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REVIEW

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# Targeting immune response with therapeutic vaccines in premalignant lesions and cervical cancer: hope or reality from clinical studies

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**ABSTRACT**

Human papillomavirus (HPV) is widely known as a cause of cervical cancer (CC) and cervical intraepithelial neoplasia (CIN). HPVs related to cancer express two main oncogenes, i.e. E6 and E7, considered as tumorigenic genes; their integration into the host genome results in the abnormal regulation of cell cycle control. Due to their peculiarities, these oncogenes represent an excellent target for cancer immunotherapy. In this work the authors highlight the potential use of therapeutic vaccines as safe and effective pharmacological tools in cervical disease, focusing on vaccines that have reached the clinical trial phase. Many therapeutic HPV vaccines have been tested in clinical trials with promising results. Adoptive T-cell therapy showed clinical activity in a phase II trial involving advanced CC patients. A phase II randomized trial showed clinical activity of a nucleic acid-based vaccine in HPV16 or HPV18 positive CIN. Several trials involving peptide-protein-based vaccines and live-vector based vaccines demonstrated that these approaches are effective in CIN as well as in advanced CC patients. HPV therapeutic vaccines must be regarded as a therapeutic option in cervical disease. The synergic combination of HPV therapeutic vaccines with radiotherapy, chemotherapy, immunomodulators or immune checkpoint inhibitors opens a new and interesting scenario in this disease.

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## Introduction

Human papillomavirus (HPV) causes one-third of infection-associated cancers. Although prophylactic vaccines provide protection against HPV-16 and HPV-18, the genotypes causing about 70% of cervical cancer, vaccination programs are disappointing in several countries, and many women remain at risk of developing cervical cancer. Moreover, the frequency of HPV-associated cancers continues to increase at anatomical sites other than the cervix, such as vagina, vulva, oropharynx, and anus, thus helping feed the problem [1].

HPV is a double-stranded DNA virus that exhibits unidirectional transcription. To date, more than 200 HPV genotypes have been identified, even if only few cause cancer [2] Six early proteins (E1, E2, E4, E5, E6, and E7) and two late, capsid proteins (L1 and L2) are synthesized by HPV during replication through the differentiating strata of epithelia. HPV virus can infect the basal epithelial cells in the cervical transformation zone, causing their proliferation and leading to epithelial hyperplasia or papillomatosis. E5, E6, and E7 are considered oncoproteins and are responsible for continuous cell proliferation, DNA amplification, and impairment of DNA repair mechanisms, with consequent accumulation of mutations, rearrangements, and aneuploidies. The integration of viral DNA into the host cell genome in selected cases is a crucial step, resulting in deletion of E2 gene and adjacent regions (frequently E4, E5, and L2 genes). The E2 deletion, a transcriptional regulator of E6 and E7, leads to overexpression of E6/E7 that, in turn, induce degradation of p53 and pRB, respectively. In addition, E6/E7 alter expression of genes involved in S phase of the cell cycle, interact with p21 and p27 that inhibits cyclin-dependent kinases regulating the transition from G1 to S phase, and interfere with pro-apoptotic functions of some proteins such as c-myc, BAK, p53. As a consequence, this complicate and finely tuned process may lead to malignant transformation [3–6].

Consequences of HPV persistent infection are squamous intraepithelial lesions (cervical intraepithelial neoplasia, CIN) that are considered as precursors of cancer, graded into three progressive risk group according to the proportion of epithelium affected by abnormalities: CIN1, CIN2, CIN3 [7]. CIN1 can evolve

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in a small percentage of cases into CIN2–3, with a continuous proliferation and an increase in chromosomal instability reflected by increasingly mitotic figures, nuclear abnormality and clumps of pyknotic cells [8,9]. In CIN3, these cell alterations are distributed throughout the full thickness of epithelium rendering histological diagnosis quite easy.

Currently, it is estimated that 32–43% of CIN2/3 lesions undergo spontaneous regression [10,11]. However, we still do not have prognostic biomarkers that can distinguish the likelihood of regression at the individual patient level.

Standard treatment for patients with CIN2/3 is surgical excision (cold knife conization or loop electrosurgical excision). It is to note that a considerable morbidity is associated with CIN treatment including low birth weight, pre-term births, increased deaths, and more caesarian section [12]. Thus, other therapeutic options are needed. Among the non-surgical hypothetical therapeutic options, several immunotherapeutic strategies have been explored in pre-clinical and clinical trials, with some promising results [13,14].

Prophylactic HPV vaccines act by inducing neutralizing antibodies against the L1 capsid proteins of HPV-16 and HPV-18 [15,16], whereas therapeutic vaccines generate cell-mediated HLA restricted adaptive T-cell immunity for killing virus infected cells. Since appreciable levels of L1/L2 capsid antigens are not expressed by cervical cancer cells, therapeutic vaccines need targets other than L1/L2, and since HPV E6 and E7 are necessary for the initiation and maintenance of malignant transformation, these antigens represent attractive targets for therapeutic vaccines. Several therapeutic vaccines, employing vector-based peptide or protein-based, nucleic acid-based, viral/bacterial vector based, and cell-based therapeutic vaccines targeting the HPV-16 E6 and/or E7 antigens have been tested in order to increase viral specific immune responses [17,18]. Achieving a valid immunotherapy against HPV-related pathologies has to face several obstacles including major histocompatibility complex (MHC) class I down-regulation, impaired antigen-processing, homing of T-cell killer, and immunosuppression due to Treg infiltration, and secretion of immunosuppressive cytokines [14]. Immunosuppression is less effective in pre-cancer lesions rendering more efficacious immunotherapeutic intervention. However, recent discovery of monoclonal antibodies against immune check-points has completely changed the immunotherapy of tumors by altering the immunosuppressive milieu of cancer [19]. Pre-clinical data from mouse models on large already established HPV tumors indicate the extremely effectiveness of immunotherapy in combination with immune check-point blockade [20,21].

