Technical Advance

Short Fragment Polymerase Chain Reaction Reverse Hybridization Line Probe Assay to Detect and Genotype a Broad Spectrum of Human Papillomavirus Types

Clinical Evaluation and Follow-Up

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The purpose of this study was to detect and genotype 16 different human papilloma virus (HPV) types simultaneously using a short fragment polymerase chain reaction (SPF) hybridization line probe assay (LiPA). 152 women who were referred to the gynecologist because of abnormal cervical smear underwent colposcopic examination and repeat cervical smear. In addition, the cervical scrapes were analyzed for the presence of HPV by a novel general HPV polymerase chain reaction assay followed by a single reaction genotyping assay allowing for a simultaneous detection and identification of 16 different HPV types. HPV DNA was detected in 38% of normal follow-up cervical scrapes, 51% of scrapes with atypical squamous cells of undetermined significance, 78% of scrapes with mild dysplasia (low grade squamous intraepithelial lesions), 86% of scrapes with moderate dysplasia (high grade squamous intraepithelial lesions), and in 88% of scrapes with severe dysplasia and carcinoma *in situ*. One case of invasive squamous cell carcinoma was positive for HPV 16. Overall, a single HPV type was detected in 56% of HPV positive scrapes, with HPV 16 being the most common and accounting for 45% of all single infections. Forty-four percent of the positive scrapes contained multiple HPV types, of which double infections prevailed. Follow-up results proved the reproducibility and reliability of SPF HPV

LiPA. In conclusion, we have used and evaluated the SPF-HPV-LiPA system for the detection and genotyping of HPV infections. The combined detection-typing method proved to be sensitive, specific, simple, and fast, making mass screening of cervical scrapes accessible for routine practice and facilitating individual patient management. (Am J Pathol 1999, 155:1473-1478)

Worldwide, cervical cancer is the second most common cancer affecting women. Although screening programs to identify precursor lesions of cervical cancer have reduced significantly the mortality and morbidity of this disease, 500,000 new cases of invasive cancer of the cervix are diagnosed annually.¹ Epidemiological and molecular studies over the past two decades have demonstrated convincingly that certain types of human papillomaviruses (HPVs) are etiologically related to the development of most cases of cervical cancer.²⁻⁴ To date, more than 85 types of HPV types have been identified, of which 30 different HPVs have been found to infect the genital mucosa.⁵ Several HPV types, such as HPVs 16, 18, 31, 33, and 35 have been implicated in cervical carcinogenesis,⁶ whereas other types, such as HPVs 6 and 11, are frequently detected in benign lesions such as condylomata acuminata. Women infected with high risk HPV types are considered to be at a higher risk for the development of cervical cancer than those who are not infected with HPV or are infected with low risk HPV

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types.^{7,8} Several studies have shown the potential relevance of HPV testing in cervical cancer screening program and management of patients with atypical squamous cells of undetermined significance.^{7,9-13} Incorporation of HPV tests into screening programs might identify women at risk for developing invasive cervical cancer. Furthermore, absence of high risk HPV in the cervical smear would permit less aggressive management of women with mild or equivocal cytological abnormalities.

General polymerase chain reaction (PCR) assays with a broad spectrum specificity for HPV are widely used for the detection of the virus in clinical specimens.^{9,14-16} However, additional typing by hybridization or type-specific PCR assays is required to identify the individual HPV type. This is laborious and time-consuming. In addition, for many of the newly described HPV types, specific primers are not available. For these reasons, it is important to pursue further development of genotyping systems applicable for mass screening of cervical scrapes. In this report we describe the practical application of a reverse hybridization line probe assay (HPV-LiPA), recently developed for genotyping of a broad spectrum of HPV types in a single assay.¹⁷ Combined with a novel general (SPF) PCR assay, amplifying a fragment of only 65 bp, this SPF-HPV-LiPA permits a highly sensitive, simultaneous detection of 16 individual HPV types in a single reaction scheme.¹⁸ Cervical scrapes obtained from women who were referred to the gynecologist because of an abnormal result of their cervical smear were analyzed by SPF-HPV-LiPA to determine its practical usefulness in detection and identification of HPV in large numbers of clinical samples.

