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DETECTION OF HUMAN PAPILLOMAVIRUS DNA IN GYNAECOLOGICAL SWABS BY FILTER IN SITU HYBRIDIZATION

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Summary. - Gynaecological smears from the endo- and ectocervix of women with and without cytological and colposcopic abnormalities of the epithelium were investigated for human papillomavirus (HPV) types 6, 11, 16, and 18 by filter *in situ* hybridization (FISH). The data were compared with cytological, colposcopic, and histological findings. Of the 266 gynaecological smears, HPV DNA was detected in 84 (32 %); of 101 cytologically and colposcopically HPV negative cases, HPV DNA was found in 10 %. Of 56 women, cytologically and colposcopically positive for HPV infection, HPV DNA was detected in 68 %. The sensitivity of the method was controlled by comparing the results of FISH with those of Southern-blot analysis of five cervical tumour biopsies. The data presented demonstrate the necessity of FISH for identification of the HPV type that might be of prognostic value in cervical pathology. Cytological and colposcopic positivity is a reliable sign in about 70 % of the cases where HPV infection was proved by FISH.

Key words: human papillomavirus; DNA/DNA hybridization; cervical cytology

Introduction

Available data inplicated human papillomaviruses (HPVs) as aetiological agents of genital cancer (zur Hausen, 1986; Broker and Botchan, 1985; Pfister, 1987). HPV DNA of specific types have been identified by techniques of molecular hybridization. Types 16, 18, 31, 33, and 35 have been found in all grades of cervical intraepithelial neoplasia (CIN I-III) as well as in invasive cervical

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cancer, whereas HPV 6 and 11 were identified predominantly in *condylom accuminata* (Dürst *et al.*, 1983; Boshart *et al.*, 1984; Lorincz *et al.*, 1986; Giss mann and Schneider, 1986). It has been demonstrated that HPV infection wit types 16 and 18 represents an increased risk for a mild cervical dysplasia t progress through CIN into carcinoma *in situ* (CIS) and to invasive cancer. Thi risk is relatively low when associated with types 6 or 11 (Dürst *et al.*, 1985 Campion *et al.*, 1986; Syrjänen *et al.*, 1986).

In benign lesions and in CIN the viral DNA persists mostly extrachromoso mally; it may be integrated into the host cell genomes in invasive cancers and i tumour derived cell lines (Dürst *et al.*, 1985; Schwarz *et al.*, 1986; Mincheva *e al.*, 1987). In most cervical lesions a few cells containing high number of HP DNA copies can easily be detected as spots by DNA hybridization (Wagner *e al.*, 1984). There are various procedures for detecting HPV DNA sequences i clinical specimens (scrapes or smears) (Gissmann *et al.*, 1987), from whic FISH is one of the widely used assays (Wagner *et al.*, 1984). However, FISH i expensive and a special training is necessary before being routinelly used. Th commercially available hybridization kits for determining HPV types are use mainly for research purposes. In many laboratories cytological, colposcopic and histological criteria are still used for screening of HPV infection.

Here we report data for the presence of HPV DNA in gynaecological swab from patients with or without cytological or colposcopic features associate with HPV infection. The samples were hybridized with ^{35}S-or ^{32}P-labelled HP DNAs of the most commonly detected types - HPV 6, 11, 16, and 18 in th female population screened for the presence of HPV DNA. This allowed us no only to screen for presence of HPV DNA in the cervical smears but also to tes the reliability of the cytological and colposcopic criteria for HPV infection i comparison with the hybridization results in the same sample.

Materials and Methods

Patients. Two hundred and sixtysix smears from ecto- and endocervix were collected from 22 women aged from 15 to 64 years (mean age 27) attending the Institute of Obstetrics and Gynaeco logy of Medical Academy in Sofia for several reasons: previous positive cytology, obvious cervica lesions, vaginal discharges, sterility, ovarian disfunction, early pregnancy.

Colposcopy. When colposcopy was performed for diagnosis of HPV infection account was take of the following: exophite lesions are obvious; flat and inverted lesions have characteristic colpos copic patterns in 20 % of the cases - the presence of micropapillar projections in the atypical zone characteristic vascular patterns, hyperkeratous mosaicism or so-called satellite lesions. Th common satellite lesions are: *cervicitis et colpitis condylomatosa, vestibulitis papillomatosa* an condylomata on the vagina and vulva. Colposcopically conducted biopsies were taken an examined.

Cytology. Two specimens were collected by rotating a cotton tipped swab in the endo- and ecto cervix or from other colposcopically abnormal areas of the genital tract. One sample was taken fo conventional cytology. The cells were fixed in 97 % ethanol and stained routinely with haematoxi lin-eosin. Cytological evidence for HPV infection was based on the presence of three cellula

types: koilocytes, dyskeratocytes or condylomatous parabasal cells (Meisels et al., 1976; Meisels *et al.,* 1977). Another sample was taken from the same place and cells were resuspended in 4 ml of phosphate buffered saline (PBS) and stored at -20°C until tested by FISH.

