Current strategies against persistent human papillomavirus infection (Review)

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Abstract. Human papillomavirus (HPV) is the most common sexually transmitted infection, exhibiting a tropism for the epidermis and mucosae. The link between persistent HPV infection and malignancies involving the anogenital tract as well as the head and neck has been well-established, and it is estimated that HPV-related cancers involving various anatomical sites account for 4.5% of all human cancers. Current prophylactic vaccines against HPV have enabled the prevention of associated malignancies. However, the sizeable population base of current infection in whom prophylactic vaccines are not applicable, certain high-risk HPV types not included in vaccines, and the vast susceptible population in developing countries who do not have access to the costly prophylactic vaccines, put forward an imperative need for effective therapies targeting persistent infection. In this article, the life cycle of HPV, the mechanisms facilitating HPV evasion of recognition and clearance by the host immune system, and the promising therapeutic strategies currently under investigation, particularly antiviral drugs and therapeutic vaccines, are reviewed.

Contents

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1. Introduction

Human papillomaviruses (HPVs) are a family of non-enveloped viruses with cutaneous and mucosal tropism, causing the most common sexually transmitted disease (1). The association of HPV infections, particularly persistent infections, with a series of malignancies has been well-established, exemplified by anogenital (cervical, vulvar, vaginal, penile and anal) cancer, head and neck cancer (oropharyngeal squamous cell carcinoma affecting the tonsils, tonsillar fossa, tongue, base of the tongue and soft palate), non-melanoma skin cancer in patients with epidermodysplasia verruciformis (EV), and malignant progression of recurrent respiratory papillomatosis (2). These malignancies generally account for ~4.5% of all cancers (3), among which cervical cancer is a major concern. It is estimated that ~530,000 new cases and 275,000 deaths from cervical cancer occur annually worldwide, causing a major global disease burden and loss of life years, particularly in developing countries (4-6).

Over the past decades, with the elucidation of the natural history of HPV and HPV-associated diseases, as well as technical progress, effective screening strategies and robust prophylactic vaccines have been developed. As the most groundbreaking scientific discovery in the fight against cervical cancer, prophylactic vaccines have an excellent safety and efficacy profile, conferring type-specific immunity against HPV infection (7). Prophylactic vaccines are virus-like particles (VLP) self-assembled by L1 capsid without viral genome, which trigger neutralizing antibody production, thus blocking the adherence and internalization of HPV by basal cells in the epithelium. These vaccines appear to be a promising approach to decreasing the morbidity and mortality of HPV-associated benign and malignant diseases.

However, despite the prophylactic effect of currently available vaccines, they are not effective in eradicating pre-existing HPV

infection and associated lesions. In addition, these vaccines merely induce immunity specific to certain HPV types, but are unable to fend off other types of the virus; furthermore, their immunization longevity, which is presumably not lifelong, has yet to be evaluated. Finally, the inaccessibility to vaccines and screening programs in resource-poor regions exposes local populations to a high risk of HPV-associated malignancies, which have already been proven to be responsible for a substantial proportion of the worldwide cancer burden. These unresolved issues necessitate screening programs and further exploration of therapeutic modalities for persistent HPV infection and associated lesions.

However, given the fact that most HPV infections that are accompanied by simultaneous epithelial dysplasia undergo spontaneous clearance under immunological surveillance within 1-2 years (8), not all HPV infections require treatment. Therefore, it is advisable to differentiate persistent HPV infection from transient infection through biomarkers or lesion characteristics, which, unfortunately, have not yet been fully elucidated. What is currently known is that higher-grade lesions have a lower probability of spontaneous regression, and the process of oncogenesis, from low-grade squamous intraepithelial lesion (LSIL) through high-grade squamous intraepithelial lesion (HSIL) to invasive cervical cancer (ICC), is consecutive. Hence, a wait-and-watch approach is usually adopted for patients with LSIL to determine whether there is spontaneous regression or progression, while HSIL is mostly treated by physical ablative or surgical modalities (9). Such strategies are practicable, but cannot address the anxiety of patients with LSIL during the long wait, or exclude the possibility of LSIL progression. Furthermore, the currently available therapeutic modalities, primarily surgical treatment, are somewhat destructive and costly, and are characterized by a high recurrence rate, several side effects and complications, restricting their applicability in LSIL management. Therefore, there is a need for non-invasive interventions, such as medications, that are appropriate for both LSIL and HSIL, or even ICC, as well as transformation of the overall concept from treating cancer to treating infection.

The aim of this review article was to discuss the extensive previous and ongoing investigations into antiviral agents, therapeutic vaccines and immunomodulators, along with their respective advantages and drawbacks.

2. HPV-associated diseases

A certain group of diseases were demonstrated to be associated with HPV infection; these may be divided into benign and malignant lesions, according to their prognosis, or into mucosal and cutaneous lesions, according to their primary location. Specifically, mucosal and cutaneous lesions in anogenital sites resulting from HPV infection are classified together into one category due to their similar natural history and etiological relevance. Hence, HPV-associated diseases may be classified as anogenital, aerodigestive and non-genital cutaneous infections.

All HPV-associated diseases share dysplasia of the epithelium as the common pathological characteristic. In particular, dysplasia of the stratified squamous epithelium in anogenital sites is further classified into grade 1, 2 and 3 intraepithelial neoplasia, corresponding to mild, moderate and severe dysplasia, respectively, with grade 3 intraepithelial neoplasia also representing carcinoma *in situ*. The term LISL in cytopathology is equivalent to grade 1 intraepithelial neoplasia and HSIL refers to grade 2 and 3 intraepithelial neoplasia.

HPV infection in anogenital sites. Although most HPV infections in anogenital sites, regardless of the HPV type, result in low-grade dysplasia, which may take the form of a benign condylomatous lesion highly likely to regress spontaneously within 2 years (10), persistent infection with high-risk HPV types has been recognized as a strong carcinogenic factor.

