

Human Papillomavirus E6/E7 mRNA Testing Has Higher Specificity than Liquid-Based DNA Testing in the Evaluation of Cervical Intraepithelial Neoplasia

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OBJECTIVE: To examine the specificity of human papillomavirus (HPV) E6/E7 mRNA testing for intraepithelial precursor lesions and invasive carcinoma of the uterine cervix in 358 women and compare the results with those of the most widely used DNA technique.

STUDY DESIGN: For HPV E6/E7 mRNA testing an amplification assay was used. For DNA determination a hybridization assay was applied. Both techniques were used simultaneously in patients with normal morphology (150), cervical intraepithelial neoplasia (173) and invasive carcinoma of the cervix (35).

RESULTS: HPV DNA positivity rates were significantly higher than E6/E7 mRNA in women with normal morphology (21–7%), cervical intraepithelial neoplasia (CIN) 1 and 2 (75–43%), and CIN 3 (93–63%). In invasive cervical carcinoma, both methods tested equally high (94% vs. 97%). Considering that E6/E7 up-regulation represents the initial step in cervical carcinogenesis, it can be assumed that this test allows a more specific detection of lesions with a potential for progression.

CONCLUSION: HPV E6/E7 mRNA may serve as a more specific discriminator between transient cervical dysplasias and potentially progressive lesions. Accordingly, testing for high-risk HPV E6/E7 mRNA might reduce the psychologic burden associated with HPV-DNA testing. (Anal Quant Cytol Histol 2011;33:311–315)

Keywords: cancer screening specificity, cervical cancer screening, cervical carcinoma, human papillomavirus DNA, human papillomavirus RNA, molecular testing.

Human papillomavirus (HPV) infections of the anogenital tract are associated with the development of both benign genital warts and cervical intraepithelial neoplasia (CIN) 1–3 and are considered essential in the etiology of cervical carcinoma.¹ More than 110 different HPV types have been isolated, showing a strong, type-specific tropism for epithelia or mucosa.² Approximately 30–40 of them

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may infect the human cervical epithelium.^{3,4} A division into low- and high-risk HPV types has been suggested for clinical purposes.² In Western Europe and North America ~95% of all HPV-positive invasive cervical carcinomas are associated with only five types, that is, 16, 18, 31, 33, and 45.

HPV is a very common virus among young, sexually active women, yet most infections are transient and asymptomatic.^{6,7} Only a small fraction of persistently infected women will subsequently develop cervical precursor lesions or carcinoma with a mean latency period on the order of 8–20 years.⁸ Therefore testing for HPV DNA has poor specificity and a low positive predictive value⁹ for the detection of significant precursor lesions of cervical carcinoma. In addition, HPV positivity carries a considerable psychologic burden for healthy patients in a screening situation.¹⁰ Therefore a more specific test to identify women with an increased risk for developing cervical carcinoma is needed.

Overexpression of the viral oncogenes E6 and E7 is essential for cell transformation and immortalization.^{11,12} E6 and E7 gene products initiate viral DNA replication and cell growth even in differentiated cells and abrogate programmed cell death mechanisms.¹³ Therefore the measurement of up-regulated E6 and E7 gene expression via elevated E6/E7 mRNA transcripts may serve as a more specific molecular marker in cervical carcinogenesis.^{14–16}

The aim of this study was to test a commercially available RNA-based real-time amplification (NASBA) assay (PreTect HPV Proofer, NorChip AS, Klokkarstua, Norway), for the detection of an early up-regulation of viral E6 and E7 mRNA. For comparison with DNA detection of HPV infections, the most widely used method (Hybrid Capture 2 system; Qiagen, Hilden, Germany) was used.

Materials and Methods

Study Population

The study protocol was approved by the Committee for Medical Research Ethics of each region involved and written informed consent was obtained from each study participant before enrollment.

Women were recruited by six hospital-based gynecologic clinics (Freiburg, Homburg, Greifswald, Wuppertal, Stuttgart, and Lahr) and two practicing gynecologists (Neu-Ulm and Stralsund) in Germany. A total of 429 women were initially enrolled into the study. After completion of the reevaluation process of all cytologic and histologic specimens, 71 women were excluded because of unavailability of

cell material for reevaluation ($n=11$), incomplete clinical data for study purposes ($n=11$), inadequate inclusion criteria for enrollment (e.g., vaginal or vulvar intraepithelial neoplasia [$n=37$]), current or previous systemic chemotherapy or radiotherapy with the risk for falsifying effects of cell material ($n=3$), ambiguous cytologic results without available review material ($n=2$), a malignant tumor of the cervix other than primary cervical carcinoma (e.g., cervical metastases of carcinoma of the colon [$n=7$]). Ultimately, 358 women met the inclusion criteria and were enrolled.

