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Papillomaviruses in Lesions of the Lower Genital Tract in Israeli Patients

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Abstract—*Human papillomaviruses (HPVs) have been strongly associated with benign lesions of the genital tract (condylomata) and with genital cancer of the vulva and cervix. Since the incidence of these lesions in Israel is considered to be low, we have studied the presence of HPV* 6, 11, 16 *and* 18 *DNAs in benign, premalignant and malignant tissue samples or gynecological swabs of the lower genital tract. HPV sequences were detected in* 48 *out of* 66 *condylomatous lesions* (72%), 5/11 *grades I–II intraepithelial neoplasia* (45%), 4/6 *grade III intraepithelial neoplasia (carcinoma* in situ) (66.6%) *and* 8/22 *invasive carcinoma* (36%). *The latter included six cases of vulvar carcinoma which were all negative for HPV sequences. No additional HPV types could be detected in any of the tissue biopsies examined. HPV* 18 *DNA has been found in one vulvar condyloma where it persisted as an episomal molecule, this being the first report of that specific viral DNA in a condylomatous lesion.*

In all the benign and premalignant lesions containing HPV, the viral sequences were maintained in an episomal state. In two cases of invasive carcinoma, the HPV 16/18 *related sequences were integrated in the cellular genome, but in five cases (three containing HPV* 16/18 *related DNAs and two containing HPV* 6/11 *related DNAs) the viral sequences were episomal. HPV* 16/18 *related sequences detected in one out of three cases of vaginal carcinoma were also found to be episomal. This data indicates that human papillomavirus sequences are indeed found in genital lesions of Israeli patients, although to a lesser extent than in other countries, especially for benign lesions and invasive carcinomas.*

Although HPVs may have a causative role in the development of genital lesions, also in this low tumor incidence area, other factors should be also considered in the etiology of these lesions.

INTRODUCTION

HUMAN PAPILLOMAVIRUSES (HPV) have been closely associated with human genital lesions. Epidemiological studies have linked specific types of this group of viruses to human cancers of the lower genital tract, implying that they may play a key role in the etiology of genital cancer [1, 2]. Molecular hybridization studies have demonstrated the presence of HPV genomes in 20–50% of premalignant lesions [3], and in about 85% of malignant lesions [4], predominantly HPV 16 and HPV 18 related sequences. Moreover, HPV 16 and HPV 18 DNA sequences were also detected in cell lines derived from cervical cancer [2, 5–7]. In these cell lines as well as in the HPV positive genital carcinomas, the viral sequences have been found to be integrated in the cellular genome [8, 9]. Two other papillomavirus types, HPV 6 and HPV 11, are mostly associated with benign genital lesions (condylomata) [10, 11]. Approximately the same proportion of positive materials have been found in different countries, including those with an extremely high incidence of cervical cancer [1].

The incidence of cervical cancer in Israel is thought to be one of the lowest. The rate of the disease is five to ten times lower than in high incidence areas [12]. Therefore, it is of particular interest to analyze genital lesions in this country, for the presence and prevalence of specific HPV types. Such studies can contribute to the clarification of the role of certain HPV types by supporting

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Table 1. Presence of HPV DNA in benign genital lesions

Histological diagnosis	Number of samples	HPV 6/11 positive	HPV 16 + 18 positive	HPV 6/11 and 16 + 18 positive
Cervical condyloma	26	16	1	1
Vulvar condyloma	29	22	2	1
Penile condyloma	8	5	1	
Rectal condyloma	1	1		
Pararectal condyloma	2	2		
Total	66	46	4	2

their causative role in tumor development or, alternatively, by strengthening the possibility that additional factors are mandatory for cancer development.

In this study we report a survey on benign, premalignant and malignant genital lesions in Israeli patients tested for the presence and physical state of HPV 6, 11, 16 and 18 related DNAs, by Southern blot and *in situ* filter hybridization analysis.

