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Human papillomavirus deoxyribonucleic acid detection in mildly or moderately dysplastic smears: A possible method for selecting patients for colposcopy

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OBJECTIVE: Current screening protocols for cervical cancer dictate that patients with smears read as mild or moderate dysplasia of the uterine cervix undergo colposcopy, although approximately half these women do not prove to have high-grade squamous intraepithelial lesions. The aim of this study was to determine whether human papillomavirus testing is capable of discriminating between high- and low-grade squamous intraepithelial lesions so as to be useful in reducing the number of colposcopic examinations.

STUDY DESIGN: We tested 190 consecutive patients with smears read as mild or moderate dysplasia for the presence of human papillomavirus deoxyribonucleic acid by use of two different polymerase chain reactions with the consensus primer pairs CPI/IIG and MY09/11. Typing was carried out by direct sequence analysis of the CPI/IIG amplicons. The MY09/11 amplicons were detected in enzyme-linked immunosorbent assay format with the SHARP (Solution Hybridization Assay for PCR Products) Signal System with two probe mixtures (A and B) to detect nononcogenic and oncogenic human papillomavirus types. The human papillomavirus test results were compared with the histologic diagnosis, which was regarded as the reference standard.

RESULTS: Fifty-six of the 190 patients had high-grade squamous intraepithelial lesions. The sensitivity was 96% for the CPI/IIG test and 95% for the MY09/11 polymerase chain reaction plus SHARP Signal System when probe B only was used. The specificity was 33% for the CPI/IIG test and 40% for the MY09/11 polymerase chain reaction plus SHARP Signal System when probe B was used.

CONCLUSION: A negative CPI/IIG or SHARP Signal System probe B test can select, respectively, 44 or 54 of the 134 patients without high-grade squamous intraepithelial lesions. The use of these human papillomavirus tests as a secondary triage in patients with smears that were read as mild or moderate dysplasia could prevent those patients from undergoing unnecessary colposcopy. However, respectively, 2 or 3 of the 56 patients who have high-grade squamous intraepithelial lesions would be missed by human papillomavirus testing. (*Am J Obstet Gynecol* 1997;177:548-53.)

Key words: Low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, human papillomavirus deoxyribonucleic acid test, colposcopy

High-grade squamous intraepithelial lesions of the cervix have the potential to progress to invasive carcinoma.¹ Thus the rationale for cervical cancer screening programs is that considerable morbidity and mortality could be avoided by early cytologic detection and treatment of these lesions. The majority of low-grade squamous intraepithelial lesions will regress to normal,² and

some authors consider it unnecessary to treat these lesions.³

Overdiagnosis on the basis of cytologic diagnosis is a growing problem.^{4, 5} Referral for colposcopically directed biopsy is certainly justifiable in patients with smears read as severe dysplasia because the majority⁶ will indeed prove to have high-grade lesions. The most frequent cytologic screening abnormality is mild or moderate dysplasia, and here the prevalence of high-grade squamous intraepithelial lesions is about 50%.^{6, 7} In The Netherlands it is recommended to restrict colposcopy only to those patients with two consecutive mildly or moderately dysplastic smears.

Because of the limited value of mildly or moderately dysplastic smears in predicting histologic high-grade squamous intraepithelial lesions, it could be useful to

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develop a secondary triage that would identify those patients most likely to have high-grade lesions before colposcopy, as was suggested by Reid and Lorincz.⁸ A similar challenge is encountered in patients with smears read as atypical squamous cells of undetermined significance according to the Bethesda classification, in whom the rate of high-grade lesions after colposcopy is even lower.⁹ Because the incidence of atypical squamous cells of undetermined significance is increasing in the United States, a secondary triage has been recommended for these patients as well.⁹

Several studies have examined the role of tests for human papillomavirus (HPV) in screening for cervical cancer.^{6, 7, 9, 10} The rate of HPV detection has been shown to vary, depending on which test is used.¹¹ HPV deoxyribonucleic acid (DNA) can be detected in 81% of abnormal cervical smears by use of a test based on the amplification of DNA (polymerase chain reaction) but in only 60% of abnormal smears if a nonamplification test such as hybrid capture is performed.¹¹

