Review Article

Anti-human papillomavirus therapeutics: Facts & future

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Received February 16, 2009

Even after 25 years of establishing Human Papillomavirus (HPV) as the causative agent for cervical cancer, effective treatment of HPV infection still unavailable. Comprehensive efforts especially for targeting HPV infection have been made only in recent years. Conventional physical ablation of HPV-induced lesions such as cryo-therapy, photo-therapy, LEEP, laser cone-biopsy and localized radiotherapy are shown to be effective to some extent in treating localized lesions where the removal of diseased tissue is associated with removal of transforming keratinocytes harboring HPV. Apart from currently available prophylactic vaccines which prevent the viral entry and should be given prior to viral exposure, several attempts are being made to develop therapeutic vaccines that could treat prevailing HPV infection. In addition, immunomodulators like interferons and imiquimod that have been shown to elicit cytokine milieu to enhance host immune response against HPV infection. Also, antiviral approaches such as RNA interference (RNAi) nucleotide analogs, antioxidants and herbal derivatives have shown effective therapeutic potential against HPV infection. These leads are being tested in pre-clinical and clinical studies. Present article provides a brief overview of conventional therapies for HPV-associated diseases. Potential of non-ablative anti-HPV treatment modalities that could prove useful for either elimination of HPV in early stages of infection when the virus is not integrated into the host cell genome or suppression of the expression of viral oncogenes that dysregulate the host cell cycle following transformation is discussed.

Key words Cervical cancer - herbal therapeutics - human papillomavirus - immunotherapy - RNA interference - therapeutic vaccines - transcriptional regulation

Introduction

The plurality of HPV genotypes is associated with its ability to cause a wide spectrum of epithelial proliferative lesions¹,². The global burden of HPV associated diseases is quite high, and is estimated to be about 5.17 per cent of total cancer burden. Among the HPV-associated diseases, cervical cancer which is the second most common cancer among women world over tops the list. Papillomaviruses exhibit a high degree of cellular tropism for squamous epithelial cells of different organ sites and have been associated with various clinical manifestations ranging from benign hyperplastic epithelial proliferative innocuous lesions (warts, papillomas) to invasive carcinomas (Table I). On the basis of their association with disease types, papillomaviruses are classified into high-risk (HR) and low-risk (LR) types. HR-HPV types (HPV 16, 18, 31, 35, 39, 45, 51, 52, 56, 59, 66, 68, 69 and 73) are often associated with high grade lesions and invasive cancer, whereas the LR-HPV types (HPV 6, 11, 40, 42, 43, 44, 53, 54, 57, 64 and 65) are often associated with low-grade lesions and non-invasive cancers.
Persistent infection of specific types of HR-HPVs particularly the type 16 and 18 is a prerequisite for the development of cervical intraepithelial neoplasia (CIN), and cervical cancer. However, HPV infection is a transient phenomenon. Following infection, the virus may persist over time, leading to development of low grade cervical lesions where the virus is present in non-integrated form, progressing in course of time to cancer in a well-characterized process that takes 15-20 years. Though the natural history of HPV infection demonstrates spontaneous clearance of HPV infection in a large number of cases, persistence of high risk HPV infection for a year or more confers an increased risk of progression to cancer. It has also been shown that it takes about two years to clear type-specific HPV infection. Although HPV genome, which is a ~ 8Kb circular DNA, codes for about 6 early (E1, E2, E4, E5, E6 and E7) and two late genes (L1 and L2), expression of E6 and E7 of the HR-HPVs can immortalize human squamous epithelial cells in vitro, the discovery that lead to establishment of the role of these viruses in development of cervical carcinoma. Virus-encoded E6 and E7 oncoproteins interact specifically with important cell cycle regulatory proteins. The E7 protein of the genital tract HPVs, similar to the adenovirus ElA proteins and the large tumour antigens of the polyomaviruses, can complex with the product of the retinoblastoma tumour suppressor gene, pRB, and disrupts the association of E2F transcription factor with pRB. The E7 proteins of the “high risk” HPVs, such as HPV-16 and HPV-18, bind pRB with ~10-fold higher affinity than do the E7 proteins of the “low risk” HPV types 6 and 11, and this difference in binding affinity correlates with the transforming potential of the different E7 proteins. Like simian virus 40 (SV40) large tumour antigen and adenovirus E1B, the E6 protein of the “high risk” HPVs can complex with the p53 protein. Primary role of E6 interaction with p53 is formation of a ternary complex alongwith a ubiquitin ligase called E6AP. This ternary complex formation leads to the ubiquitination and degradation of

