Characterization of Human Papillomavirus Type 45, a New Type 18-related Virus of the Genital Tract

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SUMMARY

DNA of human papillomavirus (HPV) type 45, a new HPV type 18-related papillomavirus of the genital tract, was cloned from a recurrent cervical lesion displaying mild to moderate dysplasia with koilocytosis. HPV-45 DNA was identified in paraffin sections of biopsies of both the initial and recurrent lesions of the patient, taken 7 months apart. HPV-45 DNA hybridized efficiently to that of many different HPV types under low and moderate stringency conditions (Tm = 37 °C to Tm = 25 °C) but with only HPV-18 DNA under high stringency conditions (Tm = 17 °C). HPV-45 DNA was distinguished from HPV-18 DNA by (i) differences in restriction enzyme digest patterns, (ii) lack of hybridization at Tm = 17 °C between HPV-18 and some fragments of HPV-45, (iii) a value of 25% in liquid reassociation kinetics between HPV-18 and HPV-45 and (iv) differences in intensities of hybridization with selected tissue DNAs. The prevalence of HPV-45 infection in the genital tract was low. In tests of over 600 tissue DNAs from female genital tract lesions, HPV-45 sequences were detected in three additional tissues, one each of invasive cervical carcinoma, condyloma, and normal cervical epithelium. HPV-45 is a newly recognized papillomavirus which rarely infects the genital tract and is associated with lesions across a wide histological spectrum.

INTRODUCTION

Over 40 individually distinct human papillomaviruses (HPVs) have been identified, many of which are associated with specific clinical lesions (Orth et al., 1977; zur Hausen, 1980; Gissmann & Schneider, 1986). More than ten HPV types are detected in the genital tract. Among this group, HPV-16 and HPV-18 have been consistently associated with premalignant and malignant lesions of the female genital tract (Gissmann & Schneider, 1986; Reid et al., 1987) and their DNAs have been found in an integrated state in invasive cancers (Boshart et al., 1984; Dürst et al., 1985). Many established cervical carcinoma cell lines carry HPV DNA sequences. For example, the HeLa cell line initiated in 1951 from a cervical cancer contains multiple integrated copies of HPV-18 (Boshart et al., 1984; Schwarz et al., 1985). We report here the identification and distribution of a new HPV-18-related papillomavirus of the genital tract which is designated HPV-45.

METHODS

Histology, immunocytochemistry and in situ hybridization. Paraffin-processed tissue sections from the patient's initial and recurrent lesions were examined for pathology after haematoxylin and eosin staining and for viral capsid antigen by immunoperoxidase tests with the genus-specific antiserum (Gupta et al., 1983). The sections were also tested for the presence of HPV-45 DNA by in situ hybridization with a 35S-labelled DNA probe, as described (Gupta et al., 1985).
**Extraction of cellular DNA and DNA–DNA hybridization.** Total cellular DNAs extracted from exfoliated cervical cells and from freshly frozen tissue of the recurrent lesions were digested with different restriction endonucleases and examined for HPV DNA sequences by sequential Southern transfer hybridization with [32P]-labelled HPV probes (Naghashfar et al., 1985). Hybridization was performed at 45 °C with 1 M NaCl, 5 × Denhardt’s solution (Denhardt, 1966), 10 mM EDTA, 0.5% SDS with 20% formamide for low stringency (Tm = 37 °C) and with 36% formamide (with a wash at Tm = 17 °C) for high stringency. The formamide concentrations for other conditions of hybridization were calculated from the equation Tm (°C) = 81.5 + 16.6 (log10 M) + 0.41 (%G+C) - 0.72 (%formamide), where Tm is the melting temperature of the DNA, M is the molarity of monovalent salt, and %G+C is the percentage of guanine plus cytosine residues in the DNA (McConaughy et al., 1969). The average G+C content of HPV DNA was taken as 41% on the basis of the nucleotide sequence of HPV-6 (Schwarz et al., 1986). Low stringency washes were done in 4 × SSC at 55 °C (Tm = 37 °C) and high stringency washes in 1 × SSC at 65 °C (Tm = 17 °C) (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate).