In this review, we report preliminary results and ongoing studies on therapeutic vaccines in pre-invasive and invasive cervical cancer, focusing on vaccines that have reached the clinical trial phase. Table 1 summarizes the main characteristics of these trials, while details on relevant ongoing clinical trials are reported in Table 2. Figure 1 shows methodologies for production and delivery of HPV therapeutic vaccines as well as their immunological activity.

## Cell-based approaches

### *Dendritic cell-based vaccines*

Dendritic cells (DCs) have strong ability to initiate and control T-cell response, being ideal candidates for immunotherapy strategies. They can be divided into DCs pulsed with peptides/proteins, or transduced with DNA or viral vectors encoding target antigen. A pilot study was carried out in 15 advanced cervical patients treated with autologous monocyte-derived DCs pulsed with recombinant HPV-16 E7 or HPV-18 E7 oncoprotein, showing T-cell response and good tolerability, but no objective responses were observed [22]. Other studies used a DC vaccine pulsed with recombinant HPV-16/18 E7 antigens and IL-2, or keyhole limpet hemocyianin (an immunologic tracer molecule), with strong immunological response but no clinical effect [23,24]. Since preliminary reports with DC cancer vaccines consisting of *ex vivo* generated DCs loaded with tumor antigens did not show clinical responses in advanced cancer patients, the combination with cytokine to favor DC maturation and antigen presentation have been tested in various tumors [25–28]. Thirty-two patients with HPV-16/18 positive advanced cervical cancer were treated with HPV-16 E6 (arm A) or HPV E7 peptide (arm B) pulsed on peripheral blood mononuclear cells (PBMCs) cultured for 5–7 days in GM-CSF and IL-4 (pre-immature dendritic cells), to obtain immune response against the relevant peptide on both arms. ELISPOT and 51Cr release assays showed immune response against the relevant peptide in 63% of the patients in arm A and in 58% of the patients in arm B. No objective responses were observed, and treatment was well tolerated. The administration of pre-immature DCs pulsed with HPV-16 E6 or E7 was feasible and induced a specific immune response, thus deserving further investigations [29].

Gene-transduced DCs vaccines represent a promising strategy, since HLA restriction may be bypassed, and several pre-clinical studies have been carried out with DC vaccines transduced with adenoviral vector carrying a mutated E7 protein with encouraging results in animal models [30].

Cytokine-induced killer (CIK) cells, generated from PBMCs by culturing in the presence of various cytokines, combined with DCs through a co-culture of DC and CIK, have the advantage of fast proliferation, high cytotoxicity and a broad tumor killing spectrum [31]. In early cervical cancer, a phase II randomized trial was performed in 79 surgery-treated, high-risk cervical cancer patients. They received as adjuvant treatment cisplatin alone versus cisplatin combined with DC–CIK cells. The immune function was significantly improved in the experimental arm (lymphocyte ratio and expression of perforin, GraB and CD107a of PBMCs), and the cumulative recurrence rate in the experimental arm was significantly lower than that in the control arm [32].

Despite some promising results DC-based therapies have several limitations, such as labor-intensive and expensive procedures and, moreover their short half-life and absence of proliferation limit long-lasting immune response.

### *Tumor-cells-based vaccines*

Tumor-cell-based vaccines comprise delivery of whole tumor cells in order to stimulate immune system to recognize tumor-associated antigens, and are often genetically modified *in vitro* with genes encoding cytokines (IL-2, IL-12, GM-CSF) to increase immune response [33–35]. At present, the use of tumor cells (harvested from the patients) as vaccination is not considered in CIN/early stages of the disease due to safety concerns.

Table 1. Main therapeutic vaccines for cervical pre-neoplastic lesions and cervical cancer.

