Sequence rearrangements in the upstream regulatory region of human papillomavirus type 6: are these involved in malignant transition?

Hidero Kitasato, Hajo Delius, Harald zur Hausen, Karin Sorger, Frank Rös and Ethel-Michele de Villiers

Human papillomavirus type 6 (HPV-6) was isolated from a tongue papilloma which subsequently progressed to an invasive carcinoma. Three biopsies were taken from the same patient at different intervals during the tumour development. The HPV-6 genome in all three biopsies contained a GT-rich 94 bp insertion at nucleotide 7350 in the upstream regulatory region (URR). In comparison to previously published HPV-6 DNA isolates, this insertion seems to be the most prevalent and constant modification, not present in the prototype HPV-6b, and allows an improved alignment with the sequence of the HPV-11 genome. The possible biological significance of these GT-rich clusterings at the beginning of the URR, present not only in these HPV-6 isolates but observed in all other 'genital' HPVs also, is discussed.

Human papillomavirus type 6 (HPV-6) DNA was isolated and cloned from a condyloma acuminatum (Gissmann & zur Hausen, 1980; de Villiers et al., 1981). The viral DNA is frequently detected in benign anogenital lesions, as well as in laryngeal papillomas and papillomatous lesions of the oral cavity (de Villiers, 1989). Based on their clinical association mainly with benign lesions, HPV-6 as well as the closely related HPV-11 have been regarded as 'low-risk' HPV types. Nevertheless, these HPV types are found occasionally in premalignant proliferations, such as in rare giant condylomata or Buschke-Löwenstein tumours. These are verrucous genital tumours which grow invasively but rarely metastasize (Buschke & Löwenstein, 1925). Clinically they are regarded as malignant tumours. Other tumours in which HPV-6 DNA has been detected include verrucous carcinoma of the vagina (Okagaki et al., 1984) and of the vulva (Rando et al., 1986a; Kasher & Roman, 1988), cervical carcinoma and one case of bladder carcinoma (Oft et al., 1993), a primary carcinoma of the urethra (Grussendorf-Conen et al., 1987), a condyloma of the mamilla (Kulke et al., 1989), a case of lung carcinoma (DiLorenzo et al., 1992), as well as a tonsillar carcinoma (Bercovich et al., 1991). In all these cases, analyses of the HPV-6 DNA genomes revealed insertions and/or duplications in the upstream regulatory region (URR). Detailed studies of the HPV-6 DNA in a number of benign lesions demonstrated similar genomic alterations (Kulke et al., 1989; Hrisomalos et al., 1990; Farr et al., 1992).

HPV-6b (de Villiers et al., 1981) has been regarded as the prototype for HPV-6, and the DNA sequence of its genome (Schwarz et al., 1983) has been used for comparative analyses of different HPV types. In screening larger series of biopsies from condylomata acuminata, it soon became evident that a number of additional subtypes exist. Their definition was based on different methods varying from the use of restriction enzyme(s), through to applying the PCR technique and performing partial or complete sequence analyses.

Gissmann et al. (1983) reported the presence of HPV-6a, -6b and -6c in 26, six and five condylomata acuminata, respectively. This subdivision was based on DNA fragment sizes resulting from digestions with the restriction enzyme Psfl. Similarly, HPV-6d was identified in a Buschke-Löwenstein tumour (Boshart & zur Hausen, 1986). HPV-6a seems to be the most prevalent subtype. In another study using restriction enzyme cleavage (Rübben et al., 1992), HPV-6a was present in 42 condylomata acuminata and in four Buschke-Löwenstein tumours. The main difference between the prototype HPV-6b and -6a is the presence of an additional 120 bp (approx.) in the URR of the latter. These 120 bp are located in the FnuDII–HinfI fragment spanning nucleotides (nt) 7271 to 7377 (Boshart & zur Hausen, 1986). The URR of HPV-6d also contained these additional 120 bp, as did an HPV-6 isolate from a condyloma of the mamilla (HPV-6-ma) (Kulke et al., 1989).