MATERIALS AND METHODS

Biopsies and DNA extraction

Tissue specimens were obtained from genital lesions of Jewish Israeli patients at Hadassah Hospital in Jerusalem and Rambam Hospital in Haifa. The specimens were divided and either placed in formaldehyde solution (10%) for histological evaluation or immediately frozen at −70°C until high molecular weight DNA was extracted as previously described [13].

Gynecological swabs

In some patients the cellular material for human papillomavirus identification was obtained from cervical or vulvar swabs, resuspended in phosphate-buffered saline, and denatured *in situ* on a nitrocellulose filter prior to hybridization with the corresponding probes [14]. Two filters were prepared from each swab, each containing about 10^5 cells.

Restriction enzyme cleavage and blot hybridization

Sheared cellular DNA (by passage through a 20 gauge needle 20 times) or DNA cleaved with restriction endonucleases was electrophoresed in 0.6% agarose gels and transferred to nitrocellulose filters [15]. Radiolabeled DNAs were prepared by the method of Rigby *et al.* [16]. Recombinant plasmids containing papillomavirus DNA, HPV 6, HPV 11, HPV 16 and HPV 18, were kindly supplied by Dr. L. Gissmann, Deutsches Krebsforschungszentrum, Heidelberg, F.R.G. Prior to radiolabeling the cloning vector pBR322 was separated from the viral sequences.

Hybridizations were performed at 65°C for high stringency conditions (T_m −20°C). For non-stringent conditions (T_m −40°C) hybridizations were performed in the presence of 20% formamide at 42°C [17].

RESULTS

Detection of HPV sequences in benign lesions

Four major HPV types, HPV 6, 11, 16 and 18, have been associated with benign and malignant lesions of the genital tract, although some other types can also be detected, occasionally. Therefore we have undertaken to screen a number of clinical specimens for the presence of HPV DNA sequences by the Southern blot hybridization technique. DNA isolated from genital lesions of Israeli patients were hybridized either with HPV 6 or HPV 11 (since these two DNAs crosshybridize to a high degree) and in parallel with a mixture of HPV 16 and 18 DNAs. DNA was extracted from benign lesions, including condylomata acuminata of the vulva and cervix, and some samples of rectal, pararectal and penile condylomata. Twenty micrograms of sheared DNA or DNA digested with BamHI, a restriction enzyme which linearizes the viral genome of HPV 6, 11 and 16, were run on agarose gel and blotted simultaneously onto two nitrocellulose filters, for hybridization with either of the two probes. In some cases there was not enough material for DNA extraction; thus gynecological swabs were analyzed, as described in Materials and Methods. From certain patients both biopsy material and gynecological swabs were examined. There was a complete correlation between the results obtained by both methods (data not shown). The results of this analysis are summarized in Table 1. HPV DNA related sequences were detected in 72% of the specimens (48/66), with HPV 6/11 related sequences in 70% of the cases (46/66) and HPV 16/18 in 6% of them (4/66). In two cases we detected a mixed infection. One of them, a vulvar condyloma, contained HPV 6/11 related sequences together with HPV 18 DNA (Fig. 1). Cleavage with BamHI and hybridization with HPV 11 revealed one band of 7.9 kilobase (kb) (Fig. 1b). Cleavage with EcoRI and hybridization with HPV 16 + 18 DNAs as a probe revealed also

