

REVIEW ARTICLE

Cancer of the uterine cervix and human papillomavirus infection

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Human papillomaviruses (HPVs) have emerged as the principal sexually transmitted causal agents in the development of cancer of the uterine cervix in women. They also cause a variety of benign lesions, warts, intraepithelial neoplasia and anogenital, oral and pharyngeal papillomas. Presently, more than 100 HPV genotypes have been identified in humans, and about one-third of them have been sequenced. Of these, while HPV types 16 and 18 are considered to be the high-risk types, HPV 6 and 11 are the low-risk types in the development of cervical cancer. Evidence for causal role of HPV in the development of cervical neoplasia comes from the etiological and epidemiological observations together with the experimental findings of the molecular pathways elicited by HPV-transforming genes. Further evidence in favour of papillomavirus as the carcinoma virus comes from the findings of presence of HPV infections in cancers of oral, esophageal, larynx and non-melanoma skin cancers. The oncogenic potentials of the virus have been attributed to its *E6* and *E7* genes. The products of these two genes stimulate cell proliferation by activating the cell-cycle-specific proteins and interfere with the functions of cellular growth-regulatory proteins, *p53* and *Rb*. Identification and characterization of several human pathogenic HPV types warrant prevention of viral infection through vaccination or therapeutic intervention which could eventually control infection and expression of human pathogenic papillomaviruses.

CANCER of the uterine cervix is the second-most common malignant tumour in the world, but is the number one cancer in Indian women, posing a major public health problem. In India, about 100,000 women develop this cancer every year^{1,2}, constituting about 16% of the world's annual incidence^{3,4}. It has been estimated that about 1,40,000 women might develop cervical cancer by the turn of this century⁵. For over a century, it was believed that cervical cancer is associated with 'sexual behaviour', indicating involvement of a sexually transmissible infectious agent. Initially, herpes simplex viruses type 2 (HSV-2) was considered as the possible candidate, but the absence of HSV-2 DNA in most cervical tumours together with the results of several epidemiological studies amply demonstrated that HSV-2 was not directly involved in

cervical cancer development. It was only in the early 1980s that the prevailing controversy over the involvement of human papillomaviruses (HPVs) was settled following cloning of several HPV genomes, including the most prevalent HPV-16 from cervical carcinomas and genital warts⁶⁻⁹. However, it took more than a decade before the causal role of specific types of HPVs in cancer of the cervix and their precursor lesions was accepted^{10,11}. Although papillomavirus particles were first observed by electron microscopy in human warts in 1949 (ref. 12), their carcinogenic potential was first demonstrated in Shope papillomavirus¹³ (later named as cottontail rabbit papillomavirus (CRPV)). Ito and Evans¹⁴ successfully induced carcinoma in domestic rabbits with CRPV DNA extracted from carcinomas. In 1963, the structure of the papillomavirus genome was analysed¹⁵. Thus it was established that infection of specific types of HPV was indeed essential for the development of cervical cancer. However, to result in malignancy, the probable^{16,17} involvement of other risk factors and/or cellular events, in addition to viral infection, was considered a requirement.

Epidemiological studies did demonstrate the association of several risk factors with the development of cervical cancer. The risk factors include sexual promiscuity and multiplicity of sexual partners¹⁸, exposure to sexual intercourse at an early age^{19,20}, number of pregnancies^{21,22}, cigarette smoking^{23,24}, use of oral contraceptives²⁵, dietary and other factors²⁶⁻²⁸. In addition to these epidemiological risk factors, the interaction between the genes encoded by the papillomaviruses and the host cell genes appears to play a crucial role during tumorigenic progression, including cellular transformation and interference with the cellular growth-regulatory tumour suppressor genes, *p53* and *Rb*. Since there is an active involvement of HPVs in these mechanisms, an understanding of immunology of HPV together with the mechanisms of HPV-induced-carcinogenesis for developing vaccines against HPVs has assumed significance.

In this review we present a brief account of the present understanding of HPV infections, emphasizing cancer of the human uterine cervix in India where prevalence of both HPV and cervical cancer is reportedly the highest in the world.

Uterine cervical cancer

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The cancers that develop from uterine cervix are of two types: (i) squamous cell carcinomas, which develop from squamous epithelium, cover mostly visible part of cervix; and (ii) adenocarcinomas, which arise from glandular lining of endocervical canal. About 85% to 90% of cervical cancers are squamous cell carcinomas, and the rest 10–15% are adenocarcinomas. Over 90% of these cancers are diagnosed at advanced stages IIB, IIIB and IV, the majority of which are presented at 35–64 years of age²⁹. Squamous cell carcinoma (SCC) is preceded by well-recognized epithelial changes, the precancerous lesions, which develop through several grades: cervical intraepithelial neoplasia (CIN) I to III; or low grade squamous intraepithelial lesions (LSIL) to high grade SIL or a mild, moderate and severe dysplasia leading to carcinoma-*in-situ* (CIS). These lesions may progress to malignancy, or persist, or regress to normalcy. The distinct premalignant or dysplastic changes are generally being detected by a simple exfoliated cytological screening, the 'Pap-test'³⁰. The uniqueness of cervical cancer is that the precancerous lesions may take a few months to several years (10–15 years) to progress to the stage of invasive cervical cancer. Therefore, early diagnosis of premalignant cervical lesions plays a pivotal role in controlling cervical cancer.

