REVIEW OF LITERATURE
The lesions of uterine cervix in sexually active women generally involve inflammatory changes referred to as non-specific cervicitis and cervical tumours represented by benign polyps and invasive carcinoma. It has been estimated that about 90,000 women develop cervical cancer annually in India (Luthra, 1983). The occurrence of high frequency of cervical cancer in India has also been supported by Das et al. (1992).

In the normal stratified squamous epithelium such as fore-skin and cervix, the diploid cells cease to divide and undergo an ordered pattern of differentiation after leaving the basal layer. In contrast, the cervical neoplasm and its precursors show a total disarray of this pattern. The benign hyperplastic lesions of cutaneous epithelium of vulva, penis or perianal area are hard and keratinized and are referred to as condyloma acuminata as against the flat lesions of mucosal epithelium of vagina, cervix and rectum. In these flat lesions, the supra-basal layers of epithelium reveal proliferation either due to continued cell division or delayed maturation of the cells.

Any abnormality in the cellular growth and development of the tissue is known as dysplasia. Such a change in the cervical tissue is termed as cervical intraepithelial neoplasia (CIN) which pathologically is suggestive of premalignant change in the tissue. The grading of preinvasive lesions (dysplasia, CIN and carcinoma in situ) depends on the extent to which the full thickness
epithelium is replaced by basal and parabasal cells and on the degree of cytological atypia. In the invasive carcinoma of cervix there is extension of the neoplastic epithelium into the stroma through the plane of basement membrane and based on the extent of invasion it has been staged as follows:

Stage 0. CIN III
Stage I. Carcinoma confined to the cervix.
Stage II. Carcinoma extends beyond the cervix but not into the pelvic wall. It involves the vagina but not the lower third.
Stage III. Carcinoma with extension onto pelvic wall. It involves the lower third of vagina.
Stage IV. Carcinoma extending beyond the pelvis involving mucosa of bladder and/or rectum. The metastatic dissemination may be seen in lymph nodes, liver, lungs and bone-marrow.

1.1 DIAGNOSTIC AIDS OF CERVICAL NEOPLASIA AND ITS PRECURSORS

In sexually active women, periodic Papanicolaou (Pap) smear helps in screening the patients requiring a diagnostic work up. Any suspicious area on the cervix, indicated as aceto-white patch and showing highly abnormal vascular pattern, can be identified by colposcopic examination. The coating of cervical cells with Schuller’s iodine further helps in locating abnormal cells which are depleted of glycogen and hence fail to stain with iodine.
The normal cells of cervical epithelium are rich in glycogen and stain as mahogany brown. A final diagnosis is made after histopathological evaluation of the colposcopically directed biopsies.

1.2 ETIOLOGY

Squamous cell carcinoma and its precursors have not been reported in virgins and nuns and a high correlation with promiscuity of women and their male partners suggests that a sexually transmitted agent(s) may be responsible for the disease. Thus several sexually transmitted diseases (STDs) were investigated for their role in cervical neoplasia. In as early as 1968, Rawls et al. reported the association of Herpes simplex virus-2 (HSV) with the patients of cervical carcinoma. This was further supported by the detection of antibodies to this virus in these patients. Viral sequences were detected in the neoplastic tissue and were found to be transcriptionally active (Frenkel et al., 1972). The attenuated HSV was capable of transforming the cells in vitro (Duff and Rapp, 1973). The etiological role of HSV was further substantiated by the detection of HSV-antigens in human premalignant and malignant cervical biopsies and explants.

The studies on patients infected with Chlamydia trachomatis (Schachter et al., 1982) and Trichomonas (Boyle et al., 1989) did not show any correlation with cervical neoplasia. Based on the epidemiological data, Herpes
simplex virus and Human papilloma virus (HPV) appear to be etiologically important. The association of HPV with cervical cancer was first suggested by Zur Hausen et al. (1974) and later supported by Meisels and Fortin (1976) and Meisels et al. (1977). HPV, which was originally known to produce condylomatous lesion, was linked to the appearance of white flat lesions in the genital tract. These lesions were considered to be the precursors of cervical carcinoma. The localization of HPV particles in the nuclei of cells from these lesions with the help of electron microscope confirmed the association of HPV with cervical neoplasia (Laverty et al., 1978). The presence of HPV in the cervical lesions was also shown by immunoperoxidase staining for HPV-capsid antigen (Jenson et al., 1980; Shan et al., 1980).

Human papilloma virus emerged etiologically the most prominent agent with 70% of the cervical tumours showing the presence of HPV types 16 and 18 (Zur Hausen, 1987). With the identification of new HPV types and the use of highly sensitive techniques like PCR, the association of HPV with cervical carcinoma has gone up to 93% (Das et al., 1992). The molecular studies on HSV-2, however, failed to provide a compelling data for its etiological association with cervical carcinoma (Zur Hausen, 1983). A similar observation was made by Vonka et al. (1984) in a prospective epidemiological study on 10,000 women with cervical neoplasia.
Till date more than 60 HPV types have been described of which HPV-6 and 11 have been implicated with benign lesions (condyloma) and low grade dysplasia while HPV-16 and 18 with neoplastic lesions and their precursors (Table-1). Thus these are designated as low and high risk types respectively (Crawford, 1986; Howley, 1991). The other less common HPV types associated with moderate to severe dysplasia and invasive carcinoma are HPV-31, 33, 35, 39, 45, 51, 52 and 56 (Werness et al., 1991).

1.3 MANIFESTATIONS OF HPV

Koss (1987) reported that koilocytosis represented the cytopathic effect of HPV and was characterized by the presence of large squamous cells occurring singly or loosely clustered in superficial and intermediate layers of epithelium and enlarged hyper-chromatic nuclei surrounded by cytoplasmic clearing. The perinuclear clearing was due to the necrosis of cytoplasm proceeding from perinuclear zone to cell periphery. The condensation of cytoplasmic fibrils gave a sharp demarcation to the perinuclear zone. Such cell abnormalities in cervical smears were referred to as koilocytic atypia by Koss and Durfee (1956).