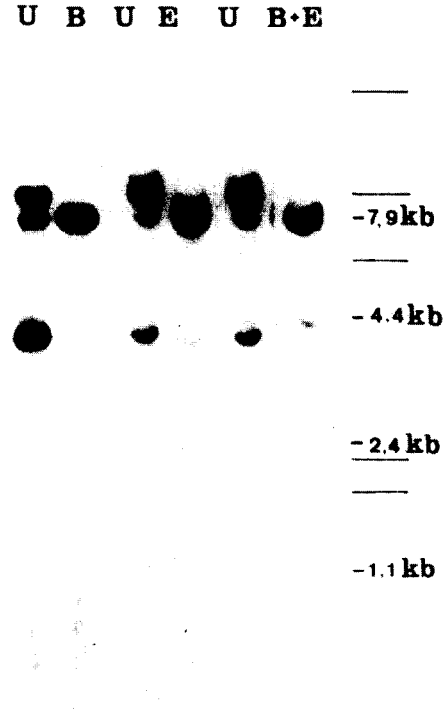

Fig. 1. Blot hybridization of ^{32}P*-labeled HPV* 11 *DNA, a mixture of HPV* 16 + 18 *DNAs and HPV* 18 *DNA to cellular DNA purified from a vulvar condyloma biopsy. Cellular DNA* (20 μg) *was gently sheared by passage through a* 20 *gauge needle* 20 *times* (*U*), *or restricted with the indicated endonucleases* (*B = BamHI; E = EcoRI*). *The DNA was transferred to two nitrocellulose filters by a modification of the procedure of Southern. The blots were hybridized to* 5×10^6 *cpm of the indicated* ^{32}P*-labeled probes at* 65°C *for* 20 h. *Filters were exposed to X-ray film either for* 2 h *at room temperature* (*lanes a and b*) *or for* 72 h *at* −70°C (*lanes c, d, e and f*). *The bars indicate the size marker fragments of* λ *DNA cleaved by Hind III* (23.1, 9.4, 6.5, 4.4, 2.2 *and* 2.0 *kb*). *The sizes of the linear fragments detected are indicated.*

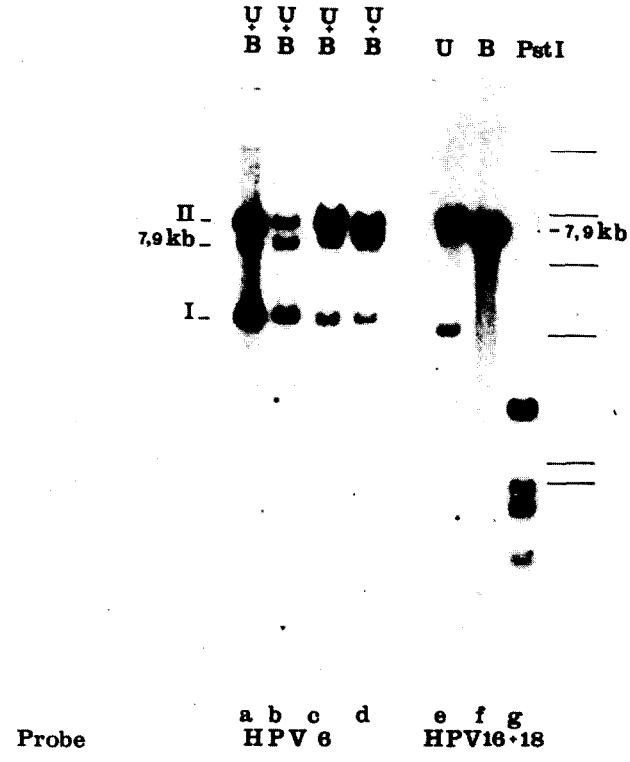

Fig. 2. Representative blot hybridization of ^{32}P-labeled HPV DNA to cellular DNAs purified from condylomata and premalignant lesions. ^{32}P-Labeled HPV 6 DNA and HPV 16 + 18 DNAs were hybridized to cellular DNAs purified from three condylomata (lanes a, b, c), from one specimen of CIN II (lane d) and from one specimen of VIN III (lanes e, f, g). Lanes a, b, c, d: 10 µg of sheared DNA (U) and 10 µg of BamHI restricted DNA (B) were loaded together on a 0.6% agarose gel. VIN III DNA (20 µg) was sheared (lane e), BamHI restricted (lane f) and Pst I restricted (lane g). Blotting and hybridization were performed as described in Fig. 1. Filters were exposed to X-ray film for 72 h at −70°C. The bars indicate the size marker fragments of λ DNA cleaved by Hind III.

