

A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions

(human papillomaviruses/low-stringency hybridization/molecular cloning/genital tumors)

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Communicated by Gertrude Henle, March 21, 1983

ABSTRACT DNA from one biopsy sample of invasive cancer of the cervix contained sequences hybridizing with human papillomavirus (HPV) type 11 DNA only under nonstringent conditions. This DNA was molecularly cloned in λ phage. Under stringent conditions of hybridization it cross-hybridized to a minor extent (less than 0.1%) with HPV types 10, 14, and 15 and showed no homology with DNA of other human HPV types. We therefore propose to designate it tentatively as HPV 16. HPV 16 DNA was used as a probe to test additional cancer biopsy samples from cervical, vulval, and penile cancer, as well as benign genital warts (condylomata acuminata) and cervical dysplasias for the presence of homologous sequences. In 61.1% (11/18) of cervical cancer samples from German patients sequences were found hybridizing with HPV 16 DNA under conditions of high stringency. In contrast, only 34.8% (8/23) of cancer biopsy samples from Kenya and Brazil revealed this DNA. Vulval and penile cancer biopsy samples hybridized to 28.6% (2/7) or 25% (1/4), respectively. Only 2 out of 33 condylomata acuminata contained HPV 16 DNA. Both positive tumors harbored in addition HPV 6 or HPV 11 DNA. The data thus indicate that HPV 16 DNA prevails in malignant tumors, rendering an accidental contamination with papillomavirus DNA from adjacent papillomas rather unlikely. The rare presence in benign genital papillomas in addition to common genital papillomaviruses suggests a dependence of HPV 16 replication on helper virus.

There is increasing evidence that papillomaviruses may be involved in the etiology of certain cancers in animals and humans (for review see ref. 1). This accounts for carcinomas induced by the Shope papillomavirus in rabbits, fibrosarcomas and esophageal carcinomas induced by bovine papillomaviruses, ocular carcinomas in cattle and cutaneous carcinomas in sheep, and malignant conversions observed in epidermodysplasia verruciformis as well as occasionally in laryngeal papillomatosis. It is a common feature of malignant conversion after papillomavirus infection that it appears to require additional interaction with initiating events (2).

Human genital cancer reveals epidemiological characteristics of infectious events (3). Two virus groups have been found to play a role: herpes simplex viruses (4, 5) and human papillomaviruses (HPVs) (6, 7). Because our group consistently failed to detect herpes simplex DNA in cervical cancer biopsy specimens but provided evidence for initiator-like functions of these virus infections (ref. 8; unpublished data), we focused our interest on the persistence of papillomavirus DNA within genital tumors.

Two types of genital papillomavirus infections have been regularly demonstrated in genital papillomas (condylomata ac-

uminata and atypical condylomata of the cervix): HPV 6 and HPV 11 (9–11). There may exist additional types within benign genital tumors (12).

Recently, three groups reported the presence of HPV DNA in some genital tumors: Green *et al.* (13) reported that 2 out of 31 cervical carcinomas and 2 out of 10 vulval carcinomas contained sequences related to a virus that probably represents HPV 10. Zachow *et al.* (14) reported DNA hybridizing with HPV 6 DNA under stringent conditions in two verrucous carcinomas of the vulva and one carcinoma *in situ* of the vulva. Under nonstringent conditions DNAs from one additional verrucous carcinoma and three carcinomas *in situ* of the vulva hybridized with DNA used by Green *et al.* (13) in their study. Gissmann *et al.* (refs. 10 and 15; unpublished data) detected DNA cross-reacting with or identical to HPV 6 or HPV 11 DNA in 6 Buschke-Löwenstein tumors (nonmetastasizing verrucous carcinomas) and in 5 out of 27 cervical cancer biopsy samples (3 of them representing invasive cancer and 2 carcinomas *in situ*). In the present study DNA hybridizing with HPV 11 under nonstringent conditions in one of these tumors was molecularly cloned and used as a probe for testing benign and malignant genital tumors for the presence of homologous sequences. The data reveal a startling prevalence of this DNA in malignant tumors and its very occasional presence in benign papillomas.

