Biotechnology for Development
Edited by M.R. Das, State Committee
on Science & Technology, Govt of Kerala, 1996

VIRAL ONCOGENESIS AND HUMAN ORAL CANCER

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Oral cancer is one of the ten most common cancers in the world. In countries like India, Pakistan, Bangladesh and Sri Lanka it is the most common cancer, which accounts for about 30-40% of all malignancies. In Kerala, oral cancer is the most common cancer among men and the second most prevalent cancer among women (Report, 1993). Epidemiological studies have attributed squamous cell carcinoma of the oral cavity to abuse of tobacco and alcohol but a multifactorial aetiology for this disease is more probable.

Herpes viruses have been associated with neoplastic diseases in several species. Epstein-Barr virus (EBV) is widely known for its association with two human malignancies; Nasopharyngeal Carcinoma in Chinese population and Burkitt's Lymophoma in Africa. The association of EBV with Nasopharngeal Carcinoma has been found to be virtually 100% (Klein, 1979). The carcinoma cells carry multiple copies of EBV DNA and express EBV nuclear antigen (Klein and Klein 1984; Anderson et al, 1979). Sero-epidemiological studies have demonstrated consistently elevated levels of anti-EBV antibodies in Burkitt's Lymphoma (Yadav et al, 1984). Burkitt's lymphoma cells were found to carry one of the three specific chromosome translocations: all of them involving the distal fragment of chromosome 8 which is transposed to the gene carrying the information for IgG heavy chain on chromosome No.14 (Zech et al, 1976). In the remaining 10% cases, the same fragment of chromosome 8 which becomes transposed to the kappa locus carrying chromosome No.2 or the lambda locus carrying chromosome No. 22 (Bernheim et al, 1981). This translocation leads to the activation of the oncogene, c-myc, which is the cellular homologue of the transforming gene of Avian Leukemia Virus, and is the final step in the malignant transformation of the EBV infected cell (Klein and Klein, 1984).

Human Papilloma Virus:

Human Papilloma viruses (HPV) belong to the Papova Virus Group. They are DNA viruses and are about 55 nm in diameter. They are generally epitheliotropic, and are generally producing benign epithelial proliferations such as warts, condylomas and papillomas.

Compelling evidence, obtained through investigations during the past decade, suggests the association of specific human papilloma viruses (HPV) with premalignant

and malignant lesions of the anogenital tract (zur Hausen et al, 1987; Howlwy, 1991). DNA of specific HPV types is detectable in about 90% of cervical carcinomas, the most prevalent being HPV-16 and HPV-18. A recent study in India has demonstrated HPV-16 DNA in about 64% of the cervical carcinomas and HPV-18 DNA in 3% (Das et al 1992). In vitro studies have clearly demonstrated the oncogenic potential of specific HPV types, especially HPV-16 and HPV-18 (Matlashawski et al, 1988; Pirisi et al, 1987; Woodworth et al, 1989). The studies using molecular biological techniques, such as DNA hybridization and molecular cloning, became all the more important because a suitable culture system or animal host system has not been developed to grow the HPVs. During the past decade more than 66 genotypes of HPV have been isolated and characterized, from pathological lesions at different sites, including skin, genital mucosa and laryngeal mucosa (Yeudall, 1992).

The first evidence for a consistent association of HPV with a cancer came from the studies by Orth et al (1980) on skin cancers in epidermodysplasia verruciformis (EV) patients. EV is a rare, hereditary skin disease of life-long persistence, characterized by defective cell-mediated immunity and disseminated skin warts (Lutzner, 1978). Some of the warts, mainly at the sites exposed to UV radiation, in about 30% of EV patients, develop into squamous cell carcinoma of skin. Orth and his co-workers (1980) demonstrated the presence of HPV-5 DNA in these cancers. Subsequently, other workers (Pfister et al, 1983; Orth, 1987) also reported HPV DNA in carcinomas and metastatic lesions from EV patients.

