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High-risk human papillomavirus E6/E7 mRNA and L1 DNA as markers of residual/recurrent cervical intraepithelial neoplasia

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Abstract. The aim of this study was to assess the use of human papillomavirus (HPV) E6/E7 mRNA testing in the follow-up of women treated for cervical intraepithelial neoplasia (CIN) by conization and to compare the prognostic value of HPV E6/E7 mRNA to HPV L1 DNA and cytology. One hundred and forty-three women underwent cytological/histological testing, HPV DNA genotyping by Linear Array, and HPV E6/E7 mRNA testing by APTIMA HPV assay during follow-up after surgical treatment for histologically verified CIN. High-grade residual/recurrent disease ($CIN2^+$/$HSIL^+$) was identified in 7 (4.9%) women, and low-grade disease (CIN1/LSIL) in 25 (17.5%). At the inclusion visit 33 (23%) women were HPV DNA-positive; 13 (9.0%) were HPV E6/E7 mRNA-positive. HPV E6/E7 mRNA did not identify three women with high-grade disease. Presence of high-risk HPV DNA at the inclusion visit predicted 100% (95% CI 64.6-100) of high-grade residual/recurrent disease, with a specificity of 80.9% (95% CI 73.5-86.6); cytology had a sensitivity of 85.7%, and a specificity of 87.5%. HPV E6/E7 mRNA testing was a poor predictor of treatment failure, with a sensitivity of 57.1% (95% CI 25.0-84.2), but high specificity (93.4%; 95% CI 87.9-96.5). Detection of high-risk HPV DNA after treatment by conization identified 100% of women with residual/recurrent high-grade disease, whereas HPV E6/E7 mRNA testing was a poor predictor of treatment failure. This study suggests that a negative HPV mRNA result cannot exclude the risk of malignant progression, and that HPV E6/E7 mRNA testing by APTIMA HPV assay is not useful in the follow-up of women treated for CIN.

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Introduction

Cervical cancer is the third most common malignancy among women worldwide. Approximately 530,000 women develop cervical cancer and 275,000 die from it every year (1). The introduction of cytology-based screening programs has resulted in a significant decrease in the incidence and mortality rates of cervical cancer (2). Indeed, the disease is highly preventable as it is preceded by a well-recognized premalignant stage that can be identified by cytological/histological examination and treated (3). The two most common histological types of cervical cancer are squamous cell carcinoma (SCC) and adenocarcinoma.

High-risk (HR) human papillomavirus (HPV) is present in over 99% of analyzed SCC, and in most precancerous lesions (4). However, HR HPV infection is very common, and is transient in most women; only an estimated 10% of those with an HR HPV infection are subject to persistent infection, which is believed to cause SCC through expression of the viral oncoproteins E6 and E7 (5). These oncoproteins play a significant role in malignant transformation, and are consistently expressed in malignant tissue. Their mechanism of action is centered on the inactivation of the tumor suppressor proteins p53 and pRb (6,7).

Increasing levels of HPV E6/E7 mRNA cause genetic instability, and imply a risk of cellular changes, resulting in

a selective growth advantage (8,9). Therefore, the presence of viral HPV E6/E7 mRNA transcripts could identify women at risk for residual/recurrent cervical intraepithelial neoplasia (CIN) with higher sensitivity and specificity than HPV DNA detection (10,11). In screening programs, detection of full-length E6/E7 mRNA of a number of HR HPV types has been shown to be highly associated with high-grade squamous intraepithelial lesions (HSIL) and SCC than HR HPV DNA detection (12-14).

Women who have been treated for CIN are considered to be at high-risk of developing invasive cervical cancer for many years after treatment, necessitating a long-term follow-up strategy to detect residual/recurrent CIN. Compared to cytology, HPV DNA testing allows quicker identification of residual/recurrent CIN (15-18), and has high sensitivity and negative predictive value (NPV) (18).

