Oral Oncol, Eur J Cancer, Vol. 28B, No. 1, pp. 61–66, 1992
Printed In Great Britain

0964–1955/92 $5.00 + 0.00

Human Papillomaviruses and Oral Neoplasia

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INTRODUCTION

WHILE SQUAMOUS cell carcinoma of the oral cavity has traditionally been attributed to abuse of tobacco and alcohol, recent work suggests that viral factors may contribute to the aetiology of these malignant neoplasms. During the past decade much tumour virus research has been focused on members of the human papillomavirus group, resulting in a greater understanding of their biology, interactions with cellular factors and their likely role in the pathogenesis of a wide range of epithelial malignancies, including oral cancer.

PAPILLOMAVIRUSES IN NON-ORAL MALIGNANCIES

More than 66 types of human papillomavirus (HPV) have been isolated from mainly benign pathological lesions at various sites, including skin, genital mucosa and laryngeal mucosa [1]. However the first evidence of the oncogenic potential of HPVs came from clinical cases of epidermodysplasia verruciformis (EV), a rare inherited skin disease which persists throughout the life of the patient, characterised by defects in cell-mediated immunity and disseminated skin warts containing specific HPV types which usually appear in childhood. Around 30% of EV sufferers develop squamous cell carcinomas of the skin, mainly at sites exposed to ultraviolet radiation, after a variable timespan [2] which are locally aggressive and eventually fatal if left untreated. Various workers have demonstrated the presence of viral DNA in carcinomas [3, 4] and metastatic lesions [5] from EV patients. However, it is as yet unclear whether or not viral sequences are required for maintenance of the transformed state. Development of HPV-containing carcinomas at sites exposed to actinic radiation probably reflects the interaction of HPVs and co-factors. Other categories of immunosuppressed individuals, such as renal transplant recipients (RTRs) or patients with acquired immune deficiency syndrome, are also prone to HPV infections. RTRs are 35 times more likely to develop carcinomas than the general population [6], while HPV-associated anogenital malignancies have been documented in HIV-infected males [7].

Laryngeal papillomas in children and young adults have been associated with HPV-6 [8] and HPV-11 [9, 10]. X-irradiation of laryngeal warts has been shown to result in progression to malignancy in some cases after a variable period of 5–40 years, suggesting synergism between HPV types and X-rays [6]. Adult onset laryngeal warts have a different potential for transformation, with progression to carcinoma occurring in over 20% of cases, a likely cofactor being heavy cigarette smoking [6]. Recent studies have detected HPV-16 [11] and HPV-30 [12] DNA in laryngeal carcinomas.

Epidemiological evidence has long suggested the involvement of a transmissible agent in the aetiology of human genital cancer [13]. DNA of several different HPV types has since been isolated and characterised from anogenital malignancies [14–17], and a high percentage of such lesions harbour viral DNA which may contribute to the malignant phenotype. Two main groups of papillomaviruses are recognised, based on their incidence in benign or malignant genital lesions: those of high (including HPVs 16, 18, 31, 33, 35) or low (including HPV types 6 and 11) oncogenic potential. Viral DNA can be detected in around 90% of genital carcinomas, with HPV-16 (50%) and HPV-18 (20%) being most prevalent, and also in cell lines derived from these lesions [18].

Papillomavirus DNA in genital cancers may be episomal or integrated into the host cell genome either in single copy or in multiple tandemly repeated structures [19, 20]. Integration requires linearisation of HPV DNA, almost invariably occurring in the E2 region of the viral genome [21], which disrupts the E2 coding sequence and removes E2-mediated repression, resulting in higher levels of transcription of the E6 and E7 ORFs.

