Expression of E6/E7 mRNA from 'high risk' human papillomavirus in relation to CIN grade, viral load and p16^{INK4a}

SONIA ANDERSSON[1], BERIT HANSSON[2], INGRID NORMAN[3], VERA GABERI[1], MIRIAM MINTS[1], ANDERS HJERPE[3], FRANK KARLSEN[4] and BO JOHANSSON[2]

[1]Department for Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, [2]Division of Clinical Virology, Department of Laboratory Medicine, [3]Laboratory of Clinical Pathology and Cytology, Department of Laboratory Medicine, Karolinska University Hospital at Huddinge, Karolinska Institute, S-141 86 Stockholm, Sweden; [4]NorChip AS, N-3490 Klockarstua, Norway

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Abstract. Detection of E6/E7 mRNA expression with real-time nucleic acid sequence-based amplification assay (NASBA) method (PreTect HPV-Proofer) from high-risk types of human papillomaviruses (HR-HPV) were compared with the presence of viral load, determined with quantitative real-time PCR in 80 cervical samples. Results regarding positivity and typing were in agreement using the two methods. However, there was no correlation between viral loads for HPV 16 or 18/45 and oncogene expression. Among 15 women with low grade atypia detected at a population-based cytology screening, and scored as 'within normal limits' according to histopathology, 14% were positive for oncogene expression, whereas 71% were HR-HPV positive. A correlation was observed between HR-HPV oncogene expression and high scores of p16^{INK4a} positivity. Since HPV-Proofer detects full-length E6/E7 mRNA, a positive result should correlate with presence of integrated HPV, loss of HPV replication and stabilized E6/E7 full-length mRNA expression. Such expression from integrated HR-HPV generates a high and stable expression of full-length E6 proteins, which explains why a positive HPV-Proofer result was independent of viral load and correlate with high expression of p16^{INK4a}. Thus, E6/E7 oncogene expression analysis yielded information, which is consistent with and will complement the results from a real-time PCR method in a clinical prognostic procedure.

Correspondence to: Dr Sonia Andersson, Department for Clinical Science, Intervention and Technology, Division of Obstetrics and Gynecology, Karolinska University Hospital, Huddinge, Karolinska Institute, S-141 86 Stockholm, Sweden
E-mail: sonia.anderson@telia.com

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Introduction

Cervical carcinoma is the second most frequent neoplasm among women worldwide. In Europe, USA, and Japan, however, there is a marked decrease in incidence and mortality rates of cervical carcinomas due to efficient mass screening programs. Since the introduction of population-wide mass screening with the cytological Papanicolaou's (Pap) test, the incidence of invasive cervical cancer has sharply declined. The cytological screening with the Pap test has, however, significant limitations with regard to sensitivity, and the success depends on the performance of multiple tests in each case.

Intensive research has provided evidence for a central, causal role of certain types of HPV in the development of invasive cervical carcinoma (1-4). The presence of HR-HPV ('high-risk' HPV)-DNA identifies both women with neoplastic disease and those who are at a particular risk of progression to disease (5). HPV 16, 18, 45, 31 and 33 are the most frequently identified HR-HPV types in high-grade squamous intraepithelial lesions (HSILs) and cervical squamous cell carcinomas (6,7), of which HPV 16 predominates (8,9).

Persistent infection with HR-HPV, especially type 16, is regarded as a significant risk factor in the development of squamous cervical lesions or squamous cervical cancer (10). However, most infections with HPV regress spontaneously, and for the cases that do progress to cancer, a long period of latency is normally observed. Thus, HPV-infections are prevalent and often transient among younger women, with a peak of 20-25% at 20-24 years of age. With increasing age, there is a decline in the prevalence of HPV to about 7% at 35 years of age (11). It is likely that most of the HPV-positive women at that age represent a subset of individuals that do not manage to clear their infections spontaneously. Persistence of a high-risk HPV-infection is a prerequisite for the development of CIN and cancer. It is also likely that women having oncogene expression from the HPV E6 and E7 area represent an even smaller subset of individuals that do not manage to clear their transforming infections or cell abnormalities spontaneously. Thus, the persistence of transforming HPV infection is associated with the cause of invasive cervical carcinoma.