HPVs: Natural history and transmission

The natural history of HPV infections is not well understood. These viruses normally infect their natural host, humans, resulting in genital warts or condylomas in the external and internal genitalia. The infected epithelial cells show koilocytes, which are indicative of HPV infections. Koss³¹ reported that 50–70% of condylomatous lesions of the cervix are associated with the spectrum of CIN. These infections may not necessarily lead to visible lesions and may naturally abort or clear up within a short time. It is the high-risk HPVs which are considered to be a major risk for progression of dysplasia to invasive cancer. The relative risk for women with HPV 16/18 developing CIN is 11 (attributable risk 52%) compared to women without HPV infection³². HPV infections occur almost exclusively in rare hereditary disease, the epidermodysplasia verruciformis (EV). However, the issue of the clonality of intraepithelial lesions and warts is very much controversial^{33,34}. A clonal growth of HPV-infected cells showed a requirement for a highly specific intracellular environment for the development of lesions, possibly provided by cells that had undergone specific genetic alterations, since presence of HPV DNA has been demonstrated in clinically symptom-free epidermal and mucosal sites of the cervix, larynx and skin^{35–38}. The persistence of HPV infection is found to be significantly higher among women infected with high-risk HPV types (16/18) and perhaps it is this subset of women that subsequently develop cancer. However, the mode of

viral DNA persistence in such latent infections is still unknown.

Transmission of HPVs, causing papillomas, is facilitated by the presence of abraded or macerated epithelial surfaces³⁹. Anogenital infections are mainly transmitted by sexual contact, since HPV DNA is rarely detected in sexually inexperienced young women^{40–43}. Occasionally, it is perinatally transmitted to infants during delivery^{44,45}. Moreover, there exists a good correlation between multiple sexual partners and the prevalence of HPV infection^{46–49}. Occasionally, anogenital HPVs are also transmitted digitally from one epithelial site to the others^{50,51}. Oro-genital contact may lead to infections at oral sites by anogenital HPVs⁵². Skin infections by HPVs originate from contacts with contaminated materials, walking barefoot on an abrasive surface⁵³, or by acquiring accidental epithelial wounding with contaminated equipment⁵⁴.

HPVs: Epidemiology

A number of epidemiological risk factors for cervical cancer, including both biological as well as behavioural variables, showing a good correlation between natural history of HPV infection and cervical cancer have been established. The current epidemiological data strongly support that HPV infection is the primary risk factor playing a central role for development of benign or invasive cervical cancer. HPV infections and their associated lesions are most prevalent among young sexually active women; much less or nil in nuns or virgins. These infections may resolve spontaneously within a month or a year, or may persist and progress to high-grade lesions. The additional risk factors for progression include reproductive factors, cellular immunity, nutritional factors, socio-economic status, co-infection by other sexually transmitted infectious agents such as HSV-2, *T. vaginalis*, *C. trachomatis*, etc. The past or present infections with these pathogens may reflect sexual promiscuity and act as surrogate markers of exposure to HPV. Certain cultural/social customs or religion and personal hygiene may influence HPV infection. Jews and Muslim women show extremely low prevalence of HPV and cervical cancer. Exposure to sexual intercourse at an early age and to a large number of sexual partners increase the chances of HPV infection. Several epidemiological studies^{55–58}, world-over, have concluded that infection of HPV, particularly of high-risk HPV types 16 and 18, is the primary risk factor for cervical cancer after adjusting for all other confounding variables including smoking, oral contraceptives, age at first sexual intercourse and life-time number of sexual partners.

Most interestingly, in India, the association of the infection of high-risk HPVs with the age of marriage below 18 years has been found to increase the risk of cervical cancer by 22 fold²⁰. Integration of viral DNA

too is associated with the progression to malignancy. Smoking, as a risk factor, though varies from region to region, may cause genetic damage or suppression of immune response, leading thereby to progression of HPV lesions⁵⁹. Multiple pregnancies and the use of oral contraceptives may enhance HPV infectivity and expression of HPV-transforming gene.

HPVs: Diagnostic techniques

Diagnosis, particularly of high-risk types of HPVs is of great importance for presence of clinically latent papillomavirus infections which are highly prevalent in general population. Carriers of high-risk HPVs and those carrying infection show a high rate of progression. However conventional diagnostic techniques, generally being used, rely on cytological and histological examination and colposcopy and cannot detect the viruses.

For a majority of viruses, specific antibody-antigen reaction can be assayed in serological tests by enzyme-linked immunosorbant assay (ELISA) or western blotting. But, such assays are not possible for HPVs due to lack of good source of papillomavirus antigens, since natural occurrence of HPV virions in cervical lesions is extremely low, and moreover, this virus cannot be grown *in vitro*. Recently, expression of the HPV proteins as fusion proteins in bacteria, resulted in providing large quantities of antigens which can be used to screen human sera for antibodies against HPV types, but which of the specific viral antigens acted as the primary targets of antibody response could not be ascertained. Therefore, diagnosis of HPV is done only by molecular hybridization methods.

Several molecular methods, such as Southern blotting, dot/slot blotting, filter *in situ* hybridization (FISH), tissue *in situ* hybridization (TISH) and, recently, polymerase chain reaction (PCR) which is the most sensitive of all, are being employed for detection of HPV. The first two methods require purification of cellular DNA from cervical biopsies or scrapes, followed by the detection of viral genome by hybridization against radio- or nonradioactive-HPV probes. Of these two methods, Southern blot hybridization⁶⁰ allows identification of HPV types by the sizes of the specific restriction bands; characteristic of each HPV type (Figure 1). Dot blotting on the other hand, differentiates between the HPV types by stringency of hybridization. In the FISH technique, scraped cells are directly filtered onto a membrane, and the viral DNA is then detected by hybridization. Though this technique is less time-consuming, allowing analysis of a large number of specimens at a time, its limitations are low sensitivity and high false positivity. Tissue *in-situ* hybridization (TISH) is specifically important for detecting viral genes on tissue sections, facilitating analysis of a specimen both at morphological as well as at the molecular level. PCR⁶¹ is highly sensitive and specific compared to all the other techniques