The role of high-risk HPV infection as a prerequisite for cervical cancer development has been well established due to the work of Boshart *et al* (11,12). It is believed that almost all cervical cancer cases are caused by HPV, and that HPV-negative cases were misclassified due to the limitation of testing methods (false-negative) (13). HPV-16 is the most frequent type found in cervical cancer, followed by HPV-18, -45, -31, -33 and other high-risk types (14). HPV-18 is more common in adenocarcinoma compared with squamous cell carcinoma, while adenocarcinoma accounts for ~10% of all cervical cancer cases (15). As regards low-risk HPV types, such as HPV-6 and -11, they are mostly found in low-grade lesions, such as cervical intraepithelial neoplasia (CIN)1, but are rarely found in high-grade lesions (CIN 2, 3 and ICC).

Anal cancer ranks second in terms of correlation with HPV infection. A study in France reported that 97% of the cases of anal cancer are HPV-positive, most of which are HPV-16-positive (16). Similarly, it is estimated that 70% cases of vaginal cancer, 45% cases of penile cancer and 40% cases of vulvar cancer are attributed to HPV, particularly HPV-16 (17). Anal intraepithelial neoplasia (AIN), vaginal intraepithelial neoplasia (VAIN), penile intraepithelial neoplasia (PIN) and vulvar intraepithelial neoplasia (VIN) are deemed as precursors of the respective carcinomas, with a certain risk of progression (18,19).

HPV infection in the aerodigestive tract. Low-risk HPVs, mainly HPV-6 and -11, are more common in the aerodigestive tract; therefore, the majority of the HPV-related aerodigestive tract lesions are benign, such as papilloma of the oral cavity and recurrent respiratory papillomatosis (RRP) of the larynx (20). However, regardless of the low risk, RRP has the potential of spread and progression. Therefore, even 'low-risk' HPVs may progress to cancer.

HPV-16 is the most common high-risk type affecting the aerodigestive tract, and is considered to be associated with a small proportion of oropharyngeal cancers, such as those originating from the tonsils, tonsillar fossa, base of the tongue and soft palate. Of note, the prevalence of HPV-positive oropharyngeal cancers has markedly increased over the past decades (21).

There has always been controversy on the association between HPV infection and esophageal squamous cell carcinoma (ESCC). Numerous studies have attempted to investigate the association between HPV infection and ESCC, but contradictory results were reported. As regards studies detecting HPV DNA in ESCC samples, both negative and positive results have been reported (22-26). However, the mere presence of HPV DNA in ESCC tissues cannot confirm its

etiological role in tumorigenesis; thus, a large international study (interSCOPE) was designed to determine whether there were anti-L1 or anti-E6/E7 antibodies in the serum of ESCC patients, with only 4 samples found positive for HPV-16 E6 and E7 (27). Further evidence demonstrated no detectable level of HPV DNA integration in ESCC samples (28,29), and the status of HPV infection did not affect the prognosis of ESCC (30). These results indicate that HPV may play a less important role in the development of ESCC, but a hit-and-run mechanism may be utilized by HPV to induce ESCC. Large prospective cohort studies with long follow-up are required to draw definitive conclusions on the involvement of HPVs in esophageal carcinogenesis.

HPV infection of non-genital skin. The HPV types involved in cutaneous infection, including HPV-1, -2, -3, -4, -10, -27, -28 and -41, among others, are quite different from those involved in mucosal infection, usually causing various types of warts, such as common, flat and plantar warts (31). While cutaneous HPV infection does not ordinarily cause skin cancer, it may become complicated when there is a genetic background of EV. EV patients are susceptible to HPV infection, particularly HPV-5 and -8, and a certain proportion of EV patients eventually develop skin cancer at the location of primary lesion (32). Therefore, HPV-5 and -8 are considered as possible carcinogens. However, the role of HPVs in non-melanoma skin cancer in the normal population is yet to be fully elucidated.

3. HPV life cycle

HPVs are non-enveloped, double-stranded circular DNA viruses with a genome ~8 kb in size, which consist of three parts: Long control region (LCR), open reading frame (ORF) of six early genes (E1, E2, E4, E5, E6 and E7) and ORF of two late genes (major capsid protein L1 and minor capsid protein L2) (Fig. 1) (33). The viral capsid is an icosahedron composed of 72 pentamers of L1 (360 in total) with variable numbers of L2 buried inside the capsid surface. To date, >170 types of HPV have been identified and they may be roughly divided into cutaneotropic and mucotropic types, while certain types of HPV may be found in both cutaneous and mucosal lesions. Those mucosal HPVs are further subdivided into low-risk and high-risk groups, according to their carcinogenic potency. HPVs only infect the basal keratinocytes of human stratified squamous epithelia, such as skin and mucosae. A microwound of the epithelium is a prerequisite for the transmission procedure, which enables HPVs to reach the basement membrane (BM) and basal keratinocytes (34). Additionally, active cell division stimulated by wound healing response is also considered to be necessary for the infection process (35,36). It has been demonstrated that HPVs first bind to heparan sulfate proteoglycans (HSPGs) (37) on the BM through the L1 capsid protein, which induces subsequent conformation of L2 minor capsid protein to expose its N-terminal, where a furin cleavage site is located (38). Upon furin cleavage, viruses shed from the BM are transferred to the cell surface for secondary binding events mediated by allosteric L1 (39-41), and the RG-1 epitope on L2, which is required for L2-mediated endosomal escape from the late endosomes (42), is exposed. In addition, BM also acts as

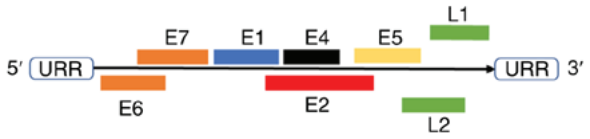

Figure 1. Genome organization of human papillomavirus. URR, upstream regulatory region.

a guidance for HPVs to identify permissive cells, i.e., basal keratinocytes (mitotically active epithelial cells) rather than non-permissive (non-dividing) cells (40). Internalization of the virions follows the secondary binding events, through α6β4 integrins (43-46), tetraspanins CD63 and CD151 (47-49) and other unidentified receptors.

As regards HPVs adhering to the cell surface through syndecans (HSPGs located on the cell membrane), it is also possible that additional components, such as epidermal growth factor (EGF) and keratinocyte growth factor (KGF), are incorporated after initial binding occurs, forming large-molecular-weight complexes. After cleavage by matrix metalloprotease, these complexes are released from the cell membrane and subsequently bind with EGFR/KGFR, which mediates the uptake of the complexes (50,51).