| Therapeutic agent | Phase | Disease | No. of PTS | Immunological effect | Clinical effect | Ref |
|---|---|---|---|---|---|---|
| Cell-based vaccines | | | | | | |
| Autologous monocyte-derived DCs pulsed with recombinant HPV-16 E7 or HPV-18 E7 oncoprotein | Pilot | Stage IV CC | 15 | Yes | NE | [22] |
| Mature autologous DC pulsed with full-length HPV-16 or -18 E7 oncoprotein, followed by subcutaneous low doses of human recombinant interleukin-2 | Pilot | Recurrent CC | 4 | Yes | NE | [23] |
| Escalating doses of mature autologous DC pulsed with full-length HPV-16/18 E7 oncoprotein and keyhole limpet hemocyanin | 1 | Stage IB/IIA CC | 10 | Yes | NE | [24] |
| HPV-16 E6 (18–26) or E7 (12–20) peptide pulsed on pre-immature dendritic cells | Pilot, R | Advanced CC | 32 | Yes | NE | [29] |
| Cisplatin chemotherapy alone or with dendritic cell-cytokine-induced killer cells | 2, R | CC | 79 | Yes | Yes | [32] |
| Tumor-infiltrating T-cells selected when possible for HPV E6 and E7 reactivity (HPV-TILs) | 2 | Advanced CC | 9 | Yes | Yes | [37] |
| Peptide–protein-based vaccines | | | | | | |
| HPV-16 E6 combined with or separated from HPV-16 E7 overlapping long peptides in Montanide ISA-51 adjuvant | 1 | Advanced CC | 35 | Yes | NE | [44] |
| HPV-16-synthetic long peptide vaccine consisting of a mix of 13 HPV-16 E6 and HPV-16 E7 overlapping long peptides in Montanide ISA-51 adjuvant | 2 | Advanced or recurrent HPV-16-positive gynecological carcinoma | 20 | Yes | No | [45] |
| Four peptides covering the HPV-type-16 E6 protein and Candida skin test reagent as adjuvant (PepCan) | 1 | CIN2-3 | 24 | Yes | NE | [46] |
| Multiple peptides cocktail vaccine; each peptide was mixed in Montanide ISA-51 adjuvant | 2 | Advanced CC/ ovarian cancer | 67 | Yes | Yes | [47] |
| Escalating doses of two HPV-16 E7 peptides and one helper peptide emulsified in Montanide ISA 51 adjuvant | 1/2 | HLA-A*0201 positive with HPV-16 positive CC | 19 | NE | Yes | [48] |
| Escalating doses of 9-amino acid peptide from amino acids 12–20 encoded by the E7 gene emulsified with incomplete Freund's adjuvant | 1 | HPV-16 and HLA-A2 positive high-grade cervical or vulvar intraepithelial neoplasia | 18 | Yes | NE | [49] |
| HLA-restricted HPV-16 E7 epitope adjuvated with very small size proteoliposomes | FiH | HPV-16 and HLA-A2 positive high grade CIN | 7 | Yes | NE | [50] |
| Mixture of HPV-16 E6E7 fusion protein and ISCOMATRIX adjuvant | 1, R | CIN 1–3 | 31 | Yes | NE | [52] |
| Escalating doses of HPV-16 L2, E6, and E7 as a single fusion protein (TA-CIN) | 1, R | Healthy volunteers | 40 | Yes | NE | [53] |
| Fusion protein (PD–E7) comprising a mutated HPV-16 E7 linked to the first 108 amino acids of Haemophilus influenzae protein D, formulated in the GlaxoSmithKline Biologicals adjuvant AS02B | 1/2 | CIN 1,3 | 7 | Yes | NE | [54] |
| Fusion protein containing an *M. bovis* BCG heat shock protein (Hsp65) covalently linked to the entire sequence of HPV-16 E7 (SGN-00101) | 2 | CIN3 | 72 | NE | Yes | [55] |
| Fusion protein containing an M. bovis BCG heat shock protein (Hsp65) covalently linked to the entire sequence of HPV-16 E7 (SGN-00101) | 2 | CIN3 | 21 | Yes | Yes | [56] |
| Nucleic acid-based vaccines | | | | | | |
| Escalating doses of ZYC101 | 1 | HPV-16 and HLA-A2 positive CIN 2/3 | 15 | Yes | NE | [59] |
| ZYC101a, including HPV-16/18 E6- and E7-derived CTL epitopes | 2, R | CIN2/3 | 127 | NE | Only in younger patients | [60] |
| Escalating doses of DNA plasmid expressing HPV-16E7 mutated at aa 24 and 26, linked to sequences coding for Sig and for HSP70 [pNGVL4a-Sig/E7(detox)/HSP70] | 1 | HPV-16 positive CIN2/3 | 15 | Yes | NE | [61] |
| DNA vaccine expressing HPV-16 E7 (DNAE7) followed by a recombinant vaccinia boost expressing HPV-16 and HPV-18 E6 and E7 (rVacE6E7; TA-HPV) | 1 | HPV-16 positive CIN2/3 | 12 | Yes | NE | [62] |
| Two DNA plasmids encoding optimized synthetic consensus E6 and E7 genes of HPV-16 and HPV-18 (VGX-3100) | 2, R | HPV-16 or HPV-18 positive CIN2/3 | 107 | Yes | Yes | [63] |
| Live vector-based vaccines | | | | | | |
| Vaccinia vector-based vaccine expressing modified forms of HPV-16 and -18 E6 and E7 proteins (TA-HPV) | 2 | Stage IB–IIA CC | 29 | Yes | NE | [65] |
| Live recombinant vaccinia virus expressing HPV-16 and -18 E6/E7 proteins | 1/2 | Advanced CC | 8 | Yes | NE | [66] |
| Modified Vaccinia Ankara virus, an attenuated replicon-deficient vaccinia strain, expressing E2 (MVA-E2) | 1/2 | CIN2/3 | 34 | Yes | Yes | [67] |
| Suspension of MVATG8042 vector particles consisting of an attenuated recombinant vaccinia virus, modified vaccinia virus of Ankara, containing the sequence coding for the modified E6 and E7 early genes of HPV-16 and human interleukin-2 gene (TG4001) | 2 | HPV-16 positive CIN2/3 | 21 | Yes | Yes | [68] |
| Recombinant adenovirus-p53 (rAd-p53) combined with chemotherapy | 2, R | Stage IIb2-IV CC | 40 | NE | Yes | [71] |
| Escalating doses of live-attenuated Listeria monocytogenes (Lm) vaccine that secretes the HPV-16 E7 antigen fused to a non-hemolytic fragment of the Lm protein listeriolysin O (LLO), Lm-LLO- E7 | 2 | Advanced CC | 15 | NE | NE | [74] |
| Live attenuated Listeria monocytogenes (Lm) bioengineered to secrete a HPV-16-E7 fusion protein targeting HPV transformed cells (ADXS11-001) | 2, R | Advanced CC | 110 | NE | Yes | [75] |

Abbreviations: CC, cervical cancer; CIN, cervical intraepithelial neoplasia; CTL, cytotoxic T-lymphocyte; DC, dendritic cell; FiH, first-in-human; N, number; NE, not evaluable; R, randomized.

