Human Papillomavirus Type 56: a New Virus Detected in Cervical Cancers

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SUMMARY

A new human papillomavirus type (HPV-56) was identified by low stringency Southern blot analysis with an HPV-31 DNA probe, in a cervical intraepithelial neoplasm (CIN). The DNA of this virus was molecularly cloned and shown to be a new HPV type based on the absence of cross-reactivity to HPV types 1 to 55 under high-stringency hybridization conditions. At low stringency, HPV-56 was most related to HPV types 30 and 45. The deduced organization of the open reading frames of HPV-56, from hybridization and partial nucleotide sequence analyses, reveals a typical HPV genome. HPV-56 was detected in two of 464 normal cervical tissues, in five of 227 cervical condylomas and CIN, and in two of 84 invasive cancers of the cervix.

More than 15 distinct human papillomavirus (HPV) types are known to infect the mucous epithelial lining of the anogenital tract (Beaudenon et al., 1986, 1987; Boshart et al., 1984; de Villiers et al., 1981; Dürst et al., 1983; Gissmann et al., 1982; Lörincz et al., 1986, 1987a, 1989; Naghashfar et al., 1987; Nuovo et al., 1988; Shimoda et al., 1988). Some of these HPVs, such as types 16, 18 and 31 (Boshart et al., 1984; Dürst et al., 1983; Lörincz et al., 1986) are commonly detected in normal, premalignant and malignant cervical tissues. These three types individually (or, rarely, in combination) are detected in approximately 70% of cervical cancers (Fuchs et al., 1988; Lörincz et al., 1987b; Reid et al., 1987). The remaining 20% of HPV-positive cancers (as revealed by Southern blot analysis) are infected with a diverse group of HPVs such as types 33, 35, 39, 45, 51 and 52, which are each found in approximately 1 to 5% of such lesions. HPV-56 is a new HPV type which was detected in two of 84 (2.4%) cervical cancers and appears to belong to this group of low-prevalence cancer-associated HPVs.

Previously we reported the cloning of six new HPV types from anogenital specimens (Lörincz et al., 1986, 1987a, 1989; Naghashfar et al., 1987; Shimoda et al., 1988). Extensive hybridization studies revealed additional putatively new HPVs. The low-stringency Southern blot method used to detect new HPVs has been described in detail (Lörincz et al., 1986). HPV-56 DNA was originally identified by low-stringency hybridization with an HPV-31 probe (Fig. 1), in a specimen (GU56) of cervical intraepithelial neoplasia (CIN) obtained from a woman in the Washington, D.C. metropolitan area. Cellular DNA from this specimen was digested with BamHI or EcoRI and analysed by Southern blotting. The digest yielded a 7-9 kb HPV DNA fragment with BamHI, and 5 kb and 2.9 kb fragments with EcoRI. In addition, specimen GU56 had several other weaker bands in both BamHI and EcoRI digests; these were later demonstrated to be due to the presence of another HPV type. The HPV-related DNA was molecularly cloned into the BamHI sites of AL47, as described previously (Lörincz et al., 1986). Hybridization of filter plaque-lifts from 2 × 10⁵ plaques revealed over 10 positive plaques. One 7-9 kb BamHI clone of GU56 was chosen and recloned into plasmid pT713 for further analysis.

To ensure that this clone represented a new HPV type, it was used as a probe to hybridize with Southern blot filters containing DNAs of all the HPV types known at that time (HPV types 1 to 55). Southern blot analysis of the HPV types that were available in our laboratory, using the HPV DNA clone from sample GU56 as probe, is shown in Fig. 2. Filters with most of the remaining HPV types were obtained from E.-M. de Villiers and G. Orth (data not shown). At
Fig. 1. Identification of a new HPV type by Southern blot analysis. Clinical specimens were digested with proteinase K and SDS to release cellular DNA, which was then extracted with phenol–chloroform and precipitated with ethanol. Five to 10 μg of DNA from each specimen was digested with PstI and electrophoresed in a 1% agarose gel. A Southern blot filter was prepared on nitrocellulose and hybridized at low stringency using 32P-labelled DNA of HPV type 31 (a). After autoradiography the filter was rehybridized with an HPV-16 probe at high stringency (b). The HPV types identified in each specimen are shown above the lanes in (a). HPV types 6, 16, 18, 31 and 42 were identified by interpretation of PstI restriction fragment patterns and by hybridizing this or a similar filter with individual DNA probes as shown for type 16 in (b) (other data not shown). The faint bands seen in lanes
Fig. 2. Analysis of homology between HPV-56 and other cloned HPVs. HPV DNAs were liberated from their plasmid vectors and 10 ng per lane was electrophoresed in a 1% agarose gel. The DNAs were transferred to Hybond and hybridized at low stringency (a) and high stringency (b) with a 32P-labelled HPV-56 DNA probe. The HPV types in each lane are shown along the top of (a). At low stringency HPV-56 was most homologous with HPV-30 and HPV-45. In contrast, at high stringency HPV-56 did not cross-react with any HPV type.

High stringency, no hybridization was detected between the GU56 clone and any other known HPV types. The Heidelberg HPV Reference Center tested the new isolate against all remaining HPV clones and found no high-stringency homology (E.-M. de Villiers and G. Orth, personal communication) thus this new HPV isolate was named HPV-56.

The genomic organization of HPV type 56 was deduced by hybridization analysis, as previously described (Lörincz et al., 1986) using subgenomic fragments of HPV-6b (Schwarz et al., 1983) as probes. HPV-56 was subjected to restriction endonuclease digestion with BamHI plus EcoRI, plus EcoRV. The resulting DNA fragments were separated by gel electrophoresis and blots were prepared on nitrocellulose. Filters containing the restriction endonuclease digests

6, 9 and 13 of (a) do not represent HPV DNAs. These bands are due to cross-reactivity of the HPV-31 probe at low stringency to the larger amounts of human DNA present in lanes 6, 9 and 13, as revealed by ethidium bromide staining of the gel prior to Southern transfer (data not shown). This cross-reactive property of the HPV-31 probe was also noted in many other Southern blot analyses. The HPV homologous DNA in the specimen analysed in lane 5 (GU65) was considered to be a putative new HPV type because it was not recognized by any of the known HPV probes at high stringency. Hence this HPV DNA was cloned into λL47. Subsequent hybridization of the GU56 specimen with the cloned HPV-56 probe at high stringency (c, lane 2) revealed that in addition to the HPV-56 DNA this specimen had another unknown HPV type, which has not yet been cloned. The presence of the unknown HPV type was inferred from the inability of the HPV-56 probe to detect under highly stringent conditions the two lower Mr bands seen in lane 5 of (a). Lane 3 of (c) shows the result of a high stringency probing of a different clinical specimen with HPV-56 DNA. This overexposed autoradiograph reveals the faint 0.45 kb PstI band of HPV-56 (arrow) predicted from the restriction endonuclease map shown in Fig. 3. The specimens in each lane are as follows. Panels (a) and (b): lane 1, CIN 1; lane 2, CIN 1; lane 3, normal cervix; lane 4, normal cervix; lane 5, CIN 1; lane 6, cervical squamous cancer; lane 7, normal cervix; lane 8, vulvar condyloma; lane 9, cervical adenocarcinoma; lane 10, normal vulva; lane 11, molecular size standards (kb); lane 12, vulvar intraepithelial neoplasia; lane 13, cervical squamous cancer; lane 14, CIN 1. Panel (c): lane 1, molecular size markers (kb); lane 2, CIN 1; lane 3, CIN 2.
of HPV-56 were hybridized at low stringency with the electrophoretically purified DNA fragments of HPV-6b generated using *BamHI* plus *EcoRI* plus *PstI* (de Villiers et al., 1981). These fragments representing the genome of HPV-6b hybridized to HPV-56, and each contiguous HPV-6b fragment hybridized with a specific contiguous HPV-56 fragment. To verify the deduced positions of the open reading frames (ORFs) shown in Fig. 3, the E6 and E7 ORFs of HPV-56 were sequenced by the dideoxynucleotide chain termination method (Fig. 4). The vertical line above the restriction endonuclease map of HPV-56 (Fig. 3) indicates the starting position of the E6 protein predicted by the nucleotide sequences. The arrow points toward the COOH terminus. Thus, the sequence arrangement of HPV-56 is consistent with the typical HPV genome.