Histology. Eightyone colposcopically conducted biopsies were examined histologically and evaluated according to the semiobjective-scoring system of Reid *et al.* (1984). The following histological features were associated with HPV infection: koilocytosis, akanthosis with papillomatosis, and dyskeratosis.

On the basis of colposcopic, cytological, and histological evaluation the patients were distributed into the following investigation groups:

1. Group with different degree of atypia of the epithelium:
CIN I-II (mild and moderate dysplasia);
CIN III (severe dysplasia) and carcinoma *in situ* (CIS);
invasive carcinoma;
2. Group without CIN but showing colposcopic patterns suspicious for HPV infection: spiked *papilloma colli uteri,* punctuated zone, flat or keratous mosaicism, a combination of punctuated zone and mosaicism with satellite lesions;
3. Group with *condylomata acuminata;*
4. Group without visible changes of the colli uteri (control group).

DNA hybridization. The smears taken with a swab were tested for the presence of HPV DNA by FISH as described by Wagner *et al.* (1984). Cells were suspended in PBS and counted; then 1 to 3 x 10^5 cells/ml were filtered through a nitrocellulose filter (0.45 μm pore size, 25 mm in diameter, Schleicher and Schull, Dassel, Germany) using a filtration unit (Schleicher and Schull). The cells on the filters were lysed and their DNA was denatured by incubation of the filters for 5 min in 1.5 mol/l NaCl and 0.5 mol/l NaOH. Following denaturation, the filters were neutralized for 10-20 min in a solution, containing 0.5 mol/l Tris-HCl pH 7.0 and 3 mol/l NaCl, and baked at 80°C for 1 hr. Each filter was cut into half and each half was separately hybridized with one radiolabelled probe. We used as a probe HPV 11, which also detects HPV 6 under the conditions used, as well as a mixture of HPV 16 and 18 (DNA probes were kindly provided by Prof. zur Hausen and Prof. Lutz Gissmann from DKFZ, Heidelberg). HPV DNAs were labelled with ^{35}S (in most of the cases) or ^{32}P by nick-translation according to Rigby *et al.* (1977) employing Amersham reagents. The labelled DNA fragments were purified utilizing Sephadex G-50 columns (Pharmacia, Sweden) as described by Maniatis *et al.* (1982). Specific activities ranged about 10^8cpm/μg (10^7 cpm of hybridization buffer). Prehybridization was performed in a solution, containing 5 x SSC (0.75 mol/l NaCl, 0.075 mol/l sodium citrate), 50 mol/l sodium phosphate buffer, pH 6.5, 5 x Denhardt's solution. 0.1 μg/ml denaturated t-RNA and 50 % formamide.

Hybridization was carried out for 48 hr under stringent conditions (T_m - 18°C) in the same solution but containing 5 - 10 ng of ^{35}S or ^{32}P-labelled HPV 11 or HPV 16+18 (16/18) DNA. After hybridization, the filters were washed 3 x 30 min at 68°C in 2 x SSC, 1 % SDS and placed in an X-ray cassete for autography. Filters were exposed for 1 — 7 days at -70°C when ^{32}P-labelled probes were used and 14 days when ^{35}S-labelled probes were used. For some specimens duplicate filters were hybridized separately with ^{35}S- and ^{32}P-labelled DNA probes.

HeLa cells filtered onto nitrocellulose membranes were used as positive controls in the HPV 16/18 hybridization and also clinically positive or negative cases were used as controls of the specificity of the test (Schneider *et al.,* 1985). The FISH was always made by the same person as well as the scoring of results.

Southernblot analysis. Nine biopsies (five from cervical carcinomas *in situ* and four control cases) were tested for the presence of HPV by FISH and by DNA extraction and Southern blot analysis (Southern, 1975). Total high molecular weight DNA from various biopsies was prepared by phenol and chlorophorm: isoamylalcohol (24:1) using standard protocols (Maniatis, 1985). Eight to ten μg of cellular DNA of each biopsy was digested with 40-50 units *Bam*HI or *Eco*RI (Boeringer, Germany) and analysed for the presence of HPV DNA by Southern blot hybridization. The blots were hybridized with ^{32}P-labelled DNA of HPV 11 and 16/18 under stringent

conditions. In this study also, in seven patients the DNA hybridization test was performed during pregnancy. In a few of these cases, the same assay was made after delivery. There were a few patients investigated after having diathermocoagulation (DTC) and hysterectomy.

Statistical analysis. The chi-square test of Pierson and Fisher was used for statistical analysis.