and can detect even a single molecule of HPV DNA out of a million cells. PCR is now being used very commonly for detection of HPVs using various primers, in variety of biological specimens including cervical, anal^{62,63}, and oral scrapes⁶⁴⁻⁶⁸; fine needle aspirates⁶⁹; urine⁷⁰, semen, etc⁷¹. An unambiguous amplification of HPV DNA sequences can be achieved after predigestion of genomic DNA with a single-cut restriction enzyme of HPVs⁷². Specific degenerating or consensus primers are employed for PCR detection of unknown HPV types^{73,74}. Primer sets from the most conserved regions such as L1, URR or E6/E7 regions of HPV genome are the common choice for detection of specific HPV types. However, the drawbacks of PCR methods are: (i) contamination or mixing of cell/DNA samples leading to detection of high frequency of HPVs, and (ii) selection and use of various primers from different regions of HPV genome may give different results. To keep such variations to minimum, proper care should be taken during each collection, transport, storage and extraction of DNA from biological specimens including all safety

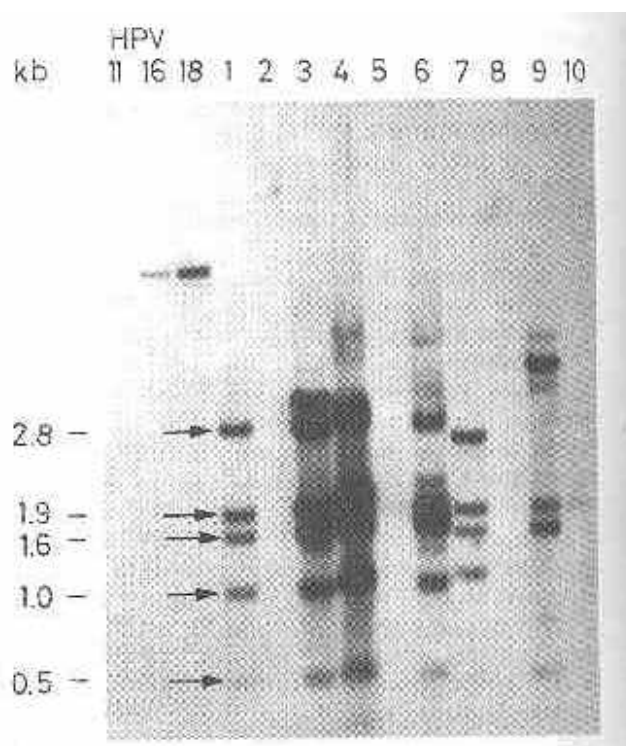


Figure 1. Southern blot hybridization analysis of HPV-16 DNA sequences in cervical biopsy specimens. Ten micrograms of cellular DNA was digested with *Pst*I, electrophoresed in a 1% agarose gel and hybridized with the ³²P-labelled HPV 16 + 18-DNA probe under stringent conditions (T_m; -20°C). Out of the 10 specimens (1-10), seven were positive (lanes 1, 3, 4, 6, 7, 9 and 10) for HPV 16. The characteristic *Pst*I cleavage pattern of HPV 16 is shown by arrows and the fragment sizes are indicated in kilobases. The first two lanes are positive control markers having 10 and 50 pg of HPV-16 and HPV-18 DNA, respectively.

precautions of running a PCR.

HPVs: Diagnosis in cervical cancer-screening programme

In developed countries, cytological screening of cervical precancers by Papanicolaou-stained cervical smears (Pap-test) and ablation of histologically confirmed lesions have helped to reduce cervical cancer-based mortality considerably. Primary cervical cancer prevention programme based on cytological screening which detects changes in cellular morphology, including HPV infections as koilocytes, however suffers from several technical limitations besides high cost, low sensitivity, inter-screener variations and diagnostic errors. Vast amount of data accumulated thus far suggest that HPV is an undisputed principal etiologic agent for the development of cervical cancer, and progression of cervical lesions is always associated with persistent infection of specific types of high-risk HPVs. Therefore, the question arises whether cervical cancer screening programme should also incorporate HPV testing to identify women at high-risk for developing cervical cancer. But HPV-DNA test cannot fully replace the Pap-test, since not all HPV infections lead to cervical cancer, and furthermore, some cancers also arise without HPV infection. Conversely, high-risk HPVs can also be detected in a substantial number of women without cytologic evidence of CIN or other lesions⁷⁵. Therefore, to achieve a significant improvements in cancer control programme worldwide, use of both these diagnostic methods is imperative to augment their sensitivity, specificity and quality-control of the Pap-test. This would facilitate identification of substantial number of women whose smears are neither normal nor abnormal or suspicious but may progress to invasive cancer if they harbour insidious infection of HPV.

It was earlier suggested that in India incorporation of HPV testing in routine screening is not possible because of the high cost involved, lack of trained man-power, etc.⁷⁶. But, now that specific HPVs have been recognized as the principal causal agent for the development of cervical cancer, and since there has been an enormous improvement and simplification in virus detection techniques, incorporation of HPV testing in control trials, particularly for confirming suspicious or negative cases, if not in routine screening programme, using the same Papanicolaou-stained slides should be considered.