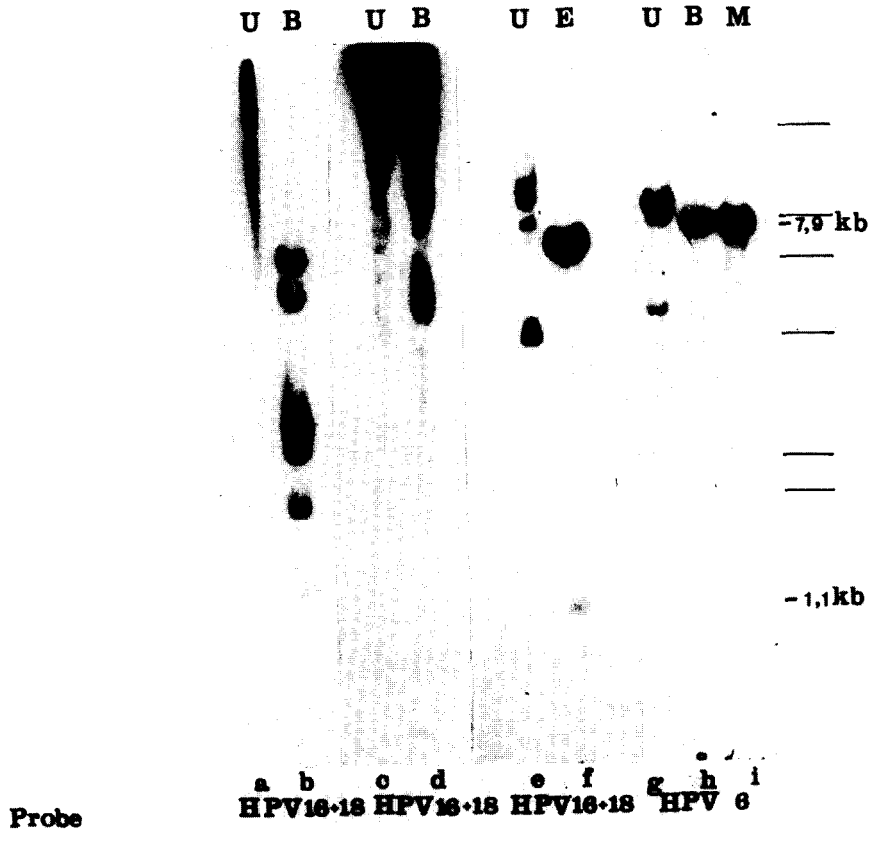

Fig. 3. Representative blot hybridization of ^{32}P-labeled HPV DNA to cellular DNAs purified from specimens of cervical carcinoma. Cellular DNAs (20 µg) purified from four specimens of cervical carcinoma were either sheared (U), BamHI restricted (B), or EcoRI restricted (E). Blotting and hybridization to ^{32}P-labeled HPV 16 + 18 DNAs and HPV 6 DNA, as indicated, were performed as described in Fig. 1. Filters were exposed to X-ray film for 72 h at −70°C. M (marker) represents 100 pg of HPV 6 linear DNA. The bars indicate the size of the marker fragments of λ DNA cleaved by Hind III.

Table 2. Presence of HPV DNA in premalignant and malignant genital lesions

Histological diagnosis	Number of samples	HPV 6/11 positive	HPV 16 + 18 positive	HPV 6/11 and 16 + 18 positive
CIN I	4	1	3	1
CIN II	3	1		
VIN I	3	—	—	
VIN II	1	1	—	
VIN III (vulvar carcinoma *in situ*)	2	—	2	
CIN III (cervical carcinoma *in situ*)	4	—	2	
Invasive cervical carcinoma	13	2	5	
Invasive vulvar carcinoma	6	—	—	
Vaginal carcinoma	3	—	1	

