Human papillomavirus DNA in biopsies of oral tissues

W. A. Yeudall and M. S. Campo*

Beatson Institute for Cancer Research, Cancer Research Campaign Beatson Laboratories, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, U.K

The DNAs of human papillomavirus (HPV) types 4, 16 and 18 have been detected in biopsies of normal and malignant human oral mucosa by Southern blot hybridization and the polymerase chain reaction (PCR). By the former technique, HPV-4, HPV-16 and HPV-18 DNAs were detected in three separate carcinomas but were found in adjacent dysplastic and normal tissue by the PCR only. The PCR technique also allowed detection of HPV-16 and HPV-18 DNA in additional carcinomas and normal samples. The oral HPV-4 DNA was molecularly cloned and extensive restriction analysis and nucleotide sequencing showed identity with the prototype HPV-4 DNA. The HPV-18 DNA detected by Southern blot hybridization showed an altered restriction pattern in the E1 region of the viral genome; however direct nucleotide sequencing of PCR products from the E6 open reading frame showed no sequence alterations in either normal or malignant samples.

Several distinct types of human papillomavirus (HPV) have been shown to be associated with both normal and abnormal oral epithelia (reviewed by Scully et al., 1988). HPV DNAs of types 2, 4, 6, 11, 13, 16, 18, 32 and 57 have been demonstrated to occur in benign proliferative lesions of oral mucosa, including papillomas, verruca vulgaris and focal epithelial hyperplasia (Adler-Storthz et al., 1986a; Naghashfar et al., 1985; Pfister et al., 1983; Beaudenon et al., 1987; de Villiers et al., 1989; Greenspan et al. (1988) found HPV-7 DNA to be most common in oral warts in immunodeficient individuals seropositive for human immunodeficiency virus.

Oral cancer biopsies have also been shown, in some cases, to contain HPV sequences. de Villiers et al. (1985) and Adler-Storthz et al. (1986b) detected HPV-2 DNA in oral squamous and verrucous carcinomas, and DNAs of HPV-16 (de Villiers et al., 1985; Maitland et al., 1987) and HPV-18 (Syrjanen et al., 1988) have been reported in other oral squamous cell carcinomas. Additionally, Maitland et al. (1987, 1989) found that a large proportion of normal oral mucosal samples, as well as carcinomas, exclusively contain HPV-16 sequences, which in most cases varied from the prototype virus in their PstI restriction pattern.

We have used Southern blot hybridization and the polymerase chain reaction (PCR) to detect the presence of HPV DNA in biopsy samples of normal, dysplastic and malignant oral mucosae, and DNA sequencing to compare oral isolates of HPVs to prototype sequences.

DNA was prepared from 39 primary oral squamous cell carcinoma biopsies and adjacent dysplastic and normal tissue, and from 25 control samples of normal buccal mucosa from cancer-free individuals by the method of Chirgwin et al. (1979). Aliquots (10 μg) of genomic DNAs were digested with PstI or HpaII and analysed by Southern blotting and hybridization to 32P-labelled probes containing the complete genomes of HPV types 1 to 7, 11, 13, 16 and 18. Hybridization to each probe was carried out first at low stringency (Tm - 40 °C) and then at high stringency (Tm - 10 °C). The DNAs of HPV types 4, 16 and 18 were identified in three individual carcinomas out of 39 (samples C4, C2 and C1, respectively) but not in dysplastic or normal tissues from the same cancer patients (Fig. 1) or in samples of normal buccal mucosa (data not shown). In each case viral DNA was present in the episomal form (data not shown) but integration of a single copy into the cellular DNA can not be ruled out. No hybridization was observed in any of the samples with the other HPV probes, although the equivalent of one copy of viral DNA per cell could always be detected in reconstruction samples.

In sample C2, which contained HPV-16 DNA, the 1·55 kb PstI fragment which maps to the L2–L1 region of the viral genome was missing, a finding similar to that of Maitland et al. (1987). In an HpaII digest the expected 2·6 kb fragment which maps to the same region was absent and an additional band of approximately 0·9 to 1·0 kb was detected (Fig. 1, C2, lanes 1 and 2). As viral DNA was in the episomal form, these data point to an alteration in the L2–L1 region of the HPV-16 genome (W. A. Yeudall & M. S. Campo, unpublished results).
In sample C1, the HPV-18 DNA contained an additional PstI site in the 6.34 kb prototype fragment (Fig. 1, C1, lane 1). Different polymorphism of PstI restriction sites of HPV-18 has previously been reported in a cervical carcinoma biopsy in the west of Scotland (Millan et al. 1986), indicating a degree of heterogeneity of this virus in the area.

