

Human papillomavirus in false negative archival cervical smears: implications for screening for cervical cancer

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Abstract

Aim—To assess the value of detecting human papillomavirus (HPV) DNA in false negative archival cervical smears in population based screening programmes for cervical cancer.

Methods—Cytomorphologically classified false negative archival Pap smears (n=27) taken from 18 women up to six years before cervical cancer was diagnosed were blindly mixed with 89 smears from hospital patients with a variety of gynaecological complaints and tested for HPV by the polymerase chain reaction (PCR). Corresponding cervical cancer biopsy specimens were also available for HPV analysis. Neither the examining cytopathologist nor the molecular biologist was aware of the study design.

Results—HPV DNA was detected in the smears of 16 patients with cervical cancer missed previously by cytology. HPV 16 and 18 were found predominantly in those smears taken up to six years before the diagnosis of cervical cancer. The smears of the two remaining patients were reclassified as inadequate for cytology or contained no suitable DNA for PCR. In 15 patients the same HPV type could be found in the smears and the cervical cancer biopsy specimens.

Conclusions—The results indicate that high risk HPV types can be detected in archival smears classified as false negative on cytology and that cytological screening errors may be reduced if combined with PCR testing for HPV.

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Keywords: PCR, human papillomavirus, cervical cancer, screening.

In industrialised countries screening for cervical cancer is based on the presence of cytomorphologically abnormal epithelial cells in cervical smears. In spite of the success of cytomorphological examination in screening for cervical cancer, major drawbacks have been recognised.¹ The percentage of false negative smears varies considerably between 15% and 50%,²⁻⁵ while the number of false positive cervical smears is about 10%. Therefore, the sensitivity of cytology in screening for cervical cancer is quite low (50-85%) and the specificity is about 90%. This unreliability is compensated for clinically by repeating the procedure within

a rather short time interval, up to three years,⁶⁻⁸ depending on the results of the Pap smears. It has been shown recently that cervical cancer is strongly associated with the presence of high risk or oncogenic human papillomavirus (HPV) types (up to 100%).^{9,10} In addition, a number of clinical follow up studies of women with cervical intraepithelial neoplasia (CIN) indicate that the presence of HPV DNA is of predictive value in progressive CIN disease.¹¹⁻¹⁴

We have shown recently in a prospective study that only patients with cytomorphologically abnormal smears containing high risk HPV types are at high risk of developing progressive CIN.¹⁵ The aim of the present study was to determine whether HPV was present in 27 archival cervical smears taken up to six years before the women were diagnosed with cervical cancer.

Methods

STUDY DESIGN

From 1982 to 1993 a triannual cervical cancer screening programme was conducted in the district of Het Gooi, an area near Amsterdam, The Netherlands. Cervical smears were taken by several general practitioners and gynaecologists. Routine cytological screening was performed in the Department of Pathology of the Gooi Noord Hospital, Blaricum, The Netherlands, according to a modified Pap classification (KOPAC)¹⁶: Pap 1, normal cells; Pap 2, inflammation; Pap 3a, mild and moderate dysplasia; Pap 3b, severe dysplasia; Pap 4, carcinoma in situ; and Pap 5, (micro)invasive cancer. With respect to quality control in the screening laboratory, retrospective analysis of cervical smears of 132 women who developed squamous cell carcinoma (confirmed by biopsy) revealed that for the 18 women who developed cervical cancer all 27 preceding smears were originally classified as normal (Pap 1). To ensure that the study was carried out blindly, the 27 smears were randomly mixed with 89 Pap smears obtained from 50 women (technical control group) attending the gynaecological outpatient department of the same hospital for a variety of gynaecological complaints including treatment of CIN lesions. In this latter group a reasonable HPV prevalence rate could be expected.⁹ Neither the investigating cytopathologist nor the molecular biologist was aware of the study design. Before starting the study, the 27 smears from the cervical cancer group were rescreened by two independent cytopathologists. The HPV testing laboratory

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personnel were unaware of the results of the original or the revised Pap test. Data were presented per patient after the HPV high risk types present had been identified. Details of the most recent smears are presented here.