Dyskeratosis is the thickening of epithelium along with koilocytosis. The superficial keratinized layers of squamous epithelium infected with HPV might shed cells with pyknotic nuclei termed as dyskeratocytes (Meisels et al., 1984). In some cases of condyloma and squamous carcinoma,
<table>
<thead>
<tr>
<th>Virus type</th>
<th>Site of Infection</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV-1</td>
<td>Skin</td>
<td>Planter warts</td>
</tr>
<tr>
<td>HPV-5</td>
<td>Skin</td>
<td>Epidermodysplasia verruciformis (EV)</td>
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<td>Laryngeal papilloma (LP) Condyloma acuminatum (CA) Cervical intraepithelial neoplasia (CIN)</td>
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<td>CIN; CA</td>
</tr>
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<td>CIN; cervical carcinoma</td>
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<tr>
<td>HPV-51 &amp; 52</td>
<td>Genital mucosa</td>
<td>CIN; cervical carcinoma</td>
</tr>
</tbody>
</table>

*Sousa et al. (1990)*
the atypical squamous cells were probably shed to form squamous 'pearls' with a keratinized center (Koss, 1979).

Heteroploidy was generally considered as a marker for HPV infection (Shevchuk and Richart, 1982) and a prognostic value was also linked to the DNA content of the lesion (Fu et al., 1981). Kashyap et al. (1990) also supported aneuploidy as high risk indicator for cervical precancerous lesions. The lesions with moderate dysplasia and aneuploidy often developed into carcinoma in situ over a period of one to six years, while the lesions with dysplasia and euploid or polyploid DNA showed regression to inflammation and normalcy during a period of one to ten years.

1.4 DETECTION AND TYPING OF HPVs

1.4.1 Clinical observations

The warty lesions of external genitalia, perineum and anus are grossly visible while the subclinical infection of cervix (dysplasia and carcinoma in situ) can be localized as aceto-white patch under colposcopic examination. The diagnosis is presumptive at this stage with no information about the presence/absence or the type of HPV that may be present in the lesion.

1.4.2 Cytomorphic/histological evaluation

Cervical smears and biopsies are evaluated for the presence of koilocytic changes, shape and size of cells and the nuclei, abnormalities in chromatin pattern, abnormal
mitoses and the presence of malignant cells. In light microscopy the presence of abnormal mitotic figures (AMF), little or no koilocytosis, higher degree of cytological atypia and the presence of undifferentiated cells and mitotic figures in upper third of epithelium were suggestive of infection with HPV-16/18 (Crum et al., 1985). However, the lesions infected with HPV-6/11 had high degree of koilocytosis, surface maturation and no AMF (Schiller et al., 1984; Winkler et al., 1984). Although certain HPV types were preferentially associated with high grade lesions, the HPV-type could not be predicted on the basis of histopathological findings alone (Kadish et al., 1986).

1.4.3 Electron Microscopy
A papilloma virus particle was shown to be about 55nm in diameter having icosahedral symmetry with 72 capsomers (Brescia et al., 1986). The ultrastructure studies were able to detect the presence of intranuclear HPV particles in cervical lesions which showed at least some highly differentiated cells. The production of mature virion was dependent on the differentiation of the epithelial cells. Thus electron microscopy was used to locate virions in koilocytes and dyskeratocytes (Hills and Laverty, 1979; Ferenczy et al., 1981; Kadish et al., 1986; Syrjanen et al., 1986) but it was not possible to distinguish between different HPV types. Moreover this technique can not be used on a day today basis.
1.4.4 Immunohistochemical studies

HPV infection can be identified by the demonstration of HPV associated antigen in the tissues. The method involves the use of antibodies to the genus specific capsid antigens that were produced only in the productive infection. These viral proteins were observed to be predominantly expressed in highly differentiated outer layers of condylomatous lesions with mild or moderate dysplasia (Alessandri et al., 1986; Kadish et al., 1986) and rarely in severe dysplasias or carcinoma in situ (Kurman et al., 1981). The detection rate of HPV-16 was found to be highest in keratinized type but none in small cell, non-keratinized type, the latter being the most undifferentiated type of squamous cell carcinoma (Hara et al., 1990). Thus it was suggested that there is a decrease in antigen positive specimens with the increase in the severity of the lesion which is also linked with the loss in differentiation (Syrjanen and Pyrhonen, 1982; Gupta et al., 1987; Sato et al., 1987)

The most commonly used method to detect HPV-capsid antigen is the peroxidase-antiperoxidase (PAP) localization of HPV using rabbit antibodies to disrupted papilloma virion (Kurman et al., 1981). However, the avidin-biotin complex (ABC) using biotinylated antibody and horseradish peroxidase was found to be two folds more sensitive in detecting PV antigen (Hsu et al., 1981). Immunohistochemical method is quite specific in nature. A 100% correlation between the
detection of HPV antigen in the cells and the observation of virions by electron microscopy was found to exist (Ferenczy et al., 1981; Kadish et al., 1986). However, this method was shown to be of little value for the typing of HPVs and was not very sensitive (Kadish et al., 1986). The low sensitivity of this method might be a reflection of sampling error, variation in the time of expression of antigen or due to the destruction of antigen during tissue processing. In addition, it was suggested that HPV-16 may produce very little capsid antigen that escapes immunohistochemical detection (Beckmann et al., 1988).

1.4.5 **Nucleic acid hybridization**

This method offers to study the prevalence, typing and transcription of the viral genome using labelled DNA/RNA probes. Hybridization under relaxed conditions can be used to detect HPV infection but stringent conditions are required for typing purposes. Roman and Fife (1989) reported that under low stringency conditions 33% of the mismatch between the two hybridizing strands was tolerated while under high stringency conditions it was only 13%. The typing of HPVs was done on the basis of the degree of homology between the two isolates. An HPV-DNA sharing more than 50% homology with a known HPV type was taken as the same type while less than 50% homology indicated a new type. HPV-DNA showing more than 50% but less than 100% homology was taken as a subtype.
1.4.5.1 Generation of probes

Hybridization studies depend on the availability of HPV DNA cloned into bacterial plasmid vectors. Durst et al. (1983) were the first to clone HPV-16 isolated from cervical carcinoma tissues into the plasmid vector and the cloning of HPV-18 genome was done by Boshart et al. (1984). A probe is generally made by labelling the known HPV type with a radioactive or a nonradioactive reporter molecule. The labelling may be conducted on the intact plasmid where the vector sequences, along with HPV genome, are also labelled. Another approach is to make HPV-DNA vector-free by appropriate restriction enzyme and then label it. Both methods work equally well if appropriate controls are used for the interpretation of the results.