MECHANISMS OF PAPILLOMAVIRUS-MEDIATED TRANSFORMATION

The continued presence and expression of HPV DNA and expression of specific viral gene products in genital cancers and cell lines derived from these indicates that papillomavirus types may play an important role in the aetiology of such tumours. Functional analysis of viral gene products *in vitro* has enabled this question to be addressed. Co-transfection of HPV-16 DNA together with an activated *ras* oncogene is sufficient for transformation of primary human fibroblasts and primary rodent epithelium, whereas neither alone results in transformation [22, 23], reinforcing the requirement for co-operative effects in HPV-mediated carcinogenesis. Transfection of HPV-16 and HPV-18 DNAs into primary cells results in their immortalisation [24, 25]; human cervical epithelial cells previously immortalised by HPV-16 but lacking tumorigenicity [26] became fully malignant when a viral Harvey *ras* oncogene was added to the system [27].

Further work using individual HPV ORFs cloned into expression vectors has localised the transforming function to the E6-E7 region of the viral genome ([28, 29] Fig. 1). Using a hormone-inducible plasmid, Crook *et al.* [30] demonstrated the continued requirement for expression of HPV-16 E7 protein for maintenance of the transformed phenotype of baby rat kidney (BRK) cells transformed with HPV-16 plus activated *ras*. Overexpression of the viral E2 protein in BRK cells was shown to transactivate the HPV-16 early promoter, increasing the transforming ability of HPV-16 plus *ras* in these cells [31].

HPV-16 is also able to co-operate with v-*fos* to transform primary cells, but is dependent on the continued presence of dexamethasone or progesterone in the culture medium when gene expression is driven from the viral promoter [32], an effect presumably mediated through the glucocorticoid responsive element in the viral long control region [33]. However at later passages these cells lose this requirement for steroid hormones, with a concurrent amplification and overexpression of c-*myc* [34] which appears to confer hormone

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Accepted 26 Mar. 1992.

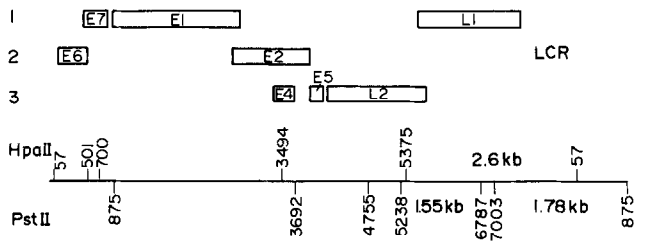

Fig. 1. Linear representation of the circular HPV-16 genome, showing the location of viral open reading frames and the relationship of restriction fragments obtained on digestion with the enzymes *PstI* and *HpaII*. Restriction fragment lengths are indicated in kilobases.

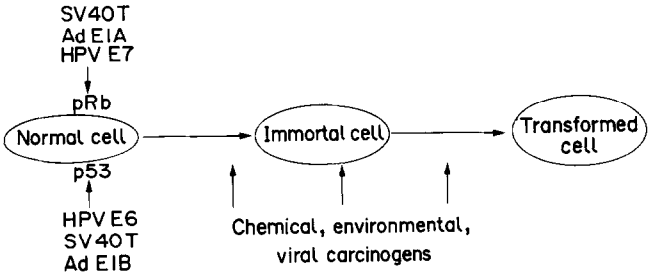

Fig. 2. Schematic representation of how the E6 and E7 papillomavirus oncoproteins may compromise normal cellular growth control by sequestration of cellular p53 and pRb. Other events are probably necessary for progression of cells to malignancy.

independence on HPV-16 transformed cell lines [35]. Interaction between viral sequences and members of the *myc* family may also occur *in vivo*. A recent report [36] has documented the integration of papillomavirus sequences close to structurally altered c-*myc* and N-*myc* genes in genital carcinomas, although it is as yet unclear whether this rearrangement is a consequence of viral integration or whether viral sequences are near enough to *cis*-activate *myc* expression.

Point-mutational analysis of the HPV-16 E7 protein has confirmed its role in HPV-mediated transformation [37]. Mutation of the "zinc-finger" motif [38] near the carboxy terminus of E7 was found to considerably decrease transforming ability, while mutations in the region of homology to adenovirus E1A and SV40 large T completely abolished the E7 transformation function. This has been confirmed in a variety of different cell lines [39].