ISOLATION OF DNA FROM ARCHIVAL CERVICAL SMEARS

The archival Pap smears were placed separately in 50 ml xylene for 24 to 48 hours to remove the coverslip. The cells were scraped from the glass using a razor blade and collected in 1 ml xylene. After a 45 minute incubation at room temperature to clean the cells from the remaining Depex mounting solution, the cells were pelleted by centrifugation and washed twice in 96% alcohol. The pellets were then air-dried at room temperature. DNA was subsequently isolated according a modified guanidine isothiocyanate GTC/silica beads protocol described by Boom *et al.*¹⁷ Briefly, cells were lysed in lysis buffer (guanidine isothiocyanate, Triton X-100, EDTA, Tris-HCl (pH 6.4)) overnight or until the solution was clear. Silica beads were then added and the mixture was incubated for 60 minutes at room temperature and centrifuged. The resulting pellet was washed twice in wash solution (guanidine isothiocyanate, Tris-HCl (pH 6.4)) and once in 70% ethanol. After drying in air for 30 minutes, the nucleic acids were eluted from the beads by incubation with sterile water for 60 minutes at 58°C. After centrifugation, the supernatant fluid was precipitated in ethanol, the resulting pellet was dissolved in 200 µl TE and 10 µl was used for PCR.

ISOLATION OF DNA FROM CERVICAL CANCER BIOPSY SPECIMENS

Formalin fixed, paraffin wax embedded biopsy specimens taken from 18 patients with cervical cancer were available for HPV analysis. Depending on the size of the sample, one to six sections (4 µm) were cut and collected in 250 µl digestion buffer (1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.45% (v/v) Tween 20, and 10 mg/ml proteinase K). The samples were incubated overnight at 37°C followed by inactivation of proteinase K at 96°C for five minutes. The samples were centrifuged and 5 µl of the supernatant fluid was used for PCR.

HPV DETECTION AND GENOTYPING BY PCR

The detection of a broad spectrum of HPV genotypes was performed by general primer

mediated PCR (GP-PCR), slightly modified from the previously described protocol.^{18,19} Experiments performed with different β-globin primers, spanning DNA fragments of different sizes, revealed that only relatively small DNA fragments (200 base pairs) could be efficiently amplified from archival Pap smears. Therefore, the HPV genotyping procedure commonly used in our laboratory^{9,19-21} was adapted. β-globin PCR positive samples were subjected to general HPV PCR in which a 150 base pair fragment is amplified. HPV 6/11, 16, 18, 31, and 33 type specific primers were designed which were internally localised in the HPV general primer mediated product and spanned no more than about 100 base pairs.

These PCRs were performed as described for the β-globin PCR. After gel electrophoresis, PCR products were blotted and hybridised with the HPV 6, 11, 16, 18, 31, and 33 probe cocktail as described previously¹⁸ at a hybridisation and washing temperature of 65°C. Samples which were positive for HPV DNA but could not be typed as HPV 6/11, 16, 18, 31, or 33 were designated HPV X. A detailed optimal protocol for detecting HPV in archival smears is described elsewhere.²²

Results

Of the 116 archival Pap smears analysed, two were β-globin PCR negative; therefore, 114 smears from 67 women were subjected to HPV analysis. Of these, 68 (59%) were HPV positive. High risk HPV types (16, 18, 31; single and multiple infections) were detected in 41 (35.9%) smears. In 25 (21.9%) of the 114 smears HPV types other than 6, 11, 16, 18, 31, or 33 were present.

Those patients with cancer were identified based on clinical data and included nine with one and a further nine with two archival Pap smears. Only one cervical smear had inadequate DNA (β-globin PCR negative) for PCR and was not included in the analysis. Of the remaining 26 cervical smears, 24 (92.3%) were HPV positive. High risk HPV types (16, 18, 31; single and double infections) were detected in 23 (88.3%) smears. HPV X was found in one (3.8%) case. In the nine patients with two smears the same HPV type(s) was present in both smears indicating persistent HPV infection. The prevalence of HPV is summarised in table 1.

One of the smears from the control group was β-globin PCR negative and was not included in the analysis. A single smear was available from 28 patients. Two of these patients were treated

Table 1 Occurrence of HPV in false negative archival smears from women with cervical cancer and the control group, which comprised 50 women with a variety of gynaecological complaints

Study group	No. of patients	No. of smears per patient	% High risk* positive patients	HPV*						Persistent HPV in last two smears	
				6/11	16	18	31	33	X		M
Cases	9	1	7/9 (78%) ^b	-	3	2	-	-	1	1 ^c	ND
	9	2	9/9 (100%)	-	3	2	-	-	-	4 ^d	9/9
Controls	28	1	5/28 (17.9%) ^e	-	2	1	2	-	5	-	ND
	22	2-5 ^f	2/22 (8.6%)	-	1	1	-	-	5	-	1/7 ^g

* Result of single or last smear; ^b one PAP smear was unsuitable for testing (Pap 0) and one was β-globin PCR negative; ^c HPV 6/11/16; ^d HPV 16/31 positive on four occasions; ^e two HPV 16 positive patients had been treated for CIN III in the past; ^f 60 smears in total; ^g persistent virus was HPV X; ND = not determined.