HPV E6/E7 mRNA is notably expressed in transforming infected squamous epithelium. Although little is known about its possible transient nature, it should be clinically interesting to monitor this expression after CIN treatment.

The aim of the present study was to assess the E6/E7 mRNA expression of 14 HR HPV types among women treated for CIN, to correlate HPV E6/E7 mRNA with subsequent cytological and histological results and to compare the prognostic value of HPV E6/E7 mRNA and HPV DNA testing.

Materials and methods

Patient selection. Women diagnosed with CIN in the regular cervical cancer screening program in Stockholm County are referred to the Department of Gynecology and Obstetrics, Karolinska University Hospital Huddinge (Karolinska), Stockholm, Sweden (a tertiary health care service) for treatment by conization. Karolinska also serves as a primary cervical cancer screening center for women residing in the surrounding area. The present study considered women treated for CIN by conization between September 1999 and June 2009 and of all treated women 149 returned for at least one follow-up visit and were invited to participate in the present study. In 2003, HPV L1 DNA testing became available at the Virology Department of at Karolinska, and the present study protocol was implemented (18). The 'inclusion visit' was defined as the first follow-up visit after conization. The study protocol was approved by the Karolinska Ethics Committee and all participants signed an informed consent form.

Treatment procedure and histological results. Of the 149 study women, 143 were treated by loop excision electrosurgical procedure (LEEP) using a C-LETZ electrode (Utah Medical Products Inc., Midvale, UT, USA) (19) between 1999 and 2009. Of the 6 study women who did not undergo LEEP, 2 were treated with cryotherapy, and 4 by cold knife or laser conization.

Histological results in the cone specimens. Diagnoses from the cone specimens of all 149 women were retrieved. Thirty-three (22%) contained CIN1, 32 (21%) CIN2, and 74 (49%) CIN3. Four contained (3%) adenocarcinoma *in situ*, two of which also contained CIN3. Six (4%) cone specimens contained no CIN, leaving 143 women (96% of 149) in the final analyses (Table I). Eighty-nine (62%) cone specimens had free margins (complete excision according to the histological findings), and the remaining 52 (36%) had positive margins (the diagnosis was not clarified due to uncertain resection margins (char/thermal artefact). In the two women (1%) treated with cryotherapy, no information was available on margin status or histology, and instead the pre-treatment histological diagnoses (CIN1 and CIN2) were used.

Table I. Conization results for 149 invited women[a] and 143 included women.

Characteristics	n (%)
Histology results in cone specimens of invited women (n=149)	
CIN3$^+$/AIS	78 (52.4)
CIN2	32 (21.5)
CIN1	33 (22.1)
No CIN[b]	6 (4.0)
Margin status of women included in final analyses (n=143)	
Free margins	89 (62.2)
Positive margins	52 (36.4)
No information about marginsc	2 (1.4)

CIN, cervical intraepithelial neoplasia; AIS, adenocarcinoma *in situ*; [a]mean age, 32.8 years, median 30, range, 21-74. [b]Excluded from analyses; [c]treated by cryotherapy.

Inclusion follow-up and subsequent visits. The inclusion visit consisted of a complete work-up, including pelvic exam, cytology testing, HPV DNA, and HPV E6/E7 mRNA testing. When indicated, colposcopy-directed biopsies were also taken. Women were divided in two groups based on when post-surgical HPV DNA and mRNA analysis were performed: 'early' (<12 months) and 'late' group (12 or more month after conization).

All 143 women had at least one additional follow-up visit after the inclusion visit (subsequent/final visit), consisting of Pap smear and, when clinically indicated, colposcopy-directed biopsies. Cytological results from these subsequent/final visits were available for 137 (96% of 143) women and histological results for 30 (21% of 143). The average follow-up time was 1,333 days (median 1,182, range 71-5,622 days), or 3.6 years.

Cytological and histological assessment. Cells from the ecto- and endocervix were collected with an Ayres spatula and a cervical brush, smeared on a glass slide, and immediately fixed in 95% ethanol and air-dried for cytological examination (20). Cytological smears were classified according to the CIN classification of the Swedish Society for Clinical Cytology (21). For the purposes of this study, cytological results were re-classified using the Bethesda system, excluding koilocytosis without nuclear atypia from the low-grade squamous intraepithelial lesion (LSIL) group (22).

Table II. Combined cytological/histological outcome during follow-up.

Diagnosis	Inclusion visit N (%)	Subsequent/last visit N (%)	All follow-up visits N (%)
WNL	114 (79.7)	124 (86.7)	110 (76.9)
Low-grade disease[a]	20 (14.0)	12 (8.4)	25 (17.5)
High-grade disease[b]	5 (3.5)	4 (2.8)	7 (4.9)
Unsatisfactory[c]	4 (2.8)	3 (2.1)	1 (0.7)
Total	143	143	143

WNL, without neoplastic lesions; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion. [a]CIN1/LSIL; [b]CIN2/HSIL; [c]histology was unsatisfactory for diagnosis.

When indicated by colposcopy, cervical biopsies were taken, preserved in formaldehyde and assessed by pathologists. In the absence of histology, the most severe cytological result (HSIL or worse, HSIL$^+$) was used, whereas cytological results of atypical squamous cells of undetermined significance (ASCUS) and within normal limits (WNL) were considered normal.

For the purposes of this study, two combinations of cytological/histological results were defined: low-grade disease, including LSIL/CIN1, and high-grade disease including HSIL$^+$/CIN2 or worse (CIN2$^+$, i.e. CIN2, CIN3, carcinoma *in situ* and adenocarcinoma *in situ*).

HPV DNA analysis. Samples for HPV DNA testing were collected at the inclusion visit in the same manner as for cytology, and suspended in PreservCyt solution (ThinPrep®, Hologic, Marlborough, MA, USA). DNA was extracted from the suspensions using the MagNA Pure LC robot (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. HPV DNA detection and genotyping were carried out using the Linear Array (LA) HPV Genotyping Test (Roche Diagnostics). Briefly, HPV DNA was amplified by PCR using a pool of biotin-labeled primers that hybridize in the L1 region (18,23,24). The 37 HPV types included in the LA test were divided into three categories: HR, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59; probable HR (PHR), HPV26, 53, 66, 68, 73 and 82; and low-risk (LR) or undetermined-risk, HPV6, 11, 40, 42, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39 and CP6108 (25). For the purposes of this study, undetermined-risk and LR HPV types were combined.

HPV E6/E7 mRNA analysis by APTIMA HPV assay. Liquid-based samples used for HPV DNA analysis at the inclusion visit were retrieved from the archives and used for HPV E6/E7 mRNA analysis. The PreservCyt sample was transferred to 2.9 ml of buffered detergent solution, and a 400 μl aliquot of the mixture was then tested by APTIMA HPV assay (Gene-Probe Inc., San Diego, CA, USA) according to the manufacturer's instructions.

The APTIMA HPV assay is a qualitative nucleic acid amplification test that detects the HPV E6/E7 mRNA of 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), and has been validated for cervical specimens collected in PreservCyt solution. The test does not differentiate between HR HPV types, and is designed not to cross-react with the LR HPV types 6, 11, 42, 43, 44, or PHR HPV53 (26-28). The APTIMA HPV assay involves three main steps. An analyte cut-off (CO) of 1.00 was used in the assay for determining HPV interpretation. All laboratory analysis were performed by the Department of Virology, Karolinska Hospital.

Statistical methods. Data were analyzed with the software STATISTICA 6.1 (Statsoft Inc., Tulsa, OK, USA). Treatment failure was defined at two disease thresholds: low-grade disease or worse, or high-grade disease or worse detected during follow-up. Accuracy parameters of the prediction of treatment failure according to these two thresholds were computed, including sensitivity, specificity, positive predictive value (PPV) and NPV.

Results

Cytological and histological diagnoses during follow-up. Each woman in the study group (mean age, 31.3 years, median: 30, range, 21-56) had at least two follow-up visits (one inclusion visit and one subsequent/final visit), and 20 women had three or more.

The combined cytological and histological results (when available) from the inclusion visit yielded 114 (79.7%) WNL, 20 (14%) women with low-grade disease, five (3.5%) with high-grade disease, and four (2.8%) with insufficient results. The results from the subsequent/final follow-up visit yielded 124 (86.7%) WNL, 12 (8.4%) women with low-grade disease, 4 (2.8%) with high-grade disease, and 3 (2.1%) with unsatisfactory samples.

When all follow-up visits were taken into account, a total of 32 (22.4%) treatment failures (25 women with low-grade and 7 with high-grade disease) were identified among the 143 study women (Table II).

HR HPV DNA and HR HPV E6/E7 mRNA results at inclusion visit. A total of 33 (23%) of the 143 study women were HR HPV DNA-positive at the inclusion visit. The most frequent HPV types detected were HPV52 (4.2%), 33 and 56 (3.5%) each. HPV16, 18, 51, 58 and 66 were equally frequent (2.8%) each. Six women had multiple HR HPV infections (4 with a double, 1 with a triple and one with a quadruple infection).

Table III. Discordant results for 26 women.

Case no.	HPV DNA	HPV mRNA	Histological/ cytological outcome
25	*66*	0	WNL
30	16, 51, 59	0	CIN2/HSIL
32	52	0	CIN1/LSIL
35	18	0	WNL
37	16	0	WNL
40	*66*	0	WNL
49	51, *73*	0	WNL
57	33	0	CIN1/LSIL
59	18	0	WNL
71	*66*	0	WNL
79	33	0	CIN2/HSIL
81	*82*	0[a]	CIN1/LSIL
103	53	0[a]	CIN1/LSIL
104	31, *66*	0	CIN1/LSIL
107	52	0	WNL
108	*66*	0	CIN2/HSIL
110	58	0	WNL
115	58	0	WNL
122	56	0	WNL
131	51	0	WNL
136	58	0	WNL
138	16	0	WNL
151	56	0	CIN1/LSIL
18	0	+	WNL
129	0	+	WNL
164	0	+	WNL

HPV L1 DNA and HPV E6/E7 mRNA by linear array and APTIMA HPV assay techniques, respectively. WNL, without neoplastic lesions; CIN, cervical intraepithelial neoplasia; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion. [a]Not detected by APTIMA HPV assay.

Only 13 (9.0%) women were HR HPV E6/E7 mRNA-positive, less than half of that for HR HPV DNA. Cases that were HPV DNA-positive for types 33, 52, 56 (three cases each), and 18 (two cases), expressed mRNA more frequently than cases HPV DNA-positive for types 16, 31, 45 and 59, where E6/E7 mRNA was expressed in one case each.

Twenty-six discordant samples were found. Three HR HPV E6/E7 mRNA-positive women were HR HPV DNA-negative and had a cytological result of WNL. Among HR HPV E6/E7 mRNA-negative women, two were HR HPV DNA-positive for types that are not included in the APTIMA HPV assay. Among the remaining 21 HPV E6/E7 mRNA-negative, HPV DNA-positive women, 3 had high-grade, and 4 had low-grade disease (Table III).

Accuracy parameters of the prediction of treatment failure. Accuracy parameters of the prediction of treatment failure were calculated for two thresholds of disease: high-grade disease or worse and low-grade disease or worse.

Presence of HR HPV DNA at the inclusion visit predicted 100% (95% CI 64.6-100) of residual/recurrent high-grade disease or worse, with a specificity of 80.9% (95% CI 73.5-86.6). Cytology at the inclusion visit had a sensitivity of 85.7% (95% CI 48.7-97.4), and a specificity of 87.5% (95% CI 80.9-92.1) (Table IVA).

