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Detection of Human Papillomavirus Types 6/11, 16 and 18 in Exfoliated Cells from the Uterine Cervices of Japanese Women with and without Lesions

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Human papillomavirus (HPV) infection of the uterine cervices of Japanese women with and without lesions was identified by the filter *in situ* hybridization method. Exfoliated cervical cells from 23 cervical papillary condylomas, 70 cervical intraepithelial neoplasia (CIN) grade I/II, 26 CIN III, 31 invasive cervical cancers and 666 cervices without evidence of disease (including 53 pregnant women) were tested for the presence of HPV types 6/11, 16 and 18. The positive rates for the detection of HPV types 6/11, 16 and 18 DNA were 47.8%, 26.1% and 8.7% in cervical condylomas, 5.7%, 15.7% and 8.6% in CIN I/II, 0, 34.6% and 0 in CIN III, 3.2%, 38.7% and 9.7% in invasive cervical cancers and 0.9%, 1.8% and 0.6% in the control cervices. These data suggest that, in Japan, HPV6/11, HPV16 and HPV18 infections are also prevalent in cervical cells with normal phenotype, and the type of HPV infection of the uterine cervix is related to the histological diagnosis.

Key words: Human papillomavirus infection — Cervical exfoliated cells — Filter *in situ* hybridization — Normal cervix — Japanese women

Many types of human papillomavirus (HPV), including HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 51, 52 and 58 are known to infect the genital tract of women. All these HPVs produce benign papillomas or intraepithelial neoplasia through sexual transmission. Only some types of HPV appear to have oncogenic potential. Four types of HPV commonly infect the genital tract. Types 16 and 18 have been detected by Southern blot analysis in 50 to 60% of invasive cervical cancers on average,[1,2] while types 6 and 11 are frequently found in benign condylomas.[3,4]

The epidemiologic investigation of HPV infections has been hampered by the lack of suitable conventional laboratory techniques to culture the viruses *in vitro*. Recently the filter *in situ* hybridization method was used to detect HPV DNA in exfoliated cervical cells collected in parallel with samples for routine cytology.[5–9] This method is a non-invasive technique that is quick and simple, and thus well suited for epidemiological studies. In all the earlier epidemiological studies by this method, HPV16 and HPV18 infections were examined together, using a mixture of HPV16 and HPV18 DNAs as the probe.

The purpose of this study was to determine, by using three HPV probes, the prevalence of HPV6/11, HPV16 and HPV18 infections in uterine cervices with and without lesions in Japan, where the occurrence of HPV in cervical cancers is lower than that reported from Europe and the USA.[1,10,11]

MATERIALS AND METHODS

Study groups and sampling procedure Over a period of 36 months, 816 samples of exfoliated cervical cells were obtained with cotton-tipped swabs in parallel with samples for routine cytology from the uterine cervices of Japanese females who attended our outpatient clinics. The first group was made up of 23 patients with papillary condylomas. The second group consisted of 70 patients with cervical intraepithelial neoplasia (CIN) grade I/II (mild to moderate dysplasia) and 26 patients with CIN grade III (severe dysplasia and carcinoma *in situ*). The third group consisted of 31 patients with invasive cervical cancers. The fourth group consisted of 666 women with normal cervical cytology (including 53 pregnant cases), attending our hospitals for routine screening. Histologic examination confirmed the diagnosis of condyloma, CIN and invasive cervical cancer in the first, second and third groups.

The cotton swabs were put into 4 ml of phosphate-buffered saline (PBS) and stored, until examination, at $-20^{\circ}C$ after preparation of slides for cytological evaluation.

Preparation of filters Cell counting confirmed that each swab contained 10^5–10^6 cells. A half of each cell suspension was examined by filter *in situ* hybridization and the remaining half was stored for further analysis. Several cell suspensions in PBS which contained different numbers of cultured cells (10^5–10^6 cells per tube) were also prepared.

After thawing, the cell suspension was blotted under vacuum onto a round nitrocellulose filter (25 mm in diameter, 0.45 μm in pore size: Schleicher & Schuell, Dassel, West Germany). To lyse and fix the cells and denature the cell DNA, the filters were gently placed on the surface of 1.5 *M* NaCl/0.5 *M* NaOH (pH 7.0) for 10 min. They were then placed twice on the surface of the neutralization solution (20×SSC/0.5 *M* Tris-HCl pH 7.0, SSC is 0.15 *M* sodium chloride-0.015 *M* sodium citrate) for 20 min each time. Each filter was cut into three pieces with a scalpel and dried on a Whatman 3MM paper at room temperature for 60 min. The filters were baked at 80°C for 15 min and stored in vacuum for up to two weeks until further processing.