The endocytosed virions are transported by retrograde trafficking sequentially through the endosomal system, where the capsid disassembles and L1 is retained in a degraded form, while L2 remains associated with viral DNA (vDNA), trans Golgi network, endoplasmic reticulum and, finally, into the nucleus during the nuclear envelope breakdown of mitosis (52).

Following the initial infection by high-risk HPVs, the viral genome tethers the cellular genome as episomes undergo transient amplification to extend to ~200 copies per cell, maintaining the viral episome at a low copy number and forming the reservoir of infection (53-55). The life cycle of intracellular viruses is closely associated with the proliferation, differentiation and maturation of keratinocytes, and the expression of viral proteins is likewise highly ordered. In the lower layers of the epithelium, where basal and parabasal cells reside, E6 and E7, referred to as the oncogenic proteins, are expressed to stimulate cell division. E6, targeting p53, mediates its ubiquitination through recruitment of E6AP and proteasome-dependent degradation (56). E7 binds retinoblastoma family proteins and, therefore, releases E2F to activate gene transcription necessary for DNA replication. Thus, coordination of E6 and E7 drives cells to re-enter the cell cycle (35). In addition, E1 helicase is required for viral genome replication, and E2, which is required for transcription activation and repression, recruits E1 at the beginning of replication. Therefore, E1 may be transiently expressed for the aforementioned initial genome amplification, but not for genome maintenance (57); by contrast, E2 is considered to be constitutively expressed for its role in transcription activation. In the middle layers, with the advent of genome amplification, the necessary proteins E1, E2, E4, E5, E6 and E7 increase in abundance. E6 and E7 are still needed, as they allow cells to re-enter the S-phase, which provides the conditions for viral genome replication (58). E5 plays a role similar to those of E6 and E7, but through stabilization of EGFR and enhancement of EGF signaling and mitogen-activated protein kinase activity (59-61).

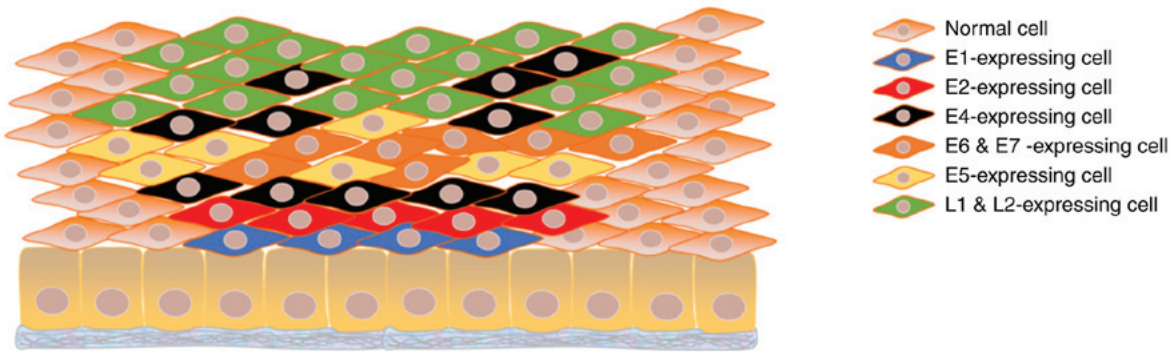

Figure 2. Viral protein expression mode in infected cervical epithelium.

In the upper layers, E4, L1 and L2 are predominantly expressed where packaging of vDNA and assembly of intact virions occur following genome amplification (Fig. 2). The virus is finally released in the superficial layers of stratified epithelium along with the shedding of senescent cells. Apart from virus release, another role of E4 is disintegration of the stratum corneum by formation of amyloid fibers, enabling repetitive infection of HPVs (62-64). On the contrary, this mode is completely changed when lesions progress (to HSIL or ICC), and the definition 'abortive infection' is often used to describe the status where most or all layers of the stratified epithelium are occupied by basal-like cells overexpressing E6 and E7. Viral genome integration is a late event, which deregulates E6 and E7 expression by loss of E2 and is highly associated with invasive lesions (58).

4. Immune evasion

HPVs have long been known to employ multiple tactics to escape recognition and elimination by the human immune system, underlying persistent infection.

The unique life cycle of HPV beyond the dermis keeps it away from immunocompetent cells. The factors contributing to the immune invisibility of HPV-infected keratinocytes include the maintenance of low profile of the viral genome in basal cells, non-secretory proteins, low profile of viral proteins via E2 as transcription repressor and suboptimal codon usage (65,66), and the absence of viremia and cell lysis.

HPVs also interfere with normal immune function through the following mechanisms. A dampened type I interferon (IFN) signaling cascade results from inhibition of TYK2 kinase activity (67) and IFN regulatory factor 3 (IRF3) transactivation (68) by E6, as well as inhibition of IRF1 (69) and IRF9 (70) by E7. An impaired antigen-presenting process via the major histocompatibility complex-I (MHC-I), also referred to as human leukocyte antigen (HLA), results from decreased expression of low-molecular-weight polypeptide (LMP)2, LMP7, transporter associated with antigen processing (TAP)1, TAP2 and MHC-I (71). Depletion of Langerhans cells (LC) in the infected epithelium results from downregulation of E-cadherin on the cell membrane of infected keratinocytes (72,73). Blocked maturation of LCs results from activation of the phosphoinositide 3-kinase (PI3K)-Akt pathway in LCs by L2 capsid protein (74,75). A shift from Th1- to Th2-response caused by HPV stimulates interleukin (IL)-10 secretion at the expense of IFN-γ (76,77). Furthermore, IL-10 is considered to downregulate the expression of classic HLA-I molecules (76) and upregulate the expression of non-classic HLA-G molecules (78), which suppress the functions of cytotoxic T lymphocytes (CTLs) (79), natural killer (NK) cells (80) and dendritic cells (DCs) (81).

Although the infected cells suffer an immune attack, the apoptosis resistance conferred by E5, which inhibits TRAIL- and CD95L-mediated apoptosis (82-84), as well as E6, which accelerates proteasome degradation of p53, FADD, procaspase-8 and c-Myc (85-87), enable their survival (88).