De Villers et al (1981) isolated the first genital HPV type, HPV-6, and characterized it by cloning its DNA. This led to the rapid isolation and characterization of several other HPV types. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52 and 56 are found in cases of moderate and severe cervical dysplasia, and in invasive cervical carcinomas, and therefore belong to the 'high risk' group (Howley, 1991). HPV-16 is the most prevalent type that is found in more than 50% of the above lesions. A recent study in India (Das et al, 1992) has demonstrated HPV-16 DNA in about 64% of the cervical cancers and HPV-18 DNA in 3%. HPV-16 or 18 DNA have been demonstrated also in several established cervical cancer cell lines (Howley, 1991).

Studies on cervical cancer tissues and on cervical cancer cell lines have found that HPV DNA usually occurs in an integrated state (zur Hausen, 1987), either as single copy or as multiple randomly repeated structures, although there are some cases where the DNA is in an episomal state. The viral genome can be found located at different sites in the host genome in different cancers and cells lines and therefore, integration is a random event as far as the site of integration is concerned. In some cell lines the integration has been found in the vicinity of known oncogenes (Durst et al, 1987). Linearisation of HPV DNA is necessary for integration and disruption almost invariably occurs in the E1/E2 region of the viral genome (Baker et al. 1987, Schwarz, 1985), which disrupts the E2 coding sequence and removes E2 mediated repression (Bernard et al, 1989; Romanizuk et al, 1990) leading to the higher levels of transcription of the E6 and E7 open reading frames (ORF).

The oncogenic potential of some HPV types has become evident in several studies. HPV-16 and 18, and a few other genital HPV types have been shown to immortalise human foreskin and cervical keratinocytes in tissue culture (Durst et al, 1987; Kaur and Mc Dougall, 1988). These cells express viral proteins and quickly become aneuploid, but are not tumorigenic in nude mice. Matlashewaski and his co-workers (1987, 1988) observed that co-transfection or HPV-16 DNA together with an activated ras oncogene is sufficient for transformation of primary human fibroblasts and primary rodent epithelium, but none of them alone can cause transformation, indicating a synergistic effect in HPV-mediated carcinogenesis. Human cervical epithelial cells previously immortalized by HPV-16, but non-tumorigenic, became fully malignant when a viral Harvey ras oncogene was added to the system. Antisense experiments targeted to E6 and E7 revealed that the expression of these genes is required for the proliferation of the HPV-18 positive cervical carcinoma cell line, C4-1 (Von Knebel-Doelberitz et al, 1988). Studies using individual HPV open reading frames (ORF) cloned into expression vectors have shown that the E6-E7 of the viral genome is the region involved in transformation. Crook et al (1989) found that the continued expression of HPV-16 E7 protein is necessary to maintain the transformed phenotype of baby rat kidney (BRK) cells co-transformed with HPV-16 and activated ras.

The HPV-16 E7 gene product is a small polypeptide of 98 aminoacids and migrates with a slow mobility in polyacrylamide gels, and has been shown to be a zinc-binding phosphoprotein. It is phosphorylated on serines by Casein kinase II (Barbosa et al,1989; 1990; Firzlaff et al, 1989). E7 has been found to be expressed in benign cervical dysplasia, cervical carcinomas and in cervical carcinoma cell lines (Smotkin and Welstein, 1986). Edmonds and Vousden (1989), by point mutational analysis, confirmed the role of E7 protein in HPV-mediated transformation, and identified 3 regions in the protein that are important for transformation. Two of the three regions in HPV-16 E7 protein have marked homology to both SV40- large T antigen and adenovirus EIA (Phleps et al, 1988; Dyson et al, 1989). Like the EIA and large T (Whyte et al, 1988; de Caprio et al, 1988), E7 is able to bind the retinoblastoma gene, pRb, product (Dyson et al, 1989). The pRb protein binding regions of EIA and TAg are known to be responsible for transformation. Interestingly, these regions are the ones which show homology with HPV E7 protein. Thus it is reasonable to infer that sequestration of cellular pRb is a possible mechanism by which HPV derails normal growth control mechanisms and contribute to carcinogenesis, and in this E7 plays the major role. Further the E7 protein of HPV-6, usually found in benign lesions, can bind only smaller amount of pRb protein (Barbosa et al, 1990). A recent study, employing chimeric E7 genes, mapped the differences in the biological activity of E7 proteins from 'high' and 'low risk' HPVs to amino-terminal sequences, showing that this region is responsible for the affinity to pRb protein, cellular transformation and abrogation of repression of the c-myc promoter by TGF-Beta (Munger et al, 1991).