Materials and Methods

Patients

Cervical scrapes were obtained from 152 women who participated in the Cervical Cancer Screening Program and who were referred by general practitioners to gynecologists for colposcopic examination after two successive cervical scrapes with atypical squamous cells of undetermined significance (ASCUS) or one smear with mild dysplasia (LSIL), moderate dysplasia, severe dysplasia, or carcinoma *in situ* (the latter three according to the Bethesda classification as HSIL). The visit to the gynecologist took place from 2 weeks to 3 months after the last cytological examination. On referral to the Department of Gynecology, during colposcopic examination, before the application of acetic acid/Lugol solution, two cervical scrapes were taken. One slide was prepared for a routine cytological diagnosis. The residual material from the first scrape and the entire material from the second scrape were collected into ethanol carbowax solution, centrifuged, and embedded in agar (Kerstens HMJ, Robben JCM, De Wilde PCM, Hanselaar AGJM, submitted for publication). The slides were cytomorphologically classified according to the standard Dutch cytological classification system and linked to the Bethesda classification as indicated.

To study the predictive value of HPV infections for short and long term progression of intraepithelial lesions, a protocol for follow-up of patients is included in the overall study design. Because of the high regression rate, the Dutch management policy for women with ASCUS, mild dysplasia (LSIL), and moderate dysplasia (HSIL) is to keep these patients under strict follow-up surveillance and not to treat these women directly for their cervical disorder. In the current study, we were able to examine repeat cytological scrapes and perform repeat HPV analysis in a small group of women after a 3-month follow-up period. SPF-HPV-LiPA tests were performed in a blind fashion without a knowledge of the results of cytological examination or previous HPV testing.

DNA Extraction from Agar Sections

A single cell section of every specimen 3 μ m thick was put into a reaction tube and incubated overnight at 56°C in 200 μ l of 10 mmol/L Tris-HCl with 1 mmol/L EDTA, 0.2% Tween 20, and proteinase K (0.3 mg/ml). Proteinase K was inactivated by 10 minutes of incubation at 100°C. The sample was centrifuged for 10 minutes at 11,000 rpm and 10 μ l was directly used for PCR analysis. A water blank control was processed with each batch of 10 samples.

PCR Amplification of HPV DNA

Broad-spectrum HPV DNA amplification was performed using a recently developed short PCR fragment (SPF-PCR) assay.¹⁸ All HPV tests were performed blinded to the cytological results and other clinical data. SPF-PCR system amplifies a 65-bp fragment of the L1 open reading frame allowing for detection of at least 43 different HPV types.¹⁸ SPF-PCR was performed in a final reaction volume of 50 μ l containing 10 μ l of the isolated DNA sample, 10 mmol/L Tris-HCl (pH 9.0), 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 0.1% Triton X-100, 0.01% gelatin, 200 μ mol/L each of deoxynucleoside triphosphate, 15 pmol each of the forward and reverse primers tagged with a biotin at the 5' end, and 1.5 units of AmpliTaq gold (Perkin Elmer). The mixture was overlaid with two drops of mineral oil and incubated for 9 minutes at 94°C followed by 40 cycles of 30 seconds at 94°C, 45 seconds at 45°C, 45 seconds at 72°C, and a final extension of 5 minutes at 72°C. Each experiment was performed with separate positive and negative PCR controls. A total 15 μ l of each PCR product was electrophoresed in a 3% agarose gel and was stained with ethidium bromide. To avoid contamination by PCR products, different steps such as sample preparation and the amplification reaction were performed in strictly separated rooms.

HPV Genotyping by Reverse Hybridization using Line Probe Assay

A poly(dT) tail was enzymatically added to the 3' end of each of 16 oligonucleotide specific for 16 different HPV types, namely HPVs 6, 11, 16, 18, 31, 33, 35, 40, 42, 43,

Table 1. HPV Occurrence in Cervical Scrapes from Women Combined with Cytological Classification Results

Classification	No.	HPV positive	HPV type																			
			6	11	16	18	31	33	35	40	42	43	44	45	51	52	56	58	66*	D [†]	T [‡]	>T [§]
Normal	39	38%	1	1	4							1								5	2	1
ASCUS	47	51%	2	3	2			1	1							2	1	2		5	3	2
Mild dysplasia	27	78%	1		4			1							1			1		9	2	2
Moderate dysplasia	22	86%	1		7		1						2		1					4	2	1
Severe dysplasia and CIS	16	88%			6	3		1										1		2	1	
Squamous cell carcinoma	1	100%			1																	
Total	152	58	5	4	24	3	1	3	1			1	2		2	2	1	4	25	10	6	

*66, HPV 66, typed by sequence analysis.