5. Chemical antivirals

Chemical antivirals are crucial for the treatment of several viral infectious diseases, such as viral hepatitis B and acquired immunodeficiency syndrome (AIDS), but little is known on the role of antivirals in HPV infections. This may be partially attributed to the fact that the targets of classical antivirals are enzymes encoded by the viral genome, while HPVs hijack the cellular replication system for their reproduction, except for E1 helicase, which provides few targets for drug design. However, several studies and clinical trials have identified and demonstrated the robust anti-HPV potential of certain acyclic nucleoside phosphonates (ANPs), among which cidofovir is the most extensively investigated.

ANPs. Cidofovir, (S)-1-(3-hydroxy-2-(phosphonomethoxy)-propyl) cytosine, was initially designed to inhibit the DNA polymerase and become incorporated into the daughter DNA, slowing down DNA replication and viral genome instability. Further studies have demonstrated its antiviral potential against herpes simplex virus (HSV), which encodes its own DNA polymerase, and against HPV, in which case no HPV-specific DNA polymerase is generated. The underlying mechanisms may involve the fact that cidofovir is more likely to be converted to its active form as triphosphorylated cidofovir in HPV-infected cells compared with uninfected cells (89), or that the single replication origin is the viral episome, in contrast to multiple replication origins in human genome, which is more susceptible to chain-terminating factors, with no substitutive origins or compensatory effects from other origins (90). A phase II clinical trial that adopted topical cidofovir in the treatment of CIN2 and CIN3 reported a 60.8% response rate in the cidofovir group vs. 20% in the control group (91). Although conization

may outperform cidofovir in terms of therapeutic efficacy, these findings have identified an alternative treatment for patients with concerns regarding postoperative complications. Similar studies have been performed on women with high-grade vulval intraepithelial neoplasia, where 4 of 10 had complete regression and 3 had a partial response (92). Another study evaluated the safety and efficacy of topical cidofovir in the treatment of PAIN and VIN in HIV-positive patients, demonstrating 15% complete response, 36% partial response, 21% stable disease and 6% progressive disease (93).

However, the two hydroxyls in the phosphonic moiety of cidofovir decrease its transmembrane activity, and it may be hypothesized that lipophilic modification of the hydroxyls will enhance its anti-HPV activity. This hypothesis has already been confirmed by adefovir and tenafovir, both resulting in significantly higher efficacy compared with their parent compounds, but exhibiting no specificity for HPV-infected cells (94), whereas GS-9191 exhibited selectivity towards HPV-infected cells with enhanced activity, which was further verified in an animal model (95). More recently, another derivative, octadecyloxyethyl benzyl 9-((2-Phosphonomethoxy) ethyl)guanine (ODE-Bn-PMEG), was designed and demonstrated to be effective in blocking HPV-11, -16 and -18 replication (90). These ANPs appear to be promising, but further studies are required to evaluate their safety and efficacy *in vivo*.

Antivirals targeting proteins encoded by HPV. In contrast to ANPs, antivirals targeting proteins encoded by HPV are characterized by higher specificity. With the exception of E1 helicase inhibitors, the majority of these antivirals are novel chemicals hindering protein-DNA or protein-protein interaction.

As previously mentioned, E1 is recruited to the origin site of HPV genome with the help of E2, followed by assembly into double hexamers to start replication, thus hindering the binding between E1 and E2, or E1/E2 and DNA, which appears to be very promising in lowering viral load. Both hypotheses have been evidenced by indandiones for the former (96-98) and polyamides for the latter (99), respectively. Indandiones were found to be more effective against HPV-6 and -11, rather than high-risk HPV types (97). Further modifications may confer anti-high-risk-HPV activities to these chemicals. In view of the inability of earlier-synthesized polyamides to penetrate the cell membrane, previous studies focused on binding modes between polyamides and DNA, while recent research has resolved this issue through the synthesis of PA1 and PA25, which have been proven effective in reducing viral load in cell experiments (100,101).

The fact that E1 is the only protein encoded by HPV that has enzymatic activity (102,103), together with the indispensability of E1 in genome replication, makes E1 the most promising target for inhibiting viral amplification. Screened out as a small molecule inhibitor of HPV6 E1 (104), biphenysulphonacetic acid affects ATP binding of E1 through allosterism involving Tyr486 (105). Therefore, the activity of biphenysulphonacetic acid appears to be dependent on the amino acid sequence (tyrosine residue) and three-dimensional structure of E1, which is somewhat type-specific. Moreover, it lacks activity in cell-based assays due to the high intracellular concentration of ATP (104), which further prevents the currently available compounds from therapeutic application.

The well-known interaction between E6 and E6AP, which mediates the proteasome degradation of p53, provides another therapeutic target for HPV infection. The recognition of the E6-binding motif on E6AP, defined as an α-helix with three leucines on one side and two negatively charged residues on the opposite side, enabled researchers to screen out small molecular inhibitors among therapeutic agents (106,107). Further medicinal development based on this finding may prove to be useful.

Other host proteins utilized by HPV as targets of antiviral therapy. Apart from the cellular replication system, several other host mechanisms usurped by HPV to facilitate its survival and reproduction may serve as targets, and corresponding agents are referred to as host-dependent viral inhibitors.

The oncoprotein E7 was also demonstrated to be associated with class I histone deacetylases (HDAC)1 and 2 (108) under the mediation of Mi2β (109), responsible for proliferation-promotion and long-term viral episome maintenance (108). HDACs decrease the acetylation state of histones, thereby inhibiting target gene transcription. The relocation of HDACs induced by E7 from proliferation-promoting genes to cell cycle-arresting or apoptosis genes leads to upregulation of the former and downregulation of the latter. Therefore, HDAC inhibitors may be able to interrupt the multiple pathogenic processes. Current HDAC inhibitors are mostly Zn^{2+}-chelating agent binding to the Zn-binding catalytic domain of HDAC, including short-chain fatty acids (110), hydroxamic acids (111,112), benzamide derivatives (113), epoxyketones and cyclic peptides (114). Although these were effective in arresting the proliferation of cervical cancer cells (115-117), they may need further optimization prior to clinical application due to their broad spectrum of cellular targets.

