HUMAN PAPILLOMA VIRUS AND UTERINE CERVICAL CANCER : STATE OF THE ART

M. RAY, S. SENGUPTA, P. BHATTACHARYA AND C. DUTTAGUPTA*

THE major cancer burden of Indian women is Uterine Cervical Cancer (CaCx). It is the third most common female cancer globally (1, 2). Each year approximately 250,000 women die throughout the world. Of these cases, 80% belong to the developing countries, which include India (3). The National Cancer Control program of India has identified uterine cervical cancer as a priority area, in the context of women's health issue. CaCx can inflict heavy burden on any society, particularly in the developing and underdeveloped countries. A good screening can reduce this burden. Cytological screening Papanicolaou (Pap smear) test, with all its limitations still is the universally accepted procedure, which can reduce the mortality from cervix cancer (4, 5).

CaCx has extensive cytopathologic database and a working hypothesis of its natural history, which indicates that there is a stepwise, time dependent progression that takes place before going into microinvasion. Thus the system lends itself to the exploration and assessment of environmental 'risk factor(s)' contributing to the incidence and development of a 'dysplastic' (preneoplastic) lesion.

The Etiology of Cervix Dysplasia

Cervix dysplasia (squamous intraepithelial lesion, SIL) is considered as a precancerous lesion of cervix cancer, which can also be detected through Pap smear screening. Histopathologically this morphological abnormality can be classified as Cervical Intraepithelial Neoplasia (CIN) grades I to III depending upon the severity of the lesion. As much as any lesion can progress at any time point, it may regress spontaneously, mild and moderate in particular (6-9). In a large scale survey in 528 women with biopsy confirmed CIN for a mean follow up time of 72 months, following have been found (7) :

	CIN I	CIN II	CIN III
Regression	56%	53%	14%
Progression	14%	21%	69%

* Biochemistry Unit, Division of Biological Sciences, Indian Statistical Institute, 203 B. T. Road, Calcutta-700 035.

The etiology of CaCx suggests that sociocultural environments such as early coitus, multiparity, promiscuity, socioeconomic condition, diet-nutrition *etc.* as life style components may influence the disease process and are considered as 'risk factor(s)' for the disease (10-15). The other risk factors that have been implicated are current smoking status (16-19), recent infection with N. gonorrhea, Clamydia trachomatis and cytomegalovirus (20) and certain immunologic factor (21).

Natural History of Genital HPV Infection

It has been more than a century that the association between CaCx and sexually transmissible agents were noted (22) and this was long before the era of all molecular investigations. In recent time, a definite sexually transmissible agent, a virus, HPV (human papillomavirus) has been implicated as an important etiologic agent in the development of CIN/CaCx (23). Approximately 85 different HPV types have already been identified (24). Women having CIN showed higher prevalence of HPV than in the normal controls (20, 25-27). Generally HPV types 6,11,42,43,44 are found to be associated with lower grade and considered as "low risk" virus; types 31, 33, 35, 51, 52 and 58 as "intermediate risks" (28); and types 16, 18, 45 and 56 as "high risk" virus (29,30). A high risk of rapid progression of HPV 16/18 cervical infection (cytologically negative) to high grade CIN in a cohort study in the US support a direct progression of HPV infection to high grade CIN (20). Similar findings were obtained from an independent study with a longer follow up time in Finland (31). A cohort study of untreated patients with epithelial abnormalities of cervix showed that detection of HPV 16 or 18 DNA sequences was associated with a significantly increased risk of disease progression (32). Or, patients who had high risk HPV type detected at the same point during a mean follow up period of 17 months were six fold more likely to develop progressive CIN than who did not. (33). The type of HPV found in the cervix seems to predict the risk of progression to high-grade cervical neoplasia. This association has been verified in both cross sectional and prospective studies (34). Most low risk type HPVs that are associated with genital warts can be eradicated by topical therapy, freezing or surgery. If left untreated, warts often regress spontaneously. The high risk

types of HPV like type 16, 18, 31, 33, *etc.* are associated with cervical lessions or CIN, which may lead to preinvasive and invasive CaCx(35). Since papillomaviruses are species specific, laboratory animals cannot be infected with HPV. The virus is also difficult to culture. Hence the association of high risk HPV with CIN and with invasive CaCx has been investigated much more extensively than the mechanisms of HPV infection (35).

Biology and Genomic Organization of HPV

HPVs belong to a large family of DNA tumor viruses, papovaviridae, and are epitheliotropic in nature (35). About 55nm in diameter the viral particles contain a double stranded close circular DNA genome of approximately 7200-8000 base pairs. The viral DNA is encapsidated with 72 capsomeres, replicating as an episome in the nucleus of the host cell.

The HPV genome (Fig 1). is divided into three segments : an early (E) region comprising of 6 genes (E1, E2, E4, E5, E6 and E7) that function primarily in replication,a late (L) region that encodes two structural proteins or the viral capsid proteins (L1, L2) and a long control region (LCR) which is the non coding region or the upstream regulatory region (URR) that separates the E and the L regions. This region contains the viral promoters as well as several enhancer elements, that control viral replication and transcription of the viral oncogenes (E6 and E7) leading to malignant transformation and maintenance of tumorigenic phenotype. The LCR constitutes about 10% of the genome (*i.e.* 800bp) and contains many cis-acting elements. Viral gene expression is generally regulated by several viral and host cell transcription factors like NF1, AP1, Oct-1, progesterone receptors, YY-1 and glucocorticoid receptors, which bind to the URR (23, 35, 36).

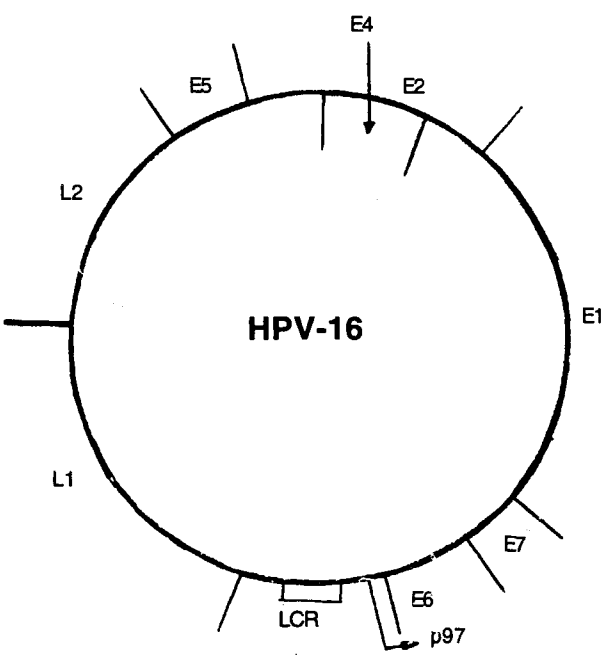

Fig. 1. Schematic Representation of the Circular Genomic Organization of HPV-16.

HPV Infection and Cervical Epithelium

Viral infection occurs through a disturbed epithelial barrier, as only basal keratinocytes are infected by HPVs. A strong candidate for the HPV receptor is an integrin, made up of α_6 and β_4 subunits that is highly expressed in basal keratinocytes (35). Expression of $\alpha_6\beta_4$ integrin is also up regulated during would healing. Since $\alpha_6\beta_4$ is attached to the extra cellular matrix, continuous endocytosis and recycling of the integrin takes place while cells migrate to cover wound sites. One possibility is that endocytosis of the integrin carries the virus with it. The exact mechanism of translocation to the cell nucleus and uncoating of viral DNA have not yet been clarified. The HPVs do not encode an unique DNA polymerase and therefore depends on the host cell replication machinery to generate viral progeny. Viral gene transcription is linked to the state of differentiation of the infected epithelial cells. Within the undifferentiated, dividing cells in the basal layer of the epithelium, HPV infection is followed by a low level of E gene transcription and a low level of viral DNA replication. When an infected basal keratinocyte divides, viral DNA is present in each daughter cell. The transcription of the late genes and assembly of virions can occur only in the more superficial layers of epithelial cells which have withdrawn from the cell division cycle and are committed to differentiate. There is no need for a lytic viral cycle to occur since the differentiated cells are ultimately shed at the most superficial layer, the stratum corneum. Virus is disseminated when it is sloughed off with the stratum corneum (35).

Host Cells and HPV Infection

The integration of the viral genome into the host genome is generally a prerequisite for malignant progression (37). After the HPV infection takes place, the viral genomes are maintained as episomes in the nucleus of normal (infected) cells. In premalignant lesions, except in carcinoma *in situ* and severe grades of CIN, the HPV DNA always persists as a non-integrated episomal molecule (37). In CIN and more frequently in cancers, HPV genomes are found integrated into the host chromosome. Integration of the viral genome takes place at fragile sites and at sites of protooncogenes in the host genome. This event involves E1 and E2 genomic sites of the virus, disrupting some portions of these regions. Disruption of E2 results in loss of transcriptional regulation of E6 and E7 genes, leading to cellular transformation. Several studies demonstrated that HPV 16 DNA is present in an integrated form in more than 70% of cervical cancers. However, absence of viral integration in about 30% of such cases indicates that integration may not be a sole pre-requisite for malignant transformation. In such cases, perhaps other events contribute to tumor progression (38).

HPV-infection and Molecular Mechanisms of Cellular Transformation

The early genes of the HPV high-risk types 16/18, *i.e.*, E6 and E7 are located at the 5'end of the early region of the HPV genome. These genes code for multifunctional proteins that interfere with cell growth and are transcribed from the same promoter, giving rise to a polycistronic mRNA (36). The proteins encoded by E6 and E7 genes of the high-risk HPV types are directly involved in cellular transformation. E6 and E7 proteins can influence transcription from different viral and cellular promoters. As a consequences of the activity of these oncoproteins, genomic instability may result, leading to the expression of a fully malignant phenotype (36, 39).

The E6 protein (of the high-risk HPV types) binds with the tumor suppressor protein p53. This association induces the ubiquitine dependant degradation of p53, removing the p53 dependant control of the cell cycle. One of the functions of p53 is to mediate cell cycle arrest in response to DNA damage. The p53 protein is an activator of gene transcription, regulating the expression of growth suppressor proteins including a cell cycle inhibitory protein called p21 and a repressor of p53 itself called Mdm-2 (35). It also regulates apoptosis by a different molecular pathway. E6, therefore, abrogates p53-related control of cell proliferation, as well as apoptosis. If HPV infected cells are allowed to continue to divide in the presence of damaged DNA, it may cause chromosomal instability and an elevated mutational rate leading ultimately to malignancy.

Complexes of cyclins and cyclin dependant kinases are thought to control the phosphorylation events of pRB and its subsequent ability to regulate the cell cycle through a family of transcription factors, called E2F (35). This particular control function of pRB seems to be critical in maintaining the normal transition of G1 to S phase that insures replication of intact cellular DNA and cell cycle progression. The nuclear phosphoprotein E7 binds to the pRB protein releasing the E2F transcription factors that are now free to activate the transcription of several genes involved in the progression of the cells through G1 into S phase of the cell cycle (36). E7 was also shown to interact with cyclin A and cyclin dependant kinase 2, disturbing the cell cycle progression, and like E6 inhibit p53 function. While E6 targets p53 for degradation, E7 can interfere with this pathway downstream of p53. In particular, E7 can interact and interfere with the functions of p21, whose expressions are activated by p53 (35). Another early gene, E5 is involved in growth stimulation and cell transformation. HPV E5 synergise with epidermal growth factor (EGF) in the stimulation of epithelial cell proliferation (36).

HPV Infection and Transcriptional Regulation

A major event, which modulates E6/E7 expression, is integration of HPV DNA into the host cell genome that often leads to an inactivation/disruption of E2 ORF. The E2 proteins thus may relieve the E6 and E7 transcriptional repression. However, in a number of CaCx situation, HPV DNA remains in an episomal form (38). This indicates that there may be involvement of other mechanisms for tumor progression, Specific cellular protein factors participate in the transcriptional of HPV on cogenes expression. A large number of host cell transcription factors like activator protein (AP-1), nuclear factor 1 (NF-1), glucocorticoid receptor and progesterone receptor are found to activate the enhancer elements of HPV 16 and other types (40).