The frequent isolations of HPV-6 DNA containing
these insertions, from premalignant or malignant lesions, raises the question of whether such rearrangements may be involved in facilitating the process of malignant conversion. To investigate this possibility, we isolated the HPV-6 DNA genomes from biopsies, taken at different stages, of a tongue papilloma which subsequently progressed to an invasive carcinoma.

Over a period of 7 years, a 72-year-old male patient had been suffering from a steadily growing tumour of the tongue. At the time of the first examination, a tumour covering approximately 80% of the tongue was histologically diagnosed as a papillomatous lesion with islands of dysplastic change of the epithelium, but with no indication of invasive growth (WV6518). Three months after removal by laser surgery, histological examination of the remaining lesion (WV6745) demonstrated papillomatous changes with premalignant areas which, after another year, progressed into a cauliflower-like invasively growing tumour (WV7346), histologically a highly differentiated, squamous cell carcinoma. Total cellular DNA was extracted from the biopsies. After detection of HPV-6 DNA by reverse blot hybridization, these results were confirmed by subjecting each sample to Southern blot analysis after digestion with the restriction enzyme PstI. The whole genomic HPV-6 DNA was cloned from the biopsy WV6745 (de Villiers et al., 1989) and subsequently sequenced by the dideoxynucleotide sequencing procedure (Sanger et al., 1977). The complete URR of the HPV-6 DNA was amplified from the cellular DNA of each of the biopsies using PCR (Saiki et al., 1988) and the products were subsequently cloned and sequenced.

The HPV-6 genomes in all three biopsies obtained from the patient described were analysed for sequence differences that could have contributed to tumour progression. In all three biopsies the same 94 bp insertion was present at nt 7350. In addition, HPV-6 was isolated, cloned and partially sequenced from a purely benign laryngeal papilloma (WV7458) from a second patient. This isolate also contained the 94 bp insertion at the same nucleotide position. The URR sequences respectively amplified and cloned from the tongue papilloma and carcinoma, as well as the laryngeal papilloma, proved (after amplification and direct sequencing of the products) to be identical to the HPV-6 in the original cellular DNA.

In view of the present analysis of HPV-6 DNA in different stages of tumour progression, these data suggest that modifications of the persisting viral genome are not responsible for the eventual emergence of HPV-6-positive carcinomas. On the one hand it is conceivable that this progression is governed rather by specific host cell modifications as postulated for high-risk HPVs (zur Hausen, 1986, 1994). On the other hand, it cannot be excluded that the observed insertions in the HPV-6 URR predispose the genome-carrying cells to malignant conversion. The frequency of such inserts in HPV-6 isolates from squamous cell carcinomas could support the latter.

The frequency of isolation of HPV-6 genomes con-

![Fig. 1. A comparison of the different URRs described for a number of HPV-6 DNA isolates. The characteristics of each isolate correspond horizontally with its designation on the left.](image-url)
Fig. 2. An alignment of the inserted sequences present in the URR of five HPV-6 isolates in comparison to the sequence of HPV-6b. The stars show the start and end positions of the sequences. The insertions of HPV-6 isolates WV6745, T70, W50 and WV7458 started at nt 7350, and that of HPV-6-vc started at nt 7348.

Fig. 3. An alignment of the DNA sequence of HPV-11 to that of HPV-6b and the HPV-6 isolate from a tongue biopsy (WV6745).