Cyclin-dependent kinase (Cdk) 2, activated by cyclin A or E, is crucial for driving the cell cycle as well as for the pathogenesis of HPV. Cdk2 is stimulated by E7 via multiple mechanisms (118) and subsequently promotes cellular proliferation. Cdk2 also accelerates viral genome amplification by phosphorylating E1 at specific sites in its N-terminal domain (119-121) and causes abnormal copy numbers of centrosome with genome instability (122-124). Phosphorylation of E1 induces its nuclear retention and, thus, facilitates the formation of hexamers that are necessary for replication initiation (125,126). Inhibitors of Cdk2, therefore, are considered to halt the proliferation of cervical carcinoma cells and restore normal centrosome replication. Cell-based assays using roscovine (127,128) and indirubin-3'-oxime (IO) (129) have already confirmed this hypothesis. Novel IO derivatives with higher specificity and potency towards Cdk2 have already been discovered (130) and, together with other, more potent Cdk2 inhibitors, such as flavopiridol, should be further evaluated to establish their role in HPV infection treatment.

The cellular transcription factor Sp1 can also bind to LCR in the viral genome of both low- and high-risk HPV types, and is involved in the transcription of HPV genes (mainly E6 and E7) independently of E2 (131,132). Inhibiting this process with derivatives of nordihydroguaiaretic acid (NDGA), i.e., tetra-O-methyl NDGA and tetra-acetyl NDGA (133), resulted

in cell growth arrest (134), apoptosis and tumor size reduction in tumor-bearing mice (134).

6. Therapeutic vaccines

The etiology of cervical carcinoma as a viral infectious disease established over the last ~50 years has enabled its prevention through prophylactic vaccination (Fig. 3). Current prophylactic vaccination, however, will not achieve a significant reduction in the morbidity and mortality of cervical carcinoma until successful world coverage by vaccination, which is an elusive goal due to the high cost of HPV vaccines. In addition, it usually takes 10-30 years (median, 23.5 years) for CIN 2/3 to progress to ICC (135); therefore, considering the size of the population with existing HPV infections and the natural history of HPV-associated precancerous diseases, tens of years may pass for the vaccines to exert their protective effects against cervical cancer. In summary, a significant decrease in the incidence of cervical cancer will not be achieved until vaccinated women enter the peak age range of cervical cancer.

The demand for clearance of established HPV infection and regression of precancerous/cancerous lesions has prompted the design of therapeutic vaccines. Apart from the humoral immunity triggered by prophylactic vaccines, therapeutic vaccines trigger cell-mediated immune responses. Among the proteins encoded by HPV, E6 and E7 are the best-characterized and the most extensively investigated due to their carcinogenic role and constitutive expression in infected cells (58). Live vector vaccines or DNA vaccines including wild-type E6 and E7 with the potential to transform cells are usually inactivated at certain sites into detox forms. Other targets include E1, E2 and E5, according to their expression mode during the life cycle of HPV. However, it must be mentioned that once viral genome is integrated, most genes are lost, except E6 and E7, which are expressed at even higher levels without repression of E2 (136-139). Several strategies for the development of therapeutic vaccines have been studied, including live vector, nucleic acid, peptide-based, protein and cellular vaccines, with several vaccine candidates currently in clinical trials (Fig. 4).

As regards the evaluation of immunization efficacy, both antigen-specific $CD4^+$ and $CD8^+$ lymphocytes are considered as indicators of cell-mediated immunity. $CD8^+$ lymphocytes further differentiate into CTLs, undertaking the main task of eliminating virus-infected cells, while $CD4^+$ lymphocytes can differentiate into T-helper type 1 (Th1) lymphocytes, playing an auxiliary role in priming antigen-specific CTLs. Other direct outcome evaluation indicators include histopathological regression or complete response rate, histopathological reduction or partial response rate, and viral clearance rate.

Live vector vaccines. Live vector vaccines utilize attenuated bacteria or viruses to transport genes of interest into cells. These microorganisms infect host cells, proliferate intracellularly and spread to surrounding cells in a restricted manner prior to immune elimination. The gene of interest is then expressed by the host protein expression system, leaving the protein at its most natural state. These allow class I MHC antigen presentation by infected cells, but inefficiently. Another more high-efficiency

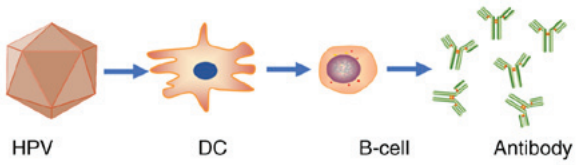

Figure 3. Immunization process of prophylactic vaccines. HPV, human papillomavirus; DC, dendritic cell.

antigen presentation pathway is achieved by dendritic cell (DC) ingestion of free antigen released by infected cells through exosomes, secretion or apoptosis. Thereafter, DCs process antigen and present it on the cell surface for T-cell recognition and activation through both class II and class I (by cross-presentation) MHC pathways. More directly, DCs residing in the vaccination sites (e.g., Langerhans cells in the dermis) may be infected by the live vectors, which simplifies the antigen presentation process. In addition, the vector itself acts as an adjuvant to enhance the immunogenicity of the vaccine due to its pathological nature, thus promoting an even stronger immune response. Unfortunately, live vector vaccines carry the risk of overwhelming infection in immunocompromised patients, and the live vector itself can induce neutralizing antibody production, thus abrogating the boost effect of repeated vaccination. In rare cases, the host may have pre-existing immunity against the live vector, leading to vaccination failure.

Bacterial vector. Bacterial vectors include *Listeria monocytogenes* (140,141), *Lactobacillus casei* (142), *Lactobacillus lactis* (143-145), *Lactobacillus plantarum* (145) and *Salmonella* species (146). Lactobacilli are non-invasive and non-commensal bacteria, which are transiently located in mucosae and express recombinant antigen priming mucosal immunity after oral or nasal administration as vaccines. Their favorable safety profile, the low possibility of immune tolerance and the convenient delivery method make Lactobacilli vectors promising candidates, while their immunogenicity remains to be further enhanced, possibly by combining with cytokines or other adjuvant agents. A recombinant *Lactobacillus casei* vaccine expressing modified HPV-16 E7 has completed its phase I/IIa clinical trial in 17 $HPV16^+$ CIN3 patients, with 9 patients experiencing disease regression to CIN2, and 5 further regressing to LSIL (147).