**Cloning of viral DNA.** The HPV-45 DNA was cloned at the unique HindIII site in phage λL47 (Loenen & Brammar, 1980) and subcloned in pGEM-1 (Melton et al., 1984) as described by Maniatis et al. (1982). One μg of cellular DNA from exfoliated cells and 1 μg of λL47 DNA were digested with HindIII. After packaging and screening of 1.3 × 106 plaques, more than 500 positive clones were obtained. In an examination of several clones, all were found to contain the same DNA segment. The viral insert in one of the clones was subcloned in pGEM-1 for further analysis.

**Reassociation kinetics.** HPV-18 DNA was labelled with 100 μCi [α-32P]dATP (2000 to 3000 Ci/mmol) by nick translation. This yielded a specific activity of 3 × 10⁶ c.p.m./μg. An analysis of the labelled DNA on a 2% agarose gel revealed a mean fragment length of about 100 bases. The unlabelled DNAs were cut to the same size by using restriction endonucleases. All specimens were derived from U.S. women and were collected in the course of previous investigations (Lorincz et al., 1986). Hybridization was performed at 45 °C with 1.0 M salt, 5 × Denhardt’s solution (Denhardt, 1966), 10 mM EDTA, 0.5% SDS with 20% formamide as described in Fig. 6 in 100 μl of 0.5 M NaCl, 0.5% SDS at 65 °C. Twenty μl aliquots were withdrawn at various times and kept at 0 °C. The percentages of radioactivity in the samples were determined by nuclease S1 digestion. Reaction kinetics were analysed according to Wetmur & Davidson (1968).

**Genital tract tissues.** Stored filters containing cellular DNAs from over 600 genital tract samples were examined for HPV-45 sequences by Southern transfer hybridization at Tm = 17 °C (Naghashfar et al., 1985). These specimens were derived from U.S. women and were collected in the course of previous investigations (Lorincz et al., 1986; Bergeron et al., 1987; Reid et al., 1987; K. Shah, unpublished results).

**RESULTS AND DISCUSSION**

The new virus was first identified, and cloned, from the recurrent cervical lesions of a 26 year old white female. The initial lesion was colposcopically seen as circumferential raised white areas within the transformation zone. The Papanicolaou smear and directed biopsies displayed mild to moderate dysplasia with koilocytosis. A laser ablation of the transformation zone was performed. Six months following the treatment, the patient was referred to the Johns Hopkins Hospital for recurrence of abnormal cytology. By colposcopy, the patient was seen to have a series of raised white lesions distributed circumferentially outside the external cervical os (Fig. 1). The directed biopsy of the recurrent lesions revealed features very similar to those of the initial biopsy taken 7 months earlier. There were focal areas of increased nuclear–cytoplasmic ratio, moderate nuclear hyperchromatism, increased mitotic activity and koilocytosis in the superficial layers of the epithelium (Fig. 2). A second laser vaporization was performed, ablating the lesions and the transformation zone. During a 2 year follow-up, the patient has shown no evidence of recurrence by colposcopy and cytology, and DNAs extracted from her cervical cells have been negative for HPV sequences.

Paraffin sections of both biopsies displayed HPV capsid antigen in immunoperoxidase tests. Two cellular DNA samples, one extracted from exfoliated cells and the other extracted from freshly frozen tissue of the recurrent lesions, were tested for HPV sequences. Both specimens gave identical results. They hybridized with an HPV-6 probe at Tm = 37 °C but the hybrids were unstable and were washed off at Tm = 17 °C. In subsequent hybridizations at Tm = 17 °C, the cellular DNAs hybridized with HPV-18 but not with HPV-16 or HPV-31. However, the restriction endonuclease digest pattern of the HPV genome in the cellular DNAs was unlike that of HPV-18 and the intensity of the signal decreased when the filter hybridized with HPV-18 was washed at Tm = 5 °C. Undigested and EcoRV-digested cellular DNAs exhibited the typical pattern of forms I, II and III, demonstrating the absence of a recognition sequence for EcoRV in
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Fig. 1. Colposcopic photograph of cervix. The entire transformation zone is seen. Raised white lesions of condylomatous texture are visible in a concentric array with the external os of the cervix at the centre. No specific vascular features are evident. Photograph taken after application of 3% acetic acid solution. Magnification × 2-4.