Table 2. Relevant ongoing clinical trials with therapeutic vaccines for cervical pre-neoplastic lesions and cervical cancer.

| Therapeutic agent | Phase | Disease | Trial ID |
|---|---|---|---|
| Peptide–protein-based vaccines | | | |
| Therapeutic human papilloma virus 16 (HPV-16) E6/E7 long peptides vaccine (ISA101) at different doses with or without IFN-α as combination therapy with carboplatin and paclitaxel | 1/2 | HPV-16 positive advanced or recurrent cervical cancer | NCT02128126 |
| HPV-16 E6 peptides combined with Candida skin testing reagent called Candin® (PepCan) | 2 | High grade CIN | NCT02481414 |
| Escalating doses of R-enantiomer of 1,2-dioleoyl-3-trimethylammonium-propane chloride plus Peptides from HPV-16 E6 and E7 (PDS0101) | 1 | Female subjects with high-risk HPV infection and biopsy-proven CIN1 | NCT02065973 |
| Escalating dose of a vaccine consisting of four HPV-16 E6 peptides in combination with Candin | 1 | High grade CIN | NCT01653249 |
| Tumor specific potentiated vaccine therapy using cyclophosphamide combined epitope peptide cocktail (five peptide vaccines of KOC1, TTK, CO16, DEPDC1, MPHOSPH1) | 1 | Progressive or relapsed solid tumors including cervical carcinoma | NCT00676949 |
| SGN-00101 (HSP-E7) fusion protein | 2, R | High grade CIN | NCT00054041 |
| Nucleic acid-based vaccines | | | |
| VB10.16 immunotherapy (DNA vaccine) | 1/2 | High grade CIN | NCT02529930 |
| HPV-16-specific therapeutic DNA-vaccinia vaccination in combination with topical imiquimod | 1 | HPV-16 related CIN3 | NCT00788164 |
| pnGVL4a-CRT/E7 (Detox) | 1 | HPV-16 related CIN2/3 | NCT00988559 |
| INO-3112 (VGX-3100 and INO-9012) delivered intramuscularly by electroporation | 1/2 | HPV-16 or -18 related invasive cervical carcinoma | NCT02172911 |
| Naked plasmid coding for HPV-16 and -18 E6/E7 (GX-188E) administered i.m. by electroporation | 1 | HPV-16 or -18 related CIN3 | NCT01634503 |
| Naked plasmid coding for HPV-16 and -18 E6/E7 (GX-188E) administered i.m. by electroporation | 2, R | HPV-16 or -18 related CIN3 | NCT02139267 |
| Change of immunogenicity and lesion condition in subjects who have enrolled and participated NCT02139267 trial | Obs | Subjects who participated NCT02139267 trial | NCT02411019 |
| Live vector-based vaccines | | | |
| Attenuated Live Listeria Encoding HPV-16 E7 Vaccine ADXS11-001 | 2 | Persistent or recurrent cervical carcinoma | NCT01266460 |
| ADXS11-001 administered following chemoradiation as adjuvant treatment | 3 | High-risk, locally advanced cervical carcinoma | AIM2CERV |

CIN: cervical intraepithelial neoplasia; Obs: observational; R: randomized.

### *Adoptive T-cell therapy (ACT)*

ACT allows a more rigorous control on the magnitude of the targeted response than tumor vaccination strategies. T-cells of a desired specificity and phenotype can be identified and expanded *in vitro* to obtain following reinfusion a number of antigen-specific T-cells that is 10-fold greater than those reachable by using current therapeutic vaccines regimens alone [36]. Recently, this ACT approach was utilized in chemotherapy-refractory metastatic cervical cancer epithelial malignancy. Tumor-infiltrating T-cells were collected from patients, expanded, and selected for HPV reactivity (HPV-TILs). Single infusion of these TILs induced objective responses in three out of nine patients and the presence of TILs in peripheral blood 1 month after treatment was associated with clinical response [37]. Although this technique cannot be easily performed, it may represent a dramatic improvement of advanced cervical cancer treatment where even new biological therapies failed to ameliorate the prognosis of these patients.

## Peptide-protein-based vaccines

Synthetic peptide vaccines can be divided into two groups: synthetic long peptides and specific epitope (short) peptides. Peptide-based therapeutic vaccines have the advantages of stability, safety and easy production [38], even if the immunogenity is low, and there is the need of identifying specific epitopes of HPV antigens for different HLA haplotypes [39].

Synthetic long peptides-based vaccines were largely tested in experimental models, and some of them tested in clinical trials [40]. The use of synthetic long peptides has several advantages over short peptides. Notably, it does not require patient selection based on MHC profile. Recently, the interest toward synthetic long peptides-based vaccines has been strengthened because of the broad range of antigenic epitopes through inclusion of immunogenic peptides or peptides which guide CD4 + T-helper or CD8+ cytotoxic responses. Results in animal models showed immunologic responses and HPV-16 tumors eradication in vaccinated animals [41,42].