Probe DNA and radiolabeling The HPV 11a and 16 DNA, each cloned into the *Bam*HI site of pBR322, and the HPV18 DNA, cloned into the *Eco*RI site of pBR322, were used as probes. After *Bam*HI digestion (HPV11a and 16) or *Eco*RI digestion (HPV 18), the HPV insert sequences were separated and purified from the flanking vector sequences by agarose gel electrophoresis and electroelution, as described by Maniatis *et al.*[12] Inserts were labeled with [α-^{32}P]dCTP by nick translation to a specific activity of 1–2×10^8 cpm/μg. pBR322 DNA was also labeled by the same procedure. Since HPV6 and

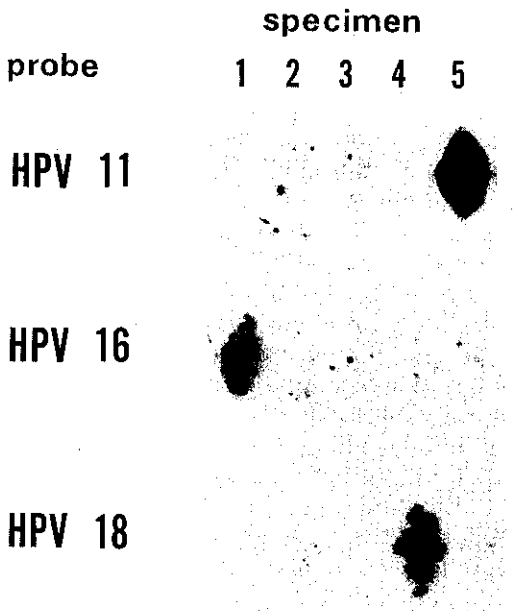

Fig. 1. Representative autoradiography of filter *in situ* hybridization. An HPV16-positive sample is in lane 1, an HPV18-positive sample in lane 4 and an HPV6/11-positive sample in lane 5. Samples in lanes 2 and 3 are negative for the three HPV probes.