Listeria, an intracellular bacterium, is able to infect macrophages and escape from phagosomal degradation with the help of listeriolysin O (LLO) (148); therefore, Listeria vectors can replicate and express recombinant proteins in the cytosol, allowing both class I and class II MHC antigen presentation (149,150). A promising Listeria-based vaccine, Lm-LLO-E7 (also referred to as ADXS11-001) was designed by fusing HPV16 E7 with LLO. A phase I study of 15 patients with metastatic, recurrent, refractory or terminal squamous cell carcinoma of the cervix, demonstrated an increase in the E7-specific T cells detected among peripheral blood mononuclear cells (PBMCs) of 3 patients and a reduction in tumor size was observed in 4 patients (151). Further clinical trials on HPV-associated cancers are currently ongoing (NCT02399813, NCT02002182, NCT02291055 and NCT01266460).

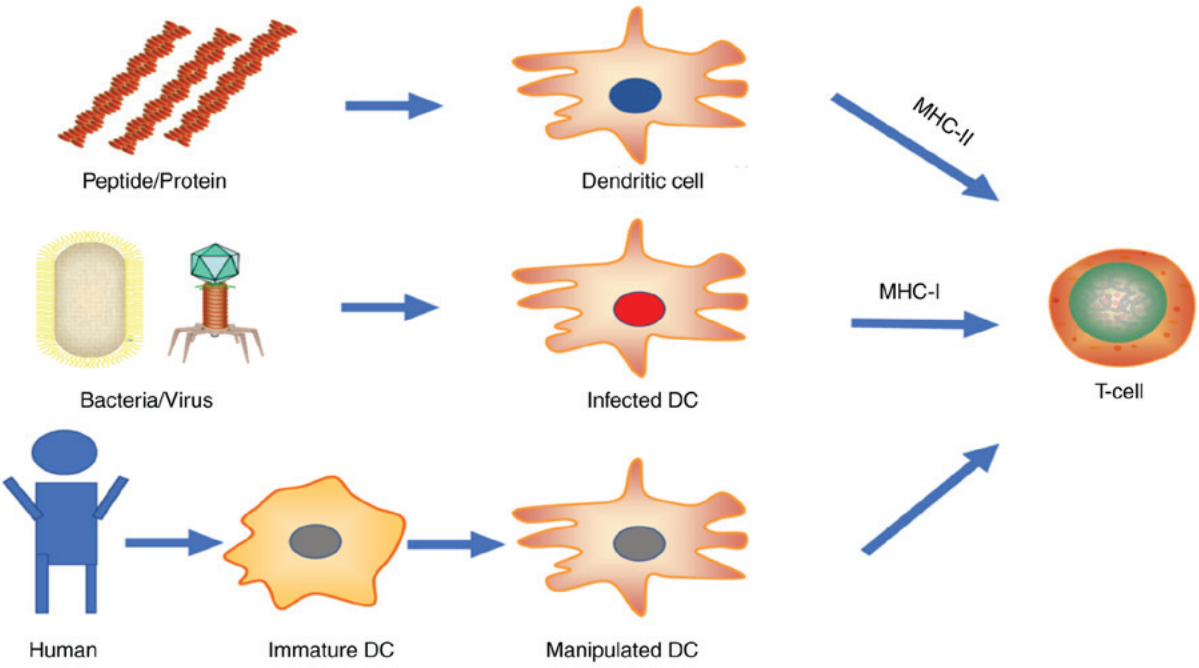

Figure 4. Immunization processes of various therapeutic vaccines. DC, dendritic cell; MHC, major histocompatibility complex.

Viral vector. Viral vectors include adenoviruses, adeno-associated viruses, alphaviruses, lentiviruses and vaccinia viruses. Alphaviruses, including Semliki forest viruses, Sindbis viruses and Venezuelan equine encephalitis viruses are RNA viruses that are transformed into RNA replicon form by substitution of their 3'-terminal structural genes with the genes of interest. These RNA replicons are capable of autonomous amplification but fail to assemble into intact virions due to lack of capsomers. In view of their similarity to nucleic acid vaccines, they will be discussed in the respective section.

TA-HPV is a recombinant vaccinia virus expressing HPV-16/18 E6/E7 with 3 completed clinical trials. A phase I/II study in patients with advanced cervical cancer reported that an HPV-specific CTL response was detected in one of three evaluable patients (152). Another phase I study conducted in patients with International Federation of Gynecology and Obstetrics stage Ib or IIa cervical cancer found that 4 of 29 patients developed an HPV-specific CTL response after a single vaccination (153). A phase II study in patients with HPV-positive high-grade VIN or VAIN with a duration of up to 15 years observed a lesion reduction of at least 50% in 5 of 12 (42%) patients, with 1 patient exhibiting complete regression (154).

TG4001 is a recombinant modified vaccinia Ankara (MVA) expressing HPV-16 E6, E7, and IL-2. A phase I study including 21 cases of HPV16$^+$ CIN2/3 patients revealed that 48% experienced disease regression, whereas 38% exhibited HPV DNA clearance (155).

MVA E2 is a recombinant MVA expressing BPV E2. In a phase III study in patients with HPV-induced anogenital intraepithelial neoplasia, a 90% clearance in female patients and 100% clearance in male patients was reported (156).

Also designed to express the fusion protein of calreticulin and HPV16 E7, adenovirus vector was demonstrated to eradicate established tumors in mice (157). Clinical trials of this vaccine, however, have yet to be conducted.

Subunit vaccines. Antigens delivered in the form of peptides or whole proteins directly are referred to as subunit vaccines. As the most classical type of vaccines, they are considered to be safer compared with live vector vaccines for lack of infectivity and persistent existence.