The HPV E6 protein has also been known to play an important role in cell transformation. The transforming properties of E6 protein was first revealed in studies using human squamous epithelial cells (Halwy-Nelson et al, 1989). HPV-16 and HPV-18 require both E6 and E7 for efficient immortalisation of primary human keratinocytes

(Hudson et al, 1990). The efficient transformation by the co-operation of E6 and E7 has been found as a property of 'high risk' HPV but not of 'low risk' HPV (Barbosa et al, 1991). The E6 protein is able to complex with p53 tumour suppressor gene product (Wernes et al, 1990). In SV-40-transformed and adenovirus 5-transformed cells, p53 level is increased due to the increased half-life of p53 (Oren et al, 1981; Reich et al, 1983). SV40 large T and E18 increase steady-state levels of p53. The situation is different with HPV in that the E6 protein stimulates the degradation of p53 via the ubiquitin dependent protease system (Scheffner et al, 1990), thereby providing an alternate loss-of-function mechanism by which the natural cell growth control is removed. A cellular factor, E6-AP, known to be present in primary human keratinocytes and cell lines and capable of forming a stable complex with E6 in the absence of p53, is shown to mediate the association of E6 and p53 (Huibregtse et al, 1991). It has also shown that the E6 proteins of both 'low' and 'high-risk' HPVs can bind to p53 through a conserved C-terminal region, but the N-terminal sequences necessary for directing the degradation of p53 is conserved only in 'high-risk' HPV E6 (Crook et al, 1991). These observations lead to the conclusion that inactivation of tumour-suppressor genes, by point mutation, gene loss, structural rearrangement or sequestration by viral proteins is important in development of anogenital carcinoma.

Human Papilloma Virus in Oral Lesions:

Several studies have reported papilloma virus structural antigens in oral papillomas and other related benign lesions of the oral cavity (Jenson et al, 1982; Syrjanen et al, 1984; Jin and Toto, 1984). Several HPV types, including HPV-2, 4, 6, 11, 13, 16, 32, 40 and 57, have been demonstrated in oral lesions by DNA hybridization (Alder-Storthz et al 1986; Beaudenon et al, 1987; Garlick et al, 1989; de Villiers et al, 1989).

De Villiers et al (1985) demonstrated HPV, DNA in 3 of 7 tongue cancers using Southern blot analysis. Two tumours were positive for HPV-16 DNA, Viral DNA in all 3 tumours showed a high copy number and were mostly in episomal state. Adler Storthz et al (1986), in an in situ hybridization study demonstrated HPV-2 DNA in 2 cases of verrucous carcinoma. Milde and Loning (1986) by in situ hybridisation demonstrated the 'high-risk' HPV-16 in 4/4 oral papillomas and 4/7 oral carcinomas. Syrjanen et al (1988) demonstrated HPV-16 DNA in 3/51 oral carcinomas and 2/22 dysplasias, and HPV-18 in 3/51 carcinomas and 1/22 dysplasia, by in situ hybridization. It is interesting to note that in this study 'high risk' genital HPV (HPV-16 and 18) were found in carcinomas, but HPV-16 and HPV-11 (low risk) were found only in dysplasias.

Yeudall and Campo (1991) demonstrated the presence of HPV-18, and variant HPV-16 in oral squamous cell carcinomas, by Southern blot hybridization and polymerase chain reaction. PCR allowed detection of viral DNA in carcinomas which were found negative in Southern blot hybridization. The variant HPV-16 was found to lack the 1.55 kb Pst I fragment and showed an altered Hpa II restriction pattern, where no 2.6 kb prototype fragment was visible, but an additional 0.9 kb fragment was detected. This study confirmed the previous report by Maitland et al (1987) on the