<table>
<thead>
<tr>
<th>Disease</th>
<th>HPV type(s) associated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign and pre-cancerous Lesions</td>
<td></td>
</tr>
<tr>
<td>Plantar warts</td>
<td>1, 2, 4, 63</td>
</tr>
<tr>
<td>Common warts</td>
<td>2, 1, 7, 4, 26, 27, 29, 41, 57, 65, 77, 1, 3, 4, 10, 28</td>
</tr>
<tr>
<td>Flat warts</td>
<td>3, 10, 26, 27, 28, 38, 41, 49, 75, 76</td>
</tr>
<tr>
<td>Other cutaneous lesions (e.g., epidermoid cysts)</td>
<td>6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73</td>
</tr>
<tr>
<td>Epidermodysplasia verruciformis</td>
<td>2, 3, 10, 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50</td>
</tr>
<tr>
<td>Recurrent respiratory papillomatosis</td>
<td>6, 11</td>
</tr>
<tr>
<td>Laryngeal papillomatosis</td>
<td>16, 11, 6, 10</td>
</tr>
<tr>
<td>Focal epithelial hyperplasia of Heck</td>
<td>13, 32</td>
</tr>
<tr>
<td>Conjunctival papillomas</td>
<td>6, 11, 16</td>
</tr>
<tr>
<td>Condyloma Acuminate (genital warts)</td>
<td>6, 11, 30, 42, 43, 45, 51, 54, 55, 70</td>
</tr>
<tr>
<td>Cancerous lesions</td>
<td></td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59</td>
</tr>
<tr>
<td>Oral carcinoma</td>
<td>16, 18</td>
</tr>
<tr>
<td>Esophageal carcinoma</td>
<td>16, 18</td>
</tr>
<tr>
<td>Laryngeal carcinoma</td>
<td>6, 11, 16, 18</td>
</tr>
<tr>
<td>Conjunctival carcinoma</td>
<td>6, 11, 16, 18</td>
</tr>
<tr>
<td>Anal carcinoma</td>
<td>16, 18, 31, 33</td>
</tr>
<tr>
<td>Vaginal carcinoma</td>
<td>16, 18, 31, 33</td>
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<tr>
<td>Vulvar carcinoma</td>
<td>16, 18, 31, 33</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>18</td>
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<tr>
<td>Bladder carcinoma</td>
<td>16</td>
</tr>
<tr>
<td>Penile carcinoma</td>
<td>16, 18, 31, 33</td>
</tr>
<tr>
<td>Non-melanoma skin cancer</td>
<td>5, 8</td>
</tr>
</tbody>
</table>

aData from3,4
bOrder indicates relative frequency; bold type indicates most frequent association
p53 by action of 26S proteasome which reduces the half life of p53 to less than 20 min in HPV infected keratinocytes. Establishment of E6 and E7’s oncogenic properties has made these viral genes the two most sought after targets for anti-HPV therapeutics.

The HPV life cycle comprises of four important steps necessary for its pathological consequences and are considered as potential targets for any anti-HPV therapeutic strategies. The viral entry is the first step in HPV pathogenesis followed by its persistence in epithelial cells. These two steps establish the viral genome into the host cell and has been the subject of therapeutic interventions through prophylactic and therapeutic vaccination. Most of the lesions maintain the virus as an episome and support complete virus replication cycle with tight regulation of viral gene expression. The third and most important step of HPV life cycle is its integration into the human genome which is frequently observed in CIN3 or higher lesions. The exact mechanism of this integration is not clearly understood, but loss of E2 ORF and release of inhibition on E6 and E7 expression and transformation/immortalization of keratinocytes follows this integration event. It is also not clear whether this integration is reversible or not. The integration of HPV genome is the key point which facilitates the progression towards malignancy. The fourth important step of viral life cycle is expression/upregulation of viral oncogene transcription in differentiating epithelium. As HPV gene expression is associated with epithelial differentiation programme and it has been controlled by specific cellular factors. Several host cell transcription factor binding sites and keratinocytes specific enhancers like AP-1, Sp1, NF-1, TEF-1, TEF-2, Oct-1, AP-2, KRF-1, YY1, STAT3 and glucocorticoid responsive elements have been identified in the upstream regulatory region (URR) of HPV, which determine the cell-type-specific viral gene expression and contribute to the tissue tropism of HPV. All these four steps provide means of interference with HPV life cycle and thus represent targets for development of anti-HPV therapeutics.

**Current treatment modalities**

**A) Conventional therapies:**

Even after establishment of causal relationship between HPV and cervical cancer, currently, presence or absence of HPV does not have any impact on deciding the treatment; the strategies are primarily anti-cancer and not anti-viral. Several therapies are available for treatment of HPV-associated diseases (Fig.). These treatment strategies are ablative in nature and aim to remove the lesion rather than specifically

**Fig.** Conventional therapies for treatment of cervical pre-cancers and cancers.
targeting the HPV infection. These treatments though effective are applicable only when the lesion is visible and does not necessarily eliminate HPV infection. Some of these procedure are also associated with significant morbidity such as profuse watery discharge, bleeding, cervical stenosis (narrowing) and in some cases cervical incompetence and pelvic infection. Even though these treatment modalities are effective, all of them are associated with considerable degree of recurrence which ranges up to 10 per cent of the cases. Studies aiming to correlate the status of HPV infection with efficacy of conization treatment for CIN 3 lesions revealed persistent HPV infection in all the cases which showed recurrence22,23 possibly due to positive resection margins24. Thus, the studies emphasized that patients with persistent HPV infection after conization for CIN 3 should be followed because they are at increased risk of developing disease recurrence.

Before the advent of colposcopy, hysterectomy was considered necessary for CIN lesions. However, the natural history of CIN has clearly shown that in a large majority of cases, CIN lesion does not progress to invasive cancer, thus, making hysterectomy an unnecessary and over-treatment procedure for CIN. At the same time, management of invasive carcinomas has little development as standard radiation or chemotherapy is applied along with surgical removal of primary tumour in advanced cancers. On the other hand, it is recommended that the earliest lesions, CIN1, should not be treated and should be followed up and treated only if they progress to higher lesion. In such cases, HPV tests are being recommended as adjunct to cytology for identification of women at risk of disease progression and selective ablation of their lesions25. In such cases, anti-HPV therapeutics may have the greatest impact.

B) Alternate therapies:

Cytotoxic agents: Though scissor excision is the most preferred methods for genital warts, topical preparations of cytotoxic compounds like Podophyllin or Trichloroacetic acid are also utilized in USA and Europe26. Similarly 5-Fluorouracil is also available but with very limited use for external genital lesions as it generates strong inflammatory reaction27. Use of Podophyllin and 5-Fluorouracil is contra-indicated in pregnancy. Most of these therapies show no or inconsistent antiviral dose response against HPV28. Recently, two most common anti-cancer agents for treatment of many cancers including cervical cancer, arsenic trioxide (As₂O₃) and carboplatin have been shown to target two most important transcription factors, AP-1 and NF-κB respectively that play a critical role in expression of HPV oncoproteins E6 and E7 though activation of HPV URR29,30 for which their anti-viral role is being reinvestigated.

Photodynamic therapy (PDT): PDT is a new treatment for a wide variety of malignancies and premalignant dysplasias, as well as some non-cancer indications. Therapeutic response to PTD is achieved through the activation of non-toxic photosensitiser located within neoplastic tissue, using visible light tuned to the appropriate absorption band of the photosensitiser molecule. This produces cytotoxic free radicals such as singlet oxygen, which result in local photo-oxidation, cell damage and destruction of the tumour cells that may induce bystander effect and activate host immunity. Topical administration of PDT though considered most appropriate for cervical and valval intraepithelial lesions, the clinical trials with 5-aminolevulinic acid has revealed variable results31-33. Though PDT is not equally efficacious for all subgroups, PDT for condyloma and intraepithelial neoplasia appears to be as effective as conventional treatments like cold-knife conization34. In contrast to cold-knife conization, PDT causes no substantial cervical stroma destruction and hence reduces the risk of a possible subsequent incompetent cervix, but the effect on HPV eradication was found to be the same as for other ablative therapies35. Subsequent study however showed that PDT could be a promising therapeutic modality as the HPV infection found in follow up studies could be a result of reinfection36.

Immunotherapy: Interferons (IFNs) are the only anti-viral drugs approved for the therapy of benign HPV-related lesions. While IFN-α, IFN-β and IFN-γ have all been tested against condyloma acuminata, most information is available on IFN-α which appears efficacious via a number of routes of administration, schedules and dosages with an acceptable safety profile. Success with IFN-α therapy, in terms of reduced recurrence rates of condylomas was reported from studies in which all visible lesions were surgically removed with subsequent administration of subcutaneous local IFN-α37. Limited data are available on the efficacy of IFNs in the treatment of HPV-related dysplasia and carcinoma. Combination therapy of IFN-α with retinoids appears promising for cervical carcinoma38,39. Though, along with retinoids, IFN-α showed synergistic anti-angiogenic effect and upregulation of HLA class I and cell adhesion
molecule, ICAM-1 in HPV-harboring tumour-cell line, the anti-viral effects of the combination have not been explored. In another study, IFNα showed no effect on HPV11 replication. The treatment is expensive, has limited efficacy and not recommended for routine clinical practice in the treatment of high-grade HPV-associated lesions.

Apart from directly injecting IFNs, immunomodulators, such as imidazoquinolones that induce production of inflammatory cytokines are being used for potentiation of innate immune mechanism mediated through macrophages and dendritic cells. Imiquimod, one of the imidazoquinolone compounds has shown efficacy and safety in clinical trials for the treatment of external HPV-infected genital warts. This class of compounds generates a cytokine milieu that activates dendritic cell migration and leads to a Th1 type of response. Recent study showed presence of HPV-specific CD4+ regulatory T cells isolated in lymph nodes of cervical cancer patients that could suppress proliferation and IFNγ & IL-2 cytokine production by responder T cells. It is likely that these compounds might have antagonizing role on these regulatory T cells.