Peptide vaccines. Peptide vaccines, with an excellent safety profile and good stability, are easy to produce and more cost-effective. However, peptides are truncated from the whole protein and, thus, may not contain the necessary epitopes for DC processing and presentation through the MHC pathway. Furthermore, the fact that each individual has his own HLA type means that epitopes recognized by MHC may differ among different individuals. Therefore, for valid immunization, the epitopes have to be identified so as to match the MHC-specificity of each individual, which limits the mass production of peptide-based vaccines (158). This was addressed by the synthesis of long overlapping peptides covering the entire sequence of the protein. Low immunogenicity is another drawback of peptide-based vaccines, which may be addressed by co-administration of adjuvants, co-expression of cytokines and fusion protein with Toll-like receptor (TLR) ligands.

HPV16-SLP (ISA101) is a peptide-based vaccine consisting of nine HPV16 E6 and four HPV16 E7 synthetic long overlapping peptides with adjuvant Montanide ISA51. In a phase II clinical trial in patients with HPV16$^+$ VIN3, 15 of the 19 patients exhibited a clinical response (79%), with a complete response in 9 patients (47%). Moreover, all patients developed a vaccine-induced T-cell response, but patients with stronger IFN-γ-associated CD4$^+$ and CD8$^+$ T-cell response were more likely to achieve complete response (159). Other studies have also demonstrated the therapeutic potential of ISA101 (160-163).

PepCan, a vaccine consisting of four HPV16 E6 synthetic peptides and Candin as an adjuvant, has completed the

dose-escalation phase of a phase I clinical study in patients with HSIL, with 50 μg reported as the most effective dose, and histological regression of disease in 45% of all patients (164).

Protein vaccines. Protein-based vaccines utilize the full-length E6 and/or E7 protein to immunize humans. Compared with peptide-based vaccines, they contain all the epitopes and exclude MHC restriction, but due to their exogenous nature mostly presented by the MHC II pathway (165), they tend to mount humoral immunity and have low immunogenicity. These problems may be overcome by fusion protein targeting them to DCs and giving them access to the MHC I antigen presentation pathway.

TA-CIN is a fusion protein of HPV16 L2, E6 and E7. As the first vaccine that combines therapeutic and prophylactic effects, it was tested on healthy subjects, demonstrating a TA-CIN-specific IgG in 24 of the 32 vaccinated patients and cell-mediated immunity in 25 of the 32 patients (166). A phase II clinical trial conducted in patients with VIN 2/3 combined topical imiquimod and TA-CIN, reporting a 63% lesion response 1 year after vaccination (167).

GTL001 (Procervix) fused the E7 of HPV-16 and -18 to the catalytically inactive *Bordetellla pertussis* adenylyl cyclase (CyaA). CyaA is an important toxin of *Bordetellla pertussis* that binds to integrins on the cell membrane and inserts its N-terminal into the cytoplasm. This characteristic of CyaA is utilized to transport antigens into the cytoplasm, subsequently initiating the MHC I antigen presentation pathway. A phase I trial of GTL001 combined with topical imiquimod in patients positive for HPV-16 or HPV-18 infection, but with normal cytology, showed effectiveness and tolerability (168). Similar strategies have been explored to fuse HPV-16 E7 to a peptide derived from the *Limulus polyphemus* anti-lipopolysaccharide factor (LALF31-52) in an *E. coli* expression system (169). LALF31-52 can penetrate cell membranes with immuno-modulatory effects increasing its immunogenicity (170). This specially designed vaccine has demonstrated its protective function in a preclinical model (169). Recently, flagellin, ligand of TLR5, was also found to form a fusion protein with HPV16 E7, and its antitumor effects were tested in a mouse model (171).

Nucleic acid vaccines

DNA vaccines. DNA vaccines are plasmid DNAs carrying genes of interest and transfecting host cells for sustained antigen expression. DNA vaccines usually do not increase neutralizing antibody production, allowing repeated vaccinations (172). However, they raise concerns regarding the risks of exogenous DNA integration, albeit without supportive evidence. Unlike viral vaccines, DNA plasmids cannot autonomously amplify or spread intercellularly, resulting in the main drawback of DNA vaccines, namely poor immunogenicity (173,174).

Intradermal administration via gene gun (175), intramuscular injection with electroporation (176), laser (177), microencapsulation of DNA (178) and fusion protein linking HPV antigens to DC targeting molecules [such as FMS-like tyrosine kinase 3 ligands (179) and heat shock protein (180)] were adopted to increase the antigen-expressing/antigen-loaded DC population. As HPV encodes proteins through suboptimal codons, codon optimization increases antigen expression, further facilitating DC uptake (181-183). Predisposition to MHC class I antigen presentation augments antigen-specific $CD8^+$ T-cell response. This can be realized through linkage of HPV antigens to molecules targeting it to endoplasmic reticulum (184) and proteasome (185), including *M. tuberculosis* hsp70 (186), calreticulin (187), heat shock protein Gp96 (188), the translocation domain of *Pseudomonas aeruginosa* exotoxin A (189) and γ-tubulin (190). MHC I single-chain trimer is another more direct option facilitating antigen presentation on the DC surface (191). Efforts to block T-cell-mediated DC apoptosis (192,193) and DC-mediated T-cell apoptosis (194) were shown to augment $CD8^+$ T-cell response.

VGX-3100, a DNA vaccine encoding HPV-16/18 E6/E7, which is administered intramuscularly with electroporation, has finished its phase IIb clinical trial in $HPV16/18^+$ CIN2/3 patients. A total of 53/107 (49.5%) patients with VGX-3100 treatment in contrast to 11/36 (30.6%) placebo subjects exhibited histopathological regression in the per-protocol analysis. In addition, 55/114 (48.2%) patients with VGX-3100 treatment in contrast to 12/40 (30.0%) placebo subjects had histopathological regression in the modified intention-to-treat analysis (195).

Other DNA vaccines, such as GX188E (196), pNGVL4a-sig/E7(detox)/HSP70 (197) and pNGVL4a-CRT/E7(detox) (198), have also demonstrated a good safety profile and effectiveness in several phase I clinical trials.