presence of variant HPV-16, which fails to demonstrate the 1.55 kb prototype Pst I fragment, in oral carcinomas. Maitland et al (1989), by using PCR, demonstrated HPV-16 DNA in about 50% of oral carcinoma biopsies and in oral cancer cultures. Oligonucleotide sequences from the E6 and L1-L2 open reading frames of HPV-16 genome were used as primers. The oral cancer biopsies and tissue cultures were found to contain DNA sequences which were identical to the HPV-16 E6 region, but only rarely contained sequences closely related to the L1-L2 region. Previous studies have shown that HPV-16 E6 gene sequences are consistently retained, whereas the late genes, L1/L2 are usually deleted in cervical carcinomas when the viral genome is integrated into the host cell genome. Yeudall and Campo (1991) in a recent study found that in the 2 oral cancer biopsies in which E6 and E7 genes of HPV-16 could be detected, but not the 1.55 kb Pst I fragment (L1-L2), some rearrangements in the L1-L2 region had occurred, possibly with a repetitive human DNA sequence. Yeudall (1992) suggested that the presence of repetitive host genome sequences in the viral genome might help integration of the viral genome with host DNA.

The exact role of HPV in the aetiology of oral squamous cell carcinoma is not well understood. Maitland et al (1989) opined that HPV cannot be considered as the single aetiological agent responsible for human oral cancer. Any large-scale studies have not so far been undertaken to establish the risk of HPV infection as far as oral premalignant and malignant lesions are concerned. To establish the role of HPV, it is essential to have information on the incidence of HPV in patients and control subjects, and also to have epidemiological data.

MATERIALS AND METHODS

Detection of papilloma virus antigens

Indirect immunofluorescence Staining: The negative control reagent used was rabbit pre-immune serum. The primary antibody was rabbit anti-bovine papilloma virus (BPV-1, common genus specific internal antigen), and the second antibody was fluorescein-isothiocyanate conjugated with goat anti-rabbit immunoglobin (Dakopats). The primary antibody was used at a dilution of 1:500 to remove non specific staining. The acetone fixed frozen sections in duplicate were incubated with the primary antibody at 37 °C for 30 minutes, rinsed in phosphate buffered saline pH 7.2 (0.1 M), drained and incubated again with FITC - conjugated anti-rabbit immunoglobulin at 1:20 dilution, at 37C for 30m minutes in a humid chamber. The slides were rinsed and observed for fluorescence.

Peroxidase - Anti-Peroxdase Technique: The Dako Pap Kit was used (Dakopatts). In every batch of test the Dako control known positive section and one section from each patient for negative serum control were included. The slides were pre-incubated with 3 percent hydrogen peroxide for 5 minutes. The sections were then covered with normal swine serum, incubated for 20 minutes and excess serum tapped off, followed by incubation with primary antibody and negative control reagent in parallel for each

sample in duplicate for 20 minutes. The slides were rinsed and sections incubated with link antibody, followed by PAP complex for 20 minutes. Unbound PAP reagent was removed by washing. The freshly prepared enzyme substrate mixture containing hydrogen peroxide and amino-thiocarbazole was placed on the sections and incubated for 40 minutes. The slides were rinsed with distilled water. Counter staining was performed by Mayer's haematoxylin.

Detection of HPV DNA sequences

Tissue DNA was extracted by the standard phenol-chloroform-isoamyl alcohol method.

Probes for HPV studies:

HPV-16 and 18 DNA, (8 kb) cloned in pBR 322, were used for the preparation of probes in this study. The vectors containing the HPV inserts were digested with appropriate restriction enzymes and electrophoresed in a 0.7% low melting point agarose gel. The HPV DNA bands were cut out and DNA extracted from the gel by electroelution. The vector-free HPV inserts were labelled with alpha ^{32}P-dATP by random priming, using 'Megaprime DNA labelling systems' (Amersham, England). The specific activity of the probes used was greater than 1-2 x 10^8 cpm/ug DNA.

Dot blotting

8 to 10 ug of DNA from oral cancer and normal tissue samples were denatured at 65°C for 30 minutes, snapchilled on ice and blotted on to a nylon membrane (Hybond-N; Amersham, U.K.) and baked at 80°C for 2 hours. Dots of positive and negative control DNA were always incorporated.

