

Human Papillomavirus DNA in Women without and with Cytological Abnormalities: Results of a 5-Year Follow-up Study

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To determine the prevalence of HPV 6, 11, 16, and 18 in a population without cytological or histological abnormalities, the cervical smears of women attending three clinics in Germany were screened over the past 5 years. The filter *in situ* hybridization method was used throughout. A total of 20,161 smears, taken from 11,667 women, were tested. When the results of only the first examination are considered, 8.8% (950/10,778) of women with normal cytology were positive for HPV DNA. If we divide the latter into age groups, 11% (852 HPV positive/7716) were below the age of 55 years and 3.2% (98 HPV positive/3062) were above this age. When the samples from patients who had undergone at least two examinations and remained cytologically negative during the 5-year period were examined (total, 2709 women), the HPV DNA positively increased to 34.7% (640/1862) for the sexually active age groups and to 9.0% (76/847) for those above 55 years of age. This study reveals that, although papillomaviral production is most pronounced in younger women, these infections are quite common in all age groups. During the period of investigation, 19 (0.65%) patients, who were diagnosed as cytologically negative at the first examination, progressed to carcinoma *in situ* or invasive carcinoma. Of these, 63.2% revealed a detectable HPV infection during the study period. The progression of HPV-positive women from normal cytology to CIN or cancer occurred at an annual frequency of 0.082%. With an infected lifespan of 45 years assumed, this results in a lifetime risk of 3.7%. © 1992 Academic Press, Inc.

INTRODUCTION

The evidence linking specific types of human papillomaviruses (HPV) to the development of anogenital cancers has become very strong [1]. Among cervical carcinomas, HPV 16 remains the most prevalent type, whereas HPV 18 predominates in adenocarcinomas [2]. Apart from these two types, 25 additional so-called genital HPVs are detected in biopsies of CIN, VIN, PIN, and vulvar, penile, and anal carcinomas [3]. Not only are these viruses present in the lesions, but an increasing amount of ex-

perimental data points to their essential role in the development of malignant proliferations (reviewed in [1]). In *in vitro* studies, transfection of human foreskin keratinocytes with, e.g., the E6 and E7 genes of HPV 16 or HPV 18 leads to immortalization [4-6] of these cells, or, in a system allowing stratification of keratinocytes (raft system), to altered differentiation, similar to *in vivo* Bowenoid changes [7,8]. A structural modification of controlling host cell genes appears to be required for the switch from immortalization/altered differentiation to high-grade intraepithelial neoplasias and the malignantly transformed cell [9].

Five years ago, very little was known about the distribution of papillomaviruses associated with genital lesions. Studies were hampered by a substantial variation in the methods used for their detection, as well as by the fact that, at that time, only four different genital HPVs had been identified and characterized. Nevertheless, attempts had been made to estimate the prevalence of the papillomaviruses, not only in diseased tissue, but also in cervical smears of symptomless, cytologically negative patients [10,11].

We made a preliminary report [11] on the screening of cervical smears obtained from 9295 women attending three clinics in Germany. The patients, with normal cytology and in sexually active age groups (<55 years), showed a positivity of approximately 10% for HPV 6, 11, 16, and 18, whereas the infection rate in older women declined to 5%. At that time we calculated our results to be an underestimation of the real rate of HPV infection by a factor of 2 to 3.

The reasons for the continuation of this study over a 5-year period were the following: (1) Will a patient with normal cytology and who is positive for HPV DNA develop CIN or carcinoma and (2) if a patient is examined several times, can a pattern in the fluctuation of HPV

positivity be identified? In this report we present the observations made after the conclusion of this study.

In order to not only maintain methodological consistency, but to handle the large series of individual probes as well, all probes were assayed by filter *in situ* hybridizations despite a higher sensitivity of other procedures (e.g., Southern blot analysis).