Whereas complete genomes or viral early regions from those HPV types frequently associated *in vivo* with genital carcinomas (HPV-16, 18 and 33) are competent in co-transformation assays of primary cells, HPV-6 and HPV-11 sequences (mainly found in benign lesions) are not [40]. Transfection of expression plasmids containing the HPV-6 or HPV-11 E7 ORF into BRK cells together with activated *ras* can, however, result in cellular transformation (albeit at a lower efficiency than HPV-16, 18 or 33 DNA), although cells transformed by HPV-6 and HPV-11 E7 are equally as tumorigenic in immunocompetent animals as those transformed by HPV-16 plus *ras* [41]. The co-transforming and transactivating activities of HPV DNA can be separated, as both HPV-11 E7 and HPV-16 E7 plasmids are able to transactivate the adenovirus E2 promoter to similar levels.

Two of the three regions of the HPV-16 E7 protein important for transformation [37] show marked homology to areas of both SV40 large T antigen and adenovirus E1A [42, 43] and, like E1A and large T [44, 45], E7 is capable of binding the product of the retinoblastoma gene, pRb [43]. The regions of E1A and T Ag responsible for binding pRb are critical for transformation and are those which share homology with papillomavirus E7. Functional similarity between these three viral oncogenes has been confirmed by using a HPV-16 E7 complementation approach to rescue cells transformed with a thermolabile SV40 T Ag from growth arrest at non-permissive temperature [46]. Thus sequestration of cellular pRb is one possible mechanism by which HPVs might compromise normal cellular growth control and contribute to development of neoplasia, with the viral E7 protein playing a major role (Fig. 2). Furthermore, the E7 protein of HPV-6, a HPV type found rarely in malignant lesions, is able to bind only much smaller amounts of the Rb protein (3.6 to 6.5-fold less [47]). A recent study using chimeric E7 genes mapped the differences in the biological activity of E7 proteins from high- and low-risk HPVs to the amino-terminal sequences, demonstrating that this region is responsible for the affinity of pRb binding, cellular transformation and abrogation of repression of the c-*myc* promoter by TGF-β [48]. The pRb binding domain also harbours a potential caesin kinase (CK) II site, and it has been shown that HPV-6 E7 is phosphorylated 2–4-fold less efficiently than either HPV-16 or HPV-18 E7 [47]. Mutations in either the pRb binding domain or the CKII site of HPV-16 E7 decreased the ability of the virus to transform NIH3T3 cells [47].

In addition to the actions of E7, the papillomavirus E6 protein also plays an important role in cell transformation. The E6 and E7 ORFs of HPV-16 were shown to co-operate in the immortalisation of primary human keratinocytes [49], whereas neither alone produced this effect. Although HPV-16 E7 was found to be the major factor able to rescue cells transformed with mutant SV40 [46], the E6 is also capable of performing this function. While the E7 protein binds pRb, the E6 protein is able to complex with the p53 tumour suppressor gene product [50], as do SV40 large T and adenovirus E1B ([51, 52] Fig. 2). The E6 oncoproteins of HPV types 16 and 18 have been shown not only to target cellular p53 but, unlike SV40 large T which increases steady-state levels of p53, to stimulate its degradation via the ubiquitin-dependent protease system [53], thus providing an alternative "loss-of-function" mechanism by which the cells may be relieved of normal growth control. Association of E6 and p53 appears to be mediated by a cellular factor, E6-AP, present in primary human keratinocytes and cell lines which can form a stable complex with E6 in the absence of p53 [54]. Further analysis has revealed that, while the E6 proteins of both low- and high-risk HPV types are able to bind p53 through a conserved C-terminal region, N-terminal sequences required for directing the degradation of p53 are conserved only in the E6 proteins of high-risk HPVs [55]. Several reports now indicate that mutant p53 and pRb are present in HPV-negative anogenital malignancies and derivative cell lines, whereas only wild-type sequences exist in papillomavirus-positive tumours [56, 57]. This implies that inactivation of these tumour suppressor genes, either by point mutation, gene loss, structural rearrangement or sequestration by viral oncoproteins, is of primary importance in development of anogenital cancer.