An interesting study in high-grade HPV-16 related vulvar intraepithelial neoplasia (VIN), where the spontaneous regression is less than 1.5%, was recently published and the authors investigated the immunogenicity and the efficacy of a synthetic long-peptide vaccine in 20 women. They received multiple three to four doses of vaccine (a mixture of long peptide from HPV-16 oncoprotein E6 and E7 in incomplete Freund's adjuvant). Toxicity was low, and at 12 months of follow-up 9 patients over 19 showed complete response (47%), which was maintained at 24 months. Moreover, patients with complete response had a stronger immunologic response [43]. A phase I trial with synthetic long peptide vaccine in advanced cervical cancer showed low toxicity and high immunogenicity [44]. Consequently, the HPV-16-synthetic long peptide vaccine was tested in 20 advanced/recurrent cervical cancer patients in a phase II trial, and HPV-16-specific T-cell response was analyzed. In 13 evaluable patients, a HPV-16-specific T-cell

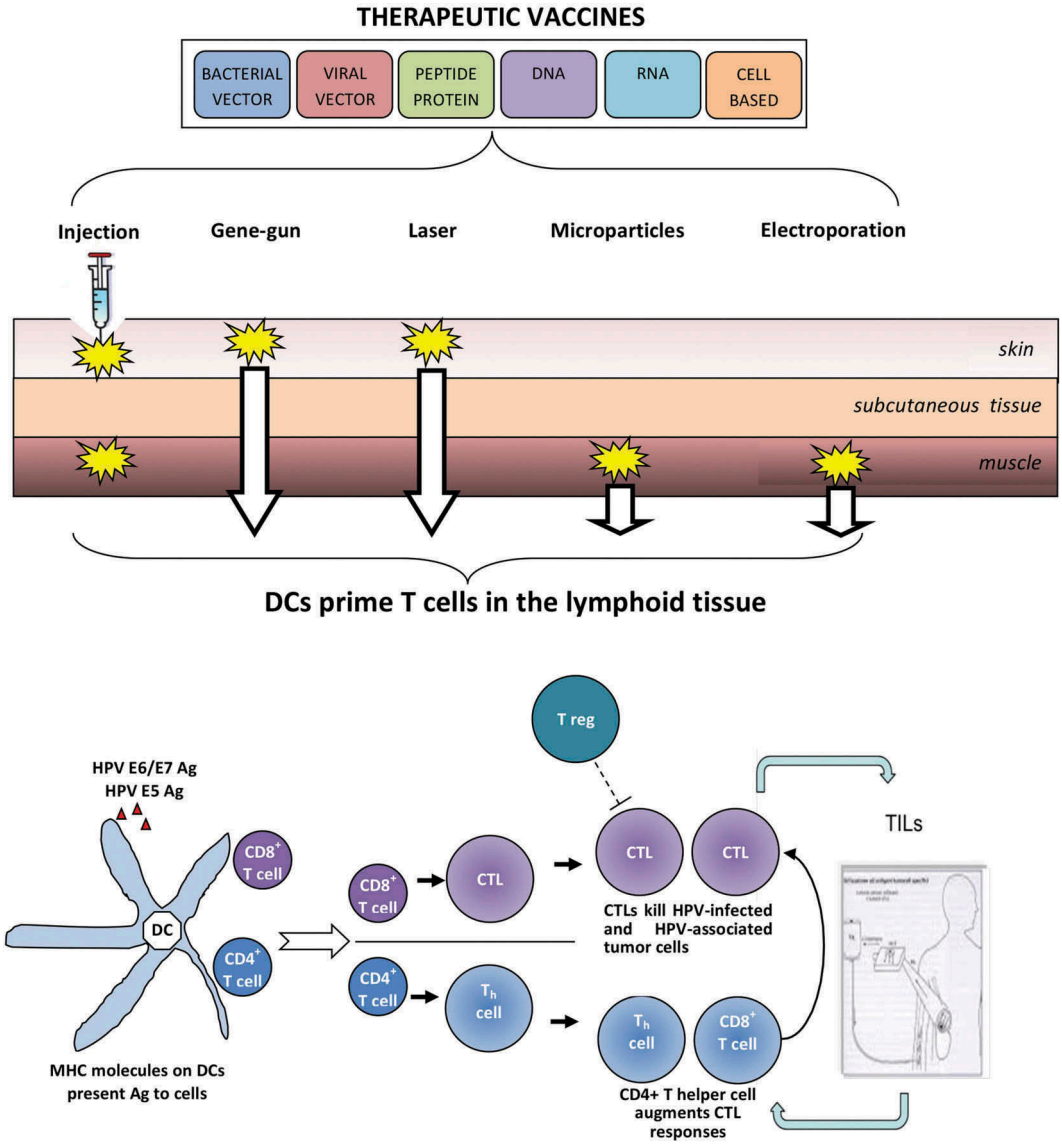


Figure 1. Methodologies for production and delivery of HPV therapeutic vaccines as well as their immunological activity. Abbreviations: Ag, antigen; DCs, dendritic cells; Treg, regulatory T-cell; Th, T helper cell.

response was recorded after two to four vaccinations, and patients failing to have a specific response also did not develop immunity against other antigens, suggesting a decreased T-cell immunocompetence. Unfortunately, although the vaccine was well tolerated, the induced specific immune response did not result in regressions of the tumor [45]. A phase I/II study with therapeutic HPV-16 long peptide vaccine (ISA101) at different doses with or without IFN-alpha in combination with carboplatin and paclitaxel in advanced cervical cancer (NCT02128126) is ongoing to determine safety and immunomodulating effects.

