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Genotyping Human Papillomavirus Type 16 Isolates from Persistently Infected Promiscuous Individuals and Cervical Neoplasia Patients

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Nucleotide sequence variation in the noncoding region of the genome of human papillomavirus type 16 (HPV16) was determined by direct sequencing and single-strand conformation polymorphism analysis of DNA fragments amplified by PCR. Individuals of diverse sexual promiscuity and/or cervicopathology were studied. In a group of 14 healthy, monogamous HPV16-positive females, only two HPV16 sequence variants could be documented. Among 17 females and 3 males with multiple sex partners and living in the same geographical region, nine sequence variants were found, whereas among 7 patients with cervical neoplasia from another region, five variants were detected. Although numbers are limited, in the group of individuals at high risk of acquiring a sexually transmitted disease or with cervical neoplasia, a larger number of HPV16 sequence variants was encountered (two types among 14 individuals versus nine types among 20; Fisher's exact test, $P = 0.07$). Seven of the individuals were sampled repeatedly over time. For these persistently infected women, no differences in HPV16 sequences were detected, irrespective of promiscuity, and persistence of a single viral variant, spread over multiple anatomic sites, for more than 2 years could be demonstrated. This indicates that viral persistence may be a common feature and that successful superinfection with a new variant may be rare, despite a potentially high frequency of viral reinoculation.

Human papillomavirus type 16 (HPV16) is implicated as a causal agent in the development of cervical and penile carcinoma. This implication is based on the results of large epidemiological studies (16, 18, 21), and numerous studies analyzing the mechanism of sexual transmission of HPV16 have been initiated (1, 2, 4, 8, 9, 13). To date, however, the precise HPV16-induced mechanisms underlying viral persistence within an individual patient and viral spread among individuals are still largely unknown. Basic to understanding viral pathology are the molecular features of viral infection and persistence. A prerequisite for characterization of viral behavior is determination of genetic variation in the genome (7, 11, 12, 14, 26–28). Genetic variants of HPV can be characterized by physical analysis of genomic regions which have been amplified by PCR. Electrophoretic typing of mutants can be performed by single-strand conformation polymorphism (SSCP) analysis (19, 23), whereas the most-detailed identification can be attained by sequence analysis (5). In this paper, we describe the application of SSCP and direct sequence analysis for investigation of PCR-amplified HPV16 DNA obtained either incidentally or longitudinally from individual patients of diverse promiscuity and cervicopathology.

The main goal of the present study was to assess whether females persistently testing positive for HPV16 were infected by the same variant for the entire screening period. Simultaneously, by comparing results for individuals of diverse sexual promiscuity, the effect of frequent superinfection could be estimated. All females positive for HPV16 were enrolled in a long-term surveillance program, irrespective of their geographical origin. Individuals were included in the present study when multiple clinical samples were still available. In addition, several single samples were used to determine the basic level of genetic variation among Dutch HPV16 strains as found in either healthy individuals or patients with cervical neoplasia.

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MATERIALS AND METHODS

Clinical samples. Clinical material was derived from individuals from Amsterdam, Delft, Nijmegen, and Groningen, The Netherlands. For female patients, scrapes from different body sites were obtained. In all cases, cervical smears were obtained from the transformation zone by using Ayre spatulas which were immersed in phosphate-buffered saline, pH 7 (PBS). Sampling of the anus, rectum, or labia minora was done with wooden spatulas or cotton-tipped applicators. Samples were transported to the laboratory in PBS and stored at −20°C. An HPV16 strain from Nijmegen was detected in an anorectal biopsy specimen obtained from a condylomatous wart from a female patient. The clinical origins of all samples are described in Table 1.

Patients. HPV16-positive individuals can be grouped according to sexual promiscuity or the presence of cervical neoplasia. Moreover, samples from these patients can be characterized as incidental or as one of a longitudinally isolated series of specimens.