Host Factors, Viral Persistence and Disease Progression

It has been observed in various studies that not only the viral infection, but the persistence of the viral infection is required for the progression to tumor development. Viral DNA can be detected in 10-50% asymptomatic women of reproductive age (41,42). However when specimens were taken from these women in follow up surveys, majority of the infection were found to be transient and only a small proportion of the women (5-10 %) tend to harbor persistent HPV infection (43, 44). Such infections are associated with an increased risk of developing CaCx. The persistence is higher among older women infected with high-risk types. The effect of HPV persistence in disease progression may be influenced by age, number of sexual partners and smoking (32) or in patients using oral contraceptive pills (OCP) for a number of years (45). Also, high parity may facilitate the persistence and/or integration of HPV through immune suppression, folate depletion or cervical trauma (46-49). The underlying mechanism of folate depletion has been attributed to irreparable double strand breaks in DNA resulting in DNA instability and chromosomal breaks that could facilitate integration of HPV (50).

The role and nature of genetic host factors in the susceptibility to HPV infection and development of cervical cancer is also currently under focus. Epidemiologic studies have revealed that people with defects in T cell mediated immunity, organ transplant recipients on immunosuppressive therapies, and HIV infected patients develop more frequent clinically apparent HPV related infections and have a tendency to develop more frequent malignant conversions of HPV positive lesions (35,51,52). As human leukocyte antigen (HLA) molecules are responsible for antigen presentation, variation at HLA genes (polymorphism) might be important, in determining the reaction to HPV infection (53). Findings from various case- control studies have suggested that class I and class II HLA alleles might be involved in the natural history of HPV- related cervical neoplasia (54). Other studies have also indicated both predisposing and protective HLA alleles of cervical cancers with the etiology of HPV infection (55-58).

The effect of host genetic variation on the oncogenic potential of the HPV E6 protein has been proposed by Storey *et al* (59). It was suggested that the susceptibility of p53 proteins towards degradation by E6 oncoproteins of high risk HPVs might depend on the polymorphic status of

p53 gene at codon 72 in exon 4. The two polymorphic forms of p53 differ in the presence of proline (CCC) or arginine (CGC) residues at this position, with the arginine form being more prone to degradation by E6 oncoproteins than the proline form. However, the results obtained so far, from various other study groups, have varied considerably (60-62).

Viral sequence variation within the major oncogenic types of HPV might affect cancer risk (63). The persistence of HPV infection has also been documented on the basis of molecular variants of a HPV type (64). Five distinct variants of HPV 16 and one variant of HPV 18 were found to be associated with persistent HPV infection so far (65). However, only one HPV 16 variant was found to persist over time, which would indicate some biological advantage that could be important in disease progression. Thus to resolve the role of viral sequence variation, there is a need to address the putative interaction between HPV sequence variation and genetic host factors such as codon 72 polymorphism, of the p53 gene as proposed by some investigators (53).

Interaction of HPV with Cofactors

HPV infections and their associated lesions are most prevalent among young sexually active women (27). The risk factors for the progression of a cervical lesion include reproductive factors, cellular immunity, nutritional factors, socioeconomic status, and co infection by other sexually transmitted infectious agents such as HSV-2, T. vaginalis, and C. trachomatis (20,66). In recent times association between HIV and cervical neoplastic lesions (and/or HPV) has been explored by a number of investigators (67-76). In a meta-analysis (14) from 15 published reports it has been found that a strong overall association exists between HPV and CaCx [OR 8.1; 95%CI: 6.5-10.1]. The association becomes even stronger in the presence of HIV infection [OR 8.8; 95%CI: 6.3-12.5].