Results

A total of 266 gynaecological swabs was analysed for the presence of HPV DNA. The results of FISH for HPV DNA of types 11 and 16/18 and the correlation with the lesions examined are shown in Table 1. Of 266 samples, HPV DNA was found in 84 (32 %); 46 (55 %) of them were positive for HPV 16/18, 19 % were positive for HPV 11, and 26 were positive for both HPV 11 and 16/18. Koilocytes were seen in 67 (25 %) of the specimens and DNA was detected in 41 (61 %).

Of all examined smears 13 % showed signs of CIN I-II; 8 % had CIN III and CIS; 11 % had an invasive carcinoma; 35 % were colposcopically suspected for HPV infection and 9 % had *condylomata accuminata.* HPV DNA was found in all investigated groups including controls, but the incidence of HPV types 16 and 18 DNAs was different in individual groups. A high incidence of these types was observed in the cases with CIN I-II (10/16; 63 %) and in the group of colposcopically suspected of having HPV infection (11/27; 40 %). Cervical smears positive both for both HPV 11 and 16/18 were found at higher rate in low grade lesions and in colposcopically suspicious cases than in CIN III and

Table 1. HPV DNA in 226 gynaecological smears detected by FISH

Investigated groups	Number of smears	With HPV DNA	Distribution of HPV types		
			6/11	16/18	6/11+16/18
CIN I-II	35	16 (45 %)	2	10	4
CIN III	20	10 (50 %)	-	5	-
Carcinoma *in situ*			-	3	2
Cervical carcinoma	29	19 (65 %)	-	16	3
Colposcopically suspicious for HPV*	94	27 (28 %)	6	11	10
Condyloma accuminata	13	7 (53 %)	4	1	2
Control group	75	5 (7 %)	4	-	1
Total	266	84 (32 %)	16 (19 %)	46 (55 %)	22 (26 %)

CIN - Cervical intraepithelial neoplasia

* To this group were included: a) two patients with multifocal lesions *vestibulitis, ectopia* and cervical papilloma; one of them - positive for HPV 6/11 and the other - positive for HPV 6/11 + 16/18; b) one patient with adenomathous polyp (HPV negative); c) one with *papillomathosis vaginae* (HPV positive for 16/18)

CIS. HPV DNA of type 11 alone was also detected in the CIN I-II and in colposcopically suspected groups of patients as well as in the women with *condylomata accuminata.* The results for the presence of HPV DNA in the different groups of patients against these of the control group showed statistically significant differences ($p < 0.001$). The rates of HPV DNA detection as related to the colposcopically suspected lesions are shown in Table 2. The highest incidence of HPV DNA was found in women with spinocellular cervical papilloma.

Comparison of cytological and colposcopic findings and HPV detection by FISH is demonstrated in Table 3. Of the 56 specimens, cytologically and colposcopically positive for HPV infection, HPV DNA was detected in 68 %. In the specimens not exhibiting cytological and colposcopical features of HPV infection, HPV DNA was found in 10 % samples. HPV 11 was found in one case, but types 16 and 18 were found in six; three of the specimens were positive for both DNA probes. Of the 81 histologically evaluated cases HPV DNA was found in 38 (47%); HPV DNA of types 16/18 was observed in 24 (63%) of the specimens but type 11 was detected in three. Eleven specimens were positive for both HPV DNA probes. Of the seven specimens of pregnant patients five were positive for HPV 16/18 but were cytologically negative for changes suggestive for HPV infection. HPV type 11 was found in one of them. In a follow up examination, after delivery, three of the positive women were nega-

Table 2. Correlation between colposcopic criteria for HPV infection and HPV DNA detected by FISH

Colposcopically suspected lesions*	Number of smears	With HPV DNA	Distribution of HPV types		
			6/11	16/18	6/11+16/18
Spinocellular papilloma of the cervix	31	7 (30 %)	2	2	3
Mosaicism	9	2 (9 %)	-	2	-
Leukoplakia	9	2 (9 %)	1	1	-
Punctation	2	1 (4 %)	-	-	1
White epithelium	3	3 (13 %)	1	2	-
Ectopia with atypical zone of transformation	32	6 (26 %)	1	2	3
Vestibulitis					
Papillomathosis	3	1 (4 %)	-	-	1
Colpitis	1	1 (4 %)	-	-	1
Total	90	23			

* To the group of „Colposcopically suspected lesions" we included also two patients with multifocal lesions (vestibulitis, ectopia, and spinocellular cervical papilloma were present simultaneously), one of them - HPV 6/11 (+) and the other - HPV (+) for 6/11 and 16/18; here were included one patient with adenomatheous polyp (HPV negative) and one with papillomathosis vaginae (HPV positive for 16/18);

Table 3. Comparison of cytological and colposcopic findings and HPV detection by FISH

Criteria for HPV infection	Total number	HPV DNA (+)
Cytology and colposcopy - positive	56	38 (68 %)
Cytology - negative Colposcopy - positive	98	36 (37 %)
Cytology - positive Colposcopy - negative	11	3 (27 %)
Cytology and colposcopy - negative	101	10 (10 %)

tive for the presence of HPV DNA at the second test.

