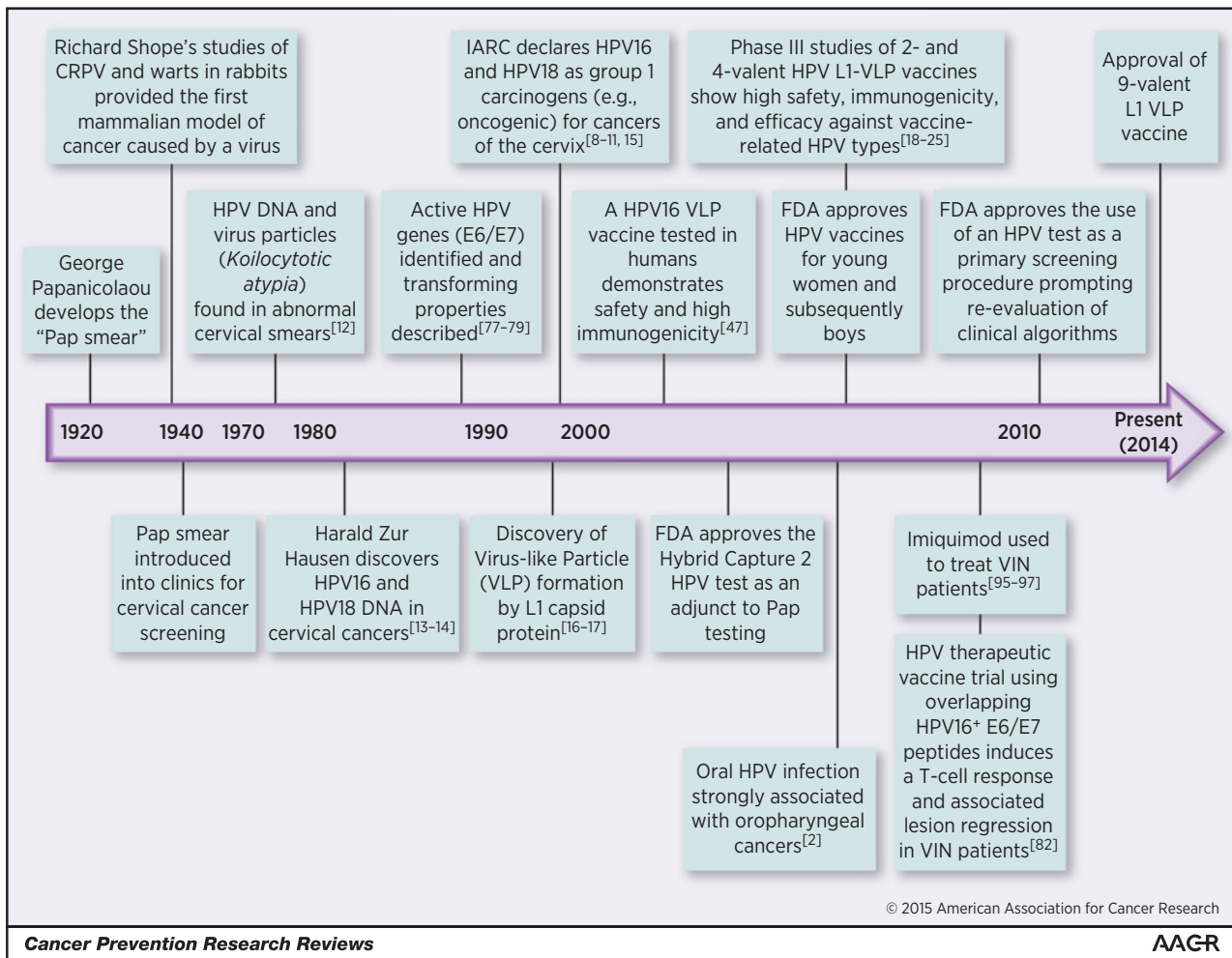


Figure 1. A summary of milestones over a century of human papillomavirus research and implementation of measures to prevent cervical cancer.