A phase I dose-escalation trial of a peptide-based HPV therapeutic vaccine with Candida skin test reagent as adjuvant (PepCan) was carried out in 24 CIN2/3 patients, showing very low toxicity, a satisfactory regression rate, and a significant immune response [46]. The phase II is ongoing (NCT02481414). HPV-16 E6/E7-derived epitopes are being investigated as liposome-encapsulated in CIN (NCT02065973). A phase I study is testing a HPV vaccine consisting of four HPV-16 peptides in combination with Candin (purified extract from Candida Albicans) to determine optimal dose, immunogenicity, maximum tolerated dose in women with HSIL lesions (NCT01653249).

A phase II study of multiple peptides cocktail vaccine for advanced cervical and ovarian patients was recently presented at American Society of Clinical Oncology Meeting, in May 2015 [47]. In 21 cervical cancer patients they observed 2 complete responses, a median overall survival of 15.4 months and a good tolerability. A phase I trial is evaluating safety and efficacy of cyclophosphamide combined with specific epitope cocktail and IL-2 for advanced cancers including cervical cancers (NCT00676949).

The use of epitope-specific short peptides-based vaccines aims at ensuring precisely targeted cytotoxic T-lymphocyte (CTL) responses through immunization with specific epitopes, and it is well known how linear short peptide epitopes can induce cytotoxic responses. This kind of vaccine is restricted to patients of a given HLA type; thus, preliminary HLA typing is

needed before vaccination. Some epitope-specific therapeutic HPV vaccines have been tested in clinical trials, with low clinical responses in VIN or CIN [48,49], even employing new delivery system [50], while other phase I trials are ongoing also in other diseases (NCT00257738; NCT01653249).

The advantage of protein-based vaccines over peptide-based is that the former can circumvent the MHC specificity limitation. However, a potential disadvantage of proteins vaccine is that they may induce antibody responses rather than CTL responses. To avoid this disadvantage and to improve immunogenicity, adjuvant have been added into vaccines and/or proteins for therapeutic vaccination have been linked to molecules able to better induce APC presentation.

Various protein vaccines have moved to clinical trials. Few protein-based vaccines have reached the clinical phase [17,51]. Immunization with HPV-16 E6/E7 fusion protein mixed with the ISOMATRIX adjuvant was shown to be safe and immunogenic in a Phase I study. In addition, enhanced CD8+ T-cell responses to both E6 and E7 were recorded in vaccinated patients versus placebo recipients [52]. A protein vaccine, TA-CIN, consisting of a fusion of HPV-16 L2, E6, and E7, was proved to be safe and able to elicit humoral response in all the treated women and cellular (T-cell specific) immunity in a subset of them [53]. A mutated HPV-16 E7 fused to part of Haemophilus influenzae protein D, mixed with adjuvant AS02B (GSK proprietary formulation) has been evaluated in and was shown to induce significant E7-specific CTL responses in Phase I/II clinical trials on CIN1–CIN3 [54]. A complete regression was reported in 22% of CIN3 patients in a phase II trial [55] employing HspE7 (a fusion protein consisting of HPV-16 E7 and Hsp65 from Mycobacterium Bovis – SNG00101). Another trial using the same vaccine in 20 CIN3 patients reported 35% of complete regression and correlation with immune response [56]. A phase II randomized (vs. standard surgical treatment) study with SGN00101 (HSP-E7) fusion protein is being tested in women with CIN3 (NCT00054041).

## Nucleic acid-based vaccines

### *DNA-based vaccines*

DNA-based vaccines are safe, can be easily produced, and are capable of inducing both CTL and Th immunity, and also B-cell immunity; moreover, they can be administered repeatedly for long-term protection, even if they lack in inducing potent antigen specific cellular response because of immune tolerance against self-antigens [57]. To increase immunogenicity, encoding xenogenic version of antigens, fusions of antigens with T-cells activating molecules, priming with DNA vectors followed by boosting with viral vectors, or the use of immunomodulators have been investigated.

A DNA plasmid (ZYC101) was tested in a phase I trial in 12 patients with anal inthraepithelial neoplasia, with a broad antigen-specific immune response [58]. The same vaccine was tested in 15 CIN2/3 patients, with five patients showing complete histological regression [59]. More interestingly, the new form of the vaccine (ZYC101a, including HPV-16/18 E6- and E7-derived CTL epitopes), was employed in a randomized, double blind, phase II trial, enrolling 127 CIN2/3 patients, with a trend in the experimental arm for complete regression, and a statistically significant difference in very young patients [60]. A phase I–II ongoing study is testing safety and immunogenicity of a DNA vaccine, VB10.16 in women with CIN3 (NCT02529930). Another DNA vaccine targeting E7, pNGVLa-Sig/E7(detox)-Hsp70, was employed in a phase I–II trial in CIN2/3 patients. The E7 sequence was fused to a chaperone (Hsp70 from Mycobacterium tuberculosis) to enhance APC uptake, processing and presentation. Significant immune response was observed in eight patients, and a complete regression was observed in three patients [61]. More recently a phase I trial with sequential pNGVLa-Sig/E7(detox)-Hsp70 with a recombinant vaccine virus encoding E6/E7 fusion protein, with or without imiquimod, an immunomodulator, was tested in 12 patients with CIN3, and five of them showed complete regression [62]. The study is ongoing (NCT00788164). Another ongoing study is testing different routes of administration of pnGVL4a-CRT/E7 (Detox) in patients with CIN2/3 (NCT00988559).