HPVs: Geographical distribution

Globally, distribution pattern of HPV appears to be similar in different countries: 60 to 65% positivity for HPV 16; 4 to 20% for HPV 18; and a low prevalence of other HPV types. An interesting population-based cross-sectional study⁷⁷, comparing HPV-prevalence rates of cervical cancer between a high-risk area (Greenland) and a low-risk area (Denmark) reported 1.5 times higher HPV 16/18 prevalence in Denmark

compared to Greenland. A comparative study on the prevalence and type-specific distribution of HPVs from cervical specimens of women undergoing hysterectomy for benign, non-neoplastic diseases, reported positivity for HPV DNA in 33% of the cases from Pakistan and 46% of the cases from Japan; while no significant difference was reported in prevalence of high-risk HPVs in cancer cases between the two countries. In a case control study, Munoz *et al.*⁷⁹ reported prevalence of HPVs among controls in California and Spain to be 3.4 vs 4.3%. But when PCR was used, a higher prevalence of 13.3% in Californian women than 4.6% in Spanish women was observed. A comparative study on HPV-prevalence rates among all newly diagnosed cervical cancer cases in Panama (USA) showed a different pattern than the one previously reported: HPV-DNA test conducted in the lowest cancer risk area (30.2 per 100,000) in contrast to the high-risk areas (51.0 per 100,000), led to the detection of HPV DNA in 70% of all cases in the former as opposed to 54% in the latter.

Reeves *et al.*⁸⁰ carried out a large case-control study in four Latin American countries using FISH and reported that 91% of invasive cancer patients were HPV positive compared to 63% in controls. This high HPV positivity of controls however indicates unreliability of the FISH technique. Lehtinen *et al.*⁸¹ in a nested case-control study of CIS and ICC from a cohort of 18,814 Finnish women who were being followed up to a period of 23 years, after the initial screening, showed that the only significant association of cervical carcinoma was with the presence of antibodies to human papillomavirus type 16. (OR: 12.5, 95% CI: 2.7–5.7). The study revealed that 76% of the CIN lesions could be attributed to HPV infection, particularly with oncogenic HPV types.

HPVs, and cervical cancer in India

Studies on cervical cancer in India show (i) as high as 98% HPV positivity in invasive cancer cases vs 20% in normal healthy controls; (ii) HPV 16 as the predominant (90%) type while the frequency of HPV type 18, is very low (3%) (ref. 82); (iii) HPV 16 is also more frequent than HPV 18 in cervical adenocarcinomas⁸³, but HPV 18 is the predominant type in other parts of the world^{84,85}; and (iv) HPV infections are at least two-times more frequent in pregnant women than in non-pregnant women^{21,86}, showing a gradual but statistically significant ($P < 0.001$) increase in HPV infection with the increasing number of pregnancies²¹. Perhaps this is why in India early marriages, normally resulting in higher number of pregnancies, is a high-risk factor for cervical cancer due to immature cervix.

In a retrospective analysis of specimens from a 12-year follow-up study, a significant association of HPV 16/18 as a risk factor for developing cervical cancer has been demonstrated. Sexual intercourse before 18 years of age has been found to increase the risk of cervical cancer by 22

fold²⁰. Detection of HPV in cervical cancer specimens from different parts of India^{82,87-90} indicates that HPV 16 is the most predominant type. Interestingly, a high frequency (80%) of HPV 16 was observed in Madras, a high-prevalence area for cervical cancer⁹¹. The lowest frequency of HPV (11%) as well as of cervical cancer were recorded from Jammu and Kashmir, while a moderate frequency ranging from 42% to 66% has been observed in rest of the country (Das *et al.* unpublished data). In precancerous lesions, the prevalence of HPV 16 and 18 is 54.28%, 52.94%, and 27.08% in severe dysplasia and CIS, moderate dysplasia, and mild dysplasia, respectively. Das *et al.* (unpublished results) have observed a gradual increase in the rate of HPV infection with increase in the severity of the lesions.

Other types of papillomavirus infection-linked cancers in humans

HPVs, particularly the high-risk types, have been detected in other anogenital cancers as well. Several additional human cancers too have been linked to human papillomavirus infection. The presence of HPVs in tumours of oral cavity⁹²⁻⁹⁸ (Das *et al.*, unpublished data), larynx⁹⁹, tonsil^{99,100}, nasal sinus¹⁰¹, and anogenital carcinomas¹⁰² including penile¹⁰³⁻¹⁰⁵ and anal carcinomas^{62,63,106,107} have been recorded in 20%–60% cases. Several reports¹⁰⁸⁻¹¹¹ on the role of HPV in esophageal cancers have also been published. Controversial reports have appeared for the presence of HPV DNA in cancer of breast^{112,113}, bladder¹¹⁴⁻¹¹⁷ and urethra¹¹⁸⁻¹²⁰. HPV has also been detected in cancer of the gastrointestinal tract (Das *et al.*, unpublished). Recently, by using consensus primers, a higher percentage of varying HPV positivity in various kinds of human cancers was detected in contrast to a low-HPV positivity in non-melanoma skin cancers^{121,122}. Quite a few HPV types, such as HPV types 1, 2, 3, 4 and 27, are associated with common cutaneous warts, plantar warts and flat warts; but these are all benign lesions, which mostly regress spontaneously. Although a number of different HPV types have been detected in several human cancers other than cancer of the cervix, their causal role in inducing these cancers, though suggestive, has not yet been proved. Therefore, there is a need to keep track of these HPV infections over a long period because the virus may not remain as a silent passenger only, but, instead may have a role as a probable co-factor in the development of these carcinomas. For the majority of human cancers associated with HPV infection that are of epithelial cell origin, and since HPV is also epitheliotropic in nature, it is quite likely that the virus may, in part, contribute to tumorigenic transformation/progression on these sites.