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 noncervical sites (9). The currently licensed HPV vaccines, Gardasil (Merck & Co.) and Cervarix (GlaxoSmithKline), are based on the major capsid protein L1, which has self-assembled into noninfectious virus-like particles (VLP; refs. 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; refs. 18–22) and Cervarix (PATRICIA and the Costa Rica HPV vaccine trial, CVT; refs. 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% before 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 before sexual debut (7). The targeting of 9- to 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 toward three 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, coadministration of current HPV vaccines with other childhood combination vaccines against multiple infectious agents should also be

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considered, as this potentially reduces the costs of vaccine administration. Indeed, studies suggest that, upon administration of an HPV vaccine with a second vaccine against another infectious agent, the antibody responses elicited by each are non-inferior to when either vaccine is 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 affects a patient's need for cytologic and/or HPV screening. Presently, because the vaccines target only two of 15 oncogenic types, cervical cytology screening continues. However, because 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 two-dose prime-boost schedule within an interval of 6 months is noninferior to the standard three-dose schedule (42). However, it is important to note that there were some findings suggesting poorer clinical efficacy with two doses that were attributed to a failure in controlling the intervals between the prime and booster immunization. WHO SAGE recommends adolescents within 9 and 13 years range for the two-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 4 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 nonvaccine hrHPV types that can also cause cervical cancer and indeed are currently responsible for approximately 30% of all cases (44). Further analysis from both preclinical and the HPV vaccine trials showed that cross-protection against hrHPV types not directly targeted by the current HPV vaccines, is generally partial, limited to a few genotypes, and of unclear duration (45–47). Therefore, it remains an important goal to extend protection to all hrHPV types without substantially driving up the 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; ref. 48) despite the introduction of the HAART therapy. Linkage studies have also shown a 2- to 22-fold increase in incidence of cervical cancer in HIV⁺ women compared with HIV⁻ women (49). Although 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 ref. 52)). In one recently completed trial (53), all HIV⁺ women (HAART naïve) were seropositive for HPV16 and 18 antibodies following vaccination with the bivalent 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 underrepresented in HIV⁺ patients, suggesting that these populations acquire more distinct HPV genotypes or they experience more multi-type infections. Although this further complicates current HPV vaccine uptake as well as policy making decision as 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 approximately 30 cutaneous types that have been proposed as a cofactor along with UV radiation in the development of nonmelanoma skin cancers (NMSC; refs. 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 seven most common oncogenic HPV types in cervical cancer and genital wart types HPV6 and 11), which has completed advanced phase III clinical trials (NCT00543543, NCT00943722, and NCT01651949). Preliminary results in a three-dose vaccine regimen report that the immune responses (with respect to HPV6, 11, 16, and 18) from V503 are noninferior to Gardasil and it was recently approved by the FDA (<http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm426485.htm>) (58). In addition, the nonavalent vaccine prevents approximately 97% of high-grade cervical, vulvar, and vaginal diseases caused by five additional oncogenic types and the vaccine is called Gardasil 9 (59). However, the cost of manufacturing this vaccine (and hence its subsequent pricing) is not expected to be cheaper than the current vaccines, and this price (estimated at \$162/dose) likely remain as a limitation toward 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 ref. 60). Delivery of L1 VLP via live recombinant vectors such as *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 preexisting immunity to the vector. In bacterial production systems, VLPs are not readily formed; rather, the expression of L1 proteins results in VLP subunits 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 ref. 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, coexpression of L1/L2 VLPs results in an L1-response only suggesting that 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). Although 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 preclinical 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

Because 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 (Fig. 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 coexpressed in all infected cells, including HPV-associated cancers (78, 79). Although 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 downregulation 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 (Fig. 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. Although immunotherapies should aim to enhance such antiviral 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 and colleagues (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 (refs. 83–87; and reviewed in ref. 88 and ref. 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 and colleagues (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 8 weeks before 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 with 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 (TCR) 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