Collection of Specimens

Conventional smears of the ectocervix and endocervix were taken using a plastic spatula and a Cervex brush (Rovers Medical Devices BV, Oss, the Netherlands) and immediately fixed with spray fixative or 100% ethanol. The remaining cell material of the spatula or brush was immersed into a methanol-based fixation medium (20 mL Preserv-Cyt Solution; Cytoc, Marlborough, Massachusetts, U.S.A.). The solution was vortexed and divided into 5 mL for DNA testing and 15 mL for mRNA testing. The specimens were blinded as to patient data.

Cytologic Protocol

Cytologic smears were available from 349 patients. In 9 patients no smear was taken because an obvious cervical carcinoma was apparent clinically. The smears were stained according to the Papanicolaou method and read by local cytotechnologists and pathologists without knowledge of the HPV result.

Histologic Protocol

Histologic material was obtained from 237 patients, immediately fixed in 4% buffered formalin for at least 24 hours, processed, and stained with hematoxylin-eosin. The material ranged from small biopsy samples to extensive surgical material (i.e., radical hysterectomy with pelvic and paraaortic lymphadenectomy). According to the approval condition of the Ethics Committees, women with inconspicuous colposcopy and a normal cervical smear did not qualify for any biopsy procedure.

Second Review of Cytologic and Histologic Samples

All cytologic smears and histologic sections were reevaluated independently by two pathologists (V.S. and J.M.) blinded to patient's name, history, HPV status, and cytologic or histologic result. The review diagnosis was considered definitive. In five

cases with discrepant results between the two reviewers, a final diagnosis was established in a subsequent interactive session.

For cytology, the Munich II terminology, mandatory in Germany, was initially used. In contrast to the Bethesda System, this terminology combines mild and moderate dysplasia into a single group and puts only severe dysplasia into the high-grade group. Although most terminologies combine CIN 2 and 3 into the high-grade group, there is evidence that CIN 2 behaves more like CIN 1, making the case for the Munich terminology.¹⁷ For histology, the current World Health Organization (WHO) classification (2003) was employed. For better comparison with other terminologies, cytologic diagnoses were translated using the WHO classification (CIN 1, 2, 3).

Amplification and Detection of HPV E6/E7 mRNA

For detection of E6/E7 mRNA, the PreTect HPV-Proofer assay (NorChip AS, Klokkestua, Norway) was used. All analyses were performed at the Institute of Virology, Charité Hospital, Berlin, Germany. PreTect HPV-Proofer is an HPV type-specific assay and detects E6/E7 mRNA of the high-risk HPV types 16, 18, 31, 33, and 45 using the real-time multiplex NASBA technique.¹⁸ The specificity of the products is increased by real-time detection by molecular beacons.¹⁹

Briefly, total RNA was extracted from 3 mL of the vortexed cervical material (15 mL) by the RNeasy Mini Kit (Qiagen). The remaining 12 mL was stored for subsequent quality control and possible retesting. Three times, 5 μ L (for each of the multiplex mixes) of the total RNA was subjected to the NASBA reaction. The signal was measured in real-time using a fluorescent reader (NorChip PreTect Analyzer). Relative signal augmentation ≥ 1.7 was considered positive and < 1.4 as the negative result. Ambiguous values between 1.4 and 1.7 were retested, and if confirmed, the result was assessed as positive.

Hybrid Capture 2: Detection of HPV DNA

For the detection of high- and low-risk HPV DNA, the Hybrid Capture 2 system was used. All analyses were performed at Labor Zimmermann, Wasserburg, Germany. This test is an *in vitro* nucleic acid hybridization assay for the qualitative detection of low-risk (6, 11, 42, 43, and 44) and high- and intermediate-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68).

Briefly, DNA was extracted from 4 mL of the vortexed cervical material, denatured and hybridized with a specific RNA probe cocktail of the Hybrid Capture 2 Sample Conversion Kit; 1 mL of the vortexed cell material was stored. Resultant RNA-DNA hybrids (150 μ L) bound to alkaline phosphatase conjugated antibodies were detected with a chemiluminescent substrate. The emitted signal was measured by a luminometer (Qiagen). Relative light units between 2 and 8 were considered positive and < 2 as negative. Results were reported using the Digene DMS2 software (Qiagen).