HPVs: Genomic organization and nomenclature

Human papillomavirus particles are about 55 nm in diameter and contain a double-stranded closed-circular DNA genome of approximately 7200 bp–8000 bp. The viral DNA is encapsidated by 72 capsomeres¹²³, replicating as an episome in the nucleus of host cells. Human papillomaviruses belong to a large family of DNA tumour viruses, papovaviridae, and are epitheliotropic in nature. The viral early genes or open reading frames (ORFs) *E1–E7*, and late genes (ORFs, *L1* and *L2*) are separated by a transcriptional long control region (LCR) or upstream regulatory region (URR) (see Figure 2). It contains viral promoters as well as several enhancer elements, which control viral replication and transcription of *E6* and *E7* genes, leading to malignant transformation and maintenance of tumorigenic phenotype^{124,125}. The LCR/URR constitutes about 10% of the genome, varying between 800 bp and 900 bp. URR forms a non-protein-coding region (NCR) of the viral genome, but it contains many *cis*-acting elements. Viral gene expression is generally regulated by several viral and host-cell transcription factors, which bind to the URR. These factors include nuclear factor-1 (NF-1), activator protein-1 (AP-1), octamer-binding factor-1 (Oct-1), progesterone receptor, Yin and Yang factor-1 (YY-1), SP-1, KRF-1 and glucocorticoid receptor, etc.^{124,126-128}.

To date, more than 100 different HPV types have been described: 30 of these types are associated with anogenital cancers, forming either the high-risk types (HPV 16 and HPV 18) that are associated with anogenital invasive tumours and their precursor lesions^{8,9} or the low-risk types (HPV 6 and HPV 11) which rarely progress to malignancy, and are mainly associated with benign growths, such as genital warts and condylomas^{7,129}. Analysis of viral genome has revealed that two early genes *E6* and *E7* of high-risk HPVs (16/18) are transforming genes which are responsible for maintenance of tumorigenic phenotype^{125,130,131}. In contrast,

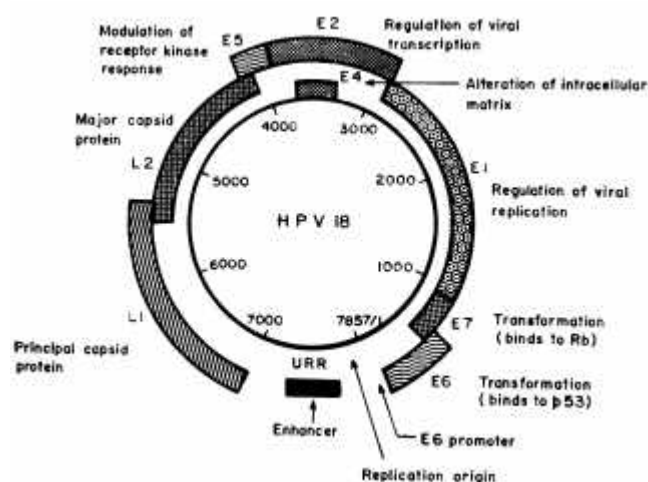


Figure 2. Genomic organization and gene functions of papillomaviruses exemplified by HPV 18.

in low-risk HPVs these genes are either nontransforming or very weakly transforming¹³²⁻¹³⁴. *In vitro* studies indicate that while proteins encoded by *E6*, of HPV 16/18, can form a similar inactivating complex with *p53* tumour-suppressor gene product, *p53* (refs 135 and 136), *E7* protein forms a stable inactivating complex with retinoblastoma-susceptibility gene (*Rb*) product, p105. Thus these oncoproteins of high-risk HPVs abrogate both transcriptional activation as well as cancer suppression (or growth control) properties exhibited by the *p53* and *Rb* gene. However, the proteins of low-risk HPVs either cannot complex or weakly complex with *p53* and *Rb*.

As for the other ORFs, while *E1* is the most conserved among different HPV types and is responsible for the HPV replication¹³⁷ as well as for the site-specific DNA binding activity¹³⁸, *E2* facilitates *E1* binding to the replication origin located proximally to the URR. The *E2* ORF codes for at least three proteins which act as the transcription factors¹³⁹. In high-risk HPV types 16/18, *E2* binds to URR and acts as the transcriptional activator. The *E2* is mostly deleted or disrupted in cervical tumours and cell lines derived from cervical cancers¹⁴⁰⁻¹⁴². This seems to facilitate integration of HPV genome into the host cell genome and leads to malignant transformation. This is supported by the observation that mutation in *E2* ORF or its binding sites within the URR leads to an enhanced immortalization activity of HPV 16 (ref. 143). The ORF coding for *E5* protein also shows transforming activity, but mostly in bovine papillomavirus (BPV) and is frequently deleted in cervical cancer¹⁴¹. The role of *E4* ORF and its product is not yet clearly understood but possibly has a role in productive infection of the virus.

In 1978, because of the observed genetic heterogeneity of human papillomavirus, for the nomenclature of a HPV type, a new type was designated and differed by more than 50% from a prototype HPV, when tested by reassociation kinetics under stringent conditions of hybridizations¹⁴⁴. Later it was decided that a difference in *E6*, *E7* and *L1* ORFs of more than 10% from the prototype HPV be used to define a new type. But in 1995, at the Annual Papillomavirus Conference in Quebec, it was decided that difference in only *L1* ORF exceeding 10% from a prototype be used to define a new HPV type.

HPVs: Physical state in host cell

Generally, for malignant progression, the integration of viral DNA into the host cell genome is considered an essential prerequisite^{142,145-147}. Analysis of the physical state of HPV DNA in cervical cancer biopsies, and cell lines derived from tumours indeed confirmed this contention in most cases^{9,142,148-153}. However, in premalignant lesions, except in carcinoma-*in situ* and severe grades of dysplasia, the HPV DNA always persists as a nonintegrated episomal molecule^{142,147,153,154}. The integrated form of HPV DNA can be revealed by presence and/or shifting of the off-sized

light bands along with HPV-specific authentic *Bam*HI- or *Pst*I-cleavage pattern which suggests integration of head to tail tandem repeats at more than one site^{142,147} (Figure 3). Several studies including ours¹⁴⁵ demonstrated that HPV-16 DNA is present in an integrated form in more than 70% of cervical cancer specimens, but absence of viral integration in about 30% of cases indicates that viral integration may not be a sole prerequisite for malignant transformation. Several studies, however, have indicated that the analysis of physical state of HPV may serve as a prognostic indicator for the preneoplastic lesions that are likely to progress to cancers^{9,147,155,156}. Das and his colleagues¹⁴⁵ compared integration of HPV using at least four different molecular approaches, including a newly developed PCR method, in more than 122 cervical specimens comprising the entire spectrum of cervical lesions, starting from cervical dysplasia to invasive carcinoma including HPV-positive normal controls. They reported that integration of HPV increases as a function of severity of the disease¹⁴². It has also been shown that integration of HPV precedes the invasive stage and usually disrupts or deletes the *E1* or *E2* ORF^{142,157-159}. Disruptions in *E2* was also reconfirmed by RNA *in situ*

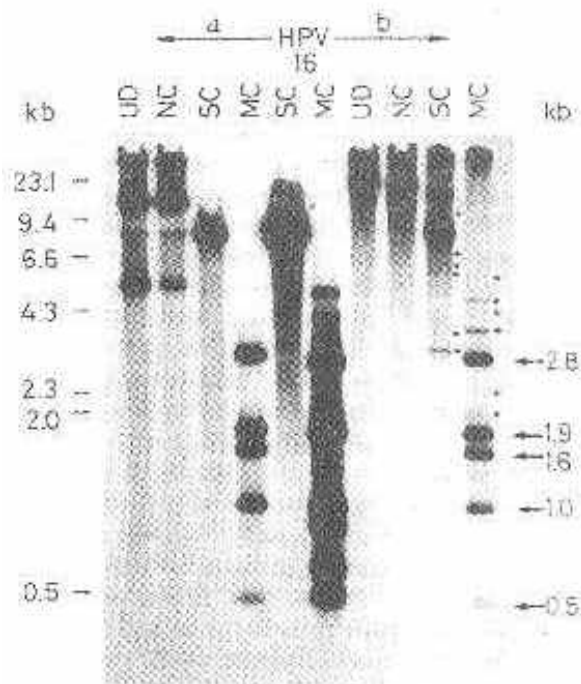


Figure 3. Southern blot hybridization of two cancer biopsy DNA, following combined digestion with no-cut (NC), *Hind*III; single-cut (SC) *Bam*HI; and multi-cut (MC), *Pst*I; in addition to lanes of undigested (UD) DNA. In the episomal form (a), it shows characteristic *Bam*HI- and *Pst*I-cleavage patterns along with three forms of high-M DNA in UD and NC, but no indication of additional off-sized fragments. In the integrated form (b) there is a shift in light off-sized fragments (thin arrows) in lanes SC and MC along with authentic *Pst*I fragments (thick arrows), suggesting integration of head-to-tail tandem repeats as more than one site.

hybridization by Daniel *et al.*¹⁵⁴. It has been shown that disruption of *E2* and other genomic regulatory elements leads to dysregulation of *E6/E7* transcription, thereby increasing the immortalization potential of HPV 16 (ref. 160). It is clear that viral DNA integration and the resultant dysregulation of oncogenic activity of *E6/E7* is not sufficient for a malignant phenotype. This is strengthened by the observation that somatic cell hybridization of carcinoma cells with normal cells resulted in a non-malignant phenotype despite the presence of the viral DNA in an integrated state^{161,162}. Also, in a sizeable proportion of cervical cancer, HPV DNA remains in an episomal form indicating thereby that other events perhaps contribute to tumour progression.

HPV-transforming genes, immortalization and tumour suppressor genes

The early genes *E6* and *E7* of high-risk HPV types 16/18, but not of low-risk types can immortalize human foreskin and cervical keratinocytes¹⁶³ after *in vitro* transfection in tissue culture. In organotropic cultures, these immortalized cells share growth characteristics with intraepithelial neoplasias¹⁶⁴. Although HPV *E6*- and *E7*-immortalized human cells are initially nontumorigenic in nude mice, long term *in vitro* cultivation results in development of malignant clones¹⁶⁵. These observations indicate that HPV infections can induce malignant growth, provided a sufficient number of cell generations are allowed for manifestation of additional spontaneous or virus gene-induced modifications. In 1984, immortalizing cells with HPVs were first attempted¹⁶⁶. Later, studies showed that HPV-16 *E7* gene can cooperate with the *ras* oncogene in transforming primary rat kidney cells¹⁶⁶⁻¹⁶⁸. It was in 1987 that immortalization of human cells with HPV 16 (ref. 169) and in 1988 (ref. 170) with HPV 18 was shown to be possible. Recent reports in both rodent and human cells show that only the expression of *E6* and *E7* genes of high-risk HPVs is essential for immortalization^{102,170-172}. But, experimental evidence indicates the additional involvement of specific host cell genes, engaged in the regulation of signalling pathways¹⁷³, as essential for immortalization^{172,174,175}.

For the high-risk HPVs, the *E6* oncoprotein binds to the product of *p53*, the tumour suppressor gene, while *E7* binds to the products of *Rb*, the retinoblastoma-susceptibility gene^{176,177}. In contrast, low-risk HPVs (6/11) showed absence of such a binding¹⁷⁸. The interaction of high-risk HPV *E6-E7* oncoproteins with cellular *Rb* and *p53* proteins could act as an important endogenous factor for progression of premalignant lesions to malignancy. On the other hand, mutations in *p53* are reported to be detected in HPV-negative cervical carcinomas and cell lines^{179,180}.