MATERIALS AND METHODS

Over a period of 5 years (1985–1989), a total of 11,667 patients attending the outpatient clinics of three hospitals in the southwestern area of Germany were screened. These were the Evangelisches Krankenhaus, Freiburg, the Frauenklinik of the University of Ulm, and the Salem Krankenhaus in Heidelberg. During the first 2 years of investigation (1985–1986), all women attending the above-mentioned clinics for routine screening were included in the study. For the following 3 years the decision was made to include “new patients” only if they were under the age of 20 years and above the age of 60 years. The number of patients examined decreased to 32.3, 21.5, and 27.4% (for the above-mentioned hospitals, respectively) of the number examined during 1985–1986. During 1989 these numbers declined further to 21, 13, and 10.8%, respectively. It was not possible to recruit patients for this study. Only those attending the hospitals on a voluntary basis could be included. In Germany the clinical history of a patient is based on personal communication and therefore is not readily available. Personal information, e.g., sexual activity, age at first intercourse, number of partners, and contraceptive practice, is therefore difficult to obtain.

A total of 3294 patients underwent at least two examinations. These included 366 patients who had displayed an abnormal cytological smear already at the first examination and 219 patients who developed a cytologically abnormal smear during the period of investigation. A total of 19 of this latter group developed carcinoma *in situ* (CIN III) or invasive carcinoma during this period. Of the 2928 women from whom a normal cytological smear was taken at the first examination and who were subjected to at least two examinations, only 2709 remained cytologically negative throughout the follow-up examinations. The intervals between examinations of all patients varied extensively.

Cervical smears were obtained as described previously and detection of HPV DNA was performed using the filter *in situ* method with radioactively labeled HPV 6, 11, 16, and 18 DNA probes [11]. As pointed out before, the main reason for the use of this method was that large numbers of samples could be handled fairly easily. During the period of investigation a total of 20,161 smears were hybridized. After the initial evaluation of results, it be-

came clear that this method had several disadvantages [11]. However, to compare all results obtained, the decision was made to continue with filter *in situ* hybridization tests throughout the 5-year period. One disadvantage of this test was the low sensitivity (a detection rate of at least 50 genome copies per cell). Hybridization results were evaluated as HPV positive, questionably positive, and negative. The reason for this grouping was that it could not be determined whether a faint intermediate hybridization signal was due to a low copy number of HPV DNA, cross-hybridization to related HPV types present in the sample, or a nonspecific binding of radioactivity to the filter because of the presence of mucus or blood in the sample. Although the HPV DNA was carefully separated from the vector DNA, it is possible that small numbers of vector DNA were trapped in the radiolabeled HPV DNA used, resulting in a cross-reaction to a possible existing bacterial infection. Only a clear signal was regarded as positive. Questionably positive was regarded as negative, unless the patient proved to be true positive in a follow-up examination. At the time of onset of the study, the only identified HPV types known to be associated with genital infections were HPV 6, 11, 16, and 18. Later studies showed that these four types remain the most prevalent in genital lesions and to compare results throughout, only these four HPV types as probes were used. A further limitation was that no true distinction could be made between HPV 6/11 or HPV 16/18 infections. Filters were found to be positive for one or the other of these two groups, or even both. Cross-hybridization could result from a large quantity of virus from the one group cross-reacting to members of the other group. A determination of specific HPV types is possible only after extraction of the DNA from the sample, with subsequent application of restriction enzymatic digestion and Southern blot analysis.

RESULTS

The distribution, in age groups, of the patients examined is shown in Fig. 1. Of the 11,667 patients, 889 (7.6%) showed cytological signs of HPV infection (koilocytosis), CIN, or invasive carcinoma. Previously it was thought that the decline in HPV positivity in the older age range could be attributed to the relatively lower number of patients examined. We therefore tried to specifically increase these numbers. In the present evaluation we could include as many as 370 patients (previously 190) in the group 75 to 79 years and 218 (83) in the group over 80 years.