The commonly used labelling method involves the nick-translation of HPV-DNA in the presence of radiolabelled nucleotides. Radioisotopes like $^{32}$P, $^{35}$S and $^{3}$H are widely used for this purpose. The radioactive probes can, however, be substituted with nonradioactive ones which make use of biotin, digoxigenin, fluorescent or chemiluminiscent molecules. The biotin-labelled analogs (dUTP and UTP) of TTP were observed to form efficient substrates for a variety of DNA and RNA polymerases in vitro and the polynucleotides containing low levels of biotin substitution showed denaturation, reassociation and hybridization characteristics similar to those of unsubstituted controls (Langer et al., 1981). In the biotinylated nucleotides, the
biotin molecule is covalently linked to C5 position of the pyrimidine ring through an alkylamine linker arm. Depending on the length of this arm, dUTP-4, dUTP-14 and dUTP-16 types of biotinylated nucleotides are available. The increased arm length in dUTP-16 was found to allow an easy access to the biotin molecule by avidin/streptavidin, thus a better signal was achieved with this nucleotide which could be detected by immunofluorescence, immunoperoxidase or by affinity binding between biotin and antibiotin-IgG or avidin/streptavidin (Brigate et al., 1983).

Some of the advantages offered by the use of nonradioactive probes are:

- These eliminate the problems of safety and disposal that are associated with radioactive probes.
- Biotinylated probes are stable over a longer period of time due to their chemical stability. Thus these can be used over the years without substantial loss in the strength of hybridization signal.
- The results are obtained in short period (< 24hr).
- Biotin-labelled probes have been found to exhibit lower non-specific binding to nitrocellulose paper than the radiolabelled DNA. Hence a unique mammalian gene sequence could be detected by using high concentration (250-750 ng/ml) of biotinylated probe in 1-2 hr of hybridization (Leary et al.,
The biotinylated probes can also have general utility in genetic studies using Southern, DNA and RNA hybridizations.

1.4.5.2 Dot blot hybridization

It is the simplest of all the hybridization procedures. A dot blot requires 0.5-1.0 µg of DNA that is used without any manipulations. The denatured DNA bound to the membrane is hybridized with a labelled probe. The results are interpreted with the help of appropriate controls. Dot blot is preferentially conducted under high stringency conditions and can be used for typing of HPVs. However, the hybridization under low stringency conditions can be used for screening purposes since it can detect the cross-hybridizing types. The hybridizing strands need not be identical over the entire length of genome to give a positive signal.

Dot blot analysis has been valuable in establishing the predominance of HPV-16 or 18 in invasive cervical carcinoma and of HPV 6/11 in invasive vulvar carcinoma and condyloma. A few cases of mixed infection of HPV-16 and 6/11 were observed in condylomas and invasive cervical carcinomas (Sutton et al., 1987). The screening of different groups of women attending health clinics but otherwise having normal Pap smears showed the presence of HPV-16 in 10% of the cases which however failed to show
progression to CIN after 3 years of follow up. The high copy number of HPV-DNA also did not correlate with the development of CIN. It was, therefore, suggested that factors other than the amount of HPV-DNA play role in triggering the neoplastic process (Wickenden et al., 1987).

From another study on patients with cytological abnormalities it was evident that the distribution of different HPV types was heterogenous. The dot blot analysis of the paired samples (smear/biopsy) from these patients revealed a higher prevalence of HPV-18 and 31 in scrapes than HPV-16, thus suggesting a more superficial nature of infection with HPV-18 and 31 (Hallam et al., 1989).

Dot blot analysis is ideal for screening those clinical samples that contain HPV-DNA levels undetectable by other methods. Lindh et al. (1992) employed biotinylated genomic HPV-DNA of types - 6, 11, 16, 18, 31, 33 and 35 for this purpose and the positive samples were further subjected to Southern blot analysis using the subgenomic probes specific for each type. These subgenomic probes were, however, found to be too insensitive for dot blot test. This study also promoted the association of HPV with the development of CIN and its persistence.

1.4.5.3 Southern blot analysis

It is the most relied upon method for checking the presence and typing of HPVs. The total cellular DNA (~10 μg) is digested with restriction enzyme. The fragments are
separated by electrophoresis and then denatured, neutralized and are transferred to nitrocellulose/nylon membrane prior to hybridization with the probe. The digestion pattern is characteristic of each type and is slightly altered in the subtypes. Pst I is the most informative restriction enzyme for typing purposes. HPV-DNA was detected in 90% of the squamous cell carcinomas of cervix by pooling the results of low and high stringency hybridization (Boshart et al., 1984). With this method, using $^{32}$P labelled probe, it was possible to detect picogram quantities of HPV-DNA homologous to the probe. However, the sensitivity was found to decrease by 20-100 folds for the detection of heterologous DNA (Ostrow et al., 1987).

In addition, Southern blot analysis made it possible to examine the physical status of HPV in the infected tissue (Durst et al., 1985). The integration was generally associated with high risk HPV types and the low risk types preferably showed the episomal existence. The event of integration was marked by the appearance of off-sized fragments which could be the virus-cell junctions (Matsukura et al., 1989; Couturier et al., 1991). The distinction between the multimeric episomal forms and integrated linear HPV fragments was made by 2D gel electrophoresis (Durst et al., 1992).