one band of the same size (Fig. 1d), indicating that a papillomavirus related to HPV 18 was present, since EcoRI recognizes two sites in HPV 16 but only one site in HPV 18. To confirm this result, we cleaved the DNA with EcoRI and BamHI simultaneously and hybridized the resulting segments with radiolabeled HPV 18 DNA only (Fig. 1f): four bands were observed. Three of them, 4.4, 2.4 and 1.1 kb long, correspond to the expected sizes of HPV 18 DNA digested with these enzymes [5]. The fourth band was 7.9 kb long. It may represent either linear molecules of HPV 18 which have not been cleaved to completion or linear molecules of linear HPV 6/11 related sequences, also present in this specimen, which crosshybridize with the HPV 18 probe, because of the extremely high copy number of HPV 6/11 molecules present; in Fig. 1a and b, representing the DNA of this biopsy hybridized with HPV 11 DNA, the blot has been exposed to X-ray film for 2 h at room temperature. In contrast, in Fig. 1c, d, e and f, representing the same DNA hybridized with HPV 16 + 18 or only with HPV 18, the blots have been exposed for 3 days at −70°C.

In order to test for additional HPV types possibly involved in these lesions, all the DNAs that did not hybridize with HPV 6/11 and HPV 16 + 18 probes were rehybridized with the same probes at non-stringent conditions. No bands were detected in the previously negative samples (data not shown).

Screening of premalignant and malignant genital lesions

We have analyzed seven specimens of CIN I–II and four specimens of VIN I–II (Table 2). Forty-five per cent of these premalignant lesions contained HPV sequences. We were able to detect HPV 6/11 related sequences in one case of CIN I, one case of CIN II and one case of VIN II. Three cases of CIN I were positive for HPV 16/18 sequences. In two specimens of VIN III, we identified HPV 16/18 related sequences, as well as in two out of four cases of CIN III.

All six cases of invasive carcinoma of the vulva failed to hybridize with HPV sequences and seven out of 13 biopsies of invasive cervical carcinoma contained HPV sequences: two of them revealed HPV 6/11 related sequences, and five cases containing HPV 16/18 related DNA. From three cases of vaginal carcinoma, one was positive for HPV 16/18. Altogether 36% of the invasive carcinomas contained HPV sequences (8/22), 9% were positive for HPV 6/11 sequences and 27% for HPV 16/18 related DNAs.

All the negative samples were subsequently hybridized with the same probes at non-stringent conditions. No bands were detected (data not shown).

Physical state of HPV DNAs in genital lesions

The physical state of HPV genomes detected in the various genital lesions could be determined by analyzing their Southern blots (Table 3). In all the HPV positive DNAs extracted from benign lesions we could detect two bands corresponding to Form I and Form II of the HPV episomal molecule (Fig. 2). In addition, after cleavage with BamHI which usually recognizes one site in the HPV genome (two in HPV 18 genome), we could detect one single band, 7.9 kb pair long, corresponding to the linear form of the HPV genome. This indicates that in these lesions the viral genome replicates as an independent episomal molecule. The same migratory pattern was observed in the premalignant lesions analyzed (Fig. 2) containing either HPV 6/11 or HPV 16/18 sequences. We have also analyzed seven cases of invasive cervical carcinoma containing HPV sequences (Fig. 3). The HPV 6/11 sequences detected in two of them were episomal as were the HPV 16 sequences detected in three other specimens. In two cases containing HPV 16/18 related DNAs the viral genomes migrated with the high molecular weight cellular DNA. After cleavage with BamHI, multiple bands were observed, and their migratory pattern was consistent with integration events, thus indicating that the viral

Table 3. Physical state of HPV DNAs in genital lesions

Histological diagnosis	Number of samples containing HPV	HPV DNA type	Physical state	
			Episomal	Integrated
Condylomata	48	HPV 6/11,16/18	48	—
CIN I, II	4	HPV 6/11,16/18	4	
VIN II	1	HPV 6/11	1	
CIN III	2	HPV 16/18	2	
VIN III	2	HPV 16/18	2	
Invasive cervical	2	HPV 6/11	2	
carcinoma	5	HPV 16/18	3	2
Vaginal carcinoma	1	HPV 16/18	1	

sequences were very likely integrated at multiple sites in the cellular genome.