Southern blotting:

About 10 ug of DNA from each sample was digested with Bam HI or Pst I, and electrophoresed in 1% agarose gel in TAE buffer (2 M Tris, 0.05 M EDTA, adjusted to pH 8 with glacial acetic acid) containing ethidium bromide (1 ul of 10 mg/ml solution/ 60 ml agarose solution). Electrophoresis was conducted for 16-20 hr, and the gel was photographed under UV. The gel was then treated with denaturing buffer (1.5 M NaCl + 0.5 M NaOH) and neutralised Tris with (0.5 M Tris-HCl + 1.5 M NaCl, pH 7.5). The DNA bands were transferred overnight on to Hybond-N nylon membrane by capillary blotting. The membrane was washed with 6 x SSC (0.09 M trisodium cirtrate, 0.9 M NaCl; pH 7.0), airdried and baked at 80°C for 2 hrs. 10 pg of viral DNA, approximately equivalent to one viral genome per cell, that was incorporated into the served as positive control.

Hybridization for HPV DNA: The blots were prehybridised in prehybridization solution (50% formamide, 5 x SSC, 0.02% Denhardt's solution, 50 ug/ml DNA,

50 mm trisodium phosphate and 1% SDS) for 2 hr at 42C. Hybridization was carried out for 16 to 20 hr under stringent conditions, at 42C in the hybridisation solution containing 50% formamide, 5 x SSC, 0.02% Denhardt's solution, 50 ug/ml DNA, 1% Dextran sulphate, 50 mM trisodium phosphate and the radiolabelled probe. After hybridisation, the membrane were subjected to stringent washes and then autoradiographed.

RESULTS AND DISCUSSION

HUMAN PAPILLOMA VIRUS ANTIGENS:

The papilloma virus antigens could be detected by immunofluorescence and peroxidase techniques (see Table 1). By both techniques, no papilloma virus antigen could be seen in the normal cervical tissues. In the chronic cervicitis, 5% expressed the virus antigen which could probably be non-specific due to heterophile antibody staining or due to the antibodies on the mononuclear inflammatory cells infiltrated into the cervical lesion. Among the cervical cancer, 38 % and 44% of the samples expressed papilloma antigens in the superficial layers of the cervical epithelium by the PAP and immunofluorescent staining respectively. While in severe cervical dysplasia it was 8% and 12% respectively. The lower layers were negative. The antigen distribution was focal and uniform in some while in others the antigen positive nuclei occurred in diffuse patches.

Table 1. DETECTION OF PAPILLOMA VIRUS ANTIGEN IN ORAL AND CERVICAL LESIONS BY IMMUNOFLUORESCENCE (IF) OR PEROXIDASE ANTIPEROXIDASE (PAP) TECHNIQUES

Test groups	Technique	No.tested	No.+ve	% +ve
Normal cervical tissue	IF	20	0	0
Normal cervical tissue	PAP	20	0	0
Chronic cervicitis	IF	29	2	5%
Chronic cervicitis	PAP	29	1	3%
Cervical dysplasia	IF	24	3	12%
Cervical dysplasia	PAP	24	2	8%
Cervical carcinoma	IF	64	28	44%
Cervical carcinoma	PAP	64	24	38%
Normal oral tissue	PAP	20	0	0
Oral cancer	PAP	54	28	52%

In yet another series of experiments, 54 oral cancer tissue samples were studied for papilloma virus structural antigens by PAP technique and 28 samples (52%) were positive (see Table 1). The nuclear staining was mainly confined to superficial layers of the squamous epithelium. The antigen distribution was usually in a focal and uniform

pattern, but in a few cases the antigen positive nuclei were observed in diffuse patches. None of the 20 normal samples studied were positive for the viral antigens.

HPV DNA DETECTION

52 DNA samples from oral squamous carcinoma were studied for HPV-16 and HPV-18 DNA sequences by dot blot hybridisation. 31 (60%) samples were positive for HPV-16 and 4 (8%) for HPV-18 (See Table 2). Two samples were positive for both HPV-16 and HPV-18. None of the 18 DNA samples from normal oral mucosa, studied for both HPV-16 and 18, was found to be positive for the viral DNA.