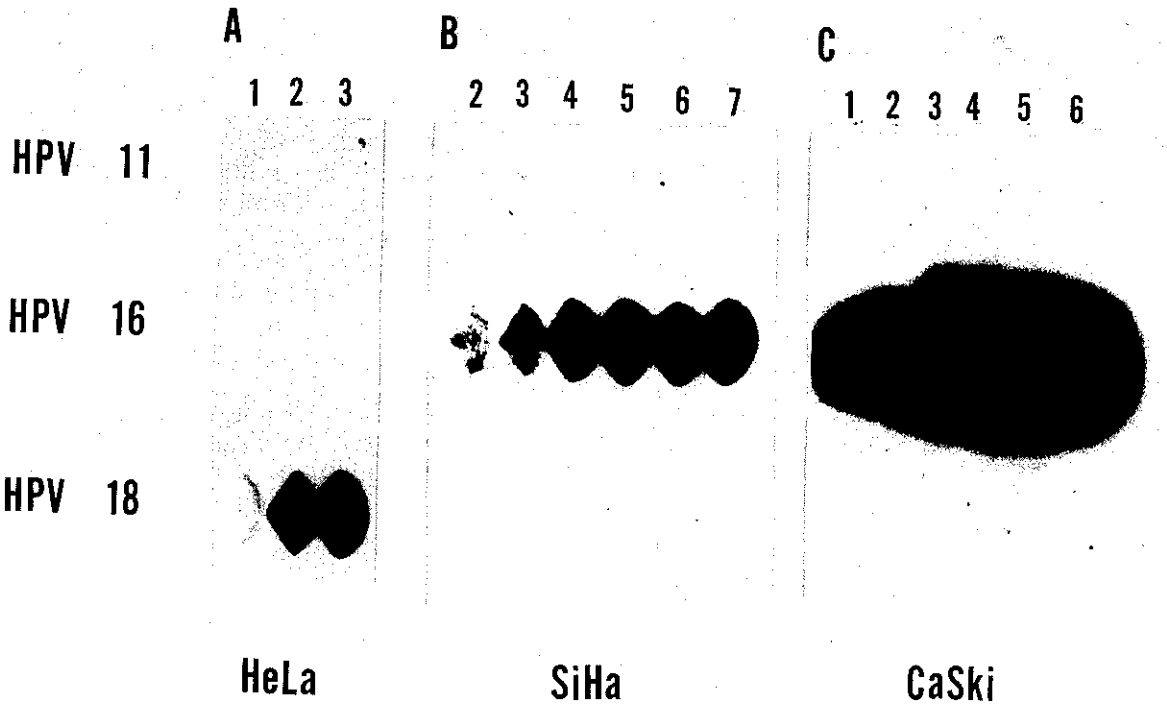

Fig. 2. Filter *in situ* hybridization of cultured cell lines (A, HeLa cells; B, SiHa cells; C, CaSki cells). Cells were blotted onto a round filter (5×10^4, 1×10^5, 5×10^5, 1×10^6, 5×10^6, 1×10^7 and 5×10^7 cells in lanes 1, 2, 3, 4, 5, 6 and 7, respectively).

HPV11 have a high degree of homology, we used only HPV11a DNA as the probe for detecting HPV types 6 and 11 DNAs.

Filter *in situ* hybridization Filter *in situ* hybridization was performed by a modification of the method described by de Villiers *et al.*[7] Prehybridization was performed overnight at 42°C in a slowly rolling 50 ml Corning tube filled with 50% formamide, 0.5 mg/ml tRNA, 5×SSC, 50 m*M* sodium phosphate buffer, 0.1% polyvinylpyrrolidone, 0.1% Ficoll and 0.1% bovine serum albumin. Tubes were sealed with a wrap-film without air bubbles. Up to 60 pieces of cut filters were processed in each tube. Hybridization was carried out for 16 h with ^{32}P-labeled HPV11, 16 or 18 DNA (4×10^5 cpm/cm^2 filter) under the same conditions as described above (Tm −18°C). Filters were washed twice in 2× SSC/0.1% sodium dodecyl sulfate for 15 min at room temperature and three times in 2×SSC/0.1% sodium dodecyl sulfate for 30 min at 68°C. After being washed, filters were dried at room temperature for 60 min on a Whatman 3MM paper and arranged according to sample number (in rows) and type of probe HPV DNA (in columns). For autoradiography, Kodak RP X-ray films were exposed to the arranged filters with intensifying screens at −70°C for 5 days. Judgement of positivity of HPV DNA was made by comparing the autoradiogram intensity among samples in rows and among probes in columns. Filters were read as "positive," when clear spots or darkening could be distinguished from background signals. Fig. 1 gives examples of negative and positive results. To exclude false-positivity generated by hybridization of bacterial sequences contaminating the samples with the small amount of pBR322 DNA present in the probes or by non-specific binding of radioactivity to the filter as a result of the presence of blood or mucous, all positive filters were retreated with ^{32}P-labeled pBR322 DNA (4×10^5 cpm/cm^2 filter). The level of detection in this study was about one copy or more of HPV genome per cell, as determined by experiments using serial dilutions of three HPV-positive cell lines: SiHa cells (containing one or two copies of HPV16 DNA), CaSki cells (containing 500 copies of HPV16 DNA) and HeLa cells (containing 10 copies of HPV18 DNA) (Fig. 2).

RESULTS

Filters that hybridized with only a single HPV probe (HPV11, 16 or 18 DNA) were negative or weakly positive for pBR322 probe, while the filters positive for two or three HPV probes (about 3% of samples) showed much stronger positivity for pBR322 probe (4×10^5 cpm/cm^2 filter) compared with positivity for the HPV probes (4×10^5 cpm/cm^2 filter). These filters positive for two or three HPV probes were confirmed to be negative for HPV DNA or to be positive for one HPV DNA by Southern blot analysis using the cell DNA extracted from the remaining half of the cell suspensions (data not shown). We included these false-positives in the HPV-negatives, because these signals were considered to be due to plasmid or other sequences in bacteria contaminating the samples or due to non-specific binding of radioactivity.

Table I lists the incidence of detectable HPV infections in filter *in situ* hybridizations.

HPV in cervical condylomas Of 23 swabs from cervical papillary condylomas, 11 (47.8%) were HPV6/11-positive, 6 (26.1%) were HPV16-positive and 2 (8.7%) were HPV18-positive. Thus, HPV DNA sequences were detected in 83% of cervical condylomas (Table I). Ten of the 23 women had no vulvar lesions, but the remaining 13 also had vulvar condylomas. Occurrence of HPV16 or HPV18 was significantly higher in cervical condylomas without vulvar lesions than in cervical condylomas with vulvar lesions (60% versus 15%) (Table II).