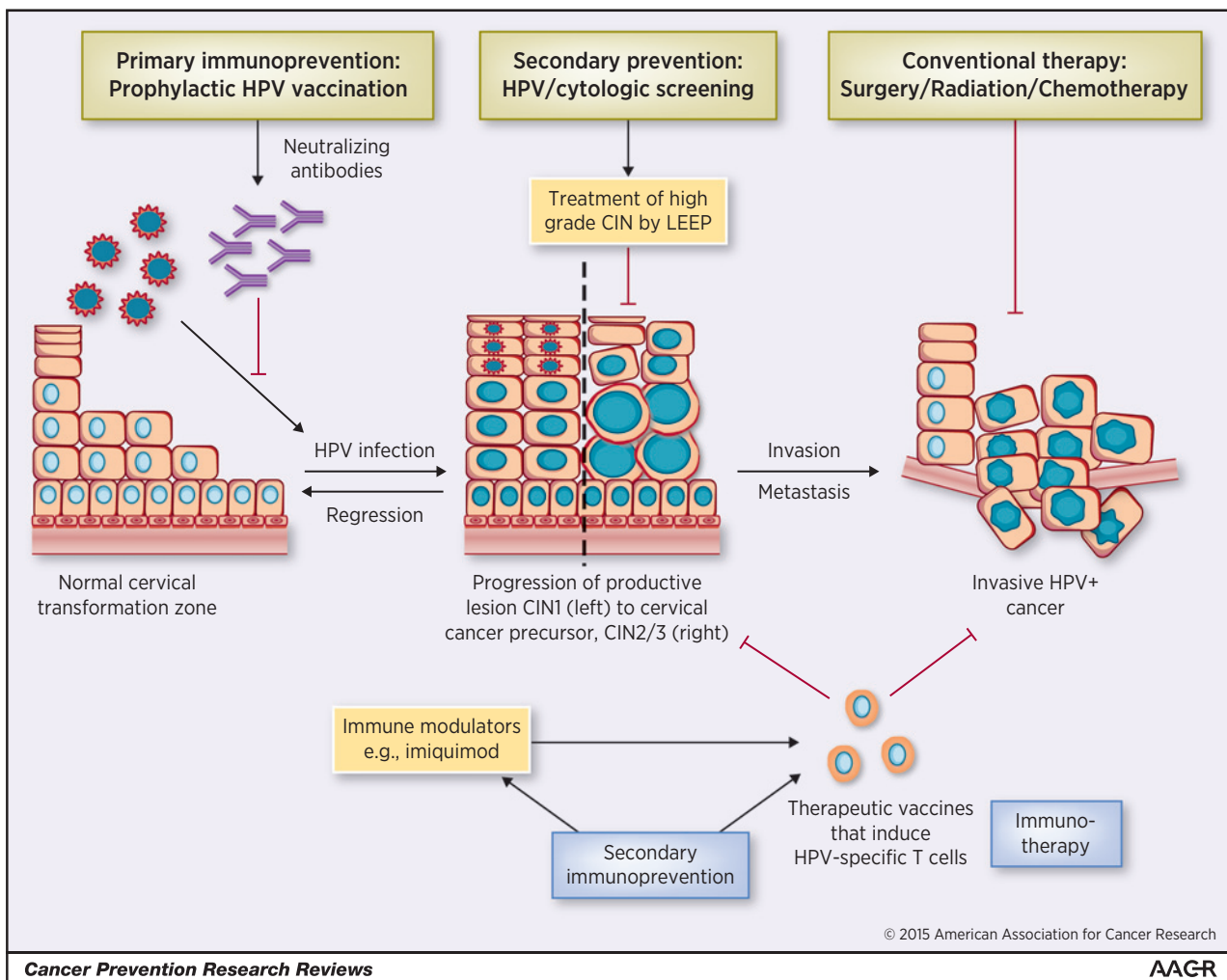


Figure 2.

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

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 preinvasive 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. On the basis of earlier studies of tissue predictors of clinical outcomes in unvaccinated CIN2/3 lesions that demonstrated downregulated 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 (87), and that it is beneficial to monitor cellular immune responses systemically and at the site of infection (92).

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 ref. 93 and ref. 94). 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 (95, 96). However, as the HPV disease enlarges and progresses, the effects of imiquimod become limited (97). 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 preclinical models, conventional cytotoxic therapies either prime or enhance preexisting or HPV therapeutic vaccination induced antitumor-specific immune responses (98–100), 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 (Tregs) or tumor-associated macrophages (TAM; refs. 101, 102) are also important contributors to immune-suppression locally in the tumor-microenvironment. Studies in cervical cancer also indicate a central role for CD4⁺ Tregs in immune evasion (86, 102). Currently, there is also much interest in using monoclonal antibody-based systemic therapies that target coinhibitory receptors such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) or PD-1 (programmed cell-death protein 1) on CTLs. Recent studies show that 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 coreceptors, 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 (103, 104). Surprisingly, however, most cervical cancers are PD-L1-negative (105), implying differences in the immune microenvironment of cervical cancer and HPV-HNSCC.

Endpoints

In HPV vaccine studies, protection against or clearance of high-grade CIN (CIN2/3) is typically a primary endpoint as it is the recognized precursor lesion of cervical cancer (Fig. 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 endpoints 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 as 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%; refs. 106, 107), 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 endpoints as well as what constitutes "efficacy" of current prophylactic vaccines. These regulatory and policy-making decisions profoundly

affect vaccine development and uptake (ref. 108, reviewed in ref. 109). 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 biosimilar 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 noninferiority 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 nonvaccine 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 antiviral 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. Although 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 nonvaccine HPV types after vaccination (110). As there are insufficient data currently to further substantiate such findings (111), 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 ages >26 years (based on the assumption that they have had an active sexual history), and vaccination is recommended for 9 to 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 nonvaccine types. However, these screening programs must be reevaluated as the predictive value and cost-effectiveness of screening will be significantly lower in vaccinated women (112; reviewed in ref. 113), 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 refs. 114, 115). These HPV DNA detection assays are more sensitive for disease as compared with PAP screening; however, in terms of determining true disease, the assays are at least 10% less specific than the Pap smear (116). Nonetheless, cohort studies have subsequently shown that there is actually minimal overdiagnosis 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; refs. 117, 118). Indeed, the U.S. FDA has recently approved the Roche cobas HPV test methodology as the first HPV DNA test for primary cervical cancer screening (there are current four FDA-approved assays but only one 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 be 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 with 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 with 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 ref. 119). While primary prevention via vaccination should prove promising in the long term (120), there is currently no secondary prevention option. Recently, a strong association between HPV16 E6 serum antibodies and HPV-associated oropharynx cancers was reported (121). 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 (122) is a particularly promising approach for screening, although more work is needed to understand the predictive value of this biomarker 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 three licensed prophylactic vaccines (Fig. 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 noncervical 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.

Disclosure of Potential Conflicts of Interest

W.K. Huh is a consultant/advisory board member for Merck and TheraVax. C.L. Trimble is a consultant/advisory board member for Merck. Richard Roden is an inventor of L2-related patents licensed to Shantha Biotechnics Ltd., GlaxoSmithKline, PaxVax, Inc. and Acambis, Inc. Richard Roden has received research funding from Sanofi Pasteur, Shantha Biotechnics and GlaxoSmithKline and is a co-founder of and has an equity ownership interest in Papivax LLC. Richard Roden owns Papivax Biotech Inc. stock options and is a member of Papivax Biotech Inc.'s Scientific Advisory Board. Under a licensing agreement between Papivax Biotech, Inc. and the Johns Hopkins University, Richard Roden is entitled to royalties on technologies described in this review. This arrangement has been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies. No potential conflicts of interest were disclosed by the other authors.

Grant Support

This work was funded by Public Health Service grants P50 CA098252, RO1 CA133749, and CA118790 (<http://grants.nih.gov/>), and the V foundation (<http://www.jimmyv.org/>).

Received September 23, 2014; revised November 6, 2014; accepted December 1, 2014; published OnlineFirst December 8, 2014.

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