[†]D, double infection.

[‡]T, triple infection.

[§]>T, more than triple infection.

44, 45, 51, 52, 56, and 58. The tailed probes were applied as horizontal lines to the membrane strips. A biotinylated poly(dT) control for conjugate was applied. The HPV-LiPA genotyping assay was performed as described previously.¹⁷ Briefly, equal volumes (10 μ l each) of the biotinylated products of PCR and the denaturation solution (400 mmol/L NaOH, 10 mmol/L EDTA) were mixed in test troughs and incubated at room temperature for 5 minutes, after which 1 ml of the prewarmed (37°C) hybridization solution (3X SSC (1X SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 0.1% sodium dodecyl sulfate) was added, followed by the addition of one strip per trough. Hybridization was performed for 1 hour at 50 $\pm$ 0.5°C in a closed water bath with back-and-forth shaking. The strips were washed twice with 1 ml of wash solution (3X SSC, 0.1% sodium dodecyl sulfate) at room temperature for 20 seconds and once at 50°C for 30 minutes. Following this stringent washing, strips were rinsed twice with 1 ml of a standard rinse solution.¹⁷ Strips were incubated on a rotating platform with an alkaline phosphatase-labeled streptavidin conjugate diluted in a standard conjugate solution at 20 to 25°C for 30 minutes. Strips were then washed twice with 1 ml of rinse solution and once with standard substrate buffer, and color development was initiated by addition of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium to 1 ml of substrate buffer.¹⁷ After 30 minutes of incubation at room temperature, the color reaction was stopped by aspiration of the substrate buffer and addition of distilled water. After drying, the strips were visually interpreted using a grid.

Sequence Analysis

In 4 scrapes only the conjugate control line on the HPV-LiPA-strip showed reaction indicating presence of HPV DNA; however, no reaction with any of the 16 HPV type specific probes was observed. To identify the HPV type involved the SPF PCR amplimers were cloned and sequenced.

PCR products were ligated into pCR2.1 vector immediately after amplification using the Original TA Cloning Kit (Invitrogen Corporation) and purified using Wizard plus Minipreps DNA purification systems (Promega) according to the manufacturer's instructions. The PCR fragments were sequenced using the Ampli Cycling sequencing kit (Perkin Elmer) and M13 forward primer for the pCR2.1 vector. All four samples were found to be positive for HPV 66.

Results

Correlation of Cytology with HPV Detection

The results of cytological examination correlated with the results of HPV DNA detection as shown in Table 1. In 39 of the women the follow-up smear was negative. However, in 38% of these negative scrapes HPV DNA was detected. HPV DNA was also detected in 24 of 47 (51%) cervical scrapes reported as ASCUS, in 21 of 27 (78%) scrapes with mild dysplasia (LSIL), in 19 of 22 (86%) scrapes with moderate dysplasia, and in 14 of 16 (88%) scrapes with severe dysplasia and carcinoma *in situ*. One scrape with invasive squamous cell carcinoma was positive for HPV 16.

Single versus Multiple HPV Types

The results of HPV genotyping and type distribution are summarized in Table 1. An example of the results of HPV-LiPA genotyping assay is shown in Figure 1. As seen in Figure 1, interpretation is very easy and multiple infections can readily be identified. In 53 of 94 HPV positive scrapes (56%) a single HPV type was detected. HPVs 6, 11, 16, 18, 31, and 33 accounted for 75% of the single infections, with HPV 16 being the most common and accounting for 45% of all single infections.

