Presence and integration of human papillomavirus type 6 in a tonsillar carcinoma

Jorge Andres Bercovich, 1 Carlos Ries Centeno, 2 Osvaldo González Aguilar, 2 Saúl Grinstein 1 and Tomas Kahn 3.

1 Virology Laboratory, Buenos Aires Children's Hospital, Gallo 1330, 1425 Buenos Aires, 2 Head and Neck Surgery Department, Oncology Hospital of Buenos Aires, Buenos Aires, Argentina and 3 Deutsches Krebsforschungszentrum, Institut für Virusforschung, Im Neuenheimer Feld 506, 6900 Heidelberg, Germany

Human papillomavirus type 6 subtype a (HPV-6a) was detected in a human invasive tonsillar carcinoma. Southern blot hybridization analysis showed the presence of additional bands when using non-cutting and single-cut restriction enzymes. Molecular cloning yielded two recombinant clones of 8-0 and 1-4 kb in size. The first represents the complete HPV-6a genome. Sequence analysis of the second clone showed a 0-6 kb DNA sequence corresponding to the L2 region of HPV-6a, whereas the rest belongs to cellular sequences. These data show the presence of a usually low risk HPV type in an invasive carcinoma, at an unusual infection site, with viral DNA integrated into the host genome. These findings add evidence in support of the hypothesis of a relationship between HPV infection and at least some ororespiratory cancers.

A strong association exists between human papillomavirus (HPV) and some human cancers. HPV types 16, 18, 31, 33 and 35 and a few others account for more than 90% of cervical carcinomas (zur Hausen, 1989) whereas the genome of HPV type 5 and more rarely also types 8, 17 and 20 are found in almost all malignant lesions of individuals suffering from epidermodysplasia verruciformis, a disease in which 30 to 50% of patients develop skin carcinomas (Orth, 1986).

The DNA of HPV types 16 and 18 is usually found integrated into the cellular genome of cell lines derived from cervical carcinomas (zur Hausen, 1986; Baker et al., 1987). Episomal and integrated molecules have been shown to coexist in cervical carcinomas, and a few carcinomas were devoid of detectable integrated sequences (Matsukura et al., 1989; Cullen et al., 1991).

Conversely, HPV types 6 and 11 have been proposed to be causative agents of predominantly benign genital and laryngeal lesions (Gissmann, 1984). Although they are regarded as non-oncogenic, they have been found in the rare verrucous carcinomas of the vulva and penis and Buschke-Löwenstein tumours (Gissman et al., 1983; Boshart & zur Hausen, 1986), and in other respiratory and genital cancers in the absence of specific promoting factors (Rando et al., 1986; Byrne et al., 1987). Except for one perianal squamous cell carcinoma from an immunosuppressed patient, where integration was deduced from a two-dimensional gel electrophoresis analysis (Manias et al., 1989), the physical state of the viral genome was reported to be always episomal.

The relationship between HPV infection and ororespiratory cancers is not as evident as it is at the genital level. Only a small percentage of individual biopsies have been shown to be positive using the DNA of known HPVs as probes in high stringency conditions in Southern blot hybridizations (Byrne et al., 1987; Ishibashi et al., 1990). One new HPV type was cloned from a laryngeal carcinoma, but this type has not been found in other laryngeal cancers up to now (Kahn et al., 1986).

In this report we describe the presence of HPV-6a in a human tonsillar carcinoma, and show, using molecular cloning, sequencing and Southern blot hybridization, the integration of this viral DNA into the host genome.

After the resection of a lymph node metastasis from the neck, the primary tumour was detected in a tonsil of a 61 year old woman. The tumour was described as an infiltrating, poorly differentiated epidermoid carcinoma. The patient did not consume alcoholic drinks, was not subjected to radiotherapy and had smoked an average of three cigarettes per day for 30 years.

Total DNA from the tumour biopsy was cleaved with PstI and analysed by Southern blot hybridization (Southern, 1975) at high stringency conditions (Tm -18 °C) with the use of an HPV-11 DNA probe labelled with 32P by the random primer technique (Feinberg & Vogelstein, 1983). The pattern characteristic of HPV-6a
was observed. Additional digestions were performed using two single-cut enzymes for HPV-6a (HindIII and BamHI), two non-cutting enzymes (XhoI and EcoRI) and PstI again, and hybridized with an HPV-6b DNA probe (Fig. 1). The PstI, XhoI and BamHI patterns were as expected, indicating the presence of the HPV-6a genome as an 8 kb episome. The hybridization patterns obtained after digestion with EcoRI and HindIII, however, showed the presence of additional bands (Fig. 1) which could represent fragments from either another HPV or integrated HPV-6 sequences. To test the hypothesis that these additional bands were due to the presence of another HPV in the same lesion, tumour DNA was hybridized against HPVs 6, 16, 18, 30, 31, 33 and 35 used as a probe at conditions of high (T_m = 18 °C) and reduced (T_m = 35 °C) stringency. In addition, tumour DNA was labelled with ^32P, and used as a probe in hybridizations against Southern blots containing DNA from HPV types 1 to 57 (provided by Dr E.-M. de Villiers). These hybridizations were also performed under conditions of high and reduced stringency. The results of these experiments confirmed the presence of HPV-6 in the tumour, but provided no evidence for the presence of any additional HPV type in it. Based on Fig. 1 and these hybridization experiments, the most likely interpretation is that most of the viral DNA in the tumour is present as monomeric episomal HPV-6 molecules, and that the additional bands probably represent fragments of integrated HPV-6 sequences. Since integration has never been demonstrated by sequence analysis of cloned viral–cellular junction fragments for HPV-6, the work was focused on this particular aspect.

Tumour DNA was digested with HindIII and a library was constructed using λ NM 1149 as a vector. HindIII was chosen as a cloning enzyme in order to enable cloning of both the 8 kb and the integrated fragments from a single genomic library. Screening experiments using HPV-6b DNA as a probe were performed. Two types of recombinant phages were detected. One, containing an 8.0 kb insert representing the complete HPV-6a genome, was not analysed in greater detail. The second type revealed the presence of a 1.4 kb insert after cleavage with HindIII (not shown). This band showed the same electrophoretic mobility as the most prominent additional band observed in the Southern blot experiment (Fig. 1). This 1.4 kb fragment was subcloned into the pBluescript plasmid yielding the TonB clone. Sequencing of the 1.4 kb insert from the TonB clone was performed by the dideoxynucleotide chain termination method using a T7 sequencing kit (Pharmacia). For primer walking, synthetic oligonucleotides were made using an Applied Biosystems oligonucleotide synthesizer. The primers used were located at positions 358, 615, 831, 1070, 1240 (insense) and 1086, 764, 460 and 60 (antisense) of the TonB clone, in addition to the ‘universal’ and T3 primers. The sequences were analysed with the aid of the Biological Sequence Analysis (BSA) program library developed at the Deutsches Krebsforschungszentrum and with the HUSAR (Heidelberg University Sequence Analysis Resources) program library.

Five-hundred and three base pairs were closely similar to those of the L2 region of HPV-6b (nucleotide positions 4907 to 5410) (Fig. 2). Due to the mutations G to A and G to T, a HindIII site not present in HPV-6b was created,
Fig. 2. Nucleotide sequence of the virus–cellular junction. The upper line represents the nucleotide sequence at the virus–cellular junction on the 1.4 kb TonB clone. Bold typed nucleotides correspond to the viral component of the sequence. Numbers above the sequence indicate the nucleotide position within the 1.4 kb TonB clone. The lower line represents the corresponding HPV-6b L2 sequence. Numbers below the sequence indicate the nucleotide position in the published HPV-6b sequence (Schwarz et al., 1983). Vertical bars indicate nucleotides identical in both sequences.

and constituted the cloning site: AAGCTT instead of AGGCTG. Since the L2 open reading frame (ORF) from HPV-6b starts at position 4378 (ATG at position 4423) and stops at nucleotide 5799, one junction of the cloned HPV-6a integrate falls within the L2 region. The sequence contained only conservative mutations when compared to the published HPV-6b sequence (Schwarz et al., 1983) (Table 1). One flank of the virus–cellular DNA junction is shown in Fig. 2. Part of the cellular component (positions 1 to 813) of the clone was subcloned into pBluescript as a HindIII–MnlI fragment, and hybridized at $T_m - 18^\circ C$ with a blot containing human placenta DNA cleaved with different restriction enzymes (Fig. 3).