Table 2 Characterisation of the cervical cancer patient group with cytomorphologically false negative archival Pap smears

Patient number	First smear (=Pap 1)			Second smear (=Pap 1)			HPV type in biopsy specimen ^c
	Time interval ^a	HPV	Revised cytology ^b	Time interval ^a	HPV	Revised cytology ^b	
1	1 year	ND ^d	Pap 3b/4	ND ^c			HPV 16
2	3 months	HPV negative	Pap 0/0	ND			ND (β-)
3	2 months	HPV 16	Pap 3b/4	ND			ND (β-)
4	3 months	HPV 18	Pap 4/4	ND			HPV 18
5	1 year	HPV 16	Pap 4/4	ND			ND (β-)
6	1 year	HPV 18	Pap 5/5	ND			HPV 18
7	1 year	HPV X	Pap 4/4	ND			HPV X
8	2 years	HPV 16	Pap 3b/3b	ND			HPV 16
9	2 years	HPV 6, 11, 16	Pap 5/5	ND			HPV 16
10	2 years	HPV 16/31	Pap 3b/3b	1 year	HPV 16/31	Pap 3b/3b	HPV 16,31
11	3 years	HPV 16	Pap 3b/3b	2 years	HPV 16	Pap 3b/3b	HPV 16
12	4 years	HPV 16	Pap 4/4	3 months	HPV 16	Pap 4/4	HPV 16
13	4 years	HPV 18	Pap 3b/4	2 years	HPV 18	Pap 3b/4	HPV 18
14	4 years	HPV 31	Pap 3a/3a	3 years	HPV 16/31	Pap 3a/3a	HPV 16/31
15	5 years	HPV 16	Pap 3b/3b	3 months	HPV 16/31	Pap 3b/3b	HPV 16
16	5 years	HPV 16	Pap 3b/3b	2 years	HPV 16/31	Pap 3b/3b	HPV 16/31
17	6 years	HPV 16/18	Pap 0/0	6 years	HPV 16	Pap 0/0	HPV 16
18	7 years	HPV negative	Pap 0/0	3 months	HPV 18	Pap 4/4	HPV 18

^a Time interval: period (years or months) before cervical cancer was diagnosed. ^b Cytology was revised independently by two cytopathologists (separated by dashes). ^c Not determined because smears were not available. ^d Not determined because β-globin PCR was negative. ^e Biopsy specimens were histologically classified as poor to well differentiated squamous cell carcinoma.

for CIN III in the past and eight patients underwent subsequent uterus extirpation (five with leiomyoma, two with menstrual disorders, one with uterine prolapse). Twenty two patients contributed two to five smears (10 with leiomyoma, seven with endometrial disorders, four with uterine prolapse, and one unknown).

A high risk HPV type was detected in five (17.9%) of 28 patients in the group with a single cervical smear. Smears from the two patients with CIN III lesions contained HPV 16. Of the 22 patients with two to five smears, two (8.6%) had a high risk HPV type detected in their final smear. HPV X was detected in all three smears from one patient, which is suggestive of persistent infection. A consistent pattern was not detected in successive smears from most patients.

The results of HPV detection in the Pap smears and cervical cancer biopsy specimens, and the revised cytology of the 18 patients with cancer are summarised in table 2. Of the nine patients with only one available Pap smear, the time of collection of the smear varied from two months to two years before cervical cancer was diagnosed. Smears from two patients were HPV negative. The smear from one of these patients was β-globin PCR negative (patient no. 1), while that from the other (patient no. 2) was reclassified twice as cytomorphologically inadequate (Pap 0/0). Of the remaining seven patients with one available cervical smear, HPV DNA (HPV 16 in three patients, HPV 18 in two, HPV 16, 6 and 11 in one, and HPV X in one) was present up to two years before cervical cancer was diagnosed.