HPV in CIN We detected HPV6/11 DNA in only 4 (5.7%) of 70 CIN I/II and none of 26 CIN III; and HPV16 DNA and HPV18 DNA in 11 (15.7%) and 6 (8.6%) of 70 CIN I/II and 9 (34.6%) and none of 25 CIN III. HPV16 and HPV18 infections were increased and HPV6/11 infections decreased in the cases of CIN III, in comparison with CIN I/II (Table I).

HPV in cervical cancers In 31 cervical cancers, we detected HPV6/11 DNA, HPV16 DNA and HPV18 DNA in 1 (3.2%), 12 (38.7%) and 3 (9.7%), respectively (Table I).

HPV in clinically and cytologically normal cervices Of 666 control cases, 6 (0.9%) were HPV6/11-positive, 12

Table I. The Incidence of HPV Infections in the Cervix

Diagnosis	No. of cases	HPV6/11-positive	HPV16-positive	HPV18-positive
Papillary condylomas	23	11 (47.8%)	6 (26.1%)	2 (8.7%)
CIN I/II	70	4 (5.7%)	11 (15.7%)	6 (8.6%)
CIN III	26	0 (0.0%)	9 (34.6%)	0 (0.0%)
Invasive cancer	31	1 (3.2%)	12 (38.7%)	3 (9.7%)
Normal controls	666	6 (0.9%)	12 (1.8%)	4 (0.6%)

Table II. HPV Types in Cervical Condylomas with and without Vulvar Lesions

Vulvar lesion	No. of cases	HPV6/11-positive	HPV16-positive	HPV18-positive
+	13	8 (61.5%)	2 (15.4%)	0 (0.0%)
			15.4%	
−	10	3 (30.0%)	4 (40.0%)	2 (20.0%)
			60.0%	

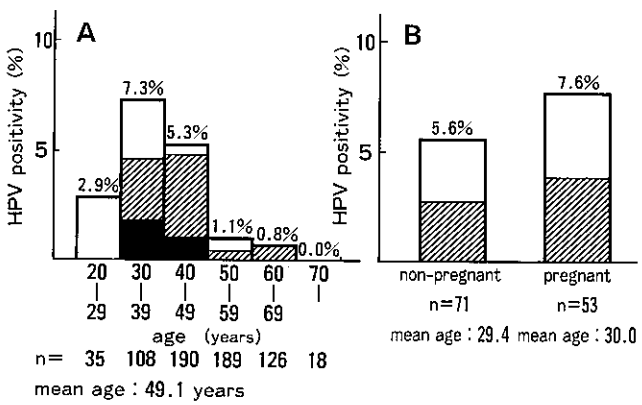

Fig. 3. Incidence of HPV infections in cytologically normal women. A: Age distribution. B: Comparison between the pregnant population and the non-pregnant population under age 35 years. Fifty-three pregnant and 71 non-pregnant women are included in the 666 controls. □, HPV6/11; ▨, HPV16; ■, HPV18.

(1.8%) were HPV16-positive and 4 (0.6%) were HPV18-positive. In this group, the total positivity of these HPVs was 3.3% (22/666) (Table I). As shown in Fig. 3A, occurrence of HPV DNA was distinctly higher in the younger group of women (under 50 years) than in the older group (50 or more) (5.7% versus 0.9%). The positivity of HPVs in 53 pregnant women was slightly higher than that of the non-pregnant aged under 35, but the difference was not significant (7.6% versus 5.6%) (Fig. 3B).

DISCUSSION

We examined HPV6/11, HPV16 and HPV18 infections by filter *in situ* hybridization in the cervices of Japanese women with and without lesions and found HPV DNA in 83% of condylomas, 30% of CIN I/II, 35% of CIN III, 52% of invasive cancers and 3.3% of the normal controls. About 3% of samples positive for two or three HPV probes were observed. However, these samples showed specific or non-specific reactions with pBR322 probe and no hybridization with the HPV probes by Southern blot analysis. Thus, infections with two or three types of HPV were not found in our materials. Lorincz *et al.*[13] reported that pBR322-related sequences were found by Southern blot analysis in about 12% of exfoliated cervical cells. Filter *in situ* hybridization using exfoliated cervical cells by itself alone seems not to be suitable for diagnosis of infections with multiple types of HPV.