The prediction of high-grade squamous intraepithelial lesions has been studied with different HPV tests,^{7, 10} whereas the prediction of low-grade lesions has been investigated only with the hybrid capture test.^{3, 9} Cox et al.⁹ concluded that there is a role for HPV testing by hybrid capture in selecting patients with atypical squamous cells of undetermined significance for colposcopy.⁹ They reported that 14 of 15 patients with high-grade lesions were HPV positive and 90 of 125 patients with low-grade lesions were HPV negative by the hybrid capture test. With use of the hybrid capture method to test 96 patients with cytologic low-grade dysplasia for the presence of HPV, Hatch et al.³ found histologic high-grade lesions in 37 patients, of whom 29 were HPV positive. These authors recommended against the use of hybrid capture HPV testing as a triage method for patients with mildly or moderately dysplastic smears because patients with a high-grade lesion were missed.

The aim of the current study was to determine whether HPV testing by polymerase chain reaction could discriminate between the presence and absence of high-grade squamous intraepithelial lesions in patients with cytologically mild or moderate dysplasia. The results of two polymerase chain reactions were compared to ascertain which one would be most effective in predicting the absence of high-grade lesions and thereby possibly minimizing unnecessary colposcopic examination.

Material and methods

Study population. The study population included consecutive patients referred for colposcopy to the outpatient clinic of the Academic Medical Center in Amsterdam or to the De Heel Hospital in Zaandam. The included patients had a single or repeated mildly or moderately dysplastic smear, PAP IIIA as classified ac-

cording to the Papanicolaou system, which is used in the Netherlands. Patients with a history of colposcopy or previous treatment of dysplasia were not included.

After informed consent was obtained from the patients, cervical smears for HPV DNA detection were taken with a cotton swab and placed in 1 ml of Virapap transportation medium in Virapap collection tubes (Digene Diagnostics). They were kept at +4° C for short-term storage and at -20° C for long-term storage according to the specifications of the Virapap determination protocol.

Colposcopy was performed after application of 3% acetic acid. Punch biopsies were taken from the most abnormal area of the cervix. An endocervical curettage was performed if the transformation zone was not entirely visible. The material was fixed in formaldehyde. The colposcopists were unaware of the HPV diagnosis at the time of examination.

HPV DNA analysis. For DNA purification 100 µl of Virapap transportation medium containing scraped cervical cells was taken. DNA was extracted once with phenol-chloroform and precipitated with ethanol. Finally, the precipitated DNA was dissolved in 100 µl of 10 mmol/L Tris-hydrochloric acid and 1 mmol/L ethylenediaminetetraacetic acid (pH 7.0) and stored at -20° C until use.

Detection of HPV DNA by polymerase chain reaction amplification was performed with the degenerated consensus primer pair CPI and CPII, which amplifies a 188 bp fragment in the E1 open reading frame of a broad spectrum of genital HPV types. The polymerase chain reaction with the primer pair CPI/IIIG was performed as described before.^{11, 12} The 100 µl polymerase chain reaction mixture consisted of 10 mmol/L Tris-hydrochloric acid (pH 8.8), 50 mmol/L potassium chloride, 3.6 mmol/L magnesium chloride, 0.1 mg of bovine serum albumin per µl, 0.2 mmol/L of each deoxynucleoside triphosphate, 150 ng of each primer, 1.5 U of *Taq* polymerase (Amplitaq), and 10 µl of the DNA sample. Forty-step cycles (1 minute at 94° C, 1 minute at 55° C, and 2 minutes at 72° C) were performed and the amplification product was analyzed on a 2% ethidium bromide-stained agarose gel after phenol-chloroform extraction and ethanol precipitation.