1.4.5.4 Reverse blot hybridization

It is basically the reverse of Southern blot
procedure involving the labelling of cellular DNA instead of HPV DNA (De Villiers et al., 1986). A modification of this method involved the use of Pst I digested HPV-DNA to know the relatedness of the labelled cellular DNA to the full length HPV and/or its subgenomic fragments (Webb et al., 1987).

The results obtained with this technique were comparable to those of Southern blot analysis for the detection of HPV in invasive carcinoma of vulva (Kasher and Roman, 1988). In another study the findings of reverse blot to detect and type the HPV in condyloma acuminata, invasive carcinoma of cervix and vulvar carcinoma were consistent with those of dot blot analysis. The sensitivity of reverse blot was found to be lower than the dot blot (Sutton et al., 1987).

1.4.5.5 In situ hybridization (ISH)

This technique involves the direct probing of the histological sections (tissue hybridization) or metaphase chromosomes (chromosomal in situ hybridization) to detect the viral DNA without the extraction of DNA from the specimen (Beckmann et al., 1985). The unique property of this technique is to allow the simultaneous detection and localization of the viral DNA in the cell and study the pathology. Thus the presence of virus could be directly correlated with the histopathology of the lesion (McNeil et al., 1991). According to Stoler and Broker (1986) ISH is a
sensitive and specific method to investigate the interplay of papilloma virus replication, gene expression, cellular differentiation and neoplastic transformation.

The utility of ISH has been widely increased with the use of nonradioisotopic detection systems. Biotin-ISH was used for the early detection of HPV-DNA in the exfoliated cervico-vaginal cells (Sato et al., 1987). In addition such an analysis can be of value to monitor latency, regression, recurrence and post-treatment response of lesion with regard to HPV infection as suggested by Pao et al. (1989). The sensitivity of DNA in situ hybridization (DISH) using biotin-labelled probes was found to be dependent on the proteinase-K treatment given to the cells prior to hybridization. The increase in proteinase treatment increased the lower limit of HPV-16 detection from 30-40 HPV-DNA copies to 20 copies per carcinoma cell. This increased treatment, however, resulted in poor morphology of the cells (Walboomers et al., 1988). The detection limit of HPV-DNA in CIN and condylomas was found to be 800 copies per cell (Crum et al., 1986).

In addition to the detection of HPV-DNA by ISH, Durst et al. (1992) used this technique to study the RNA expression of HPV-16 in premalignant and squamous cervical cancers. This has complemented various biochemical analyses for the identification of viral mRNA without the extraction of total RNA from the specimen. The only limitation was the inability of ISH to distinguish between the various mRNA
transcripts due to extensive overlapping especially in 3' early regions of HPV.

1.4.5.6 Filter in situ hybridization (FISH)

It is a simple, rapid and non-invasive procedure for typing the infecting HPV and has a diagnostic value (McNicol et al., 1989). FISH involves the direct application of cells from scrapes to the membrane on which they are treated to release the nucleic acids followed by their denaturation and neutralization prior to hybridization. The sensitivity of this method was found to be 1/2 to 1/3 of the Southern blot procedure (De Villiers et al., 1987). The lower limit of detection of HPV by this method was found to be $10^5$ Caski cells (Cornelissen et al., 1988). A positive reaction by FISH was also observed with 100 bovine papilloma virus (BPV) transformed cells containing 100 copies of BPV per cell (Wagner et al., 1984) and 100 cells with 500 copies of HPV-16 per cell in Caski cell line (Caussy et al., 1988). In fact FISH could detect even a single viral genome in 20 cells (Fukushima et al., 1985).

DNA hybridization studies are generally performed on fresh or frozen samples. The formalin-fixed tissues are unsuitable because of strong cross-linking between DNA strands, between DNA and proteins and also between DNA and RNA due to the formaldehyde (Gantt et al., 1985; Dubeau et al., 1986). FISH has, however, made it possible to use
formalin-fixed, paraffin-embedded cervical tissues for the detection of HPV (Demeter et al., 1988). In another study, Kulski et al. (1990) also showed the validity of FISH to detect HPV DNA in a variety of formalin-fixed histological specimens besides the cervical tissue. These findings were comparable to the cyto-FISH analysis performed on the fresh cells taken from the same patients.

Though FISH has been suggested as a rapid method for the screening of formalin-fixed biopsies and the study of archival pathological samples there are, however, certain limitations of this technique which include its inability to give the cellular distribution of the viral DNA and high number of false positive results (De Villiers et al., 1987).

1.4.5.7 Polymerase chain reaction (PCR)

It is a promising method for the enzymatic amplification of specific DNA sequences in vitro from a few copies to a level detectable by gel or Southern blot analysis (Saiki et al., 1985). The method is quick, simple and semiautomatic and can be performed without the use of radioisotopes. But its use is limited to only those target DNAs whose sequences are known. It was possible to detect even one HPV DNA molecule in the sample with PCR (Li et al., 1988). The level of sensitivity was found to increase 10 to 30 folds as compared to Southern blotting and FISH respectively by using smaller fragments of DNA.
For the detection of HPV-16 in the samples, the primer sequences complementary to the upstream regulatory region (URR) or E6 and E7 open reading frames (ORFs) should be used. It was observed that these regions were conserved after the integration of HPV-16 into the host genome (Tidy et al., 1989). The event of integration generally occurred at E1/E2 ORF resulting in the deletion or disruption of these ORFs (Choo et al., 1987).

The applicability of PCR for screening and typing of HPV in clinical samples has increased with the use of broad range primers. Primer pU-1M/pU-2R was used for the detection of malignant (HPV-16, 18, 31, 32 and 52b) and pU-31B/pU-2R for benign (HPV-6/11) types (Fujinaga et al., 1991).

PCR was employed to evaluate the physical status of HPV by using primer homologous to E2 ORF which was deleted or disrupted in integrated form but was intact in episomal form. The inference was based on the formation of 1139 bp fragment in PCR corresponding to the intact E2 ORF. For this purpose, PCR was found to be comparable to the conventional methods like Southern blot, 2D gel electrophoresis or chromosomal-ISH (Das et al., 1992).