From the study of 23 cervical papillary condylomas with and without vulvar lesions, we found a close relationship between the HPV type and the distribution of the lesions. Cervical condylomas without vulvar lesions had a higher incidence of HPV16 and HPV18 infections in comparison with those with vulvar lesions (60% versus 15%) (Table II). We found HPV6/11 DNA in all 21 samples of exfoliated cells from condylomas localized in the vulva (unpublished data). In general, papillomaviruses have host-specificity and tissue-specificity, and show habitat segregation. These data suggest that cervical condylomas without vulvar lesions have preferential association with HPV16 and HPV18 compared with condylomas with cervical and vulvar lesions and condylomas only with vulvar lesions.

The incidence of HPV16 and HPV18 infections in histologically proven CIN and cervical cancers was 32.3% (41/127). This is fourteen-fold higher than that in the control group (2.3% (15/666)). HPV16 and 18 positivity increased gradually with the severity of cervical neoplasia (CIN I/II: 24.3%, CIN III: 34.6% and invasive cancer: 48.4%). These data suggest that CIN containing HPV types 16 and 18 is a precancerous lesion with a high risk of progression to cancer. The occurrence of HPV types 16 and 18 in cervical cancers in this study was lower than what was reported from Europe and the USA.[1, 10, 11] However, these results are consistent with the data obtained by Southern blot analysis in Japan.[2, 14, 15] Recently, Saito *et al.*[14] and Yajima *et al.*[15] reported that the lower positivities of HPV16 and HPV18 DNAs in cervical carcinomas from Japanese women may be due to associations of other types of HPV.

We found HPV6/11, HPV16 and HPV18 infections in 0.9%, 1.8% and 0.6% out of 666 normal control cases,

Table III. Geographic Distribution of HPV Prevalence in Cytologically Normal Women

Country	No. of cases	HPV6/11-positive	HPV16-positive	HPV18-positive
Japan (This study)	666	0.9%	1.8%	0.6%
Brazil[8]	2189	4.1%	4.2% (HPV16+HPV18)	
The Netherlands[9]	1033	0.3%	0.6% (HPV16+HPV18)	
West Germany[7]	8755	9% (HPV6/11+HPV16+HPV18)		

respectively. HPV16 infection was the most common in normal controls. In previous studies on HPV infections in normal controls using filter *in situ* hybridization, the total occurrence of HPV 6/11, HPV16 and HPV18 ranged from 0.9% to 9% and the occurrence of HPV16 and HPV18 ranged from 0.6% to 4.2% (Table III).[7-9] The incidence of HPV infections in normal controls in this study was comparable with that of the previous studies in other countries, though we cannot compare the individual incidences of HPV16 and HPV18 infections with the previous studies.

The relationship of HPV6/11, HPV16 and HPV18 infections to age in the control group is shown in Fig. 3A. High incidence of the infections was observed in 30 to 49 age groups, and the incidence became apparently lower in women over 50. Since this control group includes only a small number of women under 30, we could not study the incidence of subclinical HPV infections in women in their teens or twenties. The age distribution of subclinical HPV infections seem to lean toward older age than that of papillary condylomas, which is known to have peak incidence in women in their twenties from an epidemiologic study.[16] These data indicate that HPV16 and 18 infections are likely to persist subclinically and that the persistance of HPV16 and 18 infections up to perimenopausal age might be related to sex hormone excretions or sexual activities of hosts.

In our study there was no significant difference in the occurrence of HPV between pregnant women and non-pregnant women aged under 35 (7.6% versus 5.6%) (Fig. 3B). Schneider *et al.*[17] reported that HPV infections were 2.3 times more frequent in pregnant women than in the non-pregnant (28% versus 12.5%). In their study, the pregnant cases in the second or third trimester (beyond the 12th week of gestational age) had increased prevalence of HPV infections (29% (25/85)), but the cases in the first trimester had almost the same incidence of HPV infections as non-pregnant cases (14% (1/7)). The absence of any difference of the incidence of HPV infections between the pregnant and non-pregnant cases in this study might be due to the fact that all our pregnant cases were in the first trimester.

We identified HPV6/11, 16 and 18 infections by filter *in situ* hybridization in exfoliated cells from cervical precancerous lesions, cervical cancers and normal control cervices of Japanese women. It is important to investigate further the natural history of HPV infections in the female genital tract in order to establish the exact role of HPVs in the generation of cervical cancers.

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