A remarkable finding was the high prevalence of multiple HPV types in cervical scrapes. Forty-one of 94 HPV

Figure 1. HPV genotyping of SPF amplimers by line HPV probe assay (HPV-LiPA). The grid on the right indicates the positions of different HPV probes. Positive control indicates the conjugate control line. The probe a31 reacts with products after PCR amplification of HPV 31 as well as HPV 40 and HPV 58. HPV 40 and HPV 58 have specific probes in a different location on the strip. Presence of HPV 31 can be identified when an additional band in location b31 is present. The numbers above the LiPA strips indicate consecutive samples. The numbers below the strips indicate HPV type(s) detected in the assay. In the last four strips multiple HPV types are detected.

positive cervical scrapes contained two or more HPV types, accounting for 44% of the HPV positive scrapes (Table 1). The distribution of the individual HPV types within the multiple infections was random (statistical analysis not shown) and any combination of HPV types could be found. Multiple HPV infection was more common in smears diagnosed as LSIL. The ratio of single *versus* multiple HPV type infection in normal, and ASCUS smears was similar, 46:54 and 58:42, respectively. The ratio in smears with LSIL was 38:62, and a reverse ratio 69:31 in smears with HSIL was observed. Up to six different HPV types were detected in a single sample. Double infections were most common, accounting for 61% of the multiple infections. Infections with three HPV types were detected in 24% of the scrapes, four HPV types in 7%, five HPV types in 5%, and six HPV types in 2% in the scrapes.

Low Risk versus High Risk HPV Types

The distribution of high risk HPV types (16, 18, 31, 33, 35, 45, 51, 52, 56, and 58) and low risk HPV types (6, 11, 40, 42, 43, and 44) in the scrapes is shown in Figure 2. Prevalence of high risk HPV types was increasing with the

Figure 2. Correlation between the cytological diagnosis and the detection of low and high risk HPV types.

Table 2. SPF-HPV-LiPA Follow-Up

First sampling		Second sampling	
Cytological diagnosis	HPV type	Cytological diagnosis	HPV type
ASCUS	Negative	ASCUS	Negative
ASCUS	Negative	ASCUS	Negative
ASCUS	Negative	ASCUS	Negative
ASCUS	Negative	ASCUS	Negative
ASCUS	16	Mild dysplasia	16
Mild dysplasia	31	Mild dysplasia	18,31
Mild dysplasia	16	Mild dysplasia	16
Mild dysplasia	52	Mild dysplasia	52
Mild dysplasia	6,16,31	Mild dysplasia	6,16,31
Moderate dysplasia	52	Moderate dysplasia	52
Severe dysplasia	18	Severe dysplasia	18
Severe dysplasia	18	Severe dysplasia	18

severity of cervical smear abnormality. The ratio of high risk to low risk HPV types in normal scrapes was 1.9:1, in ASCUS 2.9:1, in LSIL 18.5:1, and HSIL 28:1.

Seven percent of all multiple infections contained low risk HPV types only, 37% contained high risk HPV types only, and 56% contained a mixture of both low and high risk HPV types. The ratio of low to high/low/high risk multiple HPV infections in normal scrapes was 1:1.6.3, in ASCUS 1:1.3.5, in LSIL 0:1.1.2, and in HSIL 0:1.0.5.

Results of Follow-Up Cytology and SPF-HPV-LiPA

The results of the second cytological examination and repeat HPV analysis in a group of 12 patients after 3 months of follow-up is shown in Table 2. SPF-HPV-LiPA testing for the presence of HPV showed a complete correlation with the results obtained during the first sampling. In 4 cases with ASCUS diagnosis and negative first HPV testing result, the follow-up with second HPV testing was also negative. In one case of ASCUS, positive for HPV 16, the follow-up HPV testing was also positive for HPV 16 indicating a persistent infection. Cytological examination revealed LSIL. In one case of LSIL, positive for HPV 31, the second sampling revealed the presence of an extra type, HPV 18, suggesting additional exposure.

Discussion

Cytological screening programs together with clinical and public awareness have resulted in a remarkable decrease in the incidence of cervical cancer and the mortality due to this disease.¹⁹⁻²¹ However, substantial overtreatment of noninvasive intraepithelial lesions is the consequence of early surgical intervention.^{22,23} It is well established that only 1% of LSIL and about 12% of HSIL progress to invasive cervical cancer. As the markers of progression of squamous intraepithelial lesions are not established, identification of high risk HPV types in cervical scrapes would help to identify patients with increased risk for development of cervical cancer. World-wide at least 99% of the cervical carcinomas are HPV DNA positive.^{2,18,24-26} In addition to HPV 16 and 18, 13

other HPV types were detected as a single HPV type in the cervical carcinomas. These HPV types have been identified as high risk HPVs. Infection with high or low risk HPV type cannot be determined on the basis of cytological examination.²⁷⁻²⁹ The importance of high risk HPV in the pathogenesis of cervical neoplasia suggests that detection of an infection with high risk HPV would be useful in patient management.³⁰ Much effort has been taken to develop HPV detection and typing assays in the last two decades, which led to the development of broad-spectrum HPV PCR assays allowing for detection of at least 34 anogenital HPV types in a single reaction.^{9,14-16} In the present study, we have used a novel, ultrasensitive SPF HPV general PCR assay. The SPF assay was shown to be more sensitive than other assays using general primer sets, My11/09 and GP5+/6+.¹⁸ The other advantage of the SPF HPV general PCR assay is the combination with the reverse hybridization line probe assay permitting the simultaneous detection of 16 different HPV types individually in a single, quick assay. When this study was prepared for publication the HPV-LiPA has been further extended for genotyping of 25 different HPV types, including the 16 probes described in this report using a slight modification of the primer content.¹⁷ Recently, a phylogenetic tree based on sequence homology was developed in which HPV types were classified in groups of high risk (HPVs 16, 18, 31, 33, 35, 45, 51, 52, 56, and 58) and low risk (HPVs 6, 11, 40, 42, 43, and 44).⁵ The classification of HPV type as high risk was further substantiated by follow-up studies showing that the persistent infection with high risk HPV correlated strongly with progression of cervical intraepithelial neoplasia.^{8,15,27,31} In the current study, 13 cases with single or multiple infection of low risk HPV was detected and 77 cases with single or multiple infection of high risk HPVs or multiple infection of combined high risk and low risk HPV was detected. The detection of high risk HPVs increased with the severity of the lesion, from 38% in ASCUS up to 84% of the smears with HSIL. This finding illustrates that HPV infection, especially high risk HPV infection, is important for progression of cervical dysplasia. We were able to perform a repeat HPV testing on a cohort of 12 women after a 3-month follow-up. The results of the second HPV testing correlated completely with the results of the first HPV testing indicating that, SPF-HPV-LiPA is a highly sensitive, specific, and reproducible assay.

We found a high incidence of HPV in women with normal cervical smears (38%) or with atypical squamous cells of undetermined significance in their cervical smears (51%) compared to other data obtained in the Dutch population or European women.¹⁶ This reflects both the higher sensitivity of the system compared to other general HPV detection systems as described previously, as well as (and probably mainly) patient selection bias.^{9,32} Women were incorporated into the study who were referred to the gynecologist after at least two successive cervical smears containing cells consistent with atypical squamous cells of undetermined significance. Indeed, this apparent difference in prevalence rate was equalized in the higher grade lesions in which the prev-

alence of HPV was not different from those described in general.

We found multiple HPV infections in 27% of the total scrapes analyzed, which accounted for 44% of the total amount of HPV positive scrapes. This prevalence is much higher than previously described in the Dutch population or in general.^{8,14,16,24} The difference may be due to the high sensitivity of the method used in the current study. The major advantage of the SPF-HPV-LiPA is the possibility to discriminate very easily whether the amplification has resulted in heterogeneous PCR clusters, which cannot be differentiated by conventional hybridization methods used for genotyping general PCR products. The results indicate that the incidence of multiple infections has been highly underestimated. Kleter et al¹⁷ reported an incidence of multiple HPV infections in 34% of cervical scrapes obtained from women with mild or moderate dysplasia using the SPF-HPV-LiPA. Very recently, a similar reverse line blot detection method has been described by Gravitt et al³³ in which 27 HPV types could be identified in a single blot system. Although this genotyping system appears to have similar specificity as the SPF-HPV-LiPA, Gravitt et al³³ found only 8.5% of multiple infections among all HPV positive specimens. The main difference between the HPV-LiPA and the reverse line blot detection method, as described by Gravitt et al,³³ is the primary step of PCR amplification. Gravitt et al³³ used the My11/09 general PCR as initial step which proved to be less sensitive than the SPF HPV general PCR, especially for HPV types 16, 35, and 45, which may have influenced the rate of detection of multiple infections,¹⁸ although a difference in patient selection may also have affected the prevalence of multiple infections.

In conclusion, the SPF-HPV-LiPA system for the detection and genotyping of HPV infections proved to be a sensitive, specific, and simple assay. This combined detection-genotyping assay makes mass screening of cervical scrapes in principle accessible for routine clinical practice and research applications.

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