HPV E6/E7 mRNA was a poor predictor of treatment failure in the present study, with a sensitivity of 57.1% (95% CI 25.0-84.2). However, the test did have a high specificity (93.4%; 95% CI 87.9-96.5), PPV (30.8%; 95% CI 12.7-57.6) and NPV (97.7%; 95% CI 93.4-99.2). Margin status and presence of $CIN2^+$ in the cone specimen were also poor predictors of treatment failure, with a sensitivity of 57.1% (95% CI 25.0-84.2) and 71.4% (95% CI 35.9-91.8), and a specificity of 64.2% (95% CI 55.8-71.8) and 22.8% (95% CI 16.5-30.5), respectively (Table IVA).

Considering low-grade disease or worse as the threshold for treatment failure resulted in lower sensitivity and higher specificity values (Table IVB).

Discussion

To our knowledge, the present study is the first to compare the HPV E6/E7 mRNA of 14 HR HPV types and HPV DNA testing in the follow-up of women treated for CIN.

HPV infection is a necessary, but not sufficient, factor in the development of cervical neoplasia (4,5,29). Certain HR HPV types (16, 18, 45, 31 and 33) are more frequently found in $CIN2^+$ and SCC of the cervix (25). The oncogenic potential of these HPV types is due to the expression of the E6 and E7 oncoproteins and to their type-specific properties, such as affinity to tumor suppressors in the host cell (5). One would therefore expect HPV E6/E7 mRNA to be expressed in transforming cells, notably those infected with HR HPV. Such expression has been demonstrated in advancing grades of CIN using different mRNA tests, such as PreTect HPV Proofer (Norchip AS, Oslo, Norway), APTIMA HPV assay, and the real-time PCR technique (30-32), with improving sensitivity. We have previously reported on the expression of HR HPV mRNA using PreTect HPV Proofer compared to viral load and the tumor marker $p16^{ink4a}$ in different grades of cervical dysplasia (33). In that study, we observed a correlation between $p16^{ink4a}$ and mRNA expression, but not viral load, nor increasing lesion severity. Probably not all HR HPV-infected cases have transcriptionally active E6/E7 expression, and absent mRNA may also represent regressing dysplasia as a consequence of E6/E7 expression being switched off (34).

Studies by Ratnam *et al* (31) and Dockter *et al* (35) reported a sensitivity of 91-95% for mRNA by APTIMA HPV assay to detect $CIN2^+$ in women with abnormal cytology, whereas the specificity was between 55 and 43%. Thus, our expectation was that an mRNA test targeting 14 HR HPV types would have a high sensitivity compared with HPV DNA testing, with a high specificity even after CIN treatment. In the present study, 23% of women harbored HR HPV DNA after treatment. Less than half of these women were also HR HPV E6/E7

Table IV. Accuracy parameters of the prediction of treatment failure for high-grade disease or worse and low-grade disease or worse.

A, High-grade disease or worse

Criteria	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
High-risk HPV DNA	100.0 (64.6-100)	80.9 (73.5-86.6)	21.2 (10.7-37.8)	100 (96.6-100)
Cytology at inclusion visit (ASCUS$^+$)[a]	85.7 (48.7-97.4)	87.5 (80.9-92.1)	26.1 (12.6-46.5)	99.2 (95.4-99.9)
High-risk HPV mRNA	57.1 (25.0-84.2)	93.4 (87.9-96.5)	30.8 (12.7-57.6)	97.7 (93.4-99.2)
Margin status	57.1 (25.0-84.2)	64.2 (55.8-71.8)	7.7 (3.0-18.2)	96.6 (90.6-98.9)
Cone containing CIN2$^+$	71.4 (35.9-91.8)	22.8 (16.5-30.5)	4.5 (2.0-10.2)	93.9 (80.4-98.3)

B, Low-grade disease or worse

Criteria	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
High risk HPV DNA	53.3 (34.3-71.7)	84.4 (76.2-90.6)	48.5 (30.8-66.5)	86.8 (78.8-92.6)
Cytology at inclusion visit (ASCUS$^+$)[a]	73.3 (54.1-87.7)	99.1 (95.1-100)	95.6 (78.1-99.9)	93.2 (87.1-97.0)
High-risk HPV mRNA	23.3 (9.9-42.3)	94.6 (88.7-98)	53.9 (25.1-80.8)	82.2 (74.5-88.3)
Margin status	36.7(19.9-56.1)	64 (54.3-72.9)	21.6 (11.3-35.3)	78.9 (69.0-86.8)

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; ASCUS, atypical squamous cells of undetermined significance. [a]Cytological results that showed more than atypical squamous cells of ASCUS were considered abnormal.

mRNA-positive. HR HPV E6/E7 mRNA testing by APTIMA HPV assay had a low sensitivity (57.1%) to predict residual/recurrent high-grade disease, but a high specificity (93.4; 95% CI 87.9-96.5) resulting in an NPV of 97.7%. According to Verguts *et al*, sensitivity and NPV are more important than specificity and PPV in the follow-up management of women at risk of developing of cervical cancer compared to a screening setting (36). Among HPV E6/E7 mRNA-negative women in our study, three cases of high-grade disease were missed by the APTIMA HPV assay, despite the observed high NPV (as high as 97.7%). We therefore concluded that the high NPV was misleading.

Negative HPV E6/E7 mRNA could have been due to the distribution of HR HPV types in archived samples. Indeed, prevalence of HPV16 and 18 was low, and HPV33, 52, and 56 were most frequent. Interestingly, these three HPV types were more frequently associated with HPV E6/E7 mRNA positivity than HPV16 and 18. False-negative HPV E6/E7 mRNA results are a possibility, although studies on the clinical performance of the APTIMA HPV assay indicate that mRNA remains stable and can be detected to the same extent as DNA (27,35). mRNA quality may be another explanation, as samples were stored at room temperature, and the time between collection and testing was sometimes as much as 6-12 months.

Our study is the first to evaluate HPV E6/E7 mRNA expression after treatment by conization using the APTIMA HPV assay, which detects the E6/E7 mRNA of 14 HR HPV types. Only one previous study has compared HPV E6/E7 mRNA expression to HPV DNA detection in follow-up after CIN treatment, using PreTect HPV Proofer (37). The aforementioned study had a shorter follow-up period, but found a similarly low sensitivity and low PPV. This could have been due to false-negative results, since 8 of the 12 histologically-verified CIN2$^+$ in the study were HPV DNA-positive for an HPV type included in the mRNA assay. The authors concluded that a negative HPV mRNA result could not exclude the risk of malignant progression (37).

HPV DNA testing is increasingly used as a screening method, either alone or in conjunction with cytology. Its high sensitivity, ease and reproducibility make it very attractive as a first-round screening tool (Fröberg *et al*, unpublished data). Indeed, the high NPV of HPV DNA allows to safely referring women back to screening (Fröberg *et al*, unpublished data). However, HPV infections are often transient, especially in younger women, making overtreatment a serious problem.

In accordance with results from our previous study (18), HPV DNA testing in post-treatment follow-up identified all residual/recurrent high-grade CIN, and presence of HR HPV predicted 100% of high-grade disease with a specificity of 80.9%. Indeed, HPV DNA-negative women in our study had only a negligible risk of treatment failure.

A summary of meta-analyses (15) concluded that a positive HPV DNA result is a better predictor of treatment failure than cytology or positive resection margins. However, based on our results, HPV E6/E7 mRNA testing by APTIMA HPV assay is not useful in the follow-up of women treated for CIN.

In the future, one could envision a follow-up strategy that includes both HPV DNA and cytology. Women who are HPV DNA-negative at 6 and 24 months after treatment could be safely referred to the usual screening program, while HPV DNA-positive women should be followed annually until negative.

Careful surveillance of women treated for CIN is still required, as these women are at a higher risk of developing cervical cancer than the general population. Meta-analyses are needed to help establish an optimal follow-up strategy for women treated for CIN.

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