Recently, a mixture of two plasmids encoding HPV-16/18 E6/E7 antigens, VGX-3100 vaccine, administered by electroporation, is being tested in phase I–II trials. A phase IIb randomized, double-blind placebo controlled trial (NCT01304524) tested VGX-3100 in CIN2/3 patients. Among 107 evaluable patients, 53 of the treated patients (49.5%) and 11 (30.6%) of the placebo arm showed complete regression ($p = 0.034$). Toxicity was low, with mild injection-site reactions in almost all the patients, and erythema significantly more common in the vaccine arm, so the VGX-3100, inducing both T-cell and antibody response to E6 and E7, is the first therapeutic vaccine to show efficacy against HPV-16 and HPV-18 related CIN2/3 [63]. This study represents a proof of principle for a therapeutic approaches distinct from surgery. Another study is ongoing, testing VGX-3100, administered i.m. by electroporation with IL-2-12-coding construct (INO-9012) and optionally combined with radiotherapy (NCT02172911). A naked plasmid coding for HPV-16 and 18 E6/E7 (GX-188E) and for fms-like tyrosine kinase-3 ligand (FLT3LG) administered i.m. by electroporation is being tested for safety in a phase I study in patients with CIN3 (NCT01634503), and with different doses in a phase I–II randomized study in patients with HPV-16 or 18 related CIN3 (NCT02139267). Another ongoing study with the same vaccine is a prospective, multicenter, observational, follow-up clinical study to determine recurrence of CIN and long-term safety (NCT02411019).

### *RNA-based vaccines*

RNA-based vaccines are created using naked RNA replicons derived from alpha-viruses to promote antigen-specific immune response. The replicon-based vectors can replicate in a wide range of cell types, with different expression of antigens. These RNA replicons are less stable than DNA. A combined approach with DNA-launched RNA replicon, termed 'suicidal' DNA was developed for HPV vaccine in preclinical models [64]. The newest mRNA-based vaccines have also been developed and the RNActive® vaccine platform from CureVac (Tübingen, Germany) is in clinical trials for prostate and non-

small cell lung cancer. RNActive vaccines were well tolerated and induced long lasting, humoral and cellular immune responses. However, information about clinical testing on HPV-associate cancers is lacking.

## Live vector-based vaccines

### Viral vector vaccines

Viral vector-based vaccines have high infection efficiency and high antigens expression, encoded by the virus in the infected cells. Many viral vectors, such as adenoviruses, alphaviruses, vaccinia viruses, fowlpox viruses have been tested, and the most interesting are vaccinia virus-based vaccines [65,66]. The first clinical trial of a live recombinant vaccinia virus expressing HPV-16 and -18 E6/E7 proteins was a phase I–II trial where eight advanced cervical cancer patients received a single dose of vaccine. No significant side-effects were observed, and 3 out of 8 patients had an HPV-specific antibody response [66]. A study tested a vaccinia vector-based vaccine expressing modified forms of HPV-16 and -18 E6 and E7 proteins (TA-HPV) in 29 patients with stage IB-IIA prior surgery, and specific CTLs and serologic response were found in a subset of patients [65]. Further positive results were observed in VIN and in VAIN.

The MVA-E2 viral-based vaccine is a modified Vaccinia Ankara virus, an attenuated replicon-deficient vaccinia strain, expressing E2, and a phase I–II study in 34 CIN2/3 patients showed complete regression in 20 patients, with the development of HPV- specific antibodies [67]. In another study 21 patients with CIN2/3 were treated with TG4001, a modified vaccinia Ankara viral vector encoding HPV-16 E6/E7 and IL-2, and 10 (48%) patients had clinical response at month 6, with seven complete responses, warranting further development of clinical trials [68]. A randomized phase II study (NCT01022346) is testing TG4001, and preliminary results reported significant differences between vaccine and placebo in a press release [69]. Twelve patients with HPV-16 associated CIN2/3 have been treated with peripheral vaccination with a heterologous DNA prime-recombinant vaccinia vector-based boost vaccination regimen before surgical resection in a phase I study. The schema included two priming vaccination with a DNA vaccine expressing HPV-16 E7, following by a recombinant vaccinia boost expressing HPV-16 and -18 E6/E7. Three dose levels have been evaluated (cohort 1, 2, 3). Toxicity was low, mostly consisting in injection site reactions, and one out of three patients in each of the one and two cohorts had complete regression of the lesion at surgery, whereas at the highest dose level, three out of six patients had complete regression [62].

Adenoviral vector (rAd)-based vaccines showed immunological response in preclinical studies, but to date no therapeutic effects have been observed [70]. Recombinant adenovirus-p53 combined with chemotherapy was tested in a phase II study presented at American Society of Clinical Oncology May 2013 [71]. Forty patients received chemotherapy plus rAd-p53 or chemotherapy alone; the response rate was 95% versus 75%, and the overall 1-year survival rates were 90% and 65%, respectively, without serious adverse events related to rAD-p53.