For the SHARP (Solution Hybridization Assay for PCR Products) Signal System (Digene Diagnostics), we used the degenerated consensus primer pair MY09 and MY11, which amplifies an approximately 450 bp fragment in the L1 open reading frame.¹³ The MY11 primer was 5'-biotinylated. Polymerase chain reaction amplification was performed in a 50 µl polymerase chain reaction mixture containing 12.5 mmol/L Tris-hydrochloric acid (pH 8.3), 62.5 mmol/L potassium chloride, 2.5 mmol/L magnesium chloride, 0.2 mmol/L of each deoxynucleoside triphosphate, 75 ng of each primer, and 1.25 U of

Taq polymerase (Amplitaq). Forty-step cycles (1 minute at 94° C, 2 minutes at 55° C, and 3 minutes at 72° C) were performed. Amplimers were detected exactly as described by the manufacturer. A 5 µl sample of polymerase chain reaction mixture was denatured in base and hybridized to two different sets of unlabeled ribonucleic acid probes. Set A contained probes for the nononcogenic HPV types 6, 11, 42, 43, and 44 and set B contained probes for the oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58. The hybridization mixture was transferred to a streptavidin-coated microplate on which the ribonucleic acid–DNA hybrids were captured. These hybrids were subsequently detected by an alkaline phosphatase-labeled antibody directed against ribonucleic acid–DNA hybrids. Colorimetric measurements were obtained with use of *para*-nitrophenylphenol as a substrate in a microtiter reader. Appropriate negative and positive controls were included as prescribed by the manufacturer.

To determine whether samples were adequate for HPV detection by polymerase chain reaction amplification, amplification of a 184 bp fragment of the *A-myb* gene (EMBL accession number A-Myb: X 13294) was performed with use of the primer pair *A-myb1* (5'-CATGGAATGCCAATTAAACG-3') and *A-myb2* (5'-CATCCCTAAGTTTCGCTGCC-3'). The polymerase chain reaction was performed in a 50 µl mixture consisting of 10 mmol/L Tris–hydrochloric acid (pH 8.8), 50 mmol/L potassium chloride, 2.0 mmol/L magnesium chloride, 0.1 mg of bovine serum albumin per µl, 0.2 mmol/L of each deoxynucleoside triphosphate, 150 ng of each primer, and 0.75 U of *Taq* polymerase (Amplitaq) with 5 µl of the DNA sample. Forty-step cycles (1 minute at 94° C, 1 minute at 55° C, and 2 minutes at 72° C) were performed and then 10 µl of the amplification product was analyzed on a 2% ethidium bromide-stained agarose gel. All samples were positive with this polymerase chain reaction. Between patient samples negative controls (water only) were used throughout the extraction procedure. The polymerase chain reaction results of these samples remained negative.

The HPV types of the CPI/IIG polymerase chain reaction–positive samples were determined by direct sequence analysis of the purified 188 bp product with use of the 3' primer (CPI) as a sequencing primer.¹¹ Polymerase chain reaction products were concentrated and purified before sequence analysis by phenol-chloroform extraction, ethanol precipitation, and agarose gel electrophoresis. The appropriate bands were extracted from the agarose gel according to a modification of the procedure described by Boom et al.¹⁴

The HPV detection and the sequence analysis were performed without prior knowledge of clinical data.

Histologic study. The cervical biopsy specimens were diagnosed according to the Bethesda classification sys-

tem¹⁵ as the absence of dysplasia, low-, or high-grade squamous intraepithelial lesions by a single pathologist who had no prior knowledge of the patients' clinical data and HPV status. Low-grade lesions were defined as mild cellular and nuclear atypia. Stratification was generally maintained and the cells were variously differentiated. High-grade lesions were characterized histologically by a very low nuclear/cytoplasmic ratio and a lack of stratification. Mitoses and abnormal mitoses might be found through the full thickness of the epithelium.

Statistical analysis. The histologic diagnosis was regarded as the reference standard. High-grade squamous intraepithelial lesions were defined as the presence of disease. Low-grade lesions and the absence of dysplasia were defined as the absence of disease. The HPV test results of each patient were compared with the histologic diagnosis, and the sensitivity, specificity, predictive values, and 95% confidence intervals were determined.

A χ^2 test was used to compare HPV test results and the prevalence of high-grade squamous intraepithelial lesions in patients with a single mildly or moderately dysplastic smear and those with repeated mildly or moderately dysplastic smears. A *p* value <0.05 was considered statistically significant.