A high prevalence of transient infections makes detection of the virus an inefficient means of identifying women at risk of developing cervical cancer (12,13). However, expression of the viral E6/E7 oncogenes is necessary for conversion to, and maintanance of malignancy in cervical tissue (14,15). It has been suggested that detection of full-length E6/E7 mRNA of a limited number of HR-HPV types, having the possibility to produce full-length E6 proteins, serves as a better risk evaluation factor than HR-HPV DNA detection for the development of high-grade squamous intraepithelial lesions (HSIL) and invasive cervical cancer (ICC) (16). It has become increasingly clear that additional requirements, other than the presence of HR-HPV *per se*, are likely to be significant for the development of cervical carcinogenesis, since only a subset of women infected with HR-HPV will eventually develop cervical cancer (17).

The integration of HR-HPV into the chromosomes of premalignant cervical cells might be a risk marker for progression of cervical cancer (18,19). Integration usually disrupts the E1 and E2 open reading frames, while those of E6 and E7 remain intact (18,20). HR-HPV E2 proteins has been shown to exhibit repressor activity for expression of oncogenic mRNA by binding to E6 promoter-proximal binding sites (21,22). Thus, integration might indirectly result in the deregulation of the transcription of E6/E7 mRNA (23) and in the transcription of human DNA sequences downstream of the integrated HPV DNA, causing stabilization of the whole transcript (24), inhibition or loss of the splicing event (25,26), an irreversible production (the B-cell defence system cannot remove integrated HPV DNA) of oncogenic E6 and E7 proteins increases the risk for gene damage and subsequent clonal expansion of cells with growth advantage. The results of this genetic instability can be seen in the light microscope as anisonucleosis and hyperchromasia, i.e. the morphological signs of aneuploidy or cervical intraepithelial neoplasia (CIN). However, the role of the E2 protein as a repressor of HPV oncogene expression has been questioned by Bechtold *et al* (27).

The detailed molecular analysis of these cellular changes in CIN identified an overexpression of the cyclin-dependent kinase inhibitor p16^{INK4a} as a marker of an active expression of the viral oncogene E7 of all HR-HPV types (28). p16^{INK4a} is involved in cell-cycle regulation, and its overexpression is correlated with a dysfunctional pRb.

In normal, non-dysplastic cells, p16^{INK4a} is expressed at a very low level, that is not detectable by immunochemical means. In a few cells, however, it can be weakly expressed physiologically during cellular stress, such as in squamous metaplasia. Klaes *et al* found that overexpression of p16^{INK4a} could assist in the identification of HR-HPV-related cervical squamous lesions (29). Sano *et al* also showed that overexpression of this tumor suppressor protein was associated with HPV infection (30). Analysis of p16^{INK4a} expression appears to be particularly useful for distinguishing reactive squamous epithelium from true CIN lesions, which can be a challenge in morphologic differential diagnosis.

In the present study, our aims were to compare expression of HR-HPV E6/E7 mRNA with: a) HR-HPV viral load; b) presence of p16 protein, and c) cytological findings. These analyses were performed using liquid based (ThinPrep) cell samples, obtained from women with previous cytological abnormalities, observed in the Swedish Health Control Program.

Materials and methods

Patients. We consecutively enrolled 80 women with any grade cytological abnormalities detected at a population-based primary screening. The women were examined with colposcopy after 2-6 months at the Gynecological Department, Karolinska University Hospital at Huddinge, Stockholm.

Clinical evaluation. Each woman underwent a Pap smear and a ThinPrep test. A sample of cells from the ecto- and endocervix was obtained by cervical brush and spread on a slide for Papanicolaou staining. Similarly, collected cells were also suspended in a vial containing PreservCyt (Cytyc Corporation, Boxborough, MA, USA), a buffered preservative fluid. The vial was then sent to the cytopathology laboratory for automated slide preparation, using the ThinPrep 2000 Processor (Cytyc Corporation) (31).

A Zeiss OMPI colposcope was used for magnification. The ectocervix and distal part of the endocervix were stained with 5% acetic acid. Punch biopsies were obtained from acetowhite areas. Acetic acid causes neoplastic epithelium to turn white and also has the same effect on reactive epithelium, although less distinct. If no acetowhite area was observed, a biopsy was taken close to the squamo-columnar junction, at 12 o'clock. The samples were evaluated by the local pathologist and classified according to the CIN classification (WHO). The most severe grade of atypia found in each biopsy determined the diagnosis with which the results of the HPV test were compared.