Prospective therapeutics

The most significant events in the HPV biology have been successful clinical trial with potent prophylactic anti-HPV vaccines, Gardasil from Merck, USA and Cervarix from GlaxoSmithKline, Belgium. However, there are a number of limitations like cost of the vaccine, efficacy in target population, sex preference and age of immunization that prevent their effective mass scale implementation in near future. In view of these bottlenecks, it becomes essential to explore alternative methods for control of HPV infection and effective management of cervical and other HPV-related pre-malignant and malignant lesions. Several therapeutic strategies are being explored which can effectively target at various phases of viral infection such as viral entry, viral latency, viral replication, and its oncogenic expression. Since low grade and high grade lesions differ with respect to the viral activity, the treatment approaches also differ and are specific to the grade of the lesions to be treated. Low grade lesions are usually homogeneous, genetically stable with permissive viral replication; in a immunocompetent individual, therapeutics are aimed to resolve these lesions and to prevent entry, replication and latency of the virus without any recurrence. In contrast, high grade disease is heterogeneous and genetically unstable with less active viral replication but with expression of oncogenic proteins. Therefore, targeting expression of these viral oncogenes either by promoting anti-HPV immunity or RNA interference or transcriptional inactivation is the norm. Prospective therapeutics can be classified as therapeutic vaccines, immunotherapies, RNA interference-based therapies, anti-retrovirals and natural/herbal derivatives. Many of these therapeutics are at later stages of their clinical evaluation.

A) Therapeutic HPV vaccines:

Vaccination with virus-like particles (VLP) such as Gardasil and Cervarix has demonstrated efficacy in HPV prophylaxis but these vaccines lack therapeutic potential. Considering the vital role of HPV16 in carcinogenesis, most of the efforts for developing therapeutic HPV vaccines have been directed towards development of vaccines against HPV16 viral oncogenes E6 and E7. Based upon the nature of immunogen the therapeutic vaccines can be divided into two major classes, (i) proteins/peptide-based vaccines, and (ii) DNA-based vaccines. Majority of the studies conducted as phase I clinical trials both in healthy individuals as well as in patients having high grade lesions or frank malignancies have used whole protein or peptides derived from HPV16 or HPV18 E6 and E7 mainly because of their oncogenic potential and they are invariably retained and expressed throughout HPV-related disease progression and carcinogenesis.

These immunogens were found to be highly effective in generating both humoral as well as cytotoxic T cell response in majority of trial subjects, but their effect on lesion clearance/regression or on HPV positivity were determined in a very few studies. Several animal studies have shown promising results indicating that therapeutic HPV vaccine may regress disease progression. Phase I trials that recorded these parameters indicate that despite effective anti-HPV immunity, lesion regression as well as HPV clearance was suboptimal and was only observed in a subset of patients. Perhaps this could be the potential reason why these therapeutics could not enter in Phase II and Phase III studies. Apart from these, there are several other limitations that remain to be overcome like subunit vaccines are costly to prepare and require cold chain and the response is short term and requires several boosters. To overcome some of these limitations, second generation, less expensive therapeutic DNA-based vaccines are being prepared against HPV16 E7. These efforts are also directed towards generation of effective immune response
<table>
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<tr>
<th>Target Antigen</th>
<th>Phase/study status</th>
<th>Disease model</th>
<th>Response</th>
<th>References</th>
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<tbody>
<tr>
<td><strong>A. Protein-based Vaccines</strong></td>
<td></td>
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<tr>
<td>HPV16 and 18 E6 and E7 (TA-HPV) in live vaccinia virus</td>
<td>Phase I/II trial</td>
<td>Late Stage Cervical Cancer (n = 8)</td>
<td>HPV-specific antibody response in 3 out of 8, presence of HPV-specific cytotoxic T lymphocytes</td>
<td>ND</td>
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<tr>
<td>HPV16 L1/L2-E7 chimeric VLPs</td>
<td>Pre-clinical</td>
<td>C57BL/6 mice injected with TC-1 expressing HPV16 E7</td>
<td>Agglutinate erythrocytes and elicit high titters of neutralizing antibodies Effect mediated through class I restricted cytotoxic T lymphocytes</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 E7 peptides (amino acids 12-20 and 86-93)</td>
<td>Phase I</td>
<td>CIN/VIN II/III (n = 8)</td>
<td>DC infiltration in 6/6 cases No positive delayed type hypersensitivity for E7 increase in cytokine response &amp; E7-specific cytolyis in 10/16 cases 3 cases had complete clearance of dysplasia and CIN 6 had partial regression</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 L2, E6 and E7</td>
<td>Phase I</td>
<td>Healthy volunteers (n = 40)</td>
<td>Induced IgG antibodies &amp; enhanced T-cell immunity in 8/11 cases</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 E6E7 fusion protein</td>
<td>Phase I</td>
<td>CIN (n = 31)</td>
<td>HPV-specific antibody response, delayed type hypersensitivity, <em>in vitro</em> cytokine release, and CD8 T cell responses to E6 and E7 proteins</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 L1-E7 chimeric virus-like particles (CVLP)</td>
<td>Phase I</td>
<td>CIN II/III (n = 39)</td>
<td>Induces with high antibody titers against L1 and low titers against E7 as well as cellular immune responses against E7 and L1 Histological improvement to CIN I or normal was seen in 39% of the patients 56% of the responders were also HPV16 DNA-negative</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16/18 E7</td>
<td>Phase I</td>
<td>Stage IB or IIA cervical cancer (n = 10)</td>
<td>Enhanced CD4+ T-cell and antibody responses, E7-specific CD8+ T-cell response, DTH responses to intradermal injections of HPV E7 antigen</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 E6 and E7 synthetic long peptides</td>
<td>Phase I</td>
<td>Cervical cancer patients (n = 6)</td>
<td>Elevated CD4+ and CD8+ T cells response against E6 and E7</td>
<td>ND</td>
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<tr>
<td><strong>B. DNA-based vaccines</strong></td>
<td></td>
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<tr>
<td>HPV16 E7 (ZYC 101)</td>
<td>Phase I</td>
<td>Anal dysplasia (n = 12)</td>
<td>10/12 subjects showed increased immune response to E7 peptide Partial histological responses</td>
<td>ND</td>
</tr>
<tr>
<td>HPV16 E7 (ZYC101)</td>
<td>Phase I</td>
<td>CIN II/III (n = 15)</td>
<td>HPV-specific T-cell responses Partial histo-pathological response Response</td>
<td>ND</td>
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<tr>
<td>HPV16 E7</td>
<td>Preclinical</td>
<td>E7-expressing tumour-bearing mice</td>
<td>Higher E7-specific CD8+ T-cell immune responses Apoptotic tumour cell death</td>
<td>ND</td>
</tr>
</tbody>
</table>

CIN, cervical intraepithelial neoplasia; ND, not determined; VIN, valval intraepithelial neoplasia.
against HPV by intra dermal administration of DNA vaccines which represents a feasible strategy to deliver DNA directly into the professional antigen-presenting cells of the skin but the efficacy of this strategy for regression or downstaging of the lesion is yet to be proven. It is likely that additional targets are needed to be identified to compensate dysregulated host cells apart from targeting only the HPV oncoproteins. Recent studies show that combining DNA vaccine with either death receptor-5 antibody or silencing of fas ligand by RNA interference can substantially enhance the therapeutic potential of these vaccines. Apart from targeting only the HPV oncoproteins. Recent studies show that combining DNA vaccine with either death receptor-5 antibody or silencing of fas ligand by RNA interference can substantially enhance the therapeutic potential of these vaccines.

Apart from therapeutic HPV vaccines, there have been attempts to pulse dendritic cells (DC) with tumour lysate expressing HPV16 antigens. Such DCs when matured with non-toxic double stranded RNA were capable of inducing class I and class II T cell immunity in a pilot clinical study. This strategy is currently being tested in a clinical trial in patients with cervical cancer.

**B) RNA interference-based therapies:**

Following identification of target genes involved in neoplastic transformation and tumour growth in HPV-associated disease, target-specific therapeutic approaches are being extensively investigated. Last decade has witnessed a major advancement in RNA interference technology specifically targeting HPV oncogenes E6 and E7 without any damage to normal cellular RNA pool, which is often lacking in conventional therapeutics. E6 and E7 oncogenic expression is detected in both upper epithelial layers as well as during early stages in advanced stage of disease and targeting these proteins will eventually lead to the treatment of active lesions. Although, there are a number of RNA interference-based therapies for several oncogenes in Phase I-III clinical trials, they are yet to be tested clinically for HPV-associated lesions. Several in vitro and in vivo studies show effective targeting of high-risk HPVs using antiviral ribozymes and antisense molecules, siRNA and shRNA (Table III).

RNA interference has been achieved by four distinct approaches primarily against HPV16/18 E6 and E7. Most well established among these are anti-sense oligonucleotides and ribozymes. Application of antisense or ribozyme molecules has a favored position because of their specificity and the requirement of continued expression of the transforming genes E6 and E7 for maintaining the malignant phenotype of cervical cancer cells. The effects of anti-sense plasmids or oligonucleotides on the cell proliferation of cervical cancer cell lines have already been demonstrated in a variety of assays. Inhibition of E6 and E7 by complementary anti-sense transcripts led to reduced growth rates, loss of the transformed phenotype of cervical and oral carcinoma cell lines, and inhibition of tumour formation in an animal model. Antisense E6 and E7 oligonucleotides also showed an anti-proliferative effect in primary tumour explants. Treatment of HPV16-infected CaSkii and SiHa cells with phosphorothioate oligonucleotides against HPV16 E6 and E7 not only reduced cell proliferation in vitro but also led to a reduction in the weight of tumours derived from SiHa cells implanted into nude mice. Expression of antisense E6/E7 genes using adenoviral gene transfer has resulted in a strong inhibition of SiHa cell proliferation and a complete suppression of tumour formation after ex vivo application. However, for therapeutic application of antisense genes, their delivery is the major problem as a high percentage of target cells has to be transduced, because no bystander effect will help to spread the therapeutic principle. In addition, the expression of anti-sense genes has to be stable because the tumour cells are not eliminated by treatment with a proliferation-inhibiting antisense construct. To address this issue, Liposome-mediated transfer of genes efficiently expressing anti-sense E7 has been tried along with co-delivery of interleukin-12 cytokine genes and this approach was found to induce tumour cell apoptosis as well as an anti-tumoural immune response which was further augmented.

On the other hand, ribozymes offer several advantages over conventional anti-sense RNA. They are capable of catalytic activity and do not require the presence of an auxiliary enzyme such as RNase H. Furthermore, one molecule of ribozyme can bind and cleave several molecules of mRNA, giving rise to an amplification of the net effect, and thus a desirable reduction in the dosage required. In vitro studies showed that Ribozymes can be used for effective cleavage of HPV transcripts and can inhibit E6/E7-mediated immortalization. Recently, a hammerhead ribozyme, Rz170, have been successfully designed that can specifically target HPV16 E6E7 transcripts and was found to be effective in inhibiting cell growth and promoting apoptosis. Rz170 also appears to be very efficient in inhibiting tumour growth in nude mice.

In contrast, achieving RNA interference using small interfering RNA is more efficient than Anti-sense
Table III. Molecular targeting of HPV gene expression using RNA interference technology

<table>
<thead>
<tr>
<th>RNA Interference approach</th>
<th>Target</th>
<th>Study type</th>
<th>Biological response</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-sense oligonucleotides (AS-ODNs)</td>
<td>HPV16 &amp; HPV18 E6 and E7</td>
<td>In vitro</td>
<td>Growth inhibition of cervical and oral cancer cells harboring HPV18 or HPV16 but had little effect on cells lacking HPV</td>
<td>57,58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vivo in nude mice</td>
<td>Inhibited formation of colonies in soft agar</td>
<td>58</td>
</tr>
<tr>
<td>Ribozymes (Rz)</td>
<td>HPV16-E6/E7 HP ribozyme (R434)</td>
<td>In vitro</td>
<td>Reduced the growth rate and prevented immortalization of E6/E7 transformed normal human keratinocytes</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>HPV6b and HPV11 E1 genes</td>
<td>In vitro</td>
<td>Efficiently cleaved E1 gene RNA transcripts</td>
<td>60</td>
</tr>
<tr>
<td>Short-interfering RNAs (siRNA)</td>
<td>HPV16 &amp; HPV18 E6 and E7</td>
<td>In vitro</td>
<td>E6 silencing induced accumulation of cellular p53 protein, transactivation of the cell cycle control p21 gene and reduced cell growth. E7 silencing induced hypo-phosphorylation of retinoblastoma protein and apoptotic cell death. Inhibited cellular DNA synthesis. Induced morphological and biochemical changes characteristic of cellular senescence</td>
<td>61-65</td>
</tr>
<tr>
<td></td>
<td>HPV16 E6 and E7</td>
<td>In vivo in NOD/SCID nude mice</td>
<td>Retarded tumour formation in nude mice</td>
<td>62,66</td>
</tr>
<tr>
<td>Short hairpin RNA (sh-RNA)</td>
<td>HPV16 &amp; HPV18 E6 and E7</td>
<td>In vitro</td>
<td>Accumulation of p53, p21, and hypophosphorylated pRb proteins in cells. Lentiviral (LV) delivery of low dose sh-RNA reduced cell growth and the induction of senescence, high-dose infection resulted in specific cell death via apoptosis</td>
<td>67,68</td>
</tr>
<tr>
<td></td>
<td>HPV18 E6 and E7</td>
<td>In vivo in NOD/SCID nude mice</td>
<td>Transplant of HeLa cells infected with a low dose of LV-shRNA into Rag-/- mice significantly reduced the tumour weight, whereas transplant of cells infected with a high dose resulted in a complete loss of tumour growth. Systemic delivery of LV-shRNA into mice with established HeLa cell lung metastases led to a significant reduction in the number of tumour nodules</td>
<td>68</td>
</tr>
</tbody>
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oligos or ribozymes. Recent studies were demonstrated that using siRNA is more rational as these molecules can induce selective silencing of exogenous viral genes in mammalian cells, and the process does not interfere with the recovery of cellular regulatory systems previously inhibited by viral gene expression\(^\text{61}\). Moreover, because E6 and E7 genes are not present in normal cells, RNAi-based therapies would not affect them. Several studies have shown that HPV E6 and E7 can be efficiently targeted by small interfering RNA, resulting in suppressed monolayer and anchorage-independent growth and induce apoptosis and replicative senescence of SiHa cells\(^\text{61,64,66,68,79}\). The most interesting feature of E6 and E7 targeting by siRNA is the stabilization of E6 target protein p53 and its nuclear accumulation which eventually leads to the expression of p21 (WAF1/CIP1)\(^\text{64,66}\), hypophosphorylation of retinoblastoma protein (pRb 105) and activation of apoptotic pathways in HPV-infected target cells. Moreover, delivery of E6/E7 siRNA into nude mice has shown significant reduction in the number of tumour nodules and retarded tumour growth of HPV16+ cells in NOD/SCID mice\(^\text{64,66}\).