MATERIAL AND METHODS

Blot Hybridization of Cellular DNA. Tissue biopsy samples were kept frozen at -20°C . DNA was prepared by phenol/chloroform/isoamyl alcohol extraction as described (10). About $10\ \mu\text{g}$ of tumor DNA was cleaved by using various restriction endonucleases, separated on agarose gels, and transferred onto nitrocellulose filters.

Hybridization was done in 0.75 M NaCl/0.075 M sodium citrate and various concentrations of formamide at 42°C (10) with tRNA instead of calf thymus DNA as carrier. ^{32}P -Labeled HPV DNA cloned in plasmid pBR322 was purified from the vector by electrophoresis prior to nick-translation. Filters were washed in 0.30 M NaCl/0.03 M sodium citrate/0.1% sodium dodecyl sulfate at the respective hybridization temperature. For screening of tumor DNA, filters were hybridized under conditions of low stringency (40°C below the melting temperature, t_m), kept moist before and during exposure, rewashed under stringent conditions (20°C below t_m), and exposed again.

Cloning of Viral DNA. HPV DNA from a cervical carcinoma (laboratory code WV 2916) was cloned in bacteriophage λ L 47 at the single *Bam*HI site (11) and subcloned in pBR322.

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Abbreviations: HPV, human papillomavirus; kb, kilobase pair(s).

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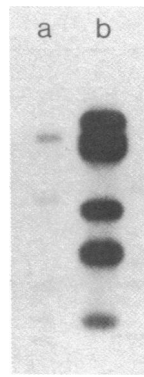


FIG. 1. Blot hybridization at low stringency of ³²P-labeled HPV 11 DNA with *Pst* I-cleaved cellular DNA from a cervical carcinoma (WV 2916, lane a) and a laryngeal papilloma containing HPV 11 DNA (lane b).

RESULTS

By screening of a large number of cervical carcinomas under nonstringent conditions of hybridization (unpublished data) with HPV 11 DNA (11), one tumor (WV 2916) was found to harbor papillomavirus sequences with an unusual *Pst* I cleavage pattern (Fig. 1) not detectable under stringent conditions. Further analysis of WV 2916 DNA (Fig. 2) showed a main band of 7.2 kilobase pairs (kb) after *Bam*HI cleavage. This fragment was shortened by cleavage with *Eco*RI and migrated with the bulk of cellular DNA after *Hind*III cleavage (see also Fig. 3).

Additional bands of different length in lower concentrations were seen in each case (Fig. 2 *Left*, lanes b–d). Washing at conditions of high stringency (Fig. 2 *Right*) removed virtually all signals in WV 2916 DNA compared to positive controls (Fig. 2, lanes a, e, and f). The construction of a genomic library and subsequent screening of plaques with ³²P-labeled HPV 11 DNA under nonstringent conditions resulted in a number of recombinant phages containing the 7.2-kb fragment. Furthermore, several positive hybridizing plaques harbored DNA inserts of various lengths corresponding to the additional fragments seen in Fig. 2. The characterization of this DNA will be reported elsewhere.

The 7.2-kb fragment was subcloned in pBR322 and char-



FIG. 3. Blot hybridization at high stringency with ³²P-labeled 7.2-kb DNA isolated from a cervical carcinoma (WV 2916) after molecular cloning. WV 2916 DNA was cleaved with *Bam*HI (one cleavage site, lane a), *Eco*RI (two cleavage sites, lane b), and *Hind*III (no cleavage site, lane c). The 7.2-kb DNA exists in the tumor in an episomal state (unpublished data) but obviously not in a monomeric form.

acterized with different restriction enzymes (unpublished data). Hybridization of WV 2916 DNA with labeled 7.2-kb DNA under stringent conditions (Fig. 3) resulted in a pattern similar to that seen in Fig. 2. However, the additional fragments were not detected with this probe, thus indicating heterogeneity of HPV sequences in this cervical carcinoma.