Results

Study population. Between February 1994 and October 1995 a total of 190 patients were enrolled in the study, of whom 141 were referred to the Academic Medical Center in Amsterdam and 49 to De Heel Hospital in Zaandam. One hundred six patients were referred for colposcopy after a single mildly or moderately dysplastic smear and 84 patients after repeated mildly or moderately dysplastic smears. The mean age of the patients was 35 years (range 16 to 65 years).

HPV DNA analysis. The CPI/IIG polymerase chain reaction was positive in 144 of 190 patients (76%). Sequence analysis of the amplimers revealed the presence of the HPV types listed in Table I.

The MY09/11 polymerase chain reaction plus SHARP Signal System was positive in 138 of the 190 patients (73%). Five of these 138 smears were positive with probe set A (3.6%), 117 with probe set B only (85%), and 16 with both sets A and B (12%). The oncogenic HPV types were detected by probe B as expected. Although set B contained probes for neither HPV-54 nor HPV-70, it was nevertheless able to detect both HPV types, apparently because of cross-hybridization with HPV type(s) present in mixture B.

Histologic study. The histologic diagnosis revealed no dysplasia in 24 patients, 110 low-grade squamous intraepithelial lesions, and 56 high-grade lesions. High-grade lesions were found in 28% of patients with a single mildly or moderately dysplastic smear and in 31% of patients with a repeated mildly or moderately dysplastic smear (*p* = 0.62).

Table I. HPV types in high- and low-grade squamous intraepithelial lesions detected by CPI/IIG polymerase chain reaction

HPV type	High-grade SIL	Low-grade SIL or no dysplasia	Total
HPV-6	0	1	1 (0.5%)
HPV-16	25	17*	42 (21.6%)
HPV-18	2	3	5 (2.6%)
HPV-31	9	14	23 (12.1%)
HPV-33	1	3	4 (2.1%)
HPV-35	1	3	4 (2.1%)
HPV-45	1	5	6 (3.2%)
HPV-51	2	2	4 (2.1%)
HPV-52	0	1	1 (0.5%)
HPV-54	3	3	6 (3.2%)
HPV-56	2	12	14 (7.4%)
HPV-58	5	9	14 (7.4%)
HPV-59	0	2	2 (1.1%)
HPV-68	0	1	1 (0.5%)
HPV-70	0	1	1 (0.5%)
HPV-X	3	13	16 (8.4%)
Test negative	2	44	46 (24.2%)
TOTAL	56	134	190 (100%)

SIL, Squamous intraepithelial lesion; HPV-X, as yet unidentified HPV type.

* One patient had both HPV-16 and HPV-18.

Relationship between HPV detection and histologic diagnosis

CPI/IIG polymerase chain reaction. Of the 56 patients with a high-grade squamous intraepithelial lesion, 54 were found to be HPV positive by the CPI/IIG polymerase chain reaction, resulting in a sensitivity of 96% (54/56) (Table II). Of the 134 patients without high-grade squamous intraepithelial lesions, 44 were determined to be HPV negative, yielding a specificity of 33% (44/134) (Table II).

HPV types 16, 18, 31, 33, and 35. When the detection of only HPV types 16, 18, 31, 33, and 35 by the CPI/IIG polymerase chain reaction was considered to be a positive test, the sensitivity was 68% (38/56) and the specificity was 70% (94/134) (Table II).

MY09/11 polymerase chain reaction plus SHARP Signal System. The sensitivity of the SHARP Signal System was 95% (53/56) with both probe sets A and B. The sensitivity remained 95% (53/56) by use of probe B alone (Table II). The specificity increased from 37% (49/134) when probe sets A and B were used to 40% (54/134) when probe B was used alone (Table II).

CPI/IIG polymerase chain reaction and MY09/11 polymerase chain reaction plus SHARP Signal System. The CPI/IIG primer-mediated polymerase chain reaction was performed in those smears that were negative with SHARP probe B alone. When the results of these two tests were combined, 55 of the 56 high-grade squamous intraepithelial lesions were found to be positive, resulting in a sensitivity of 98% (55/56) and a specificity of 28% (38/134) (Table II).

Table II. Comparative sensitivity and specificity with their 95% confidence intervals for different HPV tests

	Sensitivity and 95% CI	Specificity and 95% CI
CPI/IIG	96% (88%-100%)	33% (25%-41%)
HPV types 16, 18, 31, 33, 35	68% (54%-80%)	70% (62%-78%)
SHARP probe B	95% (85%-99%)	40% (32%-49%)
SHARP probe B plus CPI/IIG	98% (90%-100%)	28% (21%-36%)

CPI/IIG: Polymerase chain reaction with consensus primer pair CPI/IIG; HPV types 16, 18, 31, 33, 35: detection of HPV types 16, 18, 31, 33, 35 by CPI/IIG polymerase chain reaction and direct sequencing of the CPI/IIG amplicimers; SHARP probe B: MY09/11 polymerase chain reaction plus SHARP Signal System with only probe B used; SHARP probe B plus CPI/IIG: combined results of MY09/11 polymerase chain reaction plus SHARP Signal System with probe B only used and CPI/IIG polymerase chain reaction. CI, Confidence interval.

The negative predictive values were 96% for the CPI/IIG; 95% for the SHARP probe B test; 84% for HPV types 16, 18, 31, 33, and 35; and 97% for the combined results of SHARP probe B and CPI/IIG. The positive predictive values were 38%, 40%, 49%, and 36%, respectively.

The HPV test results were similar in patients with a single mildly or moderately dysplastic smear and those with repeated mildly or moderately dysplastic smears.

Comment

Our findings indicate that it is feasible to use a negative HPV test result as a basis for selecting patients who do not require colposcopy. The number of unnecessary colposcopies prevented by this secondary triage has to be contrasted with the number of patients with an HPV-negative high-grade squamous intraepithelial lesion who would not have been referred for colposcopy. On the basis of a negative CPI/IIG test, colposcopy could have been avoided in 44 of the 134 patients (33%) without high-grade lesions. The CPI/IIG test was negative in the cervical smears of two patients with a high-grade lesion who would have been missed if a negative CPI/IIG test had been used to rule out the need for colposcopy. When detection of only HPV types 16, 18, 31, 33, and 35 by the CPI/IIG polymerase chain reaction was instead used as a criterion, 94 of the 134 patients without high-grade lesions showed a negative test result, thereby increasing the specificity of testing from 33% to 70%. However, this test cannot be recommended because 18 of 112 women with negative test results proved to have high-grade lesions (Table III). The same sensitivity was achieved with the MY09/11 polymerase chain reaction plus SHARP Signal System with use of either both probe set A and B or probe set B only, whereas the specificity increased from 37% to 40% when probe B was used instead of probe A plus B. For our goal, probe A seems to be not useful. With probe B only, negative test results were obtained in 57

Table III. Number of patients who had negative results by different HPV tests

	High-grade SIL (n = 56)*	Low-grade SIL or no dysplasia† (n = 134)	Total
CPI/IIG	2	44	46
HPV types 16, 18, 31, 33, 35	18	94	112
Probe B	3	54	57
CPI/IIG plus probe B	1	39	40

CPI/IIG: Polymerase chain reaction with consensus primer pair CPI/IIG; HPV types 16, 18, 31, 33, 35: detection of HPV types 16, 18, 31, 33, 35 by CPI/IIG polymerase chain reaction and direct sequencing of the CPI/IIG amplimers; probe B: MY09/11 polymerase chain reaction plus SHARP Signal System with only probe B; probe B plus CPI/IIG: combined result of MY09/11 polymerase chain reaction plus SHARP Signal System with probe B only used and CPI/IIG polymerase chain reaction. *SIL*, Squamous intraepithelial lesions.

* False negatives.

† True negatives.

patients, of whom three had a high-grade squamous intraepithelial lesion. This test would have correctly eliminated the need for colposcopy in 54 of the 134 patients (40%) without high-grade lesions (Table III). The combined use of MY09/11 polymerase chain reaction plus SHARP Signal System with probe B only used and the CPI/IIG polymerase chain reaction would have avoided 39 (29%) unnecessary colposcopic examinations and missed only one patient with a high-grade lesion (Table III) but requires that two separate tests have to be performed. So, both the MY09/11 polymerase chain reaction plus SHARP Signal System with probe B and the CPI/IIG polymerase chain reaction performed well in predicting patients without disease. The first test seems preferable because the SHARP assay is easy to perform and is available in standardized kits suitable for the use in nonresearch laboratories as well. In this respect, it is also worthwhile to further evaluate the performance of nonamplification assays, like the recently described microplate version of the Hybrid Capture method.¹⁶

Although the identification and treatment of high-grade squamous intraepithelial lesions has been established as an effective strategy for reducing the incidence of cervical cancer, it has not been established that treating lesser grades of intraepithelial lesions will have an impact on cancer incidence.^{3,17} The majority of low-grade lesions will regress to normal or remain stable, with very few progressors.² Whether HPV detection can discriminate between progressive and nonprogressive low-grade lesions has to be assessed after follow-up of the patients in our study. Low-grade lesions without detectable HPV could appear to have a lower risk to progress compared with lesions with detectable HPV DNA even if no biopsies are taken. Remmink et al.¹⁸ did not perform

biopsies but considered the colposcopic impression as the diagnosis of the grade of dysplasia to study the natural history of cervical dysplasia. No progression was seen when no HPV or when low-risk HPV types were detected. The follow-up of patients with HPV-negative mildly or moderately dysplastic smears by repeat cytologic studies could therefore be a safe strategy, although the grade of dysplasia assessed by colposcopy alone¹⁸ does not always correlate with the grade of dysplasia as assessed by histologic diagnosis.¹⁹

The pretest probability, or prevalence, of the absence of high-grade squamous intraepithelial lesions was 71% and the posttest probability was 84% to 97%, depending on which HPV test was used. This means that HPV testing was able to increase the probability of identifying the absence of high-grade lesions before colposcopy by 13% to 26%. An improvement in the ability to predict high-grade lesions could also have important clinical implications.⁶ In this study HPV testing afforded an additional 7% to 20% benefit, increasing the probability of high-grade lesions from 29% to 36% to 49%.

In our study population there was no significant difference in HPV test results or in the prevalence of high-grade squamous intraepithelial lesions between patients who had a single mildly or moderately dysplastic smear and those who had repeatedly mildly or moderately dysplastic smears. This observation does not favour the current recommendation in The Netherlands that a second mildly or moderately dysplastic smear is required before patients are referred for colposcopy.

We used the histologic diagnosis of the colposcopically directed biopsy specimens as a reference standard for the assessment of the HPV tests. However, the reliability of histologic diagnosis may be limited by sampling errors in the colposcopically directed biopsies. Indeed, several reports have shown a 20% to 50% disagreement between colposcopically directed biopsies and methods that allow the study of the whole transformation zone, such as large loop excision, cold-knife conization, and laser cone.²⁰⁻²² Moreover, there is interobserver and intraobserver variability in the histologic diagnosis.²³ Thus the relationship between HPV infection and histologic diagnosis not only depends on which HPV test is used but also may be influenced by errors in the histologic diagnosis.

HPV testing could be used in patients with smears read as mild or moderate dysplasia to avoid unnecessary colposcopies, as was also suggested by Reid and Lorincz.⁸ Moreover, HPV testing could also play a role in identifying candidates for colposcopy who might be missed by current cytologic screening techniques. Cuzick et al.²⁴ reported high-grade squamous intraepithelial lesions in patients with HPV-positive cytologically normal smears and in those with abnormal cytologic findings. This finding suggests that HPV testing could be important in

reducing the risk of underdiagnosis as well. So changing the current screening policy to reduce overdiagnosis only seems not to be sufficient. Therefore we recommend that the performance of both cytologic testing and HPV testing be studied separately in current screening programs to determine exactly which combination of tests will offer the highest sensitivity and specificity.

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