Despite being a totally rational antiviral strategy, RNA interference operates only at post-transcriptional level to suppress gene expression of viral oncogenes and does not have any impact on viral latency. Moreover, due to lack of bystander effect and requirement of large amount of therapeutic principle, the cells with targeted oncogenic RNA tend to loose their proliferative capacity (as desired) and get diluted out in fast expanding pool of non-targeted or inefficiently
targeted cells expressing viral oncogenes. Therefore, in view of these major shortcomings RNA interference may not be useful as stand alone therapy. Keeping these inherent lacunae, a few more rational approaches have been developed by combining RNAi against HPV E6 and E7 with the current forms of treatment for cervical cancer. In a recent study, targeting HPV oncogene by siRNA and reactivation of p53 pathway was found to increase the sensitivity of tumour cells to cisplatin. This strategy also reduced the cisplatin dose requirement and the prevented occurrence of drug resistance and resulted apoptotic death in cancer cells. In another study, the use of photocross-linking reagent, 4,5',8-trimethylpsoralen, conjugated oligo (nucleoside phosphorothioate) (Ps-S-Oligo) for photodynamic anti-sense regulation of the anti-apoptotic HPV18 E6 expression along with low concentration of cisplatin, upregulated more p53 activity and induced massive apoptotic death in comparison to that induced by Ps-S-Oligo alone.

As with the other technologies, in vitro testing of siRNAs against cervical cancer has shown promising results. However, there are issues that have held up the clinical development of ribozymes and antisense molecules are target selection, specificity and delivery along with low efficiency, short-period of maintenance, and high costs. In addition, transfection with siRNA can activate stress response genes (e.g., GADD and p38), and there is evidence that in some cases, siRNA might degrade some off-target mRNAs in human cells. This non-specific effects may explain, in part, why cytotoxicity can occur after transfection with seemingly harmless siRNAs and thus underline the importance of siRNA optimization.

C) Natural/herbal derivatives:

In this age of targeted therapy, the failure of most current drug–discovery efforts to yield safe, effective, and inexpensive drugs has generated widespread concern. Successful drug development has been obstructed by a general focus on target selection rather than clinical safety and efficacy. The very process of validating the targets themselves is inefficient and in many cases leads to drugs having poor efficacy and undesirable side effects not only for HPV-related diseases but cancer in general. Since any given cancer carries an estimated 300 gene alterations, this raises an important question about how effective these targeted therapies can ever be against cancer. Thus, it has become necessary to rethink drug development strategies and emphasis is being given to natural and herbal derivatives that are safe and possess multi-hit capability not only to target against the viral oncogenes but also to inhibit the co-operative signaling and dysregulated gene expression of the host cells.

Since productive life cycle of HPV is tightly linked with the differentiation programme of infected epithelial cells, various leads are being tested for their activity at different stages of HPV’s life cycle in the host cell which include virus binding and entry, presence of viral DNA in episomal form and its replication in host cells as well as host cell-dependent expression of viral oncogenes following integration of its DNA into the host cells. The noncoding upstream regulatory region (URR) of HPV consists of myriad of transcription factor binding sites like AP-1, SP1, NFκB, NF-1, TEF-1, TEF-2, Oct-1, AP-2, KRF-1, YY-1, STAT-3 and glucocorticoid responsive elements. During differentiation, these upregulated cellular transcription factors bind to the HPV URR and facilitate the expression of viral oncoproteins and late structural proteins. As these cellular transcription factors serve as a major link between oncogenic host cell transcription and also the viral gene expression, transcription factors provides a unique target for development of anti-HPV therapeutics. Theoretically, preventing binding of these transcription factors will eventually lead to the suppression of HPV oncogenic gene expression and stop assembly of virion particles. In past few years, several herbal compounds, derivatives, antioxidant and small polyphenol plant compounds have been used for selective suppression of host cell transcription factors like AP-1, and NF-κB in HPV infected cells. The selective suppression and alteration in composition of these factors has been shown to be associated with downregulation of HPV gene expression and induction of apoptosis in infected cells. Here, in this section we have described some of the potent anti-HPV activities which are in process of development for being used as anti-HPV therapeutics (Table IV).

Curcumin: Curcumin is a potent antioxidative agent (diferuloylmethane) and an active compound of the perennial herb which also exhibits anti-inflammatory and antitumour activity. Recent studies using this curcumin on HPV positive human cervical cancer cell lines have demonstrated that curcumin downregulates the AP-1 and NF-κB expression in a dose and time dependent manner. The AP-1 plays a crucial role during the development of cervical cancer by binding to its potential cognate binding sites within HPV URR.
and is absolutely indispensable for efficient HPV oncogene expression\(^8\). AP-1 has been shown to be regulating the transcription of almost all HPV types investigated so far\(^1^{1}\). Curcumin treatment of HPV 18 positive cervical cancer cells selectively suppresses the HPV 18 transcription as well as abolished AP-1 binding to the viral URR\(^89\). In subsequent studies, curcumin has also been shown to suppress activation of transcription factor NFkB. Curcumin also selectively blockes IκB phosphorylation and degradation and lead to abrogation of NFkB activation and downregulation of NF-kB dependent COX-2 expression\(^90\). These activities of curcumin are primarily linked to its strong anti-oxidant activity. Based on these leads a polyherbal cream, Basant, has been formulated using curcumin and other herbal components\(^92\). Basant was found to not only inhibit HPV entry into the cervical cells \textit{in vitro} but also inhibited Neisseria gonorrhoeae, various species of Candida and HIV-1 in cultures\(^92\). Currently, this formulation is being tested clinically for its safety and efficacy against HPV infection in humans in a Phase II multicentric clinical trial in women having HPV positive pre-cancer (CIN1/LSIL) lesions\(^1^{12}\).