Blot hybridization of the 7.2-kb fragment with DNA from human papillomavirus types thus far characterized under stringent condition resulted in a positive reaction only with HPVs 10, 14, and 15 (G. Orth, personal communication) and one yet-unclassified isolate from an epidermodysplasia verruciformis patient (G. Orth, personal communication). The extent of cross-hybridization with these types was estimated to be less than

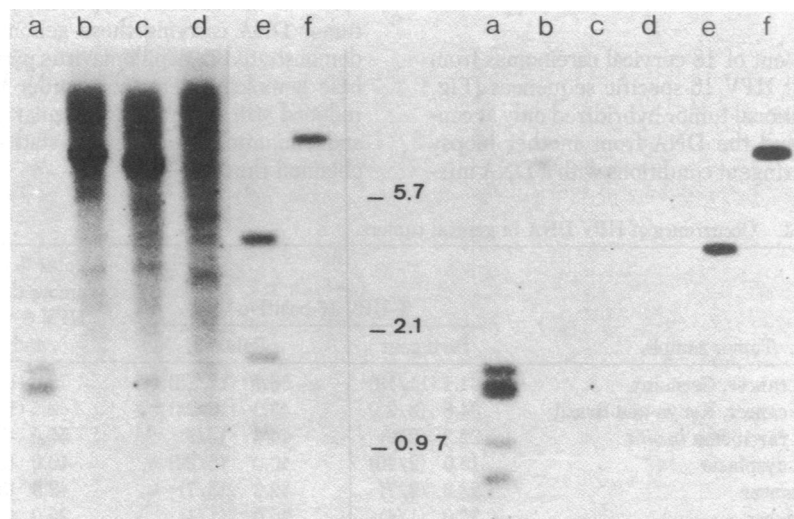


FIG. 2. Blot hybridization at low stringency of ³²P-labeled HPV 11 DNA. The DNA tested was WV 2916 DNA cleaved by *Bam*HI (lanes b), *Eco*RI (lanes c), and *Hind*III (lanes d). HPV 11a DNA (lanes a) and HPV 6a DNA (lanes e) cleaved by *Pst* I and 10 pg of linearized HPV 11 DNA (lanes f) were applied as controls. The filter was washed at 40°C below *t_m* (*Left*) and rewashed after exposure at 20°C below *t_m* (*Right*). Hybrids with HPV 6 DNA were partially melted at the high temperature (11). Length markers are given in kb.

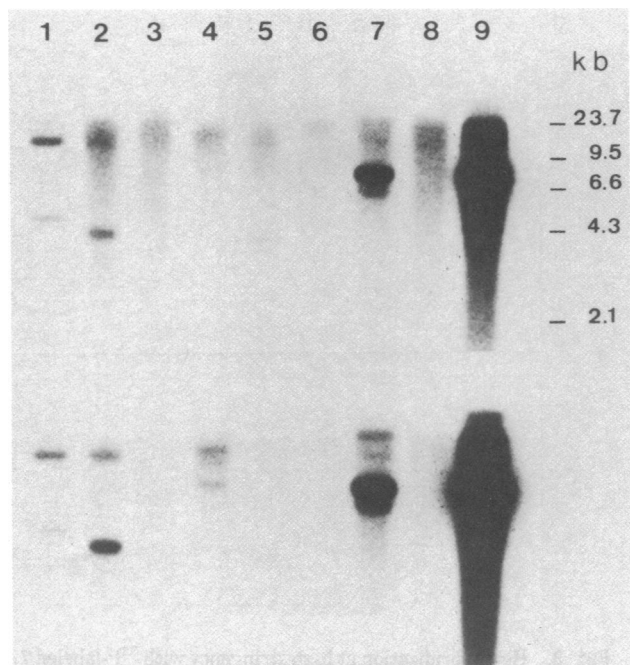


FIG. 4. Blot hybridization with ^{32}P -labeled 7.2-kb (HPV 16) DNA. Cellular DNAs from invasively growing cervical carcinomas (lanes 2, 4, 5, 7, and 9), one dysplasia (lane 6), two carcinomas *in situ* of the cervix (lanes 1 and 5), and one vulval carcinoma (lane 3) were cleaved by *Bam*HI. Hybridization and washing at low stringency (*Upper*) and rewashing at high stringency (*Lower*) are shown. Samples 1, 2, 4, 5, 7, and 9 were positive under both conditions. Positive bands in 2, 4, and 7 are seen more clearly after removal of unspecific background at high temperature.

0.1%. It was therefore concluded that the 7.2-kb fragment from WV 2916 DNA represents another papillomavirus type, tentatively designated HPV 16.

HPV 16 DNA purified from the vector was used as probe for screening of a series of genital warts, dysplasias, and carcinomas *in situ*, as well as invasively growing genital carcinomas. Hybridization at low stringency followed by subsequent washing at high stringency after a first exposure permitted the detection of HPV 16-specific as well as more distantly related papillomavirus sequences in the same experiment (Figs. 4 and 5).

As shown in Table 1, 11 out of 18 cervical carcinomas from German patients contained HPV 16-specific sequences (Fig. 4). The DNA from one additional tumor hybridized only at conditions of low stringency and the DNA from another biopsy sample hybridized under stringent conditions with a DNA mix-

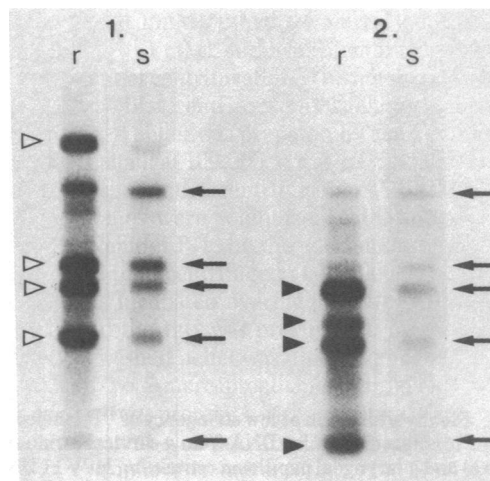


FIG. 5. Blot hybridization of *Pst* I-cleaved cellular DNA extracted from two different genital warts with ^{32}P -labeled HPV 16 DNA under relaxed conditions (r) with a subsequent wash at high stringency (s). Sample 1 contains HPV 6a DNA (\triangleright), sample 2 harbors HPV 11b DNA (\triangleright), detected under low-stringency conditions. HPV 16-specific DNA fragments (arrows) were not removed at high temperature.

ture of HPV 8, 9, 10, and 11 DNAs. The DNAs from only 8 out of 23 biopsy samples obtained from Africa and South America reacted under stringent conditions with HPV 16 DNA. The higher percentage of negative non-European tumors (65.2%) and those materials containing detectable concentrations of different papillomaviruses (8.7%) suggest that other virus types might be prevalent in tumors from patients in these areas.

HPV 16-specific and related sequences were also found in some dysplasias and carcinomas *in situ* of the cervix as well as in two vulval and one penile cancer (see Table 1). However, only 2 of 33 (6.1%) genital warts contained HPV 16 DNA as determined by the characteristic *Pst* I cleavage pattern (Fig. 5). Interestingly, these two tumors also harbored either HPV 6 or HPV 11 DNA.

DISCUSSION

Characterization of papillomavirus types is at present based exclusively on analysis of the DNA (16) after molecular cloning. Many of the classified types have been identified only from tumor DNA carrying these genomes and have not yet been demonstrated in papillomavirus particles (11). A low degree of base homology, detectable under hybridization conditions of reduced stringency (17), a similar gene organization (18, 19), and a nonintegrated episomal state (9) characterize the isolates obtained thus far.

Table 1. Occurrence of HPV DNA in genital tumors

Tumor sample	% HPV 16-positive		Total % positive tumors (including HPV 6, 8, 9, 10, and 11)
	Stringent	Relaxed	
Cervical cancer, Germany	61.1 (11/18)	66.7 (12/18)	72.2 (13/18)
Cervical cancer, Kenya and Brazil	34.8 (8/23)	43.5 (10/23)	43.5 (10/23)
Cervical carcinoma <i>in situ</i>	22.2 (2/9)	44.4 (4/9)	55.6 (5/9)
Cervical dysplasia	10.0 (2/20)	40.0 (8/20)	40.0 (8/20)
Vulval cancer	28.6 (2/7)	42.9 (3/7)	42.9 (3/7)
Penile cancer	25.0 (1/4)	25.0 (1/4)	25.0 (1/4)
Condyloma acuminatum	6.1 (2/33)	81.8* (27/33)	90.9 (30/33)

Numbers in parentheses indicate the number of positive samples and the total number of samples tested.
* Only biopsy samples containing higher concentrations of HPV 6 or HPV 11 DNA reacted with HPV 16 DNA under conditions of low stringency.