### Bacterial vector vaccines

Several bacterial vectors have been tested as therapeutic vaccines, such as Listeria monocytogenes, Lactobacillus plantarum, Lactococcus lactis, with the Listeria-based being the most extensively studied [72]. A phase I trial tested the tolerability of the Listeria-based vaccine (Lm-LLO-E7) in 15 patients with advanced cervical cancer, with some severe (grade 3) reactions in 40% of the patients, and 1 partial response [73].

The results of a prospective randomized trial employing a Listeria Monocytogenes-based vector vaccine (ADXS11-001) in 110 advanced cervical cancer patients were presented at the ASCO meeting in June 2014. Patients were randomized to three doses of vaccines or four doses with weekly cisplatin. Final 12-month's overall survival was 36%, 18-month's overall survival was 28%, response rate was 11% with six complete responses, and the authors reported 2% of grade 3 adverse events. No benefit was observed from the addition of cisplatin [74]. The NRG0265 phase II study with multiple doses of ADXS11-001 is currently ongoing [75, NCT01266460], without cisplatin, and preliminary results showed in 26 evaluable patients receiving at least one dose a 12-month overall survival of 38.5% [76]. Other trials with ADXS11-001 are ongoing in CIN2/3 or cervical cancer, and an international phase III study (AIM2CERV) of ADXS11-001 as adjuvant treatment of high-risk, locally advanced cervical cancer is under development in collaboration with Gynecologic Oncology Group.

## Conclusions

Immunotherapy is one of the most promising strategies for cancer treatment. In cervical cancer and its precursors, the use of therapeutic vaccines was associated with the regression of premalignant lesions and some clinical benefit in cancer patients. To mitigate the immunosuppressive effects of cancer microenvironment, combinatorial approaches, such as radiotherapy, chemotherapy, immunomodulators, immune checkpoint inhibitors, are being tested in cervical cancer patients. Indeed, although chemotherapy and immunotherapy have not shown to be curative as single treatment modalities in the advanced setting, growing evidence suggests a synergistic activity with immunological approaches [77].

The available literature data suggest that therapeutic vaccines for pre-neoplasia and cancer of cervix are reality (i.e. synthetic long peptide or electroporated DNA vaccines). The improvement of all therapeutic strategies and the identification of their optimal combination opens a new scenario in the treatment of cervical cancer and pre-neoplastic lesions.

## Expert commentary

About 20% of human cancers are linked to infectious diseases and among them one-third of tumors are caused by HPV. The observation that HPVs related to cancer express two main oncogenes, i.e. E6 and E7, that are essential for transformation and maintenance of transformed status, makes these

oncogenes ideal targets of cancer immunotherapy. E6 and E7 can be considered the best tumor-associated antigen, and many different therapeutic vaccines have been produced and employed in clinical trials for cervical cancer and pre-neoplastic lesions. Some of these trials have demonstrated not only safety and immunological responses to therapeutic HPV vaccines but also their clinical efficacy. Thus, HPV therapeutic vaccines must be regarded as a therapeutic option together with other currently available therapies.

## Five-year view

The HPV-E6 and -E7 antigens represent the ideal targets for therapeutic vaccines in premalignant and malignant cervical lesions. Looking to the future, applying new technologies (i.e. omics) to better define HPV infections that evolve to CIN and cervical cancer could enhance the efficacy of the vaccines by improving target selection and by reducing unwanted side effects. Since at present effective treatments are lacking mainly in advanced cervical disease, the next 5 years will be focused also on how to combine therapeutic vaccination with other conventional treatment strategies, such as chemotherapy, radiation, biological agents, and other immunologic approaches. In particular, check-point inhibitors, empowering any ongoing anti-cancer immune response that might have been too weak or exhausted, have shown extremely encouraging clinical activity. Monoclonal antibodies (mAb) interfering with CTLA-4-CD80/86, PD-1/PD-L1, TIM-3-GAL9, and LAG3-MHC-II belong to this category of check-point inhibitors and some of them are already US FDA approved for melanoma cancer treatment [20]. As the role of immunotherapy for the treatment of premalignant lesions and cervical cancer continues to evolve, several clinical trials are ongoing and in development, and further studies on the immune cellular and molecular mechanisms of action and on preclinical models are needed to better elucidate immunological background and to explore the optimal integration among treatments and combination immunotherapies. For the next 5 years, the combination or sequential application of immunotherapic agents will be the front line in the battle against cancer, including cervical cancer and its precursor. However, toxicities and prohibitive costs of immunostimulatory mAb are serious limits to combination immunotherapy and researches must be also focused on overcoming these obstacles.

## Key issues

- HPVs linked to cancer express two main oncogenes, E6 and E7, that represent an excellent target for cancer immunotherapy.
- Many therapeutic vaccines have been tested in order to increase viral specific immune responses, showing an acceptable safety profile and clinical efficacy.
- In a phase II trial, patients with chemotherapy-refractory metastatic cervical cancer epithelial malignancy were treated with adoptive T-cell therapy. Despite this technique is technically difficult, it showed some clinical activity.
- In patients with HPV16 or HPV18 positive CIN, a phase II randomized trial showed clinical activity of a nucleic acid-based vaccine.
- Multiple trials involving peptide-protein-based vaccines and live-vector based vaccines demonstrated that this approaches may be effective in CIN as well as in advanced cervical cancer patients.
- HPV therapeutic vaccines is one of the most promising strategies for cancer treatment, showing regression of premalignant cervical lesions and some clinical activity in cervical cancer patients.

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