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Clinical Performance of Human Papillomavirus *E6, E7* mRNA Flow Cytometric Assay Compared to Human Papillomavirus DNA Typing

Christine Kottaridi, M.Sc., Ph.D., **Sotirios Tsiodras,** M.D., Ph.D., **Aris Spathis,** Ph.D., **Aikaterini Chranioti,** M.Sc., Ph.D., **Asimakis Pappas,** M.D., Ph.D., **Dimitrios Kassanos,** M.D., Ph.D., **Ioannis Panayiotides,** M.D., Ph.D., and **Petros Karakitsos,** M.D., Ph.D.

OBJECTIVE: To use flow cytometry to screen cervical samples for the overexpression of human papillomavirus (HPV) E6 *and* E7 *mRNA and compare the performance of this assay with an HPV DNA array for the detection of high-grade cervical lesions.*

STUDY DESIGN: Cervical samples were analyzed for HPV DNA by clinical arrays, and the overexpression of E6 *and* E7 *viral oncogenes was monitored using an HPV mRNA detection kit that quantifies the intracellular HPV* E6 *and* E7 *mRNA on a cell-by-cell basis.*

RESULTS: HPV positivity increased with severity of histologic lesions. On the basis of histology-confirmed CIN 2+ cases the specificity of HPV assay was 73.9% (95% CI 66.07, 80.88), whereas it was 39.3% (95% CI 31.85, 47.1) for the DNA assay.

CONCLUSION: The HPV assay provides an early pre-

From the Department of Cytopathology, Fourth Department of Internal Medicine, Third Department of Obstetrics and Gynecology, and Second Department of Pathology, Attikon University Hospital, Athens University Medical School, Athens, Greece.

Drs. Kottaridi, Spathis, and Chranioti are Research Fellows, Department of Cytopathology.

Dr. Tsiodras is Associate Professor, Fourth Department of Internal Medicine.

Dr. Pappas is Consultant Obstetrician and Gynecologist, Third Department of Obstetrics and Gynecology.

Dr. Kassanos is Associate Professor, Third Department of Obstetrics and Gynecology.

Dr. Panayiotides is Assistant Professor, Second Department of Pathology.

Dr. Karakitsos is Associate Professor, Department of Cytopathology.

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Address correspondence to: Christine Kottaridi, M.Sc., Ph.D., Department of Cytopathology, Attikon University Hospital, Athens University Medical School, 1 Rimini Street, 12462 Chaidari, Athens, Greece (ckottaridi@gmail.com).

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dictor of persistent HPV infection and may improve cervical cancer screening by increasing the specificity of detecting high-grade lesions. (Anal Quant Cytol Histol 2011;33:305–310)

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Cervical cancer is the second most common cancer in women worldwide and has an increased prevalence in developing countries; it is thus a major concern of public health.[1,2] According to World Health Organization estimations, in 2008 there were an estimated 529,000 new cases and 274,000 deaths resulting from cervical cancer.[3]

The infection with human papillomavirus (HPV) oncogenic types is the most important risk factor for cervical carcinogenesis.[4] The most prevalent HPV oncogenic types worldwide include 16, 18, 31, 33, 35, 45, 52, and 58.[5]

The expression of two viral genes, *E6* and *E7*, is responsible for a transforming high-risk HPV infection. The *E6* and *E7* viral oncogenes disrupt host cell factors such as the tumor suppressor genes *p53* and *pRB*, respectively, which control cell cycle and apoptosis.[6] The regulation of mitotic spindle apparatus is disturbed, and chromosomal instability is induced.[7] Up-regulation of the *E6* and *E7* oncogenes occurs, leading to inactivation of tumor suppressor genes.[8]

Although the life-long risk for HPV infection is 80%, the percentage of women who will develop cervical cancer is < 5%.[9] HPV DNA detection methods are well established,[10,11] but they do not distinguish between transient infections and the relatively small number of infections that result in cervical cancer. Overexpression of *E6* and *E7* oncogene transcripts is a biomarker that potentially can distinguish high-risk HPV infections that are progressing to cervical cancer.[12-15]

In the present study, we used a novel technique that combines in situ hybridization with flow cytometry to screen clinical samples for the presence of HPV *E6* and *E7* mRNA of all high-risk HPV genotypes. Furthermore, we report the prognostic value of this method and its possible application as predictor of disease progression when used as a screening test.

Materials and Methods

A liquid-based cytology ([LBC]; ThinPrep Pap-Test; Cytyc, Marlborough, Massachusetts, U.S.A.) sample was taken following consent for study participation by women in a general screening population. A monolayer smear was prepared on a TP 2000 Processor and stained according to the Papanicolaou method. A trained cytopathologist diagnosed each case, according to Bethesda 2001.[16] A total of 189 histology-confirmed samples from a general screening population of > 4,000 samples were screened for the presence of HPV DNA and *E6* and *E7* mRNA transcripts in intact cells. Patients were referred to colposcopy and biopsy based on an abnormal cervical cytology.