Of the nine patients who had two cervical smears taken, HPV DNA was detected up to six years before diagnosis of cervical cancer. In eight of these patients both smears were positive for high risk HPV types. In one patient (no. 17) both cervical smears were reclassified as inadequate for cytomorphological examination (Pap 0/0), although both smears were β-globin and HPV positive. One Pap smear over seven years old (from patient no. 18) was β-globin positive and HPV negative, and was reclassified as inadequate for cytomorphological examination (Pap 0/0). The adequate Pap smear taken three

months before treatment for cervical cancer was started was positive for HPV 18. Finally, after excluding β-globin negative samples, no disagreement was found between HPV results for smears and biopsy specimens. Moreover, high risk HPVs were consistently present in the eight patients whose two smears contained amplifiable DNA.

Discussion

The number of deaths from cervical cancer has dropped dramatically in the past decade.^{23,24} This is largely thought to be because of population based cervical cancer screening programmes using Pap smears for the detection of cytomorphologically abnormal cells.²³⁻²⁶ However, the Pap smear has not eradicated the disease in any screened population to date. Reports on failures of cytology to predict cervical cancer and cumulative false negative error rates for invasive cancer of up to 50% have been published.²⁷ This complex cervical cancer detection system is prone to errors both in sampling (sampling error) and cytomorphological interpretation by the cytologist (screening error).¹ Recent epidemiological and basic virological data strongly suggest that specific HPVs play an important role in the development of cervical cancer.^{28,29} In general, HPV 6 and 11 have been associated with benign cervical lesions and are referred to as non-oncogenic or low risk HPV types, whereas HPV 16 and 18, and to a lesser extent HPV 31, 33 and 35, have been found mainly in cervical lesions with severe dysplasia (CIN III) and in more than 90% of cervical carcinomas.^{9,10,20,30,31} These latter HPVs are regarded as oncogenic or high risk HPV types. As HPV cannot be cultured in vitro, HPV genotypes must be identified by analysing nucleic acid sequences. The most sensitive HPV detection method is PCR which is theoretically able to detect one copy of a target sequence.^{32,33} Because of this sensitivity and the distribution of the different HPV types in cytomorphologically normal, dysplastic and malignant cervical smears and tissue,^{9,19,30,31} the detection of HPV in cervical smears by PCR in conjunction with cytology could potentially

improve screening for cervical cancer. With this in mind, we designed this retrospective study. Although patients with true negative smears would comprise the most appropriate control group, the expected prevalence of cervical cancer within the short time span studied (seven years) following a negative smear is so low (3% in The Netherlands) that any epidemiological control group had to be very large to be informative. This was checked in a pilot study of 40 age matched patients with cytomorphologically confirmed true negative smears over seven years old. We only found one HPV 16 positive case and no abnormal cytology in the seven year follow up period (unpublished data).

HPV DNA was detected in the early smears of those women who subsequently developed cervical carcinoma. Interestingly, in one patient both smears were unsuitable for cytomorphological examination, indicating that testing for HPV can yield good results even in cytologically inadequate smears. Another important observation is that in the same patient the same HPV type was present in both the diagnostic biopsy specimen and the smears taken seven years before the diagnosis of cervical cancer. Moreover, if two smears were available, the same HPV type was found in both. By contrast, a variety of HPV types, which subsequently disappeared during follow up, were detected in the control group. These patients did not develop cervical cancer.

This fits the hypothesis that persistent infection with high risk HPV types is a prerequisite for the development of cervical cancer²⁹ and suggests that the continuous detection of a high risk HPV type in cervical smears is a significant risk factor for the development of invasive cervical cancer. Indeed, we have recently shown, in a longitudinal, non-interventional, colposcopic study in 327 women presenting with abnormal cytology, that progression to cancer was only found in those women who had a persistent HPV infection.^{15 34}

Re-examination of smears taken from a woman who subsequently developed cervical cancer revealed that nearly all of the smears contained abnormal cells. Therefore, the detection of high risk HPV types, in conjunction with cytology, could potentially reduce the number of erroneously classified cervical smears substantially. Cytologically negative, HPV positive smears should be rescreened, thereby increasing the detection rate of abnormal cervical cells. For the Dutch population over 35 years of age, this means that 2% (the prevalence of high risk HPV types in this group) of cytomorphologically normal smears would be re-examined.²¹ In a preliminary experiment cytomorphologically abnormal cells were detected in smears from about 50% of women with normal cytology and oncogenic HPV types on rescreening (Meijer, personal communication).

As invasive cervical cancer takes about 12.7 years to develop,³⁵ the screening interval for women with cytomorphologically normal, HPV negative smears aged over 35 years could be increased to more than three years,^{36 37} as is presently the case in The Netherlands. In this

way, more effective screening with fewer screening errors could be attained. Analyses of the cost of these alternative screening approaches are currently under way.

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