The retrospective studies were facilitated by the use of modified PCR which could be conducted even on the paraffin-embedded tissues (Shibata et al., 1988). By a sandwich way of analysis involving Hematoxylin-eosin staining then PCR followed by Hematoxylin-eosin staining, it
was possible to correlate the sites of CIN lesion with the
distribution of HPV-16 in a cervical cone containing both
CIN lesions and the normal epithelium (Cornelissen et al.,
1989).

Infection by one HPV type is rarely associated with
concurrent active infection by a second HPV type. Thus one
HPV type is restricted to a very low copy number that is
undetectable by in situ or Southern blot hybridization.
However, PCR was successfully employed to find the incidence
of HPV 16/18, 33/18 and 18/58 were detected by subjecting
the amplified product to digestion with restriction enzymes.
The detection rate of two or more HPV types in a single
lesion by PCR was approximately 10 folds greater than ISH
analysis.

The sensitivity of PCR makes it vulnerable to
laboratory infections or contaminations with virus
particles, cloned plasmids and the carry over of PCR
products of previous runs leading to a rise in false
positive results. Thus appropriate controls are very
important to guard against this disadvantage. A negative
control includes the PCR reaction buffer, Taq-polymerase and
primers. In paraffin-embedded tissues the knife should be
cleaned with 70% ethanol after cutting each specimen
followed by cutting of paraffin block without tissue which
should be used for PCR amplification of β-globin and HPV-DNA
(Cornelissen et al., 1989). β-Globlin primers are used as
A new strategy against contamination by cloned-HPV plasmids is the use of anti-contamination primers flanking the unique cloning sites. The interpretation was made on the basis of the size of the product of PCR. van den Brule et al. (1989) were able to detect HPV infection even in the presence of contaminating cloned-HPV plasmid by following this approach. Cornelissen et al. (1989) used E7 and L1 ORF primers for the amplification of HPV-16 in cancer specimens where L1 ORF spanning the Bam HI site worked as an anti-contamination primer. An amplified product, 3150 bp long, was indicative of pHPV-16 DNA contamination, 152 bp fragment for colinear L1 ORF and presence of E7 was marked by 142 bp fragment.

It is thus evident that there are different methods available to detect the presence of HPV (Table-2). The use of cytological smear to check the presence of koilocytosis, indicative of HPV infection, is not very sensitive (Bistoletti et al., 1988). The immunohistochemical method is more sensitive but epitopes detected by commercially available reagents are only weakly or unexpressed in genital lesions and hence many cases may go undiagnosed. Thus the nucleic acid hybridization reactions such as dot blot, Southern blot, reverse blot, tissue or membrane in situ hybridization have become indispensable. A fresh impetus has been given by the recent addition of PCR to study and establish the role of HPV in the carcinogenesis of cervix.
<table>
<thead>
<tr>
<th>Country</th>
<th>Method</th>
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<th>Sample</th>
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<td>RL</td>
<td>Scrape</td>
<td>AC</td>
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<td>Cancer (16, 18)</td>
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RL - Radiolabelled; BL - Biotin labelled.
CIN - Cervical intraepithelial neoplasia.
AC - Abnormal cytology.
1.5 GENETIC ORGANIZATION OF HUMAN PAPILLOMA VIRUSES

The base sequence of many HPVs has been determined. Each HPV type has approximately 7900 bp long genome. All the papilloma viruses have more or less similar genome organization containing around 8-10 open reading frames (ORFs) present on the same DNA strand, while the other DNA strand contains only small unconserved ORFs and is assumed non-coding (Fig. 1; Table-3). The PV genome has two coding regions separated by a non-coding segment of 0.4-1.0 kb.

The functions of HPV ORFs were elucidated by in vitro transformation of cultured cells (Durst et al., 1987; Pirisi et al., 1987; von Knebel-Doeberitz et al., 1988; Kaur et al., 1989) and by studying the transcripts in the viral infected cell lines (Schwarz et al., 1985; Smotkin and Wettstein, 1986; Baker et al., 1987; von Knebel-Doeberitz et al., 1988). The studies revealed that the early coding region contains early genes necessary for viral replication and cellular transformation and late coding region contains L1 and L2 ORFs coding for structural proteins of the virus. The non-coding region is located between the end of L1 ORF and start of E6 ORF. It contains AT-rich regions, several RNA polymerase II promoters, constitutive and inducible enhancers, binding sites for cellular transcription factors and several copies of the palindrome ACCN’6GGT known to be the binding site for viral E2 transcriptional regulatory proteins. Thus this region is responsible for the control of viral gene expression and is
**Fig. 1:** Organization of HPV-16 genome. The open reading frames (ORFs) in all the three phases are depicted. E and L stand for early and late region ORFs, respectively. The vertical dotted line represents the first ATG codon of each ORF. Black circles show polyadenylation sites. The bold black lines indicate the long control regions.
<table>
<thead>
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<th>ORF</th>
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<tr>
<td>E 2</td>
<td>Transcriptional modulator of viral promoters</td>
</tr>
<tr>
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</tr>
<tr>
<td>E 5</td>
<td>BPV1 transforming gene</td>
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<tr>
<td>E 6</td>
<td>Transforming gene</td>
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<td>Major capsid protein</td>
</tr>
<tr>
<td>L 2</td>
<td>Minor capsid protein</td>
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</tbody>
</table>

*Campo (1988).
commonly referred to as Long Control Region (LCR) or Upstream Regulatory Region (URR).

1.6 TRANSFORMATION POTENTIAL OF HPV

HPVs have been linked with the etiology of human cervical cancer and HPV-16 is considered as the most common type found in cancer biopsies (Durst et al., 1983). The presence of viral transcripts and proteins in cancer cells suggested their roles in the development of cancer (Lehn et al., 1985; Pater et al., 1986; Kanda et al., 1988). A similar observation was made in the cell lines derived from cervical carcinoma (Schwarz et al., 1985; Lehn et al., 1985; Baker et al., 1987). Since the viral mRNA and proteins are present in low levels in the transformed cells, much of the information has come from studies based on deletion, mutation or expression under the surrogate promoter of the ORFs of interest or by selective inhibition of gene expression by antisense HPV RNA to determine their effects on the transformation of cells.