The group of people with multiple heterosexual partners, of which 70% were engaged in commercial sexual contacts, consisted of 3 men and 17 women selected from participants in a prospective study of HPV infection carried out at the Clinic for Sexually Transmitted Diseases of the Amsterdam Municipal Health Service (24). Demographic characteristics and medical history, including sexually transmitted diseases (STDs), were recorded. An additional questionnaire was used to assess the possibility of HPV-associated risk factors and sexual practices (Table 2). For 4 of these 20 individuals (prostitutes 35 through 38; Table 1), multiple samples, gathered over time, were available. For the other 16 individuals, incidental samples were obtained. These samples served as a reference panel for establishing the nature of the variants encountered in Amsterdam.

TABLE 1. Survey of sequencing data and SSCP analysis for amplified DNA from HPV16 strains isolated from patients with multiple sexual partners and less-promiscuous females involved in routine screening for cervical carcinoma[a]

Group or isolate type	Sex of patient[b] and strain no.	Geographic origin	Sample code[c]	Sampling date[d]	Mutation[e] at position:																	Sequence type[f]	SSCP type[g]
					7191	7192	7193	7231	7311	7312	7314	7357	7358	7385	7386	7405	7429	7430	7431	7433	7438	7447		
Single isolates from heterosexuals with multiple sex partners	M 1	Amsterdam	6	2/89	—	—	—	—	—	—	—	—	—	—	—	—	A	G	C	G	—	—	II	II
	F 2	Amsterdam	3	7/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 3	Amsterdam	5	5/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 4	Amsterdam	1	5/91	—	—	—	—	—	—	C	—	—	—	—	—	—	—	—	—	—	—	III	I
	F 5	Amsterdam	5	7/91	—	—	C	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	IV	I
	F 6	Amsterdam	1	6/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 7	Amsterdam	1	6/90	—	—	—	C	—	—	—	A	A	—	—	—	—	—	—	—	G	—	V	III
	M 8	Amsterdam	6	5/90	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	VI	I
	F 9	Amsterdam	5	1/90	—	—	—	—	C	G	—	—	—	—	—	—	—	—	—	—	—	—	VII	I
	F 10	Amsterdam	3	10/91	—	G	—	—	—	—	C	—	—	C	G	—	—	—	—	—	—	—	VIII	I
	F 11	Amsterdam	5	11/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 12	Amsterdam	1	12/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 13	Amsterdam	1	NK	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	ND
	F 14	Amsterdam	1	NK	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	C	IX	ND
	F 15	Amsterdam	1	NK	*	*	*	*	—	—	C	—	—	—	—	—	—	—	—	—	—	—	III	ND
	M 39a and b	Amsterdam	4, 5	3/91	—	—	—	—	—	—	C	—	—	—	—	—	—	—	—	—	—	—	III	IV
Single isolates from healthy, monogamous women	F 16	Delft	1	NK	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	VI	I
	F 17, F 18	Delft	1	NK	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 19	Delft	1	12/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 20	Delft	1	12/93	T	—	—	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	VI	I
	F 21	Delft	1	12/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 22	Delft	1	11/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Y	IX	I
	F 23, F 24	Delft	1	11/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 25, F 26	Delft	1	NK	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	ND
Single isolates from patients with cervical neoplasia	F 27	Groningen	1	93	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	C	IX	ND
	F 28	Groningen	1	93	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	ND
	F 29	Groningen	1	93	*	*	*	*	—	—	—	—	A	—	—	—	—	—	—	—	—	—	X	ND
	F 30	Groningen	1	93	*	*	*	*	—	—	C	—	—	—	—	—	—	—	—	—	—	—	III	ND
	F 31	Groningen	1	93	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	ND
	F 32	Groningen	1	93	*	*	*	*	—	—	—	—	—	—	—	—	—	—	—	—	—	C	IX	ND
	F 33	Groningen	1	92	*	*	*	*	—	—	—	—	—	—	—	C	—	—	—	—	—	—	XI	ND
Single isolate from condylomatous wart	F 34	Nijmegen	2	NK	T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	VI	ND
Persistently infected heterosexual females with multiple sex partners	F 35a, b, i and j	Amsterdam	1, 3, 4, 5	3/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 35c, d, e, and f	Amsterdam	1, 3, 4, 5	2/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 35g and h	Amsterdam	1, 3	2/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 36a	Amsterdam	1	2/89	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 36d and e	Amsterdam	1, 3	11/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 37a	Amsterdam	1	1/89	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 37b	Amsterdam	1	4/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 37c	Amsterdam	1	12/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 38a	Amsterdam	1	2/90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 38b	Amsterdam	1	6/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I

Persistently infected healthy, monogamous women	F 40a and b	Delft	1, 1	10/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 40c	Delft	1	3/92	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 40d	Delft	1	9/92	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 40e	Delft	1	2/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 40f	Delft	1	6/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 40g	Delft	1	12/93	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I
	F 41a and b	Delft	1, 1	10/91, 1/93	T	—	—	—	—	—	—	—	—	—	—	—	—	—	VI	I
	F 42a and b	Delft	1, 1	10/90, 10/91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I

[a] Strains have been grouped according to their geographic origin and whether they were obtained from persistent carriers or represented single, incidental isolates.

[b] M, male; F, female. Small letters after strain numbers indicate multiple samples.

[c] When two or more numbers are given, codes for multiple samples are indicated. Sample codes: 1, cervical smear; 2, anorectal biopsy specimen; 3, smear from labia minora; 4, and swab; 5, smear from proctum; 6, urethral swab.

[d] Given as month/year or year. Multiple dates indicate multiple samplings. Identical dates for individual patients indicate identical sampling days as well. NK, not known.

[e] The sequence previously presented by Seedorf et al. (22), GTAAGCAGGGCTCAGCTT, sequence type I, SSCP type not determined, has been used as the reference (consensus). The positions of the mutations are based on sequence numbering presented in reference 22. —, no mutation detected (identical to consensus); *, no sequence information available. The nature of the mutation is indicated in single-letter code; Y, in two separate experiments, either a C or a T was determined to be present at this position. Not shown is that in sequence 1, an inserted C was present after nucleotide 7439.

[f] The overall sequence type was deduced from the combination of mutations present in the amplified HPV16 DNA fragments.

[g] Based on the banding patterns observed for a subset of strains. ND, not determined.

For three patients, specimens collected from different anatomic sites during the same visit were positive for HPV16.

A group of monogamous, healthy women was recruited from in and around Delft. Fourteen females involved in a routine screening for cervical carcinoma were included. Again, an inventory of potential HPV risk factors and sexual practices was made. For this group, 11 incidental isolates were included. From two females (numbers 41 and 42; Table 1), HPV16-positive samples were obtained on two separate occasions, whereas from one patient (number 40), seven consecutive HPV16-positive cervical scrapes were collected over the years.

For the third group, material from seven females from Groningen was collected. This group of patients was known for having cervicopathological aberrations.

Finally, a single HPV16 strain from a patient in Nijmegen was included.

DNA isolation and amplification. The scrapings were kept frozen at −20°C until use. After thawing of the samples, tubes were vortexed rigorously for 30 s to release cells from applicators or spatulas. After removal of the spatulas and applicators, the tubes were centrifuged for 10 min at 3,000 rpm (Heddich Rodamda/AP centrifuge) and the cell pellet was resuspended in 0.5 ml of PBS. Of this fraction, a small portion (100 μl) was treated with pronase and DNA was isolated by phenol-chloroform-isoamyl alcohol extraction (17). The PCR assay for diagnosis of HPV16 infection was as described before (6). Amplified DNA was characterized by Southern blotting of analytical agarose gels and hybridization to an HPV16-specific DNA oligonucleotide probe which was previously labeled with [α-^{32}P]ATP (3,000 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) and polynucleotide kinase (Promega, Leiden, The Netherlands). Hybridization was under high-stringency conditions, and during amplification positive and negative control samples were included. Efficiency of the PCR was determined by amplification of a part of the β-globin gene (20). All specimens were positive for both HPV16 DNA and β-globin DNA. For genotyping assays, the HPV16-positive samples were subjected to a second round of PCR. The entire noncoding region of HPV16 was amplified in two fragments by using two distinct primer sets (27). The region between nucleotides 7109 and 7527 (10, 22) was amplified with primer A (CCTCATCTACCTCTACAACTGCTAAACGG) and primer B (GTTTAAACCATAGTTGCTGACATAGAAC). An overlapping 682-nucleotide fragment (the region between nucleotides 7445 and 222 [see references 10 and 22]) was amplified with primer C (GCTTCAACCGAATTCG GTTGCATG) and oligonucleotide D (CGTCGCAGTAACTGTTGCTTGCA GTACACAC). PCR was performed in 10 mM Tris · HCl (pH 9.0)–2 mM $MgCl_2$–50 mM KCl–0.01% gelatin–0.1% Triton X-100–0.5 U of *Thermus thermophilus* (*Tth*) DNA polymerase (Sphaero Q, Leiden, The Netherlands). Nucleotide triphosphates were present at 0.2 mM each. The amplification program consisted of 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 74°C, followed by a final extension step of 10 min at 74°C. Reaction products were analyzed on 1.5% agarose gels, run in 40 mM sodium acetate (pH 6.0)–1 mM EDTA (500 V · h). Gels were stained with ethidium bromide and photographed (Polaroid Polapan-60 films; Kodak, Amsterdam, The Netherlands). If reamplification was required, samples were run on low-melting-point agarose (FMC). Bands were cut out of these gels and redissolved in 0.5 ml of 10 mM Tris · HCl (pH 8.0)–1 mM EDTA, which served as a stock solution for subsequent reamplifications.

SSCP analyses. By using the purified DNA fragments (see above) as templates, increasing both the *Tth* polymerase concentration and the amount of primers by a factor of 3, and adding 0.75 μl of [α-^{32}P]dATP (3,000 Ci/μmol; Amersham plc), the AB fragment (the fragment amplified with primers A and B) could be homogeneously labeled during the PCR. After digestion with the restriction enzyme *Hha*I (27), the sample was diluted 10-fold by the addition of 96% formamide containing bromophenol blue and xylene cyanol. Samples were heated at 100°C for 10 to 15 min and immediately cooled on ice. Electrophoresis was performed on 10% polyacrylamide gels (acrylamide/bisacrylamide ratio, 15:1) containing 0.089 M Tris · borate (pH 8.0), 0.089 M boric acid, and 8 mM EDTA. The temperature was set at 4°C, the voltage was fixed to a value of 500 V, and electrophoresis was continued for 16 h. The gels were subsequently dried, and autoradiography was performed overnight at −70°C (X-Omat AR films; Kodak).

Direct sequence analysis. Sequence data were obtained for the AB fragments from clinical specimens of all patients. From a subset of clinical samples, a small region from within the CD fragment also was sequenced. DNA fragments suited for sequencing studies were generated by a nonradioactive PCR as described above. The only difference was that either primer A or primer C was derivatized with a 5′ biotin group. After PCR, the DNA fragments were bound to streptavidin-coated magnetic beads (Dynabeads M280; Dynal, Oslo, Norway) and washed twice with a buffer containing 50 mM Tris · HCl (pH 8.0), 100 mM NaCl, and 1 mM EDTA. The nonbiotinylated strand was eluted by treatment with 0.1 M NaOH. DNA was sequenced by standard dideoxy chain termination methods with the T7 DNA sequencing kit (Pharmacia, Uppsala, Sweden) by using α-^{35}S-dATP (3,000 Ci/mmol; Amersham plc) as the radioactive tracer. Sequencing products were separated on 5 or 8% polyacrylamide gels. DNA sequences were read manually from the autoradiographs, and sequence manipulations and comparisons were performed with the PC/gene computer program (Intelligenetics Inc., Mountain View, Calif.).

TABLE 2. Survey of demographic and patient-related data[a]

Patient class	Geographic origin	Age	% Smokers	Age at first intercourse (yr)	No. of sex partners[b]	STD partner (%)[c]	STD history (%)[d]	% Using oral contraceptives
Promiscuous	Amsterdam	34	82	17	78	100	100	43
Monogamous	Delft	39	66	16	1 (4)	33	33	67
Neoplasia	Groningen	40	71	18	1 (5)	NK[e]	14	57

[a] Data are given as averages. STDs monitored for were herpes simplex virus types 1 and 2, syphilis, gonorrheae, chlamydia, and trichomoniasis.
[b] Average per month calculated over the 4 months prior to interview and sampling. For monogamous females from Delft and Groningen, the average lifetime number of partners is given in parentheses.
[c] Percentage of HPV16-positive patients who had sex with a partner suffering from an STD.
[d] Percentage of HPV16-positive patients who had suffered once or more from an STD.
[e] NK, not known.

RESULTS

Comparison of patient groups. All groups of patients were comparable with respect to median age, smoking habits, age at date of first intercourse, and use of oral contraceptives (Table 2 and reference 20 give more-detailed descriptions). Among the people with multiple heterosexual partners, selected in Amsterdam, an increased frequency of infections caused by sexually transmittable pathogens is apparent. All of these individuals had a history of STD. Among the monogamous females from Delft and the gynecological patients from Groningen, only a limited number of past STD infections were documented (33 and 14% of the women, respectively). Another major difference among groups was the number of recent sexual partners, which averaged 78 in the past 4 months for the Amsterdam individuals. In the Delft and Groningen groups, the average lifetime numbers of sexual partners were 4 and 5, respectively, and all of these women had had a single partner for the previous 5 years. The females from Groningen had been diagnosed with cervical neoplasia. They were chosen at random from a larger group of cervical neoplasia patients. Upon cytological screening for cervical carcinoma, their cervical intraepithelial neoplasia classes were found to be II or higher, and patients 28, 31, and 32 underwent surgery for cervical carcinoma. This constitutes a major difference from the other groups, in which an incidental cervical intraepithelial neoplasia class I lesion provided the highest grade of cervical neoplasia (results not shown).

Amplification of HPV16 DNA from clinical samples. With the AB and CD primer combinations, DNA fragments of the correct length were synthesized when CasKi DNA was used as a template during PCR. No cross-reactivity of the primers with HPV6/11, HPV18, HPV31, and HPV33 DNA was observed, and human DNA was also not amplified (results not shown). Of the initial set of clinical materials ($n = 97$), which were all proven HPV16 DNA positive by amplification of a region within the E6 gene (24), only 69% ($n = 67$) gave a positive result when primers A and B or C and D were applied. Addition of more DNA or elevation of the primer or *Taq* polymerase concentration did not have a positive effect (results not shown). This indicates that the AB and CD PCR assays are not as sensitive as the one used for HPV16 diagnosis (17). No differences in the percentages of negative samples among the geographically diverse groups were observed. Nonamplifiable samples were excluded from the study.

Technical aspects of SSCP. For optimization of SSCP, the denaturation step had to be prolonged and performed at an elevated temperature in comparison with previously published protocols (27). An acrylamide/bisacrylamide ratio of 15:1 in the polyacrylamide gels rendered the most suitable matrix for electrophoretic strand separation (Fig. 1). The results are surveyed in Table 1. No SSCP data were generated for the HPV16 isolates from Groningen and Nijmegen since the resolution of SSCP appeared to be far less than that of DNA sequencing (see below).

Technical aspects of genomic sequencing of HPV16 amplicons. A small pilot study revealed that more sequence variability was observed among the AB fragments than among the CD amplimers. For this reason, nucleotide sequences of the AB fragments were determined for all HPV16 isolates. A fragment of about 290 bp from the region bordered by nucleotides 7177 and 7463 (10, 22) was fully characterized. No molecular cloning of the PCR fragments was performed, which may be a reason why mixed infections were missed. The direct-sequencing approach highlights only the most prevalent HPV16 variant in a mixture of viruses. Mutations are displayed in Table 2, where a sequence comparison of all HPV16 types is given. These sequencing results were not compromised by sequencing part of the CD fragment. Those data appeared, however, to be less discriminatory (results not shown). From Table 1, it can be concluded that the resolution of DNA sequencing exceeds that of SSCP. For instance, SSCP cannot discriminate among sequence types I, III, IV, VI, VII, and VIII, which leads to an underestimation of the actual number of sequence variants.

Viral epidemiology and persistence. The HPV16 strains from the promiscuous people (Amsterdam) were of four different HPV types, as identified by SSCP. Among the HPV16 isolates from the monogamous females from Delft, only a single SSCP pattern (based on the analysis of the AB fragment) was observed. This indicates the presence of a genetically more homogeneous group of HPV16 strains in the Delft population. By genomic sequencing, it was determined that the Amsterdam population harbored nine genetically distinct variants of HPV16. Only two sequence variants were detected among the 14 females from Delft. One of these DNA sequences was identical to that of the strain from Nijmegen. Among the patients from Groningen, four variants were detected. Results obtained by SSCP and sequencing of amplified HPV16 DNA from women sampled longitudinally do not vary; moreover, the two methods do not seem to detect differences among partial HPV16 sequences of isolates from various anatomical locations in individual patients (Table 1).

DISCUSSION

A large percentage of humans are infected by one of the many known HPVs. It has even been documented that a single individual can harbor multiple strains of a single viral type (11). This was not confirmed in the present study, perhaps because of the omission of a molecular cloning step. It has also been suggested that the presence of multiple HPV types in a single lesion may be dependent on the immune status of the patient involved (3). The identity of the longitudinal isolates was independent of sexual promiscuity. Whether promiscuous indi-

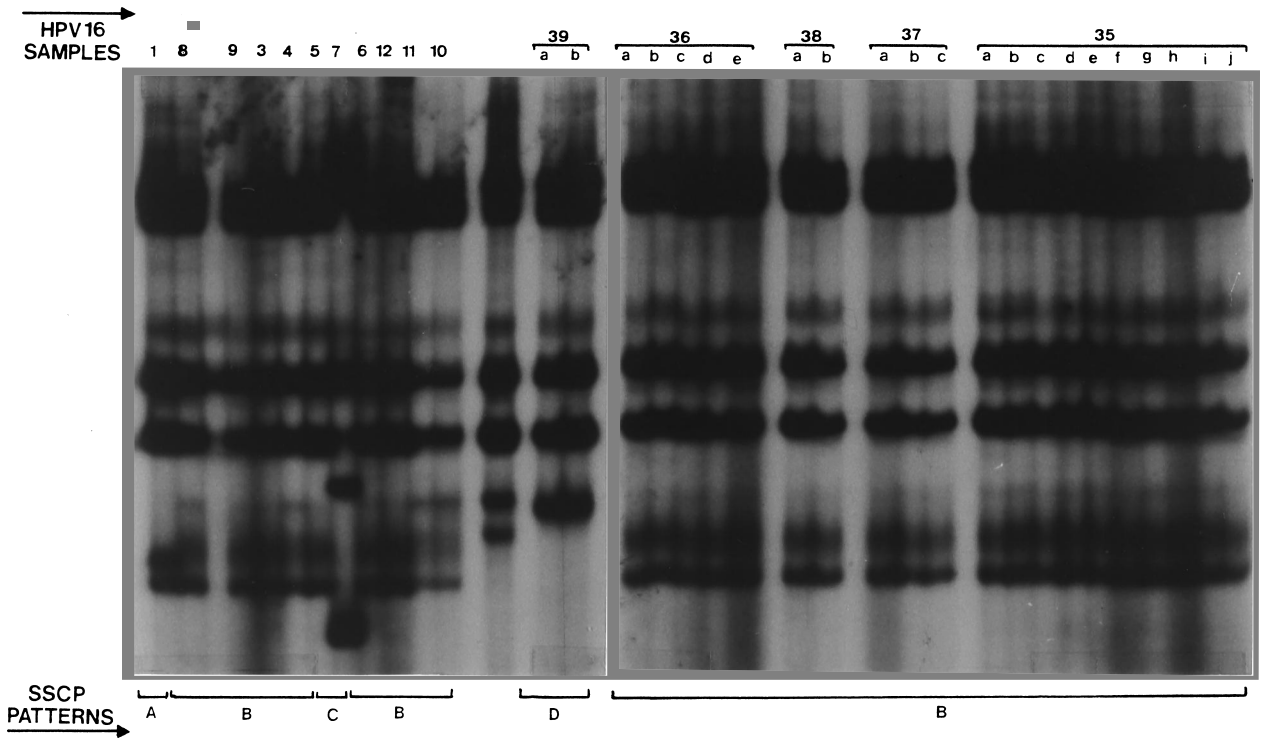

FIG. 1. Example of SSCP analysis of internally ^{32}P-labeled PCR-amplified HPV16 DNA (AB fragment) deriving from people with multiple sex partners in Amsterdam. Numbering of the lanes corresponds to numbering of patients as given in Table 1.

viduals, who remained at high risk for acquiring an HPV16 reinfection, or monogamous females are considered, HPV16 variants seem to be very persistent. Interestingly, all individuals from Amsterdam and two of three females from Delft, who were persistently HPV16 positive, were infected by a single HPV16 genotype. Whether this is a variant which specifically causes persistent infection or whether this is just the most prevalent Dutch genotype is the subject of current investigations. Since it has recently been demonstrated that high-risk HPV types can be transmitted quite easily by sexual intercourse (15), the fact that infection by HPV16 seems to preclude reinfection by another variant may have important implications for future vaccination strategies using attenuated HPV16 vaccine strains.

It is interesting that the group of HPVs collected from the monogamous females seems to be less heterogeneous than the one from the Amsterdam heterosexuals with multiple partners originating from different countries (2 types among 14 individuals versus 9 types among 20 individuals; Fisher's exact test, $P = 0.07$). This is concordant with earlier observations of geographic clustering of HPV16 variants on the basis of sequence divergence in the E7 gene (2). Moreover, it seems logical to assume that among sexually promiscuous individuals, the number of HPV16 variants encountered will be larger than that for the more monogamous population in Delft. An absence of differences in serological response between persistently and incidentally infected persons has been documented previously (25).

The mutations at positions 7191, 7192, and 7193 are bordered by a directly repeated motif (5′-TGTTTGT-3′). In the region around mutation positions 7429 to 7447, another, even larger direct repeat (5′-ATTTTGTAGC-3′) can be identified. The role of these repeats in mutagenesis is not immediately clear, but it could be that during replication, distinct repeats could form heteroduplexes with their neighboring counterparts, thereby rendering the intermittent region single stranded and vulnerable to mutagenic effects. No conclusions concerning the positions of base changes and the possible effects on promoters, enhancers, and protein binding sites in the HPV16 noncoding region in relation to biological effects (persistence, virulence, oncogenic potential, etc.) can be made as yet.

Among the promiscuous individuals from Amsterdam, SSCP revealed four genotypes of HPV16 in 20 individuals. This correlates well with previous data (27) documenting the occurrence of 12 variants in 48 patients. It was claimed that SSCP is as sensitive as direct sequencing, but in our present study we noticed that several sequence variants remain undetected by SSCP. Among the same 20 strains of HPV16, nine sequence types were found. All different SSCP types represent sequence variants, but not all variant sequences give rise to SSCP alterations. This implies that for epidemiological studies, direct sequencing of PCR products should be preferred over SSCP analysis of the same fragments.

It appears that a single genotype of HPV16 can persist over many years in a single individual. In the present study, the maximum length of follow-up was 3 years. This persistency may be an important step, ultimately leading to oncogenic transformation, and the possibility that acquisition of a strain which gives rise to persistent infection may predispose an individual to development of cytological abnormalities cannot be excluded. In order to draw definite conclusions, additional studies of HPV16 spreading and epidemiology must be performed on a larger scale.

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