RNA replicon-based vaccines and suicidal DNA vaccines. RNA replicon-based vaccines are derived from alphaviruses. They replicate intracellularly and express genes of interest with no risk of integration. However, the instability of RNA limits their application and puts forward a more stable form, namely suicidal DNA vaccines, also referred to as DNA-launched RNA replicons. In contrast to RNA replicon-based vaccines, suicidal DNA vaccines have an extra step of transcription into RNA replicons after transfection. Compared with DNA vaccines, the self-replication of these vaccines increases antigen expression, and the final apoptosis of transfected cells resulting from extensive double-stranded RNA production avoids the possibility of genomic integration. However, early apoptosis of host cells causes inadequate stimulation towards T lymphocytes and insufficient T-cell response. Co-transfection of genes encoding anti-apoptotic proteins in the vector (199) and use of flavivirus Kunjin (KUN) (200,201) have been introduced to address this issue. These vaccines appear to be highly promising for the treatment of HPV infections, but require further investigation.

Cell-based vaccines. Cell-based vaccines include extracting and isolating cells (such as DCs or T lymphocytes) from the peripheral blood or excised tumors of patients, manipulating and expanding them *ex vivo*, and finally transferring the selected and modified cells back to the patients.

DC-based vaccines. As the most robust antigen-presenting cells (APCs), DCs are mostly studied in the context of immune system activation, circumventing the necessity to access antigens to APCs *in vivo* and to use adjuvants. Antigen-loaded DCs are produced *ex vivo* through transfection by viral vectors (202,203), transduction by

DNA or RNA vectors (204,205), pulsation of antigenic peptides, proteins or tumor cell lysates (205-209). Inevitably, DC-based vaccines have certain drawbacks: First, the production of DC-based vaccines is resource-intensive and individualized, so that large-scale production and widespread use appear to be impractical; second, it is difficult to unify the culturing techniques, which leads to spotty vaccine quality and lack of standard evaluation criteria; third, in order to prime immunity against antigens, DCs have to migrate to lymphoid tissues, and this poses the question of determining the most efficient administration route among intramuscular, subcutaneous, intravenous and intranodal injection, or other options; fourth, the limited longevity of DCs caused by T-cell-mediated apoptosis weakens the magnitude of immune response, which has been partially addressed by transfecting DCs with siRNA silencing pro-apoptotic proteins (207,208,210).

In a phase I clinical study, DCs were pulsed with HPV16/18 E7 and then co-administered with IL-2 back to the patients. An E7-specific $CD8^+$ response was observed in all patients (211). Another phase I clinical trial was conducted in patients with stage Ib or IIa cervical cancer and DCs were pulsed with HPV16/18 E7 as well as keyhole limpet hemocyanin, promoting DC maturation. As a result, 8 of 10 patients exhibited an increase in E7-specific $CD8^+$ T lymphocytes (212).

Tumor cell-based vaccines. Isolated tumor cells are engineered to express cytokines such as IL-2 (213,214), IL-12 (215) and granulocyte-macrophage colony-stimulating factor (214,216). Re-administration of tumor cell-based vaccines significantly increases the immunogenicity of tumor cells, thus inducing immune elimination of lesions. Such vaccines do not need to identify certain tumor antigens, and they have been tested in clinical trials in several types of cancer (217). Given that cervical cancer has its own specific antigens, such as E6 and E7, tumor cell-based vaccines may not be the first choice for its treatment. However, tumor cell-based vaccines are associated with the drawback of implanting new cancers in patients, which limits their clinical applicability, particularly in HPV-positive patients with normal cytology or patients with low-grade lesions.

Adoptive cell transfer (ACT). ACT selects tumor antigen-specific CTLs, engineers or activates them and expands them *ex vivo*, and they are finally re-administered to the patients. A pilot study using HPV16 E6/E7-specific T cells in patients with metastatic cervical cancer reported complete regression in 2 of 9 patients (218). TCR gene-engineered T cells were also introduced to target HPV^+ epithelial cancer cells in cell-based assays and exhibited killing avidity (219).

7. Immunomodulators

Immunomodulators are agents stimulating innate and/or adaptive immunity for pathogen elimination. As regards treatment of persistent HPV infection, imiquimod is the most extensively studied and widely used immunomodulator.

Imiquimod, an agonist for TLR7, can trigger expression of cytokines and induce a local immune response. The raised levels of cytokines activate local immune cells and initiate immune clearance of HPV-infected cells. Adverse events may include itching, erythema, burning, irritation, tenderness, ulceration and pain. The antiviral as well as antitumor properties of imiquimod have been demonstrated in basal cell carcinoma (220), VAIN (221), VIN (222) and AIN (223). A more popular method is topically applying imiquimod in combination with therapeutic vaccines. However, neither of these treatments have been licensed.

IFN is widely used in the treatment of low-risk HPV-associated anogenital warts, but its role in high-risk HPV-associated pre-cancerous lesions and cancers remains a subject of debate; therefore, more large scale, double-blind, randomized controlled trials are required.

8. Future prospects

Candidate therapies for HPV infection mainly include chemical antivirals, therapeutic vaccines and immunomodulators. Therapeutic vaccines appear to be the most promising approach to eliminating HPV in terms of effectiveness, while each type of vaccine comes with its own advantages and disadvantages. Generally, most vaccines must be injected into certain sites, except for Lactobacillus-based vaccines, which are administered orally. Mucosal immunity primed by Lactobacillus-based vaccines satisfies the needs for anti-HPV immunity, as the life cycle of HPV expands beyond the BM. These synergistic factors make Lactobacillus-based vaccines a promising candidate. For all therapeutic vaccines, enhancement of immunogenicity is the common requirement for clinical application.

Antivirals robustly inhibit the proliferation of HPVs, but are unable to eradicate infection, particularly by integrated viruses. The safety profiles of HDAC, Cdk2 and Sp1 inhibitors must be further investigated, as they have numerous downstream targets. It would be preferable to verify the therapeutic effects of these inhibitors at doses not interfering with normal cell functions.

In summary, the coordinated use of various strategies may act synergistically against HPV infection. The combination of prophylactic with therapeutic vaccines, or of different types of therapeutic vaccines as in prime-booster strategy, or of therapeutic vaccines with immunomodulators, antivirals or checkpoint inhibitors, and other similar combinations, may have a profound impact on the treatment of HPV infection.

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Authors' contributions

YL and HL wrote the initial manuscript. RP created the figures and YY contributed writing material and new ideas. XZ and XQ revised the manuscript and approved the final version. All authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests to disclose.

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