DISCUSSION

Considerable epidemiological evidence has accumulated, pointing to an infectious component in the etiology of human genital cancer of the lower genital tract [18], and in the past few years to papillomaviruses in particular [19]. The malignant inducing potential of human papilloma appears to be related to specific human papillomavirus types (HPV 16, 18, 31). Precancerous conditions are regularly associated with specific types of papillomaviruses [20]. However, as reported by Doll [21], some observations may point to other factors of non-venereal origin. One such observation is the variation in the incidence of cervical carcinoma among certain populations. In particular, Jewish women have a consistent low incidence of cervical cancer. Israel, too, with a majority of Jewish women, has a very low incidence of cervical cancer. These observations were for a long time attributed to a protective effect of circumcision, but in other communities (U.S.A., Britain and Fiji) circumcision has not been protective [21]. Therefore it was important to evaluate the frequency of HPV DNA presence in genital lesions of Israeli patients, in order to assess the role of these viruses in malignant progression.

Our studies show that the same papillomavirus types, HPV 6, 11, 16 and 18, are present in the same types of genital lesions of Israeli women as in women from other parts of the world. However, these viral sequences are detected less frequently in these lesions. Moreover, no additional HPV types could be detected, as tested by hybridization of all the samples in relaxed conditions. HPV 6/11 related sequences are prevalent in benign genital lesions. However, we found HPV 16/18 related DNAs in about 6% of the condylomata biopsies that were analyzed. This figure is rather high compared to other countries (about 0.5% in Germany, L. Gissmann, personal communication), where, among benign lesions, HPV 16/18 sequences are detected mainly in Bowenoid papulosis [22].

Although HPV 16 sequences were prevalent in VIN III, CIN III and in invasive carcinoma, we could also detect HPV 6/11 related sequences in two cases of cervical carcinoma. In CIN I–II we found HPV 6/11 and HPV 16/18 related sequences at the same proportion as in other studies. It is of particular interest to note the absence of HPV DNAs in invasive vulvar carcinomas, and the presence of the virus in condylomata and premalignant lesions of the vulva is puzzling. This may indicate that the importance of HPV for neoplastic transformation of vulvar epithelium is not as central as for cervical epithelium. Screening studies of the general population are in progress to determine the frequency of human papillomaviruses in the genital tract of the normal Israeli population. If they are present in the same frequency as in other parts of the world the idea of additional factors being involved will gain further support. However, as gynecologists point out, recently there seems to be a progressive increase in the number of genital lesions observed in the outpatient clinic in Israel. This could be explained by increased sexual promiscuity in the Israeli population. Therefore, in a few years time the incidence of genital tumors in Israel might get close to the one observed in other countries.

The detection of HPV 18 DNA in a case of vulvar condyloma represents the first report of this virus type in a benign genital lesion. Furthermore, these sequences persist as 7.9 kb circular molecules, with no indication of integration. Up till now the DNA of HPV 18 had been exclusively found in malignant genital tumors integrated with the host genome [2, 9]. Similarly to others [8, 9], we have also found that in all condylomatous and premalignant lesions the HPV sequences involved are always in an episomal state. However, in contrast to the studies of Durst *et al.* [8, 9] reporting the integration of HPV sequences in invasive carcinomas, we found HPV sequences which persisted as episomal molecules in such lesions. Of course, since these lesions contain many copies of the HPV genome, it is possible that there are also a few integrated viral sequences in

these specimens. Two dimensional gels could clarify this issue. Alternatively, if the active role of HPV sequences in malignant progression is expressed through integration events, our data will again support the involvement of other possible factors and cofactors in the etiology of cancer of the low genital tract.

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