A total of 3294 patients underwent at least two examinations. Of these, 11.1% (366) had displayed an abnormal cytological smear already at the first examination and 6.7% (219) developed a cytologically abnormal smear

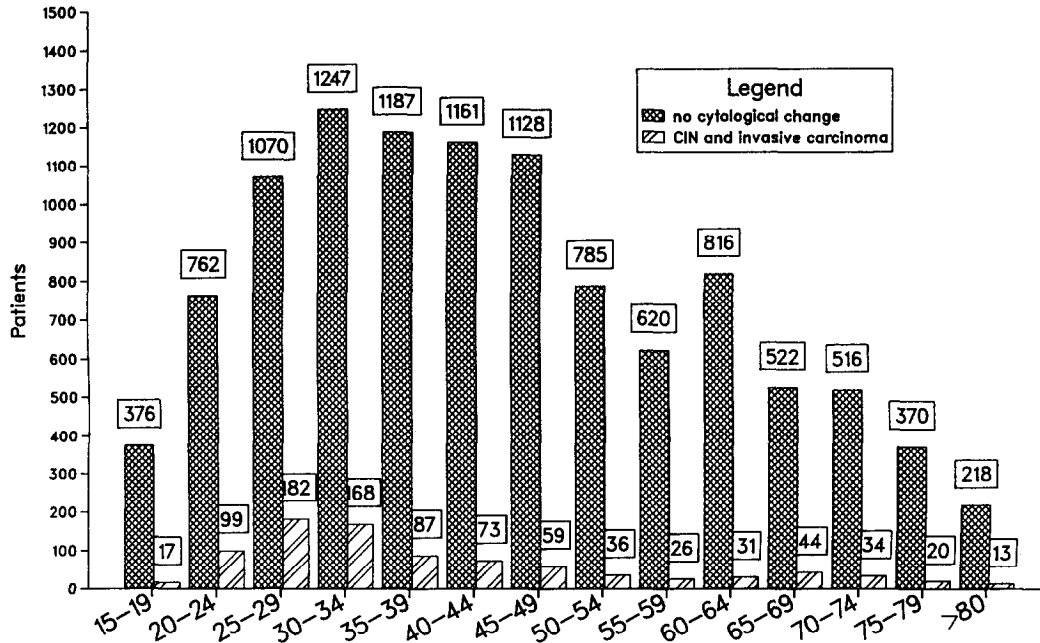


FIG. 1. Distribution, in age groups, of the patients examined.

during the period of investigation. In this latter group, 19 (8.7%) patients developed histologically confirmed carcinoma *in situ* or invasive carcinoma. Only 2709 of the women who had been subjected to at least two examinations remained cytologically negative throughout the follow-up examinations. The relative distribution in age groups of the patients examined more than once was the same as that for patients examined only once (as seen in Fig. 1), namely 65.3% of the patients were under 50 years of age and 34.7% were over 50 years.

Hybridization results were analyzed without knowledge of the cytological diagnosis. These results were, however, communicated to the clinicians. Due to the possibility of a biased interpretation of a second cytological smear when the first was HPV positive through hybridization, the category of cases with smears showing only "nonclassic" cytological signs of HPV infection was excluded from further analyses (for definition refer to [12]).

At least one cervical smear each of a total of 10,778 women with negative cytology was examined for the presence of HPV DNA. HPV DNA was detected in the smears of 950 women (8.8%) (Table 1). When these are grouped according to age (roughly according to menopausal status), the average HPV DNA positivity of 11% (852/7716) is seen in the group under 55 years of age, whereas those above this age revealed a lower degree of HPV DNA positivity (3.2% or 98/3062) (Table 2). As for the cytologically negative cervical samples taken from women who had undergone at least two examinations during the 5-year period (total of 2709), these two peaks of HPV DNA positivity remain, but the overall HPV

DNA positivity increases substantially (Table 1). The HPV DNA positivity in the sexually active age group increases to 34.4% and that for those above 55 years to 9.0%. The decrease in HPV positivity in the age groups above 55 years of age may be related to hormonal changes in the postmenopausal period, to low exposure to reinfection, or even to an inadequate number of cells obtained in the cervical sample.

In samples taken from CIN lesions or invasive carcinomas, HPV positivity remained almost at the same level regardless of age, when patients were examined only once (average, 23.5%; 209/889) (Table 1). However, for the equivalent group with at least two examinations, the average HPV positivity was higher in the age groups below 55 years (52.8%, 267/506) than in the age groups above this age (29.1%, 23/79). A possible explanation for this observation could be the low patient numbers in some of these age groups. Taking the average of all age groups, the HPV positivity increased to 49.6% (290/585). The filter *in situ* hybridization has a detection level of ca. 50 genome copies of HPV DNA per cell [13]. This figure of 49.6% is indicative of the low sensitivity of this test. If more sensitive methods, e.g., Southern blot hybridization or the polymerase chain reaction, were applied, the positivity rate would be expected to be in a higher range.

Early in this investigation, it was noted that asymptomatic patients varied quite considerably in HPV positivity when examined more than once. An attempt to clarify this question by following women over a longer period of time was made. It was not possible to specifically request that women with normal cervical cytology come

TABLE 1
HPV DNA Positivity in Relation to Age and Cytological Diagnosis

Age group (years)	Cytological diagnosis			
	Negative		CIN and CA	
	1 Examination	2+ Examinations	1 Examination	2+ Examinations
15-19	55/376 (14.6)	35/83 (42.2)	3/17 (17.6)	7/15 (46.7)
20-24	136/762 (17.8)	69/152 (45.4)	35/99 (35.4)	47/73 (64.4)
25-29	143/1,070 (13.3)	82/239 (34.3)	50/182 (27.5)	64/119 (53.8)
30-34	123/1,247 (9.9)	104/321 (32.4)	30/168 (17.9)	51/109 (46.8)
35-39	124/1,187 (10.4)	97/275 (35.3)	18/87 (20.7)	34/69 (49.3)
40-44	110/1,161 (9.5)	102/291 (35.1)	18/73 (25.7)	22/49 (44.9)
45-49	113/1,128 (10.0)	103/308 (33.4)	12/59 (20.3)	33/48 (68.8)
50-54	48/785 (6.1)	48/193 (24.9)	8/36 (22.2)	9/24 (37.5)
55-59	17/620 (2.7)	16/176 (9.1)	5/26 (19.2)	5/12 (41.7)
60-64	32/816 (3.9)	29/274 (10.6)	7/31 (22.6)	4/22 (18.2)
65-69	13/522 (2.5)	14/152 (9.2)	6/44 (13.6)	5/18 (27.8)
70-74	15/516 (2.9)	7/145 (4.8)	7/34 (20.6)	6/16 (37.5)
75-79	14/370 (3.8)	5/72 (6.9)	8/20 (40.0)	2/8 (25.0)
80+	7/218 (3.2)	5/28 (17.9)	2/13 (15.4)	1/3 (33.3)
Total	950/10,778 (8.8)	716/2709 (26.4)	209/889 (23.5)	290/585 (49.6)

Note. Numbers in parentheses refer to percentage of positivity.

to the clinic for an examination; therefore women in this group who were examined at least five times during the period of investigation, i.e., presumably attending for the sole reason of a routine examination, totaled 172. Of these, 41 (23.8%) were HPV positive in the first test, whereas 65 (37.8%) showed an HPV-positive, cytologically negative smear in at least one subsequent examination. Among all women with normal cytology and at least two examinations, 100 women (of whom 24 were subjected to more than two examinations) showed HPV positivity in every sample taken. Of the total of 270 women who had HPV-positive smears at the first examination, but tested HPV negative in all subsequent samples, 105 had undergone three or more examinations. It could not be assessed whether this is an indication of a loss of HPV infection for the following reasons: The method used for the DNA detection is too insensitive to detect single copies of persistent viral infection. The lo-

cations of the viral infection, with resulting viral production or shedding, in the genital tract could be numerous and some areas missed when a sample is taken. The same reasons would apply for those women (normal cytology) who tested negative at the first examination and positive in any subsequent examination (total of 285); i.e., no conclusion regarding the possibility of new infections in these cases can be drawn.

Another observation was the relatively high number of smears taken from patients who had undergone hysterectomy, testing positive for HPV DNA [14]. The cases in which the reason for the hysterectomy could be ascertained totaled 379. Of these 301 had suffered leiomyoma uteri or descensus uteri, whereas 78 gave the reason as precarcinomatous or carcinomatous lesions of the cervix (Table 3). No significant difference ($P = 0.38$) in the overall HPV DNA positivity between the first (16.3% or 49/301) and the second group (20.5% or 16/78)

TABLE 2
Summary of Results Shown in Table 1

Age (years)	Cytological diagnosis			
	Negative HPV + /total (%)		CIN or carcinoma HPV + /total (%)	
	1 Examination	2+ Examinations	1 Examination	2+ Examinations
15-54	852/7716 (11.0)	640/1852 (34.4)	174/721 (24.1)	267/506 (52.8)
>55	98/3062 (3.2)	76/847 (9.0)	35/168 (20.8)	23/79 (29.1)

TABLE 3
HPV DNA Positivity in Hysterectomized Patients

Age group (years)	Reason for hysterectomy	
	Myoma/descensus	Precarcinoma/carcinoma
15-19	0/0 (0)	0/0 (0)
20-24	0/0 (0)	0/0 (0)
25-29	0/0 (0)	0/2 (0)
30-34	2/10 (20.0)	1/5 (20.0)
35-39	9/26 (34.6)	3/12 (25.0)
40-44	13/38 (34.2)	4/10 (40.0)
45-49	13/57 (22.8)	6/18 (33.3)
50-54	6/51 (11.8)	0/12 (0)
55-59	2/54 (3.7)	1/6 (16.7)
60-64	4/65 (6.1)	1/15 (6.6)
65-69	0/44 (0)	0/9 (0)
70-74	0/39 (0)	1/11 (9.0)
75-79	0/19 (0)	0/2 (0)
80+	0/9 (0)	0/6 (0)
Total	49/301 (16.3)	16/78 (20.5)

Note. Numbers in parentheses refer to percentage of positivity.

could be determined, although the average percentage of positive tests exceeded that of patients with normal cervixes (HPV DNA positivity at the first examination as seen in Table 1).

During the course of the 5 years, 19 (0.65%) patients whose cervical smears had been diagnosed as cytologically negative at the first examination (out of a total of 2928 women) progressed to either carcinoma *in situ* or invasive carcinoma (Table 4). Smears were taken at every visit and hybridized against HPV DNA. Of these, 12 patients (63.2%) revealed detectable HPV DNA. Three patients had undergone only 2 examinations, two of these with intervals of 27 and 39 months between visits. The patients who had undergone more examinations (ranging from 3 to 13 visits) could be grouped into two categories: (a) those who showed normal cytology at several examinations and then in a following sample CIN III or invasive carcinoma and (b) those who, during subsequent examinations, showed a progression from normal cytology to CIN III or invasive carcinoma. In the first group, 2 patients were treated for ovarian cysts, 2 used contraceptives, 1 used an intrauterine device, and 1 was pregnant. Interestingly a relatively high number of patients were HPV negative throughout the study period. In the second group, 3 women used an intrauterine device and 1 was pregnant. Five of the six patients in this group were HPV positive. In both groups the time intervals between a cervical sample with normal cytology and that displaying CIN III or invasive carcinoma varied substantially, ranging from 3 to 23 months.

Reports over the past few years on the prevalence of HPV infection in populations showing no cytological ab-

normalities have varied greatly. The argument has been that the sensitivity of tests used varied enormously [15]. However, the application of the highly sensitive polymerase chain reaction (PCR) did not resolve this problem. The results using PCR vary from a low 6% positivity [16] to one as high as 84% [17]. When rigorous procedures to prevent contamination of samples are applied, the HPV positivity seems to lie between 30 and 40% [18].