More recent work has implicated the papillomavirus E5 as a transforming gene. Pim *et al.* [58] showed that fibroblasts expressing both HPV-16 E5 and the receptor for epidermal

growth factor (EGF) were much more sensitive to lower concentrations of exogenous growth factor, having a higher colony forming efficiency in soft agar. Leechanachai *et al.* [59] also showed that E5-transfected fibroblasts were transformed to anchorage independence. In addition, higher levels of c-*fos* mRNA were detected in E5-expressing cells in response to stimulation with EGF, PDGF (platelet-derived growth factor) and serum, and showed enhanced tumorigenicity and growth in low serum. These data suggest that the E5 protein acts as an oncogene by enhancing the activity of or signal transduction through the EGF receptor.

Several authors have reported the presence of DNA of high-risk HPV types in clinically and histologically normal cervical epithelium [60, 61]. It would seem that latent infection by papillomaviruses in genital mucosa does occur, and that the majority of HPV infections do not progress to malignancy, a fact in keeping with theories of multistage carcinogenesis. However a major difference between HPV in anogenital malignancies compared with normal tissue is the amount of viral DNA present. While viral nucleic acids are readily detected in carcinomas by Southern blot hybridisation, much of the data citing HPV DNA in healthy tissue has relied on the use of a very sensitive technique, polymerase chain reaction (PCR), for detection of papillomavirus sequences, sometimes without the use of adequate controls [18]. Thus there may be an important dose effect of HPV on genital mucosa, as well as the requirement for other co-factors.

HUMAN PAPILLOMAVIRUSES IN ORAL DISEASE

Oral papillomas and other related benign mucosal proliferations are not an uncommon occurrence. Several reports exist in the literature demonstrating papillomavirus structural antigens in such lesions [62–64]. DNA hybridisation studies have revealed that oral mucosa is subject to infection by a number of types of human papillomavirus including HPV-2, 4, 6, 11, 13, 16, 32 and 57 [65–70]. Another study reported HPV-7 DNA in a series of oral warts from patients seropositive for HIV [71], suggesting that patients with acquired immunodeficiency may be subject to infection by unusual HPV types, perhaps not surprising considering the other opportunistic infections to which this population is susceptible, and the pattern of infection in other immunosuppressed individuals (such as EV patients).

In parallel with cervical cancer, the involvement of an infectious agent has been suggested in the aetiology of oral squamous cell carcinoma [72, 73]. In view of the obvious oncogenic potential of some human papillomaviruses, the close similarity between oral and genital mucosa, and evidence from other systems of malignant progression of virally-induced oral lesions [74], the possibility of a causative role for certain HPV types in oral cancer would not seem to be remote. Initial investigations, purely at the histological level, showed features reminiscent of viral infection in pathological sections of oral verrucous carcinoma [75, 76], with positive staining of some samples using an antibody to a papillomavirus structural antigen.