All these infections reflect sexual promiscuity and act as surrogate markers of exposure to HPV (35). Certain cultural/social customs or religion and personal hygiene may influence HPV infection. Jewish and Muslim women show extremely low prevalence of HPV and cervical cancer (WHO Databank Cervix Uteri, 1977-1997). Exposure to sexual intercourse at an early age and to a large number of sexual partners increase the chances of HPV infection. In India, it has been found that the association of the infection of high risk HPVs with the age of marriage below 18 years increases the risk of CaCx by 22 fold (77). Multiple pregnancy may lead to folate deficiency that in turn may lead to genetic instability, making integration of the viral DNA into the human chromosome easy, which acts as an important factor in the progression of the disease (49). Our own data from a population based survey, however, points out to a significant correlation ($p = 0.026$) between low parity (≤ 3) and high risk HPV 16/18 infection (unpublished observation).

Mutagens and immunosuppressants present in smoke constituents may cooperate with HPVs in the induction of malignancies in different ways (78). Local immunological depletion caused by smoking or mutagenic action of smoke contents could favour viral persistence, contributing to malignant conversion.

Hormones interact with HPV genomes modifying their expression. The upstream regulatory region of HPV was shown to contain sequences similar to the glucocorticoid responsive elements that are indeed inducible by steroid hormones (36). Such induction is also observed in the presence of progesterone and progestins, the pharmacologically active component of oral contraceptives. As a result, transformation of cells with viral DNA is enhanced (46).

The association between HPV and other sexually transmitted agents like trichomonas, clamydia in the development of cervical neoplasia has been mentioned in some epidemiological studies but no clear evidence is yet available. The effects of diet and alcohol consumption in the risk of HPV associated malignant tumors also not been established.

In a number of studies, users of barrier method of contraception were found to have a low risk of cervical cancer (79). This may be due to the fact that diaphragm and condoms may protect the cervix from the venereally transmitted agents like HPV, though this has not been proven. Condom use cannot entirely prevent the spread of genital HPV infections, because HPV infections in male are not limited only to penile skin. Viral spread from scrotal and perineal lesions cannot be prevented by condom use (80). Although sexual transmission is the most important route of HPV infection, fomite transmission of HPV to the cervix appears theoretically possible based on findings of HPV DNA on underclothes and gynecologic equipments (81, 82).

Geographical Distribution of HPV

Globally, distribution pattern of HPV appears to be similar in different countries, 60-65% positivity for HPV 16, 4-20% for HPV 18 and a low prevalence of other HPV types. It was observed that prevalence of HPV 16/18 is 1.5 times higher in Denmark (low risk area of CaCx) than that in Greenland (high risk area for CaCx) (83). In Pakistan, 33% prevalence of HPV DNA was reported in non-neoplastic diseases and in Japan 46% of non-neoplastic diseases had HPV DNA though there was no significant difference in prevalence of high risk HPVs in cancer cases between the two countries. Munoz et al reported that in a case control study in California, 13.3% of the control women were HPV positive and a similar study in Spain revealed 4.6% of the control women were HPV positive (84). A comparative study on the HPV prevalence rates among cervical cancer cases in Panama led to the detection of HPV DNA in 70% of CaCx cases in the lowest cancer risk area and 54% of the CaCx cases in the highest cancer risk area (38). Reeves et al carried out case control study in four Latin American countries where 91% of invasive cancer patients were HPV positive compared to 63% in the control (85). Lehtinen *et al*

(86) also carried a case control study of carcinoma *in situ* and invasive cervical cancers from a cohort of 18,814 Finnish women who were followed upto a period of 23 years, showed significant association of CaCx with presence of HPV 16. The study revealed that 76% of the CIN lesions could be attributed to the high risk HPV infection.