HPV-4 DNA was found in sample C4 (Fig. 1, C4, lanes 1 and 2). As this is the first association of this viral type with a malignant lesion, we molecularly cloned the viral DNA for more detailed analysis. Cellular DNA from sample C4 was digested with BamHI, and the linearized 8 kb viral DNA was first cloned in the bacteriophage vector EMBL3 (Stratagene) and then subcloned in the BamHI site of pBR322. The 8 kb insert was compared to the prototype HPV-4 DNA both by restriction endonuclease digestion and by partial nucleotide sequencing. No differences were observed between the prototype HPV-4 and the newly cloned DNA using 12 restriction enzymes (data not shown) but nucleotide changes that do not create or delete a restriction site for one of the enzymes used would not be apparent.

As the complete nucleotide sequence of HPV-4 DNA has not yet been determined, we sequenced a region of the prototype HPV-4 genome immediately adjacent to the vector–viral junction using as a sequencing primer the 17-base oligomer 5' GCCGGCCACATGCCTC 3', which corresponds to nucleotides 406 to 390 of the pBR322 genome, close to the BamHI site (Sutcliffe, 1979). Using a commercially available kit (Sequenase; United States Biochemicals) and the dideoxyxucleotide chain termination method (Sanger et al., 1977), 380 nucleotides were determined, reading from the 5' end of the HindII site at map coordinate 0-018 to the HindII site at map coordinate 0-05 (Heilman et al., 1980). The first 150 nucleotides are shown in Fig. 2. Using a synthetic oligonucleotide corresponding to nucleotides 4 to 23 (Fig. 2 and 3a) as the sequencing primer, we compared prototype and oral HPV-4 and observed no nucleotide differences over the region sequenced (data not shown).

As previous reports have described the presence of HPV DNA in normal cervical and oral tissues as well as in carcinomas and dysplasias (Cox et al., 1986; Maitland et al., 1987), we analysed all the oral biopsies by the PCR, which allows detection of minute amounts of DNA. Oligonucleotides were synthesized corresponding to the HPV-4 sequence obtained, the E6–E7 region of HPV-16 (Seedorf et al., 1985) and the E6 region of HPV-18 (Cole & Danos, 1987; Fig. 3a, c and e, respectively). The E6–E7 region of HPV-16 and the E6 region of HPV-18 were chosen as they are usually retained in virus-positive carcinomas (Wilczynski et al., 1988). HPV-4 primers were expected to amplify a fragment of 136 bp, HPV-16 primers a fragment of 166 bp and HPV-18 primers a fragment of 100 bp. Amplifications were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler with 10 mM-Tris–HCl pH 8.3, 50 mM-KCl, 1·5 mM-MgCl2, 0·01% gelatin, 200 mM of each dNTP, 0·5 mg of each primer and 2·5 units of Taq polymerase. Samples were denatured for 10 min at 94 °C and cooled on ice prior to addition of enzyme. Thirty cycles of amplification at 94 °C (1 min), 50 °C (1 min) and 72 °C (1 min) were performed, followed by a final cycle of annealing at 50 °C (1 min) and extension at 72 °C for 10 min. Samples were analysed by electrophoresis in 6% acrylamide gels, stained with ethidium bromide and visualized on a u.v. transilluminator. Southern transfers of the PCR fragments were hybridized at high stringency to the complete HPV-4 genome, or oligonucleotides internal to the amplified fragments in the case of HPV-16 and HPV-18 (Fig. 3c and e). Membranes were washed three times for 20 min in 0·1 x SSC/0·1% SDS and exposed to Kodak X-Omat S film for between 1 h and 5 days. The 136 bp fragment expected for HPV-4 was amplified in sample C4 and also in the adjacent dysplastic and normal tissues.
samples D4 and N4 (Fig. 3b). None of the remaining samples was positive using this set of primers.

Of 39 cancer cases, 10 (25.6%) contained HPV-16 DNA and eight further cases (20.5%) contained HPV-18 DNA. In addition, HPV-18 DNA was detected in two of 25 (8%) cancer-free samples (Fig. 3f). Amplification was specific, as HPV-16 DNA was not amplified from an HPV-16-containing cell line (T45, derived from an oral carcinoma; Parkinson & Yeudall, 1990) using HPV-18 primers (Fig. 3f). Anticontamination primers for HPV-16 (van den Brule et al., 1989) and HPV-18, which spanned the cloning sites, were used to control against the possibility of contamination by HPV plasmids present in our laboratory. Any contamination would result in an amplification product larger than the expected one by 4.3 kb, the size of the cloning vector. In reconstruction experiments, the large (approximately 4-5 kb) fragment could be amplified from 10 pg of recombinant plasmid DNA. No evidence of such a fragment was found in any of the samples, showing the absence of contaminating plasmids. The expected 170 bp fragment of wild-type HPV-18 DNA was detected in each case found positive with HPV-18 E6 primers (data not shown), confirming that the presence of viral DNA in biopsies was not due to laboratory contamination. Using the HPV-16 anticontamination primers, which map to the late region of the viral genome, no amplification of viral sequences was observed in the HPV-16-positive cases, attributable to the extensive sequence alterations found in this region (Maitland et al., 1989; W. A. Yeudall & M. S. Campo, unpublished results).