The immortalization of cells was associated with E6 and E7 viral genes (Pirisi et al., 1987; McCance et al., 1988; Durst et al., 1992) which was further supported by the fact that these genes were conserved and expressed in HPV-16 infected premalignant and malignant tumours (Shirasawa et al., 1988; Stoler et al., 1990). The transforming property of HPV in vitro was shown to be limited to HPV types-16, 18, 31 and 33 which were also associated with cervical
carcinomas (Durst et al., 1987; Pirisi et al., 1987; Kaur et al., 1989).

Pirisi et al. (1987) studied the response of normal human fibroblasts and keratinocytes to transfection with recombinant HPV-16 DNA. These cells showed extended life span, but failed to undergo malignant conversion. A similar protocol, however, was able to induce premalignant phenotype in NIH 3T3 cells. DiPaolo (1983) also found human cells resistant to malignant transformation as compared to rodent cells by carcinogens in vitro.

The human epidermal cells, transfected with HPV-16, were found to retain the ability to stratify and express differentiation-specific keratins, but failed to differentiate normally. The histopathological changes in these cells were typical of intraepithelial neoplasia (McCance et al., 1988). HPV-16 and 18-immortalized human fore-skin keratinocytes, cultured on organotypic epidermal raft cultures at various passage levels, also displayed abnormal epidermal morphologies resembling premalignant squamous lesions (Blanton et al., 1991). The architecture of the tissue was disorganized, mitotic cells were seen throughout the depth of epithelium with abnormal mitoses, large nuclei and cells of variable sizes.

E7, in fact, was found to be the most abundant viral protein in cervical carcinomas and the cell lines derived from them (Seedorf et al., 1985; Smotkin and
Wettstein, 1986 and 1987). Tanaka et al. (1989) identified the transforming genes in HPV-16 by preparing cDNA library from mRNA extracted from transfected NIH 3T3 cells. The deletion analysis of functionally active cDNA clones showed the involvement of E7 ORF in cell transformation.

The biological significance of E6 and E7 proteins was analyzed by modulating their expression in C4-1 cells transfected with HPV-18. The gene expression and its translation was enhanced by dexamethasone resulting in substantial increase in cell growth. A marked decrease in growth was seen on the induction of anti-sense RNA to E6 and E7 in these cell lines which was directly linked to decrease in E6/E7 gene product(s) (von Knebel-Doeberitz et al., 1988). A similar approach was followed by Steele et al. (1992) using plasmids that expressed anti-sense RNA of E6 and E7 genes to study the effect of these genes on the growth of human cell lines viz. HeLa, C4-1 and 1483 containing HPV-18 DNA. Human cancer cell line 183 and vero cell line of monkey kidney were used as controls. The cells containing HPV-18 accepted the anti-sense expressing plasmids with a lower frequency, than the cells lacking HPV, which led to slowing of growth, reduced ability to form colonies on soft agar and increased serum requirement.

Vormwald-Dogan et al. (1992) reported the presence of viral RNA of anti-sense orientation in squamous cervical carcinoma having a nuclear localization. The premalignant lesions, however, did not have viral anti-sense RNA.
Despite the presence of anti-sense RNA, viral mRNA was still transported to the cytoplasm, maintaining proper level of E6 and E7 necessary for the transformed phenotype.

Hiraiwa et al. (1993) compared the transforming activities of E6 and E7 genes of HPV types - 1, 11, 16, 18 and 47 on 3Y1 cells using recombinant retroviruses. In this system it was suggested that the intensity of the morphological changes induced by E6, rather than E7 gene, correlated to the risk of malignant conversion of the lesion.

E7 gene stimulated DNA synthesis and proliferation in rat 3Y1 cells (Sato et al., 1989) as shown by the transfection of these cells by expression vector pMSN (with MMTV-LTR, responsive to dexamethasone) containing E6, E7 or E6-E7 ORFs. The mutagenic function of E7 was correlated with such activities of SV40 and adenoviruses. The amino acid sequence of E7 had a striking similarity with E1A protein of adenovirus but the relation between functional similarity is still to be worked out.

1.7 PHYSICAL STATUS OF HPV AND MALIGNANCY

HPV-6/11, associated with condyloma, existed as extrachromosomal molecules (Gissmann et al., 1982; Lehn et al., 1984) while HPV-16 was found to be integrated into the DNA of cell in majority of cervical cancers and in episomal form in benign lesions (Durst et al., 1983 and 1985). The HPV-18 infected cells of cervical cancers also showed the
integration of the viral DNA (Boshart et al., 1984). The integration of HPV-16 and 18 was also revealed by the molecular analyses of cell lines derived from cervical carcinomas (Pater and Pater, 1985; Yee et al., 1985; Smotkin and Wettstein, 1986). The integration of HPV DNA invariably led to the disruption of E1 and/or E2 ORFs while conserving the E6/E7 ORFs along with the LCR (Long Control Region) (Schwarz et al., 1985; Durst et al., 1986). Wagatsuma et al. (1990) observed the interruptions in viral genome not only at E1/E2 but also at L1/L2 ORFs indicating that integration may not take place at specific viral sites. In integrated state, the late genes were not expressed and hence the immunogenic capsid proteins were not formed. This also helped the virus to escape immuno-surveillance of the host.

An analysis of the physical state of HPV along with its mRNA transcripts was carried out to study the role of integration in the development of malignancy. Although HPV-16 sequences actively transcribed E6/E7 ORF in cervical cancers and the cell lines derived from them (Smotkin and Wettstein, 1986; Tsunokawa et al., 1986; Shirasawa et al., 1987), Lehn et al. (1985) observed that the maintenance of malignant phenotype of the cervical cancers was not dependent on the continuous transcriptional activity of HPV-16.