Studies on cervical cancer in India show 98% HPV positivity in invasive cancer cases and 20% in normal healthy controls, with the most predominant type being HPV 16 (90%) and HPV 18 being very low (3%) (87). In Chennai, a high prevalence area for CaCx, 80% of the cases were found to be HPV 16 positive (88). The lowest prevalence area for CaCx was reported in Jammu and Kashmir where prevalence of HPV 16 was only 11% while a moderate frequency from 42- 66% has been observed in rest of the country (Das *et al,* unpublished data). We have observed a 82% prevalence of HPV 16/18 infection in CaCx cases from Eastern India (communicated elsewhere, 2000). Fewer studies have been conducted till date that have identified precise risk factors and conditions for HPV infections in normal population. A Danish study showed a 15.4% prevalence of HPV in normal smears, of which 74% were oncogenic type (89). Another study from Costa Rica showed that among women with normal cytology, HPV infection peaked first at age $\leq$ 25 years and then at age $\geq$ 55 years with predominantly non-oncogenic or uncharacterized HPV types (90). Our own data showed a 9.3% prevalence of HPV 16/18 infection in a normal population (n = 774), mostly with normal cytology and age $\leq$ 35 years and age at consummation of marriage $\leq$ 20 years (communicated elsewhere). This brings up the issue of HPV testing in cervical cancer screening programmes.

HPV in Cervical Screening Programs

Cytological screening (Pap test) has helped to reduce CaCx rates dramatically since its implementation in the '50s (91). However, there are controversies in interpreting cytological findings even among experts, giving rise to high rate of false negative reports (as high as 40%) (36,91). Investigators are, therefore, looking for better methodologies of detecting high-risk subjects in their preinvasive stages. The role of HPV type specific persistent infection, specifically with conteinual high viral load, has been emphasized for the association with persistent cervical dysplasia (27). The examination of archival pap smears taken prior to the diagnosis of HPV-related CaCx has identified the viral-DNA in majority of the smears. The persistence was traced back upto 7-years time (92). Assessment of false negative archival pap smear performed upto six years preceding the diagnosis of cervical cancer showed persistent HPV 16 or 18 infection in the majority of the smears (93). Thus, the natural history of HPV has created significant impact on the outcome of CIN in terms of viral types, load and duration (25, 29). Recently a Swedish study had used archival cytology samples for HPV DNA testing from women who participated in an ongoing population based CaCx screening program (94). Their findings showed that a single positive report of HPV DNA in a Pap smear test conferred a markedly increased risk of subsequent development of CaCx. The importance of HPV testing in interpreting specifically ASCUS cytology (atypical squamous cells of undetermined significance) which may herald HSIL was further highlighted by Manos *et al* (91). All these studies implicate that the inclusion of HPV testing with cytological screening may help in reducing the number of false negative smears.

A consensus has been drawn recently (2000) by several experts in a WHO – EUROGENE joint conference. They proposed that it would not be quite logical to screen for the precursor of CaCx before the age of 25 if the woman does not fall in the "high risk" category. In the light of our country's scenario, the situation may be different.

Future Directions

Based on molecular and epidemiologic studies, conducted over the last two decades worldwide by several groups, high risk HPV infection appears to be the key etiologic factor for cervical neoplasia. The viral infection, which is sexually transmitted, may be persistent in some and transient in others. Viral persistence has been attributed to progression to high grade CIN lesions and eventually to invasive cancer. Such progression involves a number of host factors that could be genetic or epigenetic as indicated in the review. With this background, a number of avenues could be opened to understand the underlying factors that facilitate high risk HPV infection and its persistence as well as to devise modalities of prevention of the infection. Firstly, a strategy could be primary prevention of HPV infection and hence the prevention of CaCx by implementing early screening programmes involving both HPV testing and cervical cytology or testing other cost effective methods such as visual inspection of the cervix, particularly in the developing countries. Secondly, a better deciphering of the immune response to HPV infection and consequent development of vaccines, both therapeutic and prophylactic, should be a good approach. This should be chiefly beneficial in countries where the social structure makes the women to participate in sexual intercourse through marriage at an early age, go through early and multiple pregnancies (with short spacing) and where contraception through barrier use is not well utilized. Thirdly, very little is known about the nature of the risk genes in cervical cancer. Therefore, it has been proposed (95) that novel study designs involving case-control cohorts that have been carefully analysed with respect to HPV infection history could permit candidate-gene association studies using less complex phenotypes. This could potentially increase the probability of identifying the genetic risk factors involved. The identification of risk genotypes of high-risk HPV infection should be of importance for clinical management of cervical neoplasia. Lastly, control of HPV infection related lesions could be undertaken by adopting gene therapeutic approach. The research is currently ongoing.

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