Further investigations utilising hybridisation technology have enabled the viral types present in such lesions to be established. De Villiers *et al.* [77] found largely episomal HPV sequences in three of seven tongue carcinomas by Southern blot analysis, HPV-16 being present twice while one lesion harboured HPV-2 DNA, a type not previously associated with malignancy, at a high level (around 50 copies per cell). HPV-2 DNA was also detected in 3 of 9 patients with verrucous carcinoma using *in situ* hybridisation on histological material [78]. Other workers [79, 80] have reported the high-risk "genital" HPV types 16 and 18 to be present in oral squamous cell carcinomas, and some parallels seem to exist with HPV in genital lesions as Syrjanen *et al.* [80] found HPV-6 and 11 DNA in oral dysplasias but not in invasive carcinomas. HPV-16 episomes were also demonstrated in a lymph node metastasis from a primary tongue carcinoma at a level of approximately 20–40 copies per cell [81]. A recent study by Chang and co-workers [82] documented a high prevalence of HPV-16 DNA in oral carcinomas in Taiwanese patients, 76% of whom were positive for viral sequences by Southern blot analysis. Only one out of 17 controls of normal gingival mucosa contained HPV DNA; this was unable to be typed by the investigators, but was not HPV-16. The incidence of both smoking (82%) and betel quid chewing (57%) were high in cancer patients, and may reflect both a viral and chemical aetiology of oral carcinoma.

Interestingly, Maitland *et al.* [83] reported finding a variant of HPV-16 in 46% of a series of oral cancer biopsies studied by Southern blot hybridisation. HPV-16 DNA was also detected in seven out of eight biopsy specimens of lichen planus and in five out of 12 samples of normal oral mucosa, approximately the same incidence as in carcinomas. Other ill-defined benign lesions termed non-specific keratosis and reactive keratosis also harboured viral nucleic acids. These results were the first report of HPV DNA in clinically and histologically normal oral epithelium, in line with previous findings regarding papillomaviruses in human cervix. From the data presented, HPV-16 DNA appeared to be episomal in all but one case, a sample of normal oral epithelium; thus the situation may be somewhat different to that of HPV-16 and 18 in cervical tissues, where viral DNA is frequently integrated in carcinomas and may reflect malignant progression.

The variant viral DNA differed from the prototype HPV-16 DNA in its *Pst*I restriction digest pattern, although no variation was apparent on digestion with *Dra*I. The *Pst*I 'C' fragment (see Fig. 1), visible as a 1.55 kb band in digests of prototype HPV-16, was consistently absent from the majority of biopsies examined; however on reprobing with a specific subgenomic fragment the 1.55 kb band became visible, but only at very low levels. This suggests that a small minority of viral DNA present in the samples was prototype HPV-16, the remainder having some alteration in the L2-L1 region of the genome compared to the wild-type virus. A separate study [84] reported the presence of the DNAs of HPV-18, HPV-4 (not previously associated with malignant lesions) and variant HPV-16 in biopsy specimens of oral squamous cell carcinomas, confirming the lack of hybridisation of a HPV-16 probe to the 1.55 kb *Pst*I restriction fragment and demonstrating an altered *Hpa*II restriction pattern, where no 2.6 kb prototype fragment was visible but an additional fragment of 0.9 kb was detected, suggesting that any deletion or sequence variation was confined to the region encompassing the left hand region of the 2.6 kb *Hpa*II fragment where it overlaps with the 1.55 kb *Pst*I fragment.

Use of the polymerase chain reaction (PCR) to facilitate detection of HPV sequences in biopsies of oral mucosa and in cell lines derived from these supported results obtained previously, with around 50% of oral carcinomas containing HPV-16 DNA [85], although no data on prevalence of viral

DNA in normal tissue by PCR detection were discussed, and no HPV types other than HPV-16 were screened for. However, attempts by these authors to amplify DNA from the viral late region using specific primers in the PCR were unsuccessful, either due to a lack of DNA or to gross sequence divergence resulting in failure of the primers to anneal. They suggested that in the former case the virus would not be viable and while this may be correct, it would not necessarily preclude viral sequences from being involved in tumorigenesis as the E5, E6 and E7 ORFs would still be present and able to be expressed.