DISCUSSION

In the study presented here, we examined the cervical smears of 11,667 patients attending three gynecological clinics in Germany. Although we were aware of the disadvantages of the use of the filter *in situ* hybridization method to test for HPV DNA positivity, we chose to continue to apply it throughout the 5-year period. In addition, particularly in patients having several examinations at a young age, we cannot exclude that this group attended the gynecologist not just for routine smears, but for specific reasons as well (e.g., other genital infections) and may differ from other groups in certain epidemiological parameters (e.g., number of sexual partners). Although this could impede a careful evaluation of this study, the remarkable consistency of the data obtained suggests that this should be of minor or no importance.

When only one cervical smear of a patient was tested, the HPV DNA positivity in normal cervixes was 8.8% in a population of 10,778 patients. During the period of prominent sexual activity, 11% of the patients were HPV positive, whereafter it decreased to 3.2%. When multiple samples were taken from one patient over a longer period, a fluctuation between positive and negative results was obtained. This variation differed in each individual, but the possibility of obtaining a smear positive for HPV increased with increased sampling. In patients with normal cytology, 36.3% (<55 years) were HPV positive when samples were taken at least twice. The possibility of new infections cannot be excluded, although it seems to be somewhat unlikely in all the patients. Although the controlling mechanism is still unknown, the virus production most likely declines in subsequent years and the virus may be retained in a latent form in the cervical tissues. This correlates with the clinical observations of mild to moderate dysplasias regressing over a period of time [19]. Only few HPV-positive, cytologically negative cases progressed to carcinoma *in situ* or invasive carcinomas within the 5-year period. In this study, 19 (0.65%) of the 2928 patients (with normal cytology at the first visit) who had attended the clinics more than once during the 5 years developed lesions of carcinoma *in situ* or invasive carcinoma; 12 (0.41%) of those were HPV positive. After the symptomatic primary infection, a period of 20 to 50 years appears to elapse prior to development of a carcinoma

TABLE 4
HPV Positivity in Patients with Progressing Proliferative Changes

1. Two examinations only					
Patient age	Interval (months) between examinations	HPV results		Additional history	
		Normal cytology	CIN III/invasive Ca		
33	5	—	+	Vulval Ca	
27	39	—	+	—	
33	27	+	+	—	

2. Several examinations					
(a) Diagnosis of CIN III/invasive carcinoma after previous examination with normal cytology					
Patient age	Interval (months) between normal cytology and carcinoma	HPV results			Additional history
		Normal cytology	CIN III/Ca	Total period of follow-up	
34	4	—	+	+	IUD
29	16	—	—	+	—
34	5	—	—	—	Ovarian cyst
39	11	—	—	+	Contraception
37	11	—	—	—	—
27	7	+	—	+	Ovarian cyst
39	3	—	—	—	—
26	17	—	—	—	Contraception
63	12	—	—	—	—
17	3	—	—	—	Pregnancy

(b) During the total follow-up period the cytology progressed from normal to CIN III/invasive carcinoma					
To diagnosis of CIN III/invasive Ca					
Patient age	Follow-up period (months)	Number of examinations	HPV results (summarized)	Additional history	
23	8	3	+	IUD	
42	4	3	—	—	
32	23	4	+	Pregnancy	
36	23	6	+	—	
48	16	5	+	IUD	
22	10	4	+	IUD	

[9]. On the basis of a 0.41% conversion rate to carcinoma *in situ* or invasive carcinoma of HPV-DNA-positive women within a 5-year period of observation, one could therefore speculate that at least 2.9–3.7% of HPV-infected women could develop carcinoma *in situ* or cervical carcinoma during their lifetime. This figure assumes an infected lifespan of 35 to 45 years. These findings underline the hypothesis that, although HPV infection emerges as an essential factor, additional modifications of host cell genes controlling HPV expression are required for the development of a malignant lesion [9].

In conclusion, our study reveals that HPV infections are quite common in the population studied. Virus detectability and correspondingly virus production are most pronounced in younger age groups. Progression of HPV-positive women, as determined by filter *in situ* hybridi-

zation, from normal to CIN or cancer occurs at an annual frequency of 0.082%, resulting in a lifetime risk of about 3.7% (assuming an infected life span of 45 years).

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