Fig. 2. Histological sections of recurrent cervical lesion, stained with haematoxylin and eosin. (a) Full thickness of stratified epithelium of ectocervix displaying features of koilocytosis, multinucleation, nuclear hyperchromasia and abnormal maturation. Note nuclear dense region of epithelium at bottom right. Bar marker represents 100 μm. (b) Higher power view of stratified epithelium from bottom right of (a). Features of nuclear hyperchromatism, increased nuclear cytoplasmic ratio and heightened mitotic activity suggest focal mild to moderate dysplasia. Bar marker represents 500 μm.

the DNA of the putative new HPV type (HPV-45). HindIII digestion of the cellular DNAs revealed a single 8 kb band indicating that this enzyme cuts the HPV-45 genome once. There is one EcoRI site and no HindIII site in the HPV-18 genomic DNA. The HPV-45 DNA was cloned at the unique HindIII site in pLV47 and subcloned in pGEM-1. The restriction endonuclease digest pattern of the cloned HPV-45 DNA was identical with that of the HPV in the original sample. Cellular DNAs and tissues from the patient’s lesions were screened for HPV-45 sequences utilizing probes made from the cloned HPV-45 DNA. The cellular DNA from exfoliated cells of the patient was estimated to contain more than 500 copies of HPV-45 DNA per cell, whereas the cellular DNA from the biopsy taken at the same time contained about 20 copies per cell. All detectable viral DNA was present in a monomeric episomal state in both samples. HPV-45 DNA was also identified in paraffin sections of both the initial and the recurrent lesions of the patient by in situ hybridization with a 35S-labelled HPV-45 probe. The sections were non-reactive with HPV-6 and HPV-16 probes and weakly cross-reactive with an HPV-18 probe.

The physical map of HPV-45, developed by partial digests of the cloned DNA, is shown in Fig. 3. The organization of the genome was studied by hybridization of PvuII-digested fragments of HPV-45 with 32P-labelled subgenomic probes of HPV-6b as described previously (Boshart et al., 1984). The assignment of early and late regions of the HPV-45 genome is indicated in Fig. 4.

In order to examine the cross-reactivity between HPV-45 and other genital tract viruses, 100 pg of HPV-6, HPV-11, HPV-13, HPV-16, HPV-18 (provided by H. zur Hausen), HPV-31 and
Fig. 3. Physical map of HPV-45 linearized with HindIII. The following enzymes did not cut HPV-45 DNA: BclI, BgII, EcoRI, HpaI and MspI.

Fig. 4. Colinearity between HPV-45 and HPV-6b. Fragments of the BamHI-linearized HPV-6b clone were generated with EcoRI and PstI as indicated and purified from gels. HPV-45 was released from the vector, purified, digested with PvuII, and subjected to Southern transfer and hybridization under non-stringent conditions ($T_m = 37^\circ C$) with purified HPV-6b fragments as probes. Cross-hybridization between fragments of HPV-6b and HPV-45 is indicated by arrows. Open reading frames for HPV-6b DNA as determined by Schwarz et al. (1983) and representing putative genes are indicated above the homology map.