Similar features are revealed by the papillomavirus DNA isolated directly from a biopsy sample of human invasive carcinoma of the cervix. This DNA hybridizes with HPV 6 or HPV 11 under nonstringent conditions and hybridized to a very low degree (less than 0.1%) with HPV 10, 14, 15, and a yet-unclassified type isolated from an epidermodysplasia verruciformis patient (G. Orth, personal communication) at high stringency, whereas no positive reaction was obtained with the other virus types under those conditions. A comparison with HPV 7 DNA was not possible because cloned DNA of this type is not yet available. The fact that up to now this papillomavirus DNA has been found to occur exclusively in butcher's hand warts (20, 21) renders identity with the cervical papillomavirus DNA rather unlikely. The isolate described here is therefore tentatively designated as HPV 16. HPV 16 DNA exists in an extrachromosomal state in the tumor (unpublished data). The circular molecules, however, seem to be present in multimeric forms.

The presence of this DNA in more than 60% of cervical cancer biopsy specimens from German patients and its absence from most benign papillomas from the same region is a startling observation. It reveals a remarkable specificity of HPV 16 infections for malignant tissue. This renders an accidental contamination from adjacent papilloma tissue rather unlikely. The presence of this DNA in 2 out of 33 condylomata acuminata in addition to much larger quantities of either HPV 6 or HPV 11 could provide a clue for the peculiar distribution of HPV 16 DNA. Although HPV 16 exists solely as circular molecules of monomeric length in these lesions (unpublished data), one is tempted to speculate that HPV 16 DNA is defective and requires complementation by additional papillomavirus types for particle encapsidation and infectivity. This of course has to be substantiated by further experimentation.

The DNA showed some heterogeneity in cancer tissue, suggesting either the existence of various subtypes or genomic rearrangements during cancer development.

There seems to exist some geographic difference in the incidence of HPV 16 infections in human genital cancer. Only 34.8% of cervical cancer biopsy specimens obtained from Kenya and Brazil contained cross-hybridizing sequences. This may reflect the prevalence of other papillomavirus types in these regions. Indeed, at least three additional types have been demonstrated within human genital cancer biopsy samples: HPV 6 in Buschke-Löwenstein tumors or verrucous carcinomas of vulva and penis (10, 14), virus DNA probably identical with HPV 10 in 2 out of 31 cervical cancers and in 2 out of 10 vulval cancers (13), and HPV 11 DNA in a few cervical cancers (15). It is thus apparent that different types of papillomaviruses can be found in genital squamous cell carcinomas. This is further underlined by this study, which has revealed additional positive tumors by hybridizing under conditions of low stringency with other types of HPV DNA. At present, the total percentage of positive cervical cancer biopsy samples (German cases) amounts to 72%. We do expect that characterization of further types of HPV will in-

crease the percentage of positive tumors.

The regular presence of HPV DNA in genital cancer biopsy samples does not *per se* prove an etiological involvement of these virus infections, although the apparent cancer specificity of HPV 16 is suggestive of such a role. Their biological significance as well as the proposed interaction with initiating events (2) certainly requires further investigation.

Note Added in Proof. Recent data indicate that 13/18 biopsy specimens obtained from Bowen disease or bowenoid papulosis harbor HPV 16 sequences. One additional biopsy specimen contained HPV sequences that have not yet been characterized. Thus it appears that HPV 16 is also a prevalent virus infection in these lesions.

We are very grateful to Dr. G. Orth for his help in typing the HPV 16 DNA. The skillful technical assistance of Gabriele Brendel is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft SFB 31 (Medizinische Virologie: Tumorentstehung und -Entwicklung).

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