When viral DNA was present in carcinoma tissue, it was also found by PCR in adjacent dysplastic and normal tissues (Fig. 3), except in two samples, N20 and N39 (Fig. 3f). The fact that viral DNA is only detectable by PCR may reflect a low number of cells infected with virus in a much larger population of uninfected cells, thus reducing the apparent copy number per cell.

An interesting finding was the detection of HPV-18 DNA in two samples of normal oral epithelium from

---

**Fig. 3. Detection of HPV DNA sequences by PCR.**

(a) HPV-4 PCR primers derived from the nucleotide sequence in Fig. 2, expected to amplify a 136 bp fragment (not to scale). (b) Southern blot of PCR products from sample 4; the probe was HPV-4 DNA. Lanes 1 to 5, HPV-4, C4, D4, N4 and no DNA, respectively. Throughout the figure: C, cancer; D, dysplasia; N, normal. C4 has 10-fold less sample than either D4 or N4. (c) HPV-16 PCR primers expected to amplify a 166 bp fragment from the E6-E7 region and the internal oligonucleotide used as a probe in Southern blots. (d) Southern blot of PCR products from samples D2 (lane 2) and N2 (lane 3). Lane 4, control sample with calf thymus DNA; lane 5, control sample with no DNA added; lane 1, HPV-16 DNA. (e) HPV-18 PCR primers expected to amplify a 100 bp fragment from the E6 region and the internal oligonucleotide used as a probe in Southern blots. (f) Southern blot of PCR products from case samples and two external controls (N12 and N18, lanes 20 and 21). Lanes 1 to 19, HPV-18, C14, C19, C20, N20, C25, N25, C34, N34, C37, N37, C39, N39, C40, N40, C41, N41, T45 and no DNA, respectively.
cancer-free individuals. Maitland et al. (1987) previously reported HPV-16 DNA in biopsies of normal oral tissue but this is the first demonstration of HPV-18 in a similar situation. We sequenced the PCR products from the prototype HPV-18, from carcinoma sample C1 and from the two normal HPV-18-containing biopsies (samples N12 and N18; Fig. 3f). There are no alterations in base composition over this region of the E6 open reading frame between oral and cervical DNA (data not shown), as is also the case for the corresponding region of HPV-16 (Maitland et al., 1989; W. A. Yeudall & M. S. Campo, unpublished results). However, we have no sequence data on other regions of the HPV-18 genome in oral tissues and, indeed, Southern analysis of the viral DNA from sample C1 revealed an additional PstI site within the E1 region. The prevalence of HPV-16 and HPV-18 infection is approximately the same in our population, whereas Maitland et al. (1987, 1989) reported a much higher frequency of HPV-16 infection, although the overall level of viral infection is equal in both studies. This difference may reflect the different geographical origins of samples; HPV-16 may be more common in the south-west of England, whereas we note a much higher incidence of HPV-18 in the west of Scotland. This geographical difference in prevalence of HPV-18 has also been noted for cervical lesions (Millan et al., 1986).

Thus it would appear that DNAs of several HPV types may infect oral epithelium at low levels and do not necessarily result in pathology either at the clinical or histological level. The function of papillomavirus DNA in oral mucosae remains an unanswered question but the use of PCR to study transcription of viral genes, undetectable in these tissues by conventional means, will undoubtedly help to clarify this point and may shed some light on the possible role of HPV DNA in the onset of malignant oral disease.

We are grateful to R. W. Smith, D. S. Soutar and M. H. C. Webster for access to clinical material, to D. G. MacDonald for histopathological assessment of biopsies, and to D. Gillespie and S. Lowe for synthesis of oligonucleotides. We thank K. T. Smith and J. A. Wyke for helpful discussion and encouragement. We also wish to thank the Cancer Research Campaign, the Medical Research Council and the Greater Glasgow Health Board for financial support. W.A.Y. is in receipt of Research Training Fellowship G84/1855 from the MRC. M.S.C. is a CRC Life Fellow.

References


(Received 29 June 1990; Accepted 10 October 1990)