Almost 50–80% of all human carcinomas overexpress *p53* protein due to mutations in *p53* gene, coupled with a strong

tendency for selection of the mutant *p53*, suggesting a positive role for tumourigenesis, in addition to the loss of its tumour-suppressor activity. Several investigators have tried to identify the specific mutation sites of *p53* that are associated with several cancers, including cervical cancer, but no such specific mutational hotspots have been so far identified for cervical cancer, instead, both HPV-positive as well as HPV-negative cervical carcinomas show a very low frequency of mutation in *p53* gene.

HPVs: Transcriptional regulation

As early as in 1977, zur Hausen proposed that certain cellular factors control the expression of persisting tumour virus genomes. Based on this it was assumed that normal proliferating cells regulate transcription of HPV early genes, but cervical cancer cells may lack this type of control. A long persistence of HPV infections, and a very slow rate of progression of the lesions indicate involvement of a well-orchestrated modulation of viral replication and transcription. Transcripts at the start site of *E6* promoter are polycistronic in nature, and are reported to include *E2* gene. One of the events that modulate *E6/E7* expression is integration of HPV DNA into the host cell genome. HPV integration often leads to inactivation/ disruption of *E2* ORF thereby relieving *E6* and *E7* transcriptional repression by *E2* protein. But in a sizeable proportion of cervical cancer, HPV DNA remains in an episomal form indicating involvement of other mechanism(s) for tumour progression. It is now clear that specific cellular protein factors are known to participate in the transcriptional control of HPV oncogene expression. The enhancer elements of HPV 16 and other HPV types were found to be activated by a large number of host cell transcription factors. Most important of these include activator protein 1 (AP-1), nuclear factor 1 (NF-1), glucocorticoid receptor and progesterone receptor. The epitheliotropic nature of HPV, its transcriptional specificity for epithelial cells correlates perfectly with the activity of AP-1 and NF-1 transcriptional factors.

Absence of *E6/E7* transcription in basal cells of mild dysplastic lesions and its increased expression in high-grade lesions and carcinomas indicate that the tumourigenic progression is caused by the breakdown of intracellular control mechanisms of HPV-transcription in the basal cells of the cervical epithelium. Certain additional factors such as growth factor, *c-Ha-ras* proteins can stimulate *E6/E7* transcription via AP-1 which binds to URR. AP-1 can also act as a target for negative regulation of its target genes by steroid hormones and retinoic acid receptor- α

Recently, it has been shown that transcription factor AP-1, a heterodimer of *c-fos* and *c-jun* oncogenes products, appears to play a central role in transcriptional regulation of viral oncogene expression, since point mutations of the corresponding consensus sequences within the upstream regulatory region (URR) of HPV 16/18 almost completely

abolish the expression of viral-transforming genes, *E6* and *E7*. Constitutive expression of *E6* and *E7* is mainly dependent on the availability of a defined set of transcription factors derived from the infected host cells. Recent studies have demonstrated that transactivation and DNA binding activity of AP-1 or other transcription factors such as NFκB can be modulated not only by post-translational modification, such as phosphorylation or dephosphorylation, but also by alteration of the intracellular redox status. Agents such as antioxidants which can induce modification in transcription factors through changes in cellular redox status may interfere with HPV-specific transcription. Recently, treatment of a synthetic antioxidant pyrrolidine dithiocarbamate (PDTC) has been shown to result in the selective suppression of human papillomavirus gene expression *in vitro*¹⁸¹. Such studies provide a basis for development of a novel therapeutic approach to effectively control the expression of human pathogenic HPVs.

HPVs: Immunology and vaccine

The immune system of the body controls viral infections by neutralizing the virus with antibodies or by killing the virus-infected cells. These processes utilize either antigen-dependent cellular immune response or antigen-independent phagocytosis by macrophages. Clinical and histopathological observations however, indicate an important role of immune system in controlling HPV infection. It is now clear that papillomaviruses can elicit both humoral as well as cell-mediated immune responses which can be controlled by expression of specific HLA types (HLA class I and II antigens). An interesting association has been shown between expression of specific HLA types and increased rate of cervical carcinoma¹⁸². But, persistent occurrence of HPV infection in immunocompetent hosts indicates that perhaps no efficient antiviral immune response is induced, or else the infected cells escape immune surveillance.

It is known that HPV proteins have antigenic properties and immune response is immunodominant and epitope-specific. Synthetic peptide-based serology has facilitated characterization of antibody response to individual HPV epitopes. Recently, several groups have cloned and expressed HPV ORFs in expression vectors to generate bacterial fusion proteins in *E. coli*. But which viral antigens are the primary targets of the antibody response require elucidation. Also, no correlation has been established between the circulating antibodies and regression of HPV-induced lesions. Detection of antibodies to HPV proteins in cancers indicates that all antibody responses are protective, but no titer of antibody to any HPV protein has been measured after acute infection. A low prevalence of antibodies to individual proteins of specific HPV type also excludes the possibility of using serological assays for diagnostic purposes. In addition, the fusion proteins are

insoluble, and it is difficult to purify them from the contaminating *E. coli* proteins. Several investigators have generated polyclonal or monoclonal antibodies to fusion proteins in mice and have used these sera to map immunoreactive regions using segments of an ORF expressed in a bacteriophage library.