The transcriptional pattern of HPV-16, existing as episome in CIN and integrated in invasive carcinoma, was
studied by Shirasawa et al. (1988). The pattern of transcripts was uniform in CINs but varied in invasive carcinomas. The variability in the size of mRNA in invasive carcinoma was probably due to the integration of HPV into the cell DNA resulting in the separation of viral control of transcription from the E6/E7 ORF and putting the viral genes in continuation with cellular genes under the control of cellular transcription. This was further supported by the identification of viral-host fused mRNAs in a cervical carcinoma cell line QG-U containing integrated HPV-16 genome at a single site (Shirasawa et al., 1989).

Clones of HPV-16, integrated into primary cervical cancers, were isolated by Wagatsuma et al. (1990). These clones contained monomeric or dimeric HPV-16 DNA with cellular flanking sequences at both the ends. These flanking sequences from different clones did not show significant homology thus indicating the random nature of integration. However, the cellular flanking sequences of HPV-16 DNAs in the same specimen shared homology due to the monoclonality of the tumour.

Integration basically leads to dysregulation of viral transcription which may be an early step in neoplastic progression as was shown by Durst et al. (1992). E2 gene product probably acted as a repressor of the promoters of E6 and E7 genes (Romanczuk et al., 1990). In addition viral genome was liable to the influence of host specific cis-acting elements (von Knebel-Doeberitz et al., 1991) resulting
in unscheduled increase in E6-E7 gene expression in basal cells. It was proposed that fusion transcripts with altered RNA secondary structure could have altered stability leading to increased half life which increased or sustained the E6 and E7 protein activity (Schneider-Maunoury et al., 1987).

The gene product of HPV-16 E7 ORF was found to be a protein of approximately 11 kDa (Smotkin and Wettstein, 1987). It had the p105 retinoblastoma (pRB) binding domain (Dyson et al., 1989), the casein kinase II phosphorylation sites (Barbosa et al., 1990) and two zinc binding Cys-X-X-Cys motifs in the carboxyl half (Barbosa et al., 1989). pRB is a negative regulator of cell division. The binding of unphosphorylated pRB by E7 protein inhibited the interaction of the former with its natural targets. The zinc fingers were essential for the trans-activation activity of E7 and for the stability of its gene product (Phelps et al., 1988). Mutation leading to alteration in pRB binding property of E7 did not abrogate the ability of HPV-16 to immortalize the cell while mutation in one of the cysteines of Cys-X-X-Cys motifs completely eliminated the property to immortalize cells. It was suggested that this motif may be required to bring E7 in active form (Jewers et al., 1992). E6 protein of HPV formed complexes with p53 which is a growth suppressing protein found in the nucleus and promoted its degradation (Scheffner et al., 1990). The low risk HPV types 6/11 had less affinity for these cellular proteins (Dyson et al., 1989; Werness et al., 1991).
Immortalization of human cells by HPV16-E6/E7 was attributed, in part, to bypassing M1 stage of cell cycle by the removal of pRB and p53 gene products (Shay et al., 1991). Two cervical carcinoma cell lines - C33 and HT3, negative of HPV infection - had mutated p53 and pRB tumour suppressor genes which also supported the role of these proteins in the carcinogenesis by HPV (Yee et al., 1985).

The gene products of HPV may code for trans-acting factors that lead directly or indirectly to malignant phenotype. Alternatively, or additionally, papilloma virus sequences may act as insertional cis-acting promoter/enhancer mutagens that could activate nearby cellular proto-oncogenes. The possibility of integration of HPV into specific chromosomal domains was demonstrated by Durst et al. (1987) by cloning cellular flanking sequences and analyzing the location of the integration sites on the human gene map. The integration of HPV was found near the cellular proto-oncogenes C-src-1 and C-raf-1 on chromosome regions 20p ter-20q13 and 3p25-3q ter respectively in cervical tumour. In HeLa and C4-1 cell lines, HPV-18 integrated in chromosome 8, 5' of the c-myc gene and showed elevated levels of c-myc mRNA, as compared to other cervical carcinoma cell lines. Thus the integration of HPV-18 led to cis-activation of nearby proto-oncogenes. In another report the amplification of the cellular flanking sequences following integration of HPV-16 was also documented (Wagatsuma et al., 1990).

The integration of HPV was also observed near the
fragile sites (Popescu et al., 1987; Cannizzaro et al., 1988). It was proposed that for integration, HPV DNA required a replicating chromosome which was provided by the DNA at the fragile sites, often a late replicating region in the host DNA (Laird et al., 1987). A discrete loss of chromatin at the integration site of HPV-18 on chromosome 12(q11-13) in SW756 cell line was reported by Popescu et al. (1987). Deletion without re-duplication was probably responsible for the genomic alteration induced by the virus or for the loss of tumour suppressor genes.

In cervical cancer, harbouring HPV genome, the amplification and rearrangements of cellular oncogenes c-myc and/or c-Ha-ras were reported by Riou et al. (1985). The overexpression of c-myc was particularly seen in tumours at Stages III and IV (Riou, 1988). Goustin et al. (1986) suggested that amplification of genes coding for epidermal growth factor and its receptor could also occur resulting in increased growth of the cells. The mechanism of transformation is well established in retroviruses where the insertion of a foreign element near or adjacent to cellular genes controlling growth would imbalance their expression leading to uncontrolled division.

1.8 HPV RELATED CHROMOSOMAL INSTABILITY

The cytogenetic analysis of HPV-immortalized cell lines revealed that all were aneuploid. A small metacentric marker chromosome i(5p) was observed in FEA and FEH18L cell
HPV-16 immortalized keratinocytes showed cytogenetic abnormalities like translocations, deletions, achromatic lesions, homogeneously staining regions (HSRs) and double minutes (DMs) which were linked with the presence of HPV-DNA in these abnormal chromosomes. These immortalized cells were non-tumourigenic in nude mice. However the formation of HSRs and DMs was indicative of cellular gene amplification that could occur in a non-neoplastic cell as an early event in cell transformation.