Several hundred cervical specimens obtained in previous studies (Lörincz et al., 1987b; Reid et al., 1987) were analysed for the presence of this HPV DNA. DNA was extracted from these specimens and was digested with *PstI* for Southern blot analysis as previously described (Lörincz et al., 1986). HPV-56 was detected in two of 464 normal cervical tissues, in three of 122 condylomas and CIN 1, in two of 105 CIN 2 and CIN 3, and in two of 84 cervical cancers (including 11 adenocarcinomas, all negative for HPV-56).

Approximately 900 bp in the region of the E6 and E7 ORFs of HPV type 56 were sequenced. Although HPV-56 is most related to HPV types 30 and 45 by hybridization studies, these HPVs have not yet been sequenced. Thus a comparison of the partial nucleotide sequence of HPV-56 to the published sequences of HPV-16 (Seedorf et al., 1985) and HPV-31 (Goldsborough et al., 1989) is shown in Fig. 4. HPV-56 is most related to HPV-31 in the E6–E7 region in which 60% of the nucleotide bases are identical. HPV-56 and HPV-16 share 56% identical nucleotides in this region.

Analysis of the nucleotide sequences in Fig. 4 shows the predicted proteins encoded by the E6 ORFs of HPV types 16, 31 and 56 to be 158, 149 and 155 amino acids long, respectively. Comparison of the E6 ORFs (Fig. 4b) reveals that the HPV-56 E6 protein has 52% of its amino acids identical to those of the HPV-16 E6 protein, and 59% are identical to those of HPV-31. The predicted E7 proteins of HPV types 16, 31 and 56 are 98, 98 and 105 amino acids long, respectively (Fig 4c). In this region also, HPV-56 is most related to HPV-31, sharing 47% identical amino acids. HPV-56 and HPV-16 are identical at 44% of the amino acid positions.

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**Fig. 3.** Restriction endonuclease map of HPV-56 and positions of predicted ORFs. Homology mapping experiments with subgenomic fragments of HPV-6b and partial nucleotide sequence analysis revealed the genomic organization of HPV-56. The approximate midpoints of the recognized ORFs are shown below the map (URR, upstream regulatory region). The vertical line and the rightward pointing arrow indicate the positions of the initiating methionine of the E6 protein and the direction of translation respectively as deduced by nucleotide sequencing. HPV-56 was not cut by *BglI*, *HindIII*, *PvuI*, *SalI*, *SstI* or *XhoI*.

**Fig. 4.** DNA sequence in the E6–E7 region of HPV-56. To prepare DNA for sequencing, subgenomic fragments containing the E6 and E7 ORFs were made blunt by Klenow polymerase and subcloned in both orientations into M13mp19. Nested deletions were prepared by exonuclease III digestion of each subgenomic fragment. The resulting deleted DNAs were cloned and sequenced using the chain terminating dideoxynucleotide method. (a) Nucleotide sequence comparison of HPV-56 to HPV-16 and HPV-31. Sequence comparisons begin at nucleotide 1 as defined for HPV-16 (Seedorf et al., 1985).
The predicted E6 protein of HPV types 16, 31 and 56 begin at nucleotides 83, 108 and 101, and terminate at nucleotides 556, 554 and 565, respectively. The predicted E7 protein of HPV types 16, 31 and 56 begin at nucleotides 562, 560 and 571, and terminate at nucleotides 855, 853 and 885, respectively. The numbers to the right of the sequences are the numbers of nucleotides in each line. (b) Predicted amino acid sequence of the E6 protein of HPV types 16, 31 and 56. Putative zinc finger motifs (Cys-X-X-Cys) in the E6 proteins are underlined. (c) Predicted amino acid sequence of the E7 protein. The retinoblastoma protein binding motif and the zinc finger motifs are underlined.
The E6 and E7 proteins of HPV-56 have the typical features characteristic of the other sequenced HPV types. The numbers and positions of the zinc finger (Cys-X-X-Cys) motifs are conserved, as is the retinoblastoma protein binding motif in the E7 protein at positions 22 to 35, which resembles that of the other cancer-associated HPV types (Goldsborough et al., 1989). HPV-56 also has the correct splice donor and acceptor sequences to produce an E6* mRNA (Schneider-Gadicke & Schwarz, 1986). Thus on the basis of both clinical studies and molecular studies HPV-56 may be most appropriately classified with HPV types 31, 33, 35 and others as a virus with an ‘intermediate’ association with cervical cancer.

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REFERENCES


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