The results indicate unambiguously that the integration occurred in a normal, single-copy cellular sequence. We conclude that both integrated and episomal HPV-6a molecules were present in the tumour.

Information about the association of HPVs with carcinomas of the oro respiratory tract in general, and with tonsillar carcinomas in particular, is limited, because the finding of specific types of HPV in these kind of lesions is contained in isolated reports (de Villiers et al., 1985; Kahn et al., 1986; Byrne et al., 1987), although positive results after relaxed conditions of hybridization are frequent (Brandsma et al., 1986; Ostrow et al., 1987; J. A. Bercovich & T. Kahn, unpublished results). From the developing knowledge of anogenital HPV infections, one possible explanation would be that as yet unknown HPV types are responsible for these results. On tonsillar carcinomas, there exist at least two reports describing the presence of HPV-16 or HPV-16-related sequences at this location (Brandsma & Abramson, 1989; Ishibashi et al., 1990). Up to now, HPV-6 had not been found in a tonsillar carcinoma.

HPV type 6 and 11 infections are regarded as at low risk for malignant conversion (de Villiers et al., 1985). Nevertheless, they have been shown to be associated with some invasively growing tumours (Gissmann et al., 1983; Boshart & zur Hausen, 1986; Rando et al., 1986; Byrne et al., 1987; Manias et al., 1989; J. A. Bercovich & T. Kahn, unpublished results). Alterations in the

Fig. 3. A Southern blot, containing 10 µg of human placental DNA per lane, was hybridized using part of the radioactively labelled cellular component of the TonB clone as a probe (fragment HindIII–MnlI, nucleotides 1 to 813) under stringent conditions of hybridization ($T_m - 18^\circ C$). Lanes: 1, BamHI; 2, EcoRI; 3, BamHI + BglII; 4, BamHI + EcoRI + BgIII; 5, BamHI + EcoRI; 6, BgIII; 7, HindIII; 8, BamHI + HindIII; 9, PstI; 10, BamHI + PstI.

Table 1. Point mutations in TonB (HPV-6a) when compared to HPV-6b, and consequences at the amino acid level

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<tr>
<th>TonB</th>
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sequences involving the long control region (LCR) have been described in HPV type 6 and 11 genomes related to human carcinomas (Rando et al., 1986; Byrne et al., 1987). In one vulvar carcinoma (Rando et al., 1986), HPV-6 DNA has been found in an episomal state with an insertion of three sequences of 79, 19 and 15 nucleotides in the LCR. Similar observations were made on isolates from Buschke-Löwenstein tumours (Boshart & zur Hausen, 1986). In a squamous cell carcinoma of the lung associated with a laryngotracheobronchial papillomatosis, HPV type 11 was found (Byrne et al., 1987). In that case, a liver metastasis sample showed a reiteration which extended from the L1 ORF through the LCR into the E6 ORF. Although we cannot exclude the possibility of rearrangements in the HPV-6a episomal DNA, the yield of the expected PstI, XhoI and BamHI patterns suggests the absence of major alterations in these molecules.

Viral integration is a well known phenomenon in cervical carcinoma cells associated with HPV-16 and -18 (Schwarz et al., 1985; Baker et al., 1987). Manias et al. (1989) showed by two-dimensional gel electrophoresis the integration of HPV-11 DNA in a skin carcinoma as well as in metastatic tumours of an immunosuppressed patient. Here we demonstrate by molecular cloning and sequencing the integration of HPV-6a in an invasive carcinoma of an immunocompetent patient, although it has not been possible to clone the complete integration unit including the second cellular flank.

The contribution of the HPV-6 infection and integration to the development of this tonsillar carcinoma is not known. One possibility is that the episomal HPV-6 molecules may have undergone alteration increasing their oncogenic potential. Another possibility is that the integration event might have disrupted a cellular gene critical to normal cell proliferation.

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References