Chromosomal fragility of lymphocytes of women with cervical lesions produced by HPV was studied by Paz-y-Mino et al. (1992). The spontaneous fragility in patients was not high as compared to healthy women. A significant increase in chromosome fragility was, however, seen on aphidicolin treatment of patients as compared to the control group. This increase in HPV infected patients was thought to be due to integration of HPV into host DNA making the surrounding areas of genome unstable or due to chromosomal hypersensitivity to aphidicolin linked to HPV infection. A marked increase in the chromosomal fragility was found in
patients infected with HPV-16/18 as compared to HPV-6/11 which was supported by the more aggressive nature of HPV 16/18 group. Increased chromosomal fragility of peripheral lymphocytes that might be associated with the presence of virus was also proposed by Abubaker et al. (1988) and Chieco-Bianchi et al. (1988). The lack of specific chromosomal changes exclusive to carcinoma of cervix reflected the fundamental difference between the carcinogenesis of cervix seen in adults as compared to leukemias and non-epithelial solid tumours that tend to occur at an early age and show specific chromosomal changes (Atkin, 1989).

In a majority of cervical carcinomas, HPV-16 and 18 were found in an integrated state. However, the presence of free viral DNA in some of these tumours (Fuchs et al., 1989; Matsukura et al., 1989; Cullen et al., 1991; Das et al., 1992) indicated that though integration of HPV was an important event in carcinogenesis but was not mandatory. It was likely that intragenomic rearrangements or mutations in viral episomes resulted in functional alterations equivalent to integrated state (Cullen et al., 1991; Nasseri et al., 1991). Snijders et al. (1992) observed E6/E7 transcripts in all biopsies of HPV-harbouring tonsillar carcinomas independent of the physical status of the virus. In addition the presence of integrated viral sequences in histologically normal cervical epithelium and low grade neoplasia was also seen (Millan et al., 1986; Schneider-Maunvory et al., 1987).
Thus the ultimate biological effect of HPV infection was dependent on the interaction of genetic, cellular, hormonal and immunological factors (Crook et al., 1988). According to Zur Hausen (1991) viral infection and its gene expression were important but in no way sufficient for cancer induction. The genomic instability due to HPV infection with high risk types and apparently the accumulation of mutational events formed the endogenous modification which, aided by exogenous mutagens (or co-factors) might lead to malignant changes and tumour progression.

An in vitro model for human epithelial cell carcino-genesis was proposed by Durst et al. (1992). Immortalization of cells in response to HPV-16 transfection was considered as an early event followed by activation of K-ras oncogene resulting in malignant conversion of the cells. In this multistage process, hydrocortisone acted as an enhancer by stimulating the gene expression of HPV through URR of the virus which only in the presence of activated ras oncogenes showed malignant transformation. An eightfolds increase in E6 and E7 transcripts of HPV-16 was observed in response to oestrogen in SiHa cell line. Seven regions of similarity to oestrogen responsive element with consensus sequence (GGTCANNNTGACC) were detected on the genome of HPV-16. However, progesterone or dexamethasone did not show any effect on the expression of HPV-16 genes (Mitrani-Rosenbaum et al., 1989).
A high incidence of cervical carcinoma was also related to immunosuppression in Hodgkin's disease, HIV infection and pregnancy which must be considered as a temporary immunosuppressive stage. Twenty eight per cent of pregnant women as compared to twelve per cent of non-pregnant ones showed HPV infection which could be due to higher replication rate of HPV-16 during pregnancy (Schneider et al., 1986). A 14 folds increase in the incidence of CIN was reported in renal homograft recipients by Porreco et al. (1975) which might be associated with immunosuppression in them as compared to the age matched healthy controls. A significant reduction in the ratio of helper-inducer to suppressor-cytotoxic 'T' lymphocytes in young women with latent HPV infection as compared to normal uninfected controls also suggested the alteration in cell-mediated immunity associated with the development of HPV induced lesions.

The incidence of cervical cancer was also studied in relation to the smoking behaviour. The involvement of smoking as a risk factor is probably due to its well recognized immunosuppressive effect (Brinton, 1990). A high content of nicotine and cotinine (smoke constituents) was found in the serum and a high level of cotinine concentrated in the cervical mucosa of smokers (Schiffman et al., 1987). The tobacco metabolites might exert their effect by their conversion into carcinogens such as nitrosamines. The low levels of plasma β-carotenes in smokers also correlated with
the fact that nutritional deficiencies of vitamin-C and carotenoids predispose to high risk of cervical cancer (Brock et al., 1988).

It was observed that women with recent and long term (> 4 year) use of oral contraceptives were at 2.3 and 2.9 folds increased risk of developing cervical cancer, respectively (Hildesheim et al., 1990). It could be linked to the increased growth of tumour cells due to the stimulatory effect of hormones on the HPV genes. However, Negrini et al. (1990) failed to find the synergistic effect of oral contraceptive with HPV infection to increase the risk of cervical neoplasia. Young age at the time of first coitus, multiple partners, multiparity, low socio-economic background, multiplicity of infections, immunosuppression, smoking, use of oral contraceptive were some of the co-factors evident from various epidemiological studies that could favour a neoplastic role of HPV infection (Koss, 1987; Acs et al., 1989; Hildesheim et al., 1990).

1.9 VACCINES AND GENE THERAPY

Though an effective vaccine against BPV-2 has been produced, the feasibility of Human papilloma virus vaccine is at an early stage. The L1 and L2 proteins of BPV-2 were produced in E. coli as β-galactosidase fusion proteins (Jarrett et al., 1991), which could be effectively used as prophylactic and therapeutic vaccines, respectively. Prevention was associated with the production of
neutralizing antibodies suggesting the presence of B cell epitopes in L1 and the lesion rejection associated with L2 was mainly due to massive infiltration of lymphocytes at the site of tumour showing that L2 had epitopes specific for 'T' cells.

Since the selective inhibition of gene expression was often achieved by using complementary antisense messages (Inouye, 1988) and was elucidated in several cell lines infected with HPV-DNA (von Knebel-Doeberitz et al., 1988; Steele et al., 1992) it is possible to use antisense RNA technology to develop gene therapy of HPV-expressing human cancers.