Fig. 5. Extent of homology between HPV-45 and HPV-6, -11, -13, -16, -18 and -31. One-hundred pg of pHV-6, -11, -13, -16, -18, -31 and -45 DNA were digested at the insertion site, separated from the vector by 1% agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized with pHV-6 (a), pHV-16 (b), pHV-18 (c) and pHV-45 (d) under moderately stringent conditions ($T_m = 25^\circ C$). The genomes of HPV-6, -11, -13, -16, -18, -31 and -45 are seen as 8 kb bands. HPV-13 is cut more than once with BamHI; the largest fragment is seen as a 6 kb band. pBR322 vector is seen at 4.2 kb. The 3 kb Gemini vector of HPV-45 is not shown.
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HPV-45 DNAs were digested at the insertion sites to remove the HPV DNAs from their vectors and were hybridized (Fig. 5) with HPV-6 (a), HPV-16 (b), HPV-18 (c) and HPV-45 (d) probes (pHP plasmids) under moderately stringent conditions (T<sub>m</sub> = 25 °C). The HPV-45 probe detected the heterologous viral DNAs more efficiently than probes of HPV-6, HPV-16 or HPV-18. Under stringent conditions of hybridization (T<sub>m</sub> = 17 °C), HPV-45 hybridized only with HPV-18 (data not shown). In order to confirm these findings and to examine the relationship of HPV-45 to additional HPV types, Drs E. M. de Villiers and L. Gissmann (German Cancer Center, Heidelberg, F.R.G.) labelled the cellular DNA from which HPV-45 was cloned and tested it against a panel of all available previously cloned HPV DNAs, as described (de Villiers et al., 1986). Positive hybridization was seen only with the DNA of HPV-18 (E. M. de Villiers, personal communication).

The relationship between HPV-18 and HPV-45 was therefore further examined by filter hybridization of restriction enzyme fragments of HPV-45 and also by reassociation kinetics. HPV-45 DNA was cleaved with each of four different restriction enzymes (Fig. 6a, b, c, d) and hybridized with HPV-45 (lane 1) and HPV-18 (lane 2) under highly stringent conditions (T<sub>m</sub> = 10 °C). In each restriction enzyme digest, some fragments of HPV-45 showed a strong homology with HPV-18, and others showed barely detectable or no homology. It was estimated that approximately 25% of the HPV-45 genome contained sequences which were quite dissimilar to
Fig. 7. Reassociation of 100 pg of \(^{32}\)P-labelled HPV-18 DNA with 100 ng of HPV-18 (△), 50 ng of HPV-18 (■), 25 ng of HPV-18 (▲), 100 ng of HPV-45 (○) and 100 ng of *Escherichia coli* (○) DNA.

those of HPV-18. In reassociation kinetics studies, the curve of 25% sequence homology closely matched the data points obtained with the heterologous DNA (Fig. 7). A reciprocal experiment in which labelled HPV-45 DNA was reacted with unlabelled HPV-45 and HPV-18 DNAs gave comparable results (data not shown). The degree of relatedness between HPV-18 and HPV-45 in the above experiment was similar to that previously reported between HPV-6 and HPV-11 (Gissmann et al., 1982).

In order to determine the prevalence of HPV-45 infection in the genital tract, we re-tested cellular DNAs from over 600 genital tract tissues of women from the U.S.A. for HPV-45 sequences. These specimens, which included 52 squamous cell carcinomas, 90 cervical intraepithelial neoplasias I to III, 51 condylomata and over 400 normal cervical tissues, were screened in earlier investigations with probes of HPV-6, HPV-11, HPV-16, HPV-18 and HPV-31. Only three specimens (one each of cervical carcinoma, condyloma and normal cervical squamous epithelium) were found to contain HPV-45 sequences. In the previous studies, one of these specimens was diagnosed as HPV-18 and the other two were classified as 'unidentified HPV'. Cellular DNAs from 30 tissues previously shown to contain HPV-18 did not cross-hybridize with HPV-45.

The above results indicate that HPV-45 is a newly recognized HPV-18-related virus which infects the genital tract. In the patient from whom its genome was cloned, the virus was associated with recurrent cervical lesions of mild to moderate dysplasia with koilocytosis. In a survey of genital tract tissues in the U.S.A., the viral genome was found rarely, but was associated with lesions across a wide histological spectrum which ranged from normal epithelium to invasive carcinoma.

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REFERENCES
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