Recent work to analyse the apparent variation in the late region of HPV-16 genomes detected in oral mucosa has shown some promise. Using a PCR-based approach to amplify a 1.81 kb viral DNA target spanning the 1.55 kb *Pst*I restriction fragment (Fig. 3), it was possible to amplify a DNA sequence of approximately 1 kb in length from two oral carcinomas previously shown to be positive for HPV-16 E6 and E7 sequences, but not from HPV-18 positive or virus negative controls (W.A. Yeudall and M.S. Campo, unpublished observations). Southern blot experiments using the PCR amplification products to probe human genomic DNA suggested that hybridisation was occurring with highly repetitive cellular sequences. Further use of the PCR-amplified DNA to probe cloned HPV-16 DNA revealed hybridisation to the 1.78 kb *Pst*I restriction fragment but not to the 1.55 kb DNA, implying that viral sequences were present within the PCR product but that some rearrangement had occurred, possibly with a repetitive human DNA sequence. The reason for this is not yet apparent, although it may be the result of one or more illegitimate recombination events. Conceivably, the presence of a repetitive cellular sequence within the viral genome might facilitate future integration of HPV-16 DNA into the cellular DNA. Much work is required to resolve such possibilities.

While specific human papillomaviruses have the potential to effect cellular transformation together with co-factors, the precise role of HPVs in the aetiology of oral squamous cell carcinoma is not, as yet, well understood. Scully *et al.* [86] proposed that the oral cavity might serve as a reservoir of latent human papillomaviruses which could subsequently infect anogenital epithelium following oro-genital contact. However if the variant HPV-16 does indeed lack late viral sequences coding for structural proteins, as suggested by Maitland *et al.* [85], this would not represent a feasible route for infection of genital mucosa. Other workers have suggested that oral papillomavirus infection is acquired early in life, as there was little difference in the incidence of viral DNA in oral smears from adults or pre-school children [87], with both HPV-6b and HPV-16 being detected. These data imply that HPV infection of oral mucosa need not occur by sexual contact. A recent study by Kutcher and co-workers [88] examined both oral and genital samples from individuals and sexual partners, and correlated medical, dental and sexual histories with HPV infection at both sites. The results suggested that self-inoculation from genital to oral sites, or vice versa, is the most common way to transmit papillomaviruses between these two regions, although oro-genital contact between sexual partners is also likely to transmit HPVs.

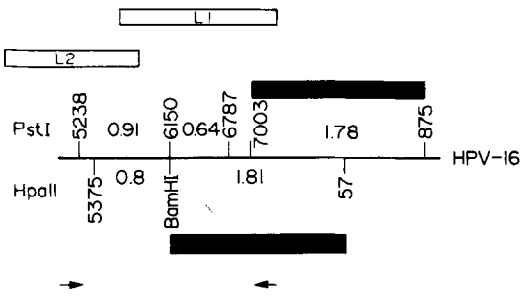

Fig. 3. Location of oligonucleotide primers (arrows) used to amplify part of the late region of HPV-16 DNA from samples of malignant oral epithelium (Yeudall and Campo, unpublished data). The shortened PCR product hybridised to the 1.81 kb *Hpa*II-*Bam*HI fragment and to the 1.78 kb *Pst*I fragment of prototype HPV-16 DNA (shaded boxes).

Although viral DNA has been demonstrated in normal oral epithelium, this does not exclude an involvement in carcinogenesis, as the effect may indeed be an early one such as an increase in cell proliferation or immortalisation. This could occur by compromising cellular p53 and pRb, allowing expansion of a population of cells which might then undergo further genetic events resulting in malignant progression. During progression viral DNA might be retained within the cells, or it may be no longer required (as for BPV-4-mediated "hit-and-run" transformation of bovine alimentary epithelium). To date, few data are available regarding the status of p53 and pRb in oral carcinomas, although both have been shown to be mutated ([89], Yeudall *et al.*, unpublished data). Functional analysis of these genes in oral squamous carcinoma cell lines is currently underway, as are studies on their possible interaction with viral oncoproteins. The results obtained may help to shed some light on the exact nature of the contribution of human papillomaviruses to the development of oral malignancy. Proof of aetiological involvement of viral agents would facilitate the design of preventive or therapeutic regimes which might help to limit the incidence and effects of what is a disfiguring and frequently fatal disease.

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