The reproducibility of FISH was controlled as performed by Schneider *et al.* (1985) by second examination of the cervical smears from the same women after an interval of one week to three months and by parallel analysis of DNA from cervical carcinoma biopsies. In our investigation three of five women were positive for HPV DNA 16/18 and were tested by FISH two times within a period of six months; three other patients (one of them positive for both HPV 11 and 16/18) were investigated three times within a period of three months. There was no change in the cytologic diagnosis as well as in HPV DNA typing of the examined cases with only one exception (a woman who was negative for HPV DNA at the first test but positive for HPV 16/18 at the second).

The results of Southern blot hybridization in all cases did not differ from the data of the FISH of the same specimens. In three cases the cells were collected in a post DTC period. The results of FISH were as follows: one of the women diagnosed CIN III, after DTC was cytologically negative but positive for HPV DNA of types 16/18; the second patient with spinocellular cervical papilloma showed no cytological changes after treatment but was positive for HPV 16/18; the third woman (diathermocoagulated because of a papillomathosis) was negative by all criteria for HPV infection. One patient, examined after laparohysterectomy because of CIN III was cytologically negative but positive for the presence of DNA HPV types 16/18.

Discussion

Our results indicate that HPV DNA of types 16 and 18 are frequently detectable in genital lesions of different severity as well as in cervical smears, cytologically and colposcopically negative for HPV infection. We confirmed initial

reports (Wagner *et al.*, 1984; Schneider *et al.*, 1985; 1987) on the reliability of the FISH for detecting HPV infection and HPV typing.

Unlike most authors who assumed that papilloma and condyloma are identical, we used histological criteria for distinguishing between papilloma and condyloma (Karageosov and Makaveeva, 1981). This led to more precise association of particular HPV type with the histologically defined lesion. Comparative analysis for presence of koilocytes was considered for most important and pathognomonic sign of HPV infection and HPV DNA proved similar to the observations of other authors (Morse *et al.*, 1988; Schneider *et al.*, 1988) that HPV infection can occur in the absence of cytological features suggestive of this sign.

Several studies have found a strong association between specific lesions and HPV types (Dürst *et al.*, 1983; Crum *et al.*, 1984; Boshart *et al.*, 1984; Mc Cance *et al.*, 1985; de Villiers *et al.*, 1987; Barnes *et al.*, 1988). It was of interest in our study, a high incidence of HPV DNA of types 16 and 18 in cases with CIN I-II. A possible explanation of this could be scored prevalence in this group of women from younger age (19 to 26 years) - positive for HPV 16/18 (data not published). We consider that a larger number of CIN I-II samples need to be analysed before drawing this conclusion. An interesting result is the rate of viral DNA detection in the group „Colposcopically suspected for HPV" in view of the significance of detecting HPV infection not yet associated with CIN (Mitchel *et al.*, 1986; Nach *et al.*, 1987; Schneider *et al.*, 1988). It is also notable that 11 of the 94 „colposcopically suspected specimens" contained HPV 16/18 and 10 of them the both DNA probes. The cases that proved to be cytologically and colposcopically negative for HPV infection but positive for HPV DNA of types 16/18 have been included in a follow-up study.

The comparison of cytological and colposcopical data with virological results showed that HPV infection in cervical cells is most securely detectable (in about 70 %) through a combination of cytological and colposcopic investigation. However, HPV DNA was detected through *in situ* hybridization in 10 % of the cases which had been previously proved cytologically and colposcopically negative for HPV infection. The results presented in this report show that the application of colposcopy in combination with cytological and histological evaluation is a reliable approach for the testing of HPV infection, without any additional investment. On the other hand, epidemiological data from some reports (Sedaghi *et al.*, 1984; Beral *et al.*, 1986; Meanwell *et al.*, 1987) showed that the number of cases with CIN is increasing with high prevalence of it among younger sexually active groups. Syrjänen *et al.* (1986) were the first to have given direct evidence, that cervical lesions positive for HPV 16 and 18 may progress through various grades of CIN into CIS and invasive cervical carcinoma. Data from many studies show that cervical HPV infections should be considered precancerous lesions and HPV 16 and 18 are the predominant types found in the higher grade of CIN as well as in cervical cancer.

Our results support the necessity of using FISH for detection of the viral type

in a particular lesion that might be of prognostic value. This cannot be achieved by routine cytological, histological, and colposcopic evaluation alone, even by an extended colposcopy.

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