Southern blot analysis was employed for 34 DNA samples from oral squamous carcinoma cases. HPV-16 DNA was noted in 18 (53%) samples and HPV-18 DNA in 3 (9%) samples. One DNA sample harboured both HPV-16 and HPV-18 DNA sequences. All the 15 samples from normal oral mucosa were found to contain no viral DNA (see Table 2).

Table 2. DNA HYBRIDIZATION STUDIES WITH HUMAN PAPILLOMA VIRUS PROBES

Technique	Probe +ve	Normal controls specimens	Oral Cancer +ve	Cervical cancer specimens +ve
Dot blot	HPV-16	0/18	31/52 (60%)	13/24 (54%)
Dot blot	HPV-18	0/18	4/52 (8%)	2/24 (8%)
Dot blot	HPV-16+18	0/18	2/52 (4%)	0/24
Southern blot	HPV-16	0/15	18/34 (53%)	-
Southern blot	HPV-18	0/15	3/34 (9%)	-

The 8kb authentic Bam HI band lighted up in the autoradiograms of all the HPV-16 DNA positive samples. Characteristic Pst I restriction fragments (2.8, 1.9, 1.6, 1 and 0.5 kb) were observed in the HPV-16 positive cases studied. However, altered intensity or size and addition or absence of the restriction fragments, indicating difference in their copy number and minor possible viral genome rearrangements could be noted in a few samples. Majority of the positive samples were found to contain a rather low level of the viral genomes. 4 samples contained very high copy number of HPV-16 DNA.

An association between HPV types and anogenital carcinomas is more or less well-established (zur Hausen and Schnieder, 1987; zur Hausen, 1988). HPV-16 and HPV-18 are known to be the most common types associated with cervical cancer, with prevalence of 50% and 20% respectively (Matlashewski et al, 1987; Woodworth et al, 1989). In a recent study conducted in India at New Delhi, by Das et al (1992), HPV-16 DNA was detected in 59.37% of the cervical cancer DNA samples by Southern blot

hybridisation. HPV-18 DNA was found in 3% of the cancer samples. In the present study, a slightly lower prevalence of HPV-16 was observed. This may be attributed to regional difference. Our results suggest a definite association between HPV-16 and cervical cancer in women from Kerala. Previous studies on cervical cancer also have reported 10-50% of the carcinoma cases to be negative for HPV DNA (Fuchs et al, 1988; Meanwell, 1988). This may be due to the presence of some unknown HPV types or to insensitivity of the detection techniques used.

There is a very high incidence of oral cancer in Kerala (Padmanabhan and Vasudevan, 1982). Our earlier studies have shown a close associationship of HSV-1 with oral cancer (Kumari et al, 1982; Kumari et al , 1985; Kumari et al, 1987). Based on a large number of HSV-1 with oral cancer by seroepidemiological and immunohistochemical studies, where 60-70% positively was observed.

Oral carcinogenesis is probably the result of a multistep process with a multifactorial etiology. Epidemiologic, clinical and laboratory studies confirm an etiologic link between tobacco chewing and oral cancer (Jussawalla and Deshpande, 1971). De Villiers et al (1985) reported HPV-16 and HPV-2 DNA in tongue carcinoma. Two in situ DNA hybridisation studies (Milde and Loning, 1986; Syrjanen et al, 1988) have noted HPV-16 and 18 DNA in oral squamous cell carcinomas. Studies made by Chang et al (1989) detected HPV-16 DNA in 76% of the oral carcinomas in a group of Taiwanese patients. HPV-18, HPV-4 and a variant of HPV-16 were detected in oral squamous cell carcinoma by Yeudall and Campo (1991). Maitland et al (1987) demonstrated a variant of HPV-16 in 46% of the oral cancer biopsies. Using polymerase chain reaction Maitland et al (1989) later demonstrated HPV-16 DNA sequences in oral cancer biopsies and in the cell lines derived from them.

In the present study is in general agreement with those of previous studies that have suggested a more than accidental association of HPVs with oral squamous carcinoma. To our knowledge, this is only report from India on the presence of HPV DNA in oral cancer.

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