**Table IV. Molecular targeting of HPV and Host cellular factors interaction using natural and herbal derivatives**

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<th>Natural/Herbal derivative</th>
<th>Anti-viral action</th>
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<td>Curcumin and curcumin-based poly-herbal formulation, Basant</td>
<td>Inhibition of oncogenic and viral transcription through inhibition of AP-1 and NF- B</td>
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<td>Stabilization of p53</td>
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<td>Silymarin</td>
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<td>Jaceosidin ( 4’,5,7-trihydroxy-3’,6-dimethoxyflavone)</td>
<td>Inhibit binding of E6 with p53 and E7 with pRb</td>
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<td>Heparin</td>
<td>Inhibition of AP-1 activity and inhibition of HPV LCR and trascription of E6 &amp; E7</td>
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<td>Supression of cell proliferation</td>
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<td></td>
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<td>Carrageenan</td>
<td>Acts primarily by preventing the binding of HPV virions to cells</td>
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<td>Inhibited HPV-16, HPV-18, and HPV-6 pseudovirion infection</td>
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**Praneem**: Similar to Basant, Praneem also is a polyherbal formulation based on purified extracts of Neem (\textit{Azadirachta indica}) leaves and other ingredients\(^1^{13}\), strongly inhibited the growth of \textit{N. gonorrhea}, multi-drug resistant \textit{E. coli} and various species of \textit{Candida} and has virucidal action against HIV-1\(^1^{13-1^{15}}\). Praneem was shown to clear and prevent \textit{in vivo} transmission of HSV2 and \textit{Chlamydia trachomatis} infection\(^1^{14}\). Praneem dispensed as pessary has completed Phase-I safety and Phase-II efficacy studies for treatment of abnormal vaginal discharge due to a variety of reproductive tract infections (RTIs)\(^1^{16-1^{18}}\). In a pilot Phase II clinical trial of Praneem in women positive for HPV 16, a 30 day application of the Praneem polyherbal tablet resulted in elimination of highly carcinogenic HPV type 16 from the uterine cervix in an overall 80 per cent of cases\(^93\). The elimination of HPV DNA was found to be accompanied by marked improvements in clinical symptoms as well as cytological abnormalities in Praneem-treated subjects. Though the mechanism(s) of action of Praneem is not known, it appears to act through immunomodulatory pathways.
Epigallocatechin gallate (EGCG): A constituent of green tea, (-)-epigallocatechin-3-gallate (EGCG) is known to possess anti-cancer and anti-proliferative properties. Several studies conducted in recent years on effect of EGCG on cervical cancer cell lines showed inhibition of proliferation of cervical adenocarcinoma cells which was associated with suppression of pKi-67 and telomerase activity and induction of apoptosis. Treatment of EGCG in organotypic culture of cervical cancer cell lines which mimics cervical cancer lesion *in vitro*, also showed decreased thickness of epithelial multi-layers and induction of apoptosis. EGCG was also tested in clinical trial along with polyphenol E of green tea in fifty-one patients with cervical lesions (chronic cervicitis, mild dysplasia, moderate dysplasia and severe dysplasia); 69 per cent response rate (35/51) was noted as compared to 10 per cent response rate (4/39) in control group. Overall these observations suggest that green tea polyphenols are effective against HPV infection and for treating cervical lesions and can be a potential drug as anti-HPV therapeutics.

**Potential leads from herbal derivatives:** With the development of newer *in vitro* drug screening assays, it is now easier to screen various agents that may have anti-viral activities against HPV. Recent *in vitro* studies have revealed a number of herbal anti-oxidants such as plant lignan, nordihydroguaiaretic acid (NDGA), silymarin, sulindac and jaceosidin or naturally occurring polysaccharides and glycoproteins like heparin, carrageenan, *Solanum nigrum* glycoproteins, silyasaponins and Sulphated *E. coli* polysaccharide derivatives to possess anti-cervical cancer and anti-HPV activities. These leads can be further tested in clinical settings, and considering the natural origin with multi hit capability against HPV and the host cell, it is expected that these formulations will be safe and effective.

**Conclusion**

Antiviral therapies based on natural/herbal derivatives have the potential to treat both inapparent HPV infection as well as visible clinical disease. A substantial number of HPV-infected, immunosuppressed individuals cannot be treated with immunotherapies or high-cost RNA-interference technology. In such patients anti-viral and anti-cancer drugs are the only option. Furthermore, antiviral agents, unlike immuno-therapies or RNA-interference, may not be HPV type restricted in their efficacy. All these reasons make development of natural/herbal derivative-based anti-HPV therapies a high priority as cheap and effective anti-viral therapies.

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