The recent attempts to control HPV-induced diseases are targeted to develop preventive immunotherapies, and HPV vaccines to prevent infection with high-risk genotypes such as HPV 16, 18, 33 and 45. At least three vaccine strategies are being developed to control HPV-associated preneoplastic and neoplastic cervical lesions: (i) therapeutic vaccines targeting transforming proteins E6 and E7, (ii) vaccines against existing HPV infection and preneoplastic lesions targeted to early proteins expressed in suprabasal stem cells, and (iii) prophylactic vaccines to immunize with virus-like particles (VLPs) to elicit neutralizing antibodies. The cellular immune system consists of two classes of T cells: T-helper cells, which help induce a B cell response and recognize foreign antigens; and T-killer cells, which can selectively kill virally infected cells and recognize foreign antigens. Immunization of mice with syngeneic non-tumorigenic cells transfected with HPV *E6* and *E7* conferred protection against transplanted HPV-positive syngeneic tumour cells. This protection is mediated by T-killer cells. Therefore, HPV E6 and E7 proteins are considered to be suitable targets for developing therapeutic vaccines. However, therapeutic vaccines have not been found too effective because the mechanism of antiviral immunity is not clear, and the mode of optimal delivery of the vaccine is uncertain.

Serious efforts are being made towards developing different effective prophylactic vaccine strategies to control HPV infection but no HPV vaccine has yet been approved for human trial. The use of L1 or L2 capsid proteins of HPV as VLPs has been emphasized because of their high level of expression in non-mammalian cells. Vaccines have also been prepared by using fusion proteins, plasmid DNA, vaccinia virus recombinants and other methods. But VLPs are most popularly being used as they lack oncogenic DNA, and produce large quantities, which are able to elicit high titres of protective antibody response in animals. These antibodies neutralize homologous virions *in vitro* and protect against experimental challenge in animal models. This justifies the initiation of human trial of HPV VLP-based vaccines which could, in principle, eliminate HPV infection, since humans are the only natural host for these viruses, but the question that has to be resolved is, will vaccination against a specific HPV type confer protection against other HPV types? Results so far obtained in animal system suggest to the contrary. Furthermore, because of involvement of at least 30 types of HPVs in anogenital cancers, initial HPV vaccine development efforts should be focused on high-risk genotypes only. Since HPV 16 is the type most prevalent throughout the world and is almost exclusively found prevalent in India, the development of

vaccine against HPV 16 would be of great help in controlling cervical cancer. Experimental evidence suggest that systemic immunization would be able to provide protection against HPV infection. The intranasal (or intravaginal) administration of VLPs in combination with non-toxic mutants of the mucosal adjuvant, cholera toxin (CT) or labile enterotoxin (LT) could result in protective immunity against mucosotropic HPVs and other established HPV infections. Alternatively, live vectors can also be used for both intranasal administration as well as delivery of therapeutic antigen vaccines. The use of plasmid DNA as a vaccine appears to be the most promising approach for induction of Th1 (T-helper cell class I) or for cell-mediated, and CTL (cytotoxic T lymphocyte) responses which are beneficial in antiviral immunity. Immunization with the plasmid expressing the *L1* protein of CRPV was found to be protective upon subsequent challenge with CRPV¹⁸³. This demonstrates that DNA vaccines can work as viable options for induction of viral protection.

But before implementation of HPV vaccination, there is a need for pre-evaluation of their safety and optimization of administration protocols. One of the most important questions is selection of target population for vaccination. Since HPV infection is acquired through sexual contact, vaccination of adolescents before initiation of their sexual activity seems to be appropriate. Also, both the partners need to be vaccinated. But it is quite unlikely that these vaccines will be useful for women who are currently HPV positive. In India, though there is no group at present working specifically on HPV vaccines, efforts should be made to develop prophylactic as well as therapeutic vaccines which could bring about substantial reduction in morbidity from this disease. However, in developing countries like India, early screening programmes using either Pap or Pap-HPV test and/or visual inspection of cervix^{184,185} remain to be the best approaches for control of cervical cancer until a safe and efficient HPV vaccine can be used in the general population.

Conclusions

Both epidemiologic and experimental data have established a central role of specific types of HPVs in the genesis of cervical neoplasia and other human cancers. But none of these virus infections can cause cancer directly. All of them seem to require involvement of certain host cell factors and/or intracellular control mechanism(s). Correlation of natural history of HPV infection with development of cervical cancer reveals a general picture that a women can have HPV infection following initiation of her sexual intercourse. The viral infection may be either transient or may cause early cervical lesions which may regress spontaneously or progress to higher grade lesions. In some percentage of women, the infection of HPV may persist and progress to high grade CIN lesions and eventually to

invasive cancer. The questions that remain to be resolved finally are:

1. Is sexual intercourse the only possible route for transmission of all HPVs?
2. For reliable screening and effective primary prevention of cervical cancer, should HPV test be incorporated along with cytologic Pap-test?
3. What is the mechanism of pathogenesis, epidemiology and clinical course of small proportion of cervical cancers that are HPV-negative, and which also show a worse prognosis?
4. What is the role of HPV in non-cervical human cancers?
5. What is the clinical relevance of HPV positivity detected by the ultrasensitive PCR methods?
6. Which vaccine strategy should be adopted for maximum efficacy, and what set of people should be vaccinated?

The literature survey indicates that for cervical cancers that arise in absence of HPV infection, an alternative pathway, involving specific cellular gene mutations, is perhaps responsible. Even in HPV-infected lesions numerous additional genetic changes¹⁸⁶⁻¹⁸⁸ including gene mutations, should occur in host cells to initiate progression. Furthermore, analysis of chromosomal abnormalities in invasive cancer cases suggest alterations in several oncogenes and tumour suppressor genes. The two HPV-transforming genes *E6* and *E7* can functionally interfere with cell cycle control by interacting with presumptive tumour suppressor gene products such as *p53* and *Rb* leading to deregulation of cell cycle. Also, the chromosomal instability and aneuploidy, which are often observed in invasive cervical cancer, may serve as biomarkers. Improved understanding of immune responses to HPV infection offers an excellent opportunity in controlling HPV infections by designing appropriate vaccines, or gene therapy strategies. Since HPV is the central cause of majority of cervical cancers, HPV vaccination could be considered as the ultimate approach for primary prevention and elimination of cervical cancer.

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