Results

Study Population

The median age of all patients was 36.8 years (range, 17–82). For patients with normal morphology it was 35.8 years ($n = 150$; range, 17–74), for CIN 1/2 34.8 years ($n = 75$; range, 21–64), for CIN 3 it was 37.3 years ($n = 98$; range, 21–82) and for invasive cervical carcinoma 46.3 years ($n = 35$; range, 26–65).

Morphologic Diagnosis and HPV Results

The comparative correlation of the HPV mRNA and DNA results is shown in Figure 1.

The definitive morphological diagnosis was based on histologic examination, available in 248 cases. In the remaining 112 cases no biopsy procedures were indicated, the final diagnosis was therefore based on cytology.

Among 150 patients with normal morphology, 32 were high-risk HPV DNA positive (21%), 11 mRNA-positive (7%). Of 75 women with CIN 1/2, 56 were high-risk HPV DNA positive (75%), 32 were positive by PreTect HPV-Proofer (43%). There were 98 cases with CIN 3 of which 91 (93%) were high-risk HPV positive and 68 (69%) were positive with PreTect HPV-Proofer.

In 35 cases with invasive carcinoma, high-risk HPV DNA and E6/E7-mRNA showed almost identical positivity rates (94% vs. 97%, respectively). The two HPV DNA negative, but E6/E7 mRNA positive cases turned out to be extensively bleeding cervical carcinomas with difficulty in taking proper cell material.

Discussion

This study compared E6/E7 mRNA detection with DNA testing for high-risk HPV in 358 women recruited in seven dysplasia clinics in Germany. A high-risk HPV DNA prevalence rate of 21% in women with normal cytology confirms that a rep-

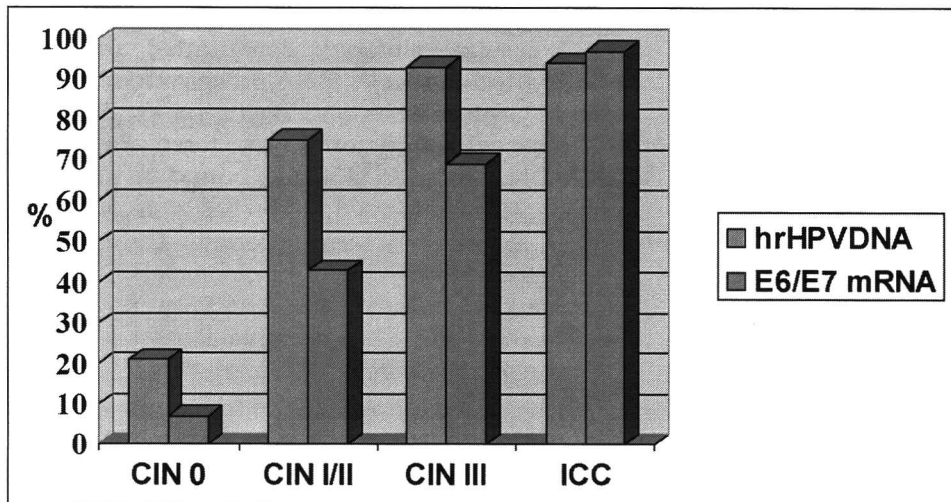


Figure 1 Comparison of high-risk HPV DNA and E6/E7 mRNA detection in CIN and invasive cervical carcinoma.

representative patient sample was recruited since it is equivalent to results of previous studies.^{5,7,9} The prevalence of E6/E7 mRNA in women with normal cytology was significantly lower (7%) than the one of HPV DNA (21%). Similarly, the positivity rate in CIN 1 to 3 was considerably lower with the E6/E7 mRNA compared with DNA testing.

E6/E7 upregulation represents the crucial initial step in cervical carcinogenesis. Therefore, it appears that E6/E7 testing allows for a more specific recognition of those intraepithelial lesions which tend to progress. Thus, high-risk HPV infected women with normal cytology and an elevated E6/E7 mRNA level had a 69.8 times higher risk of having or developing progressive dysplasia compared with a factor of only 5.7 in mRNA negative women.²⁰ Similarly, a follow-up study using PreTect HPV-Proofer in high-risk HPV infected patients demonstrated an improved specificity of 55% and higher positive predictive value for detecting histologically proven CIN 3.²¹

Therefore, measurement of oncogenic HPV E6/E7 mRNA may serve as a better discriminator than DNA testing to distinguish between transient low-grade lesions which will spontaneously regress and the small percentage of women bearing a potential transforming lesion.

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