An aliquot containing 1 mL of the LBC sample was removed and prepared for DNA extraction. HPV DNA amplification was achieved by using biotinylated primers designed to amplify a fragment within the L1 region of the virus. Hybridization of the amplified polymerase chain reaction product with immobilized matching type-specific DNA probes took place on a low-density microarray tube (Array Tube System; CLONDIAG Chip Technologies GmbH, Jena, Germany). The data obtained in each analysis were processed automatically by the system. The CLART HPV 2 kit (Genomica S.A.U., Madrid, Spain) allowed the detection of infection and coinfection of 35 different HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85, and 89.

An additional 1-mL sample aliquot removed from the LBC sample and prepared as previously described[14,17] using the commercial kit HPV OncoTect *E6*, *E7* mRNA (incellDx, Inc., Menlo Park, California, U.S.A.). Samples were run on a Partec CyFlow SL (Partec, Münster, [Deutschland] Germany) with a 488-nm argon laser, with front-scatter and side-scatter set on logarithmic scale. The forward scatter vs. side scatter dot plot was used for the distinction of ectocervical, endocervical, and polymorphonuclear cells. The distinction between these cell populations was achieved as previously reported.[14]

To set the cutoff value of the flow cytometric analysis of *E6* and *E7* mRNA, an HPV-containing cell line provided by incellDx was used as positive control and cells grown without HPV also provided by incellDx were used as negative control. In order to further verify the cutoff value of the analysis, 150 cytologically "within normal limits" samples were prepared following the described protocol and were run on the cytometer.

Sensitivity, specificity, and positive and negative predictive values (PPVs and NPVs) were calculated for abnormal cytologic results (atypical squamous cells, cannot exclude high-grade lesion [ASC-H] and high-grade squamous intraepithelial lesion [HSIL] and above) and histological diagnosis of cervical intraepithelial neoplasia (CIN) 2 and above. Furthermore the same values were calculated for abnormal flow cytometric results against abnormal cytology findings (ASC-H and HSIL and above) or abnormal histology findings (CIN 2 and above). Positive and negative likelihood ratios were also calculated.

Results

Comparison of HPV OncoTect with an HPV DNA Array for Detection of Pre–Cervical Cancer Lesions and Cervical Cancer

To determine if HPV *E6*, *E7* mRNA quantification is a useful biomarker for histologically determined cervical cancer precursor lesions, we enrolled study participants between the ages of 21 and 65 years. Squamous cells were identified using light scatter, and *E6*, *E7* mRNA was quantified on a cell-by-cell basis (Figure 1). Using a cutoff of 1.5% overexpressing squamous cells as a positive test result, the positivity of HPV OncoTect increased with the severity of the lesions and intentionally reflected a bias toward high-grade lesions. HPV OncoTect was positive in 31.1% of CIN 1, 81.8% of CIN 2, 87.5% of CIN 3, and 100% in squamous cell carcinoma. The HPV OncoTect positivity in cases with negative histology was 10.8% (Table I).

Table I *HPV DNA and mRNA OncoTect Kit Positivity within Histology Groups*

Histologic classification	HPV DNA (+) (%)	mRNA OncoTect (+) (%)	Total
Negative	16 (43.2)	4 (10.8)	37
CIN 1	88 (80.7)	34 (31.1)	109
CIN 2	21 (95.5)	18 (81.8)	22
CIN 3	16 (100)	14 (87.5)	16
SCC	4 (80)	5 (100)	5
Total	145 (76.71)	75 (39.68)	189

Overall Performance of HPV OncoTect and HPV DNA Arrays With and Without Combined Cytology Results (Co-testing)

To determine clinically relevant performance data in our study, we calculated sensitivity, specificity, PPVs and NPVs, and positive and negative likelihood ratios for abnormal cytology compared to histologic findings and for flow cytometric results (Table II). HPV DNA arrays demonstrated increased sensitivity for CIN 2+ lesions compared to HPV OncoTect (95.35% vs. 81.4%). An increased sensitivity (90.48%) for HPV OncoTect was calculated for CIN 3+ lesions, whereas for HPV DNA arrays the sensitivity remained almost the same (95.24%). The specificity of HPV OncoTect was almost 2-fold greater than HPV DNA arrays for CIN

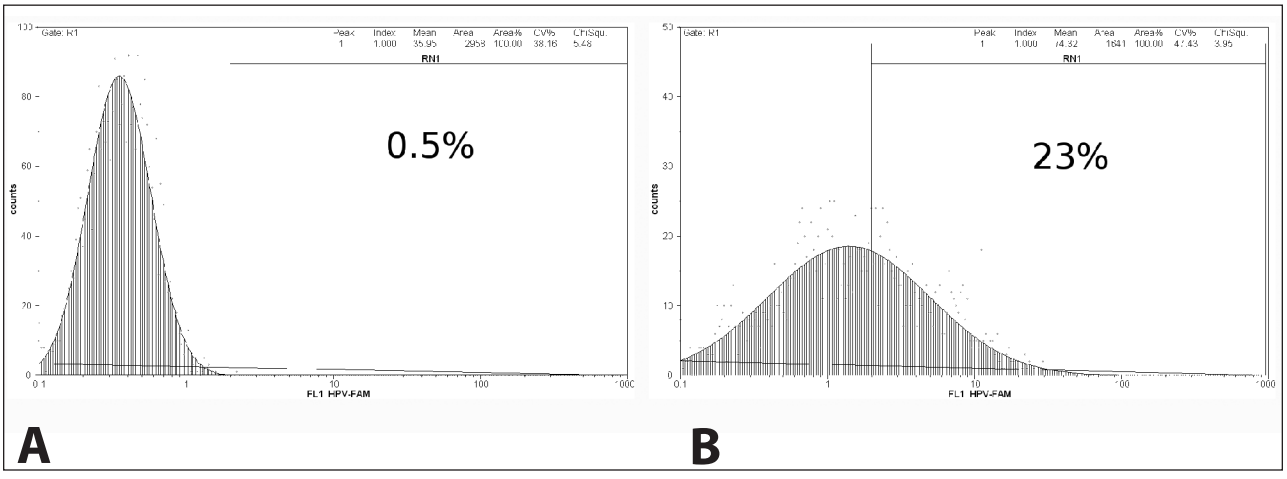

Figure 1 (A) "Within normal limits" sample. HPV *E6*, *E7* mRNA expression is quantified on a logarithmic scale on the x-axis; the percentage of *E6*, *E7* mRNA overexpressing cells is also quantified. The cutoff value for a positive test result is set at 1.5% of ectocervical cells. (B) CIN 2 diagnosed sample demonstrating *E6*, *E7* mRNA overexpression in 23% of ectocervical cells.

Table II *Sensitivity, Specificity, PPV and NPV and Positive (PLR) and Negative (NLR) Likelihood Ratios for Positive mRNA OncoTect Kit or*

Test	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)
mRNA OncoTect (+) vs. CIN 2+ histology	81.4 (66.6–91.61)	73.97 (66.07–80.88)	47.95 (36.10–59.96)
mRNA OncoTect (+) vs. CIN 3+ histology	90.48 (69.62–98.83)	83.2 (80.71–85.48)	30.16 (19.23–43.03)
ASC-H/HSIL+ vs. CIN 2+ histology	74.42 (58.83–86.48)	96.58 (92.19–98.88)	86.49 (71.23–95.46)
ASC-H/HSIL+ vs. CIN 3+ histology	95.24 (76.18–99.88)	89.88 (84.29–93.99)	54.05 (36.92–70.51)
mRNA OncoTect (+) and ASC-H/HSIL vs. CIN 2+ histology	69.77 (53.87–82.82)	98.63 (95.14–99.83)	93.75 (79.19–99.23)
mRNA OncoTect (+) and ASC-H/HSIL vs. CIN 3+ histology	90.48 (69.62–98.83)	92.26 (87.13–95.82)	59.38 (40.64–70.63)
Arrays (+) vs. CIN 2+ histology	95.35 (84.19–99.43)	39.29 (31.85–47.10)	28.67 (21.42–36.82)
Arrays (+) vs. CIN 3+ histology	95.24 (76.18–99.88)	26.79 (20.26–34.15)	13.99 (8.75–20.77)

2+ lesions (73.9% vs. 39.2%) and at least 3-fold greater for CIN 3+ lesions (83.2% vs. 26.7%). Similarly, HPV DNA was frequently positive in CIN 1 (80.7% vs. 31.1% for OncoTect) and HPV change (43.2% vs. 10.8% for OncoTect) in lesions universally left untreated. In the same table, we also depict the post test probability of disease. A combination of a positive HPV OncoTect test and high-grade cytologic lesion had a post test probability for CIN 2+ histologic lesions of 94%. When HPV OncoTect was positive in ASC-H/HSIL cytology samples, the PPV for CIN 2+ was 93.75% (95% CI 79.1, 99.2).

Discussion

In the present study, a total of 189 LBC specimens from women aged 21 to 65, were investigated by the OncoTect Kit *E6* and *E7* mRNA detection assay, which is based on flow cytometry. We report the utility of this method as a powerful tool in cervical cancer screening.

Epidemiologic and molecular data prove that the continuous presence of HPV is crucial for the development, maintenance, and progression of cervical cancer.[18] As reported, 70% of women with mean age 20 ± 3 years clear the infection within 1 year after the detection, indicating the role of the local immunity system in the clearing of an HPV infection at an earlier stage.[19] Also, only a small proportion of infected women will finally develop cancer.[20] Thus it is crucial to develop new methodology for predicting which women will develop subsequent dysplasia.

The most widely used methods for detecting HPV include extraction of total DNA and detection of genotype-specific high-risk HPV sequences. The performance of CLART HPV 2 assay, which was used in the present study to detect and genotype 20 high-risk HPV types, has been evaluated; according to the literature[11] it has very good overall agreement with Hybrid Capture 2 test (Qiagen, Hilden, Germany). Although most of the DNA methods are well established, none is able to estimate the activity of the viral oncogenes, a necessary event signaling cell transformation. Under the assumption that cervical cancer neoplastic progression occurs through HPV integration and subsequent expression of the HPV encoded oncogenes *E6* and *E7*, quantification of *E6/E7* oncogenes may be more helpful in assessing the potential for the presence and progression of lesions because the continuous expression of these proteins is necessary for the maintenance of a malignant phenotype.[21]

In the present study, we compared the performance of mRNA OncoTect with an HPV DNA array test for the detection of high-grade cervical lesions as determined by biopsy. Unlike all other commercially available HPV tests, HPV OncoTect uses liquid-based preparations for cervical cytology and keeps cells in suspension. The adequacy of specimens and particularly the distinction among ectocervical and endocervical cells and polymorphonuclear leukocytes was also determined as previously reported.[22,23] Although HPV DNA typing is a very useful tool to triage women with negative samples

Abnormal Cytology Results Versus Cytologic and Histologic Findings

NPV (95% CI)	PLR	NLR	Odds ratio (95% CI)	Post test probability (%)
93.1 (86.86–96.98)	3.12	0.25	12.43 (5.3–29.17)	48
98.41 (94.38–99.81)	2.81	0.14	47.04 (10.85–204.0)	26
92.76 (87.42–96.33)	21.73	9.49	82.04 (26.64–252.7)	86
99.34 (96.39–99.98)	9.41	0.053	177.6 (22.40–1409)	54
91.72 (86.26–95.52)	50.93	0.307	166.2 (35.61–775.2)	94
98.73 (95.47–99.85)	11.7	0.103	113.3 (23.72–540.9)	59
97.6 (89.78–99.64)	1.36	0.154	13.26 (3.10–56.72)	29
97.83 (88.47–99.94)	1.301	0.178	7.317 (0.95–56.14)	14

to longer term follow-up, the lack of specificity for high-grade lesions has led to serious concerns about sending too many women to unnecessary colposcopy and biopsy.[24]

According to the literature,[25-28] the presence of *E6* and *E7* transcripts is strongly associated with CIN 3 and invasive cervical carcinomas. Furthermore, the presence and increasing quantity of *E6* and *E7* mRNA[29,30] indicate persistent infections that could lead to cancer. In the present study, the percentage of HPV OncoTect–positive samples is, as expected, low in CIN 1 lesions because the transcriptional activity of the virus is down-regulated in the vast majority of early infections. Under such a scenario the most likely evolution of events is virologic clearance and regression of the lesions. The results presented here, are consistent with those coming from a study of Coquillard et al[14] in which the flow cytometric measurement of the percentage of cells overexpressing *E6*, *E7* mRNA in liquid-based cervical cytology samples correlates with the severity of the lesion.

Our results on the clinical performance of the OncoTect test indicate that because of its higher specificity compared to HPV DNA array; the OncoTect test would reduce the number of women referred to colposcopy unnecessarily. In the current study we compared this test to the ability of cytologic testing in predicting high-grade histologic lesions. A combination of a positive mRNA OncoTect test and high-grade cytologic lesion had a post test probability for high-grade histologic lesions of 94%. We report that a woman with high-grade cytologic lesions (ASC-H/HSIL) and a positive OncoTect test has a 93.75% PPV for CIN 2+ and can be confidently referred to colposcopy and biopsy.

In conclusion, *E6/E7* mRNA quantification using flow cytometry is a powerful tool in cervical cancer screening, with a non–time-consuming and non–labor-intensive workflow. Unlike other HPV *E6*, *E7* tests that genotype off of the *E6*, *E7* transcript, such as Aptima (Gen-Probe, San Diego, California, U.S.A.), Pretect HPV Proofer (NorChip, Klokkarstua, Norway), and NucliSeNS Easy Q (bioMérieux, Marcy l'Etoile, France), the HPV OncoTect quantifies the transcripts and thus detects the molecular event leading to cervical cancer.

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