Taining the 94 bp insertion tends to underline the suggestion (Boshart & zur Hausen, 1986) that the prototype HPV-6b represents an exception, i.e. a sequence with a deletion of a GT-rich nucleotide stretch in the URR. Additional data which argue for HPV-6a as the prototype are the following. Sequence determination of the URR of HPV-6-vc, isolated from a rapidly growing vulvar verrucous carcinoma, revealed the main differences to HPV-6b being a 74 bp insertion at nt 7348, a 15 bp insert at nt 7418, and a 19 bp insert at nt 7720 (Rando et al., 1986a). A 20 bp insertion, identical in sequence to, and inserted at the same nucleotide position as, the 19 bp insertion of HPV-6-vc was detected in HPV-6-ma (Kulke et al., 1989). Similarly, an insertion of approximately 20 bp was demonstrated in isolates from 31 of 42 condylomata acuminata and all of four Buschke-Löwenstein tumours (Rübben et al., 1992). In another study, the cleavage pattern of PCR-amplified products from the HPV-6 URR of a cervical carcinoma and a perianal condyloma respectively was the same as that determined for HPV-6-vc (Oft et al., 1993).
carcinoma of the vulva (T70) revealed the presence of a 24 bp insertion at nt 7323, an insertion of 58 bp at nt 7350, as well as a 49 bp deletion at nt 7351 (Kasher & Roman, 1988). The subsequent characterization of HPV-6 from a vulvar condyloma (W50) showed the presence of a 94 bp insertion at nt 7350. The sequence of the first 58 bp of this insert was identical to that of the 58 bp insertion in T70 (Farr et al., 1991).

A comparison of the isolates described above is presented in Fig. 1. An alignment of the 58 bp, 74 bp and 94 bp insertions in comparison to the HPV-6b sequence is presented in Fig. 2. Although the 74 bp insert (HPV-6-vc) is not identical to the other insertions, an alignment was possible. A notable difference is seen at nt 50 of the inserts. In T70 and WV6745, cytidine is present at this position in comparison to adenine in W50, WV7458 and HPV-6-vc. This change may not be of obvious functional significance, regarding the benign and malignant origins...
of the various isolates. Analyses of two condylomata acuminata and two cervical scrapes also revealed the presence of the 94 bp insertion (with nt 50 being an adenine) at nt 7350 (Hrisomatos et al., 1990). In addition, three of these samples also contained a 14 bp insertion at nt 7420 which was almost identical to the 15 bp insert at nt 7418 in HPV-6-vc (Rando et al., 1986b).

The alignment of the DNA sequence of HPV-11 with that of HPV-6b and, for example, WV6745, does however strengthen the speculation that the 94 bp fragment was at some stage deleted from the HPV-6b genome. Between nt 7315 and 7406, the HPV-11 sequence aligns with the WV6745 sequence, with 62 of 94 nucleotides being identical. This region of HPV-11 is also characterized by stretches of poly(dG-dT) (Fig. 3).

The detection of the TG stretches, which have been discussed as having regulatory functions (Hamada et al., 1984) in terms of recombination and regulation of transcription, prompted us to scan all available sequences of HPV types in order to find out whether the closely related primate viruses rhesus papillomavirus type 1 (RhPV-1) and pygmy chimpanzee papillomavirus type 1 (PCPV-1)] for the presence of a poly(dG-dT) motif (TGTTGTGTGTTGTT) (Fig. 4). The maps of the papillomavirus ORFs were grouped according to their evolutionary relationship so that the maps of the most closely related types are next to each other.

Comparison of Fig. 4(a) (‘genital’ HPV types) with Fig. 4(b) (‘skin’ types) shows a systematic difference. In the majority of the sequences from the ‘genital’ HPV group, multiple copies of the search motif or closely related sequences are present, forming clusters at the beginning of the URR, whereas the GT-rich motif is infrequent in the ‘skin’ group of papillomaviruses.

An interesting consequence of this modification by the insertion in the URR is the creation of an ORF encoding 96 amino acids (starting at nt 7295). HPV-2a and -57 also contain open reading frames in their URR with coding capacity for large polypeptides (88 amino acids and 91 amino acids in length respectively) (Hirsch-Behnab et al., 1990). Thus far, no mRNA transcripts derived from this region have been detected.

Functional analyses of these regions await further elucidation.

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