Extraction of nucleic acid. The Nuclisens NASBA Diagnostics, Magnetic Extraction Reagents (bioMérieux SA, Marcy l'Etoile, France) kit was used for extraction of total nucleic acid from the samples. In this kit nucleic acid adsorbs to silica beads in the presence of the chaotropic salt guanidiniumthiocyanate (32). From each sample 1.0-1.5 ml was added to 10 ml sterile Falcon tubes. Centrifugation was performed for 12 min at 1200 x g, and the supernatant was removed. NucliSense lysis buffer (1 ml) (bioMérieux) was added to the cell pellet, and resuspended in a Vortex. This was followed by incubation for 10 min at room temperature. Then 50 μl of the silica bead suspension was added to each sample, and this was shaken and incubated for 10 min at room temperature. After a centrifugation at 1500 x g, the supernatant was removed. Washbuffer 1 (400 μl) was added to the beads, and the resuspended pellet transferred to 1.5 ml conical Eppendorf tubes in the MiniMag tube rack. Washing in the MiniMag was performed for 30 sec using the speed step 1 setting. The supernatant was aspirated, and the wash step was repeated a second time. This wash step was repeated twice as before, but using 500 μl of wash buffer 2. The final wash step, using wash buffer 3, was performed for 15 sec. The supernatant was removed, and 50 μl of elution buffer was added. Then the tubes were shaken in a 'Thermoshaker' at 1400 rpm and 60˚C, and returned to the MiniMag tube rack. Finally, the eluted nucleic acid was removed to RNase/DNase free tubes.

Table I. Frequencies of different HR-HPV types in the real-time PCR and E6/E7 mRNA assays.

Assay	Real-time PCR							
	Pos				Neg			
E6/E7 mRNA	16	18/45	31	Total	16	18/45	31	Total
Pos	24	6	0	30	0	0	0	0
Neg	8	1	13	22	48	72	67	187
Total	32	7	13		48	72	67	

Detection of HR-HPV E6/E7 mRNA. The isothermic gene amplification method 'Nucleic Acid Sequence Based Amplification' (NASBA) has many features, which makes it well-suited for use with RNA targets. The method was performed in real-time format, using the PreTect HPV-Proofer kit to detect full-length mRNA from only HPV 16, 18, 31, 33 and 45 (NorChip AS, Oslo, Norway). A detailed description of the HPV-Proofer protocols has been published (33).

Detection and quantification of HPV DNA. The extracted DNA was quantified, and HR-HPV typed (HPV 16, 18/45, 31, 33, 52, 58/67), by using the 'Quantovir HPV' detection and quantification system. The procedure has been described in detail recently (34).

Immunocytochemistry. Liquid based cytology (LBC) slides were prepared according to protocols from the manufacturer (Cytyc Corporation) and airdryed overnight. Then, the slides were fixed in acetone PA for 10 min at room temperature and airdryed for a minimum of 30 min. All slides were subject to 'Heat Induced Epitope Retrival', using the Epitope Retrieval Solution (Dako) at 95˚C for 40 min. Staining for p16 was performed with the CINtec kit (Dako) in the DakoCytomation Autostainer, and counter-stained according to Papanicoulau. Scoring of immunocytochemistry results were performed on the basis of the staining intensity into four grades (0 to +++).

Results

In total, 80 women with cytological signs of atypia were enrolled. The mean age was 35.3 years (median 33 years, range 23-60 years). There were 36 low grade squamous intraepithelial lesions (LSIL), 23 high grade squamous intraepithelial lesions (HSIL) and 21 had atypical squamous cells of undetermined significance (ASCUS).

Histological findings. According to histopathology, 63 women had various degrees of CIN: 21 (26%) had CIN I, 17 (21%) CIN II, 23 (29%), CIN III. One case was classified as invasive cervical cancer, and one showed an unclear histological diagnosis. Seventeen women were scored as 'within normal limits' (WNL).

Frequencies of HR-HPV DNA and E6/E7 mRNA. Of the 80 samples analyzed with real-time PCR, the frequency of HR-HPV found was 65% (52/80). Double infections were found in 11% (9/80), and 6% (5/80) had triple infections with HR-HPV. Seventy-two of the samples were positive for the human control mRNA for the U1A gene, and could be included in the high-risk HPV E6/E7 mRNA analysis. In this analysis, the frequency of HR-HPV was 39% (28/72), and double infections were found in 2.8% of the cases (2/72). The frequencies of the different types of HR-HPV in the samples, from different grades of CIN, are summarized in Table I. As expected, the frequency of HPV 16 predominated, with 39% (31/80) in the real-time PCR and 32% (23/72) in the E6/E7 mRNA assay.

Analysis of the correlation between results from the real-time PCR and the E6/E7 mRNA assays, showed that of the 32 HPV 16 positive cases found with real-time PCR, 24 (75%) were also positive in the E6/E7 mRNA assay (Table II). For the combined HPV18/45 analysis, 6 of the 7 cases (86%) identified with real-time PCR were positive in the E6/E7 assay. No sample, which was negative with real-time PCR, was positive in the E6/E7 mRNA detection system.

CIN grade and HR-HPV. Results from the real-time PCR assay showed a steady increase in HR-HPV DNA positivity at the CIN I to CIN III grades (Table III). In this patient material, the cases scored as 'within normal limits' (WNL), showed a high positivity of 71%, which must be regarded in relation to previously diagnosed atypias in these women. In contrast to these figures, the E6/E7 mRNA assay showed little variability, when CIN I and III were compared. It is notable, that the percentage of positive WNL cases was considerably lower (14%) than in the real-time PCR assay. The one cervical cancer case was positive for HPV 16 in both assays.

Table II. Correlations between HR-HPV types found by using real-time PCR and oncogene mRNA detection.

	HPV type							
Assay	16	18	18/45	31	33	33 gr.	39	45
Real-time PCR	39 (31)	nd	9 (7)	16 (13)	nd	13 (10)	14 (11)	nd
E6/E7 mRNA	32 (23)	6 (4)	nd	0	1 (1)	nd	nd	4 (3)

nd, not determined as such by the assay.

Table III. Distribution of percentage positive cases in real-time PCR and E6/E7 mRNA assays in different grades of CIN.

	CIN grade (%)			
Assay	NA	I	II	III
Real-time PCR	12/17 (71)	11/21 (52)	11/17 (65)	17/23 (74)
E6/E7 mRNA	2/14 (14)	9/18 (50)	3/17 (18)	13/22 (59)

NA, no atypia. A total of 78 cases were scored in real-time PCR and 71 cases in E6/E7 mRNA analysis.

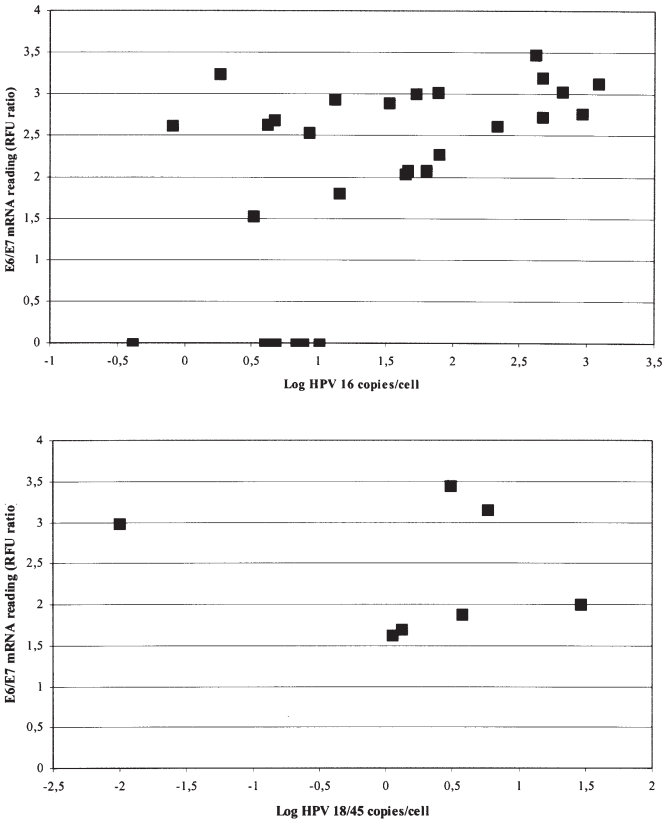

Figure 1. (A) Correlation between oncogene expression of HPV 16 E6/E7 mRNA (RFU ratio readings) and number of HPV 16 copies per cell. HPV 16 E6/E7 mRNA expression (RFU ratio readings) were compared with number of HPV 16 copies per cell, obtained from the real-time PCR assay. (B) Correlation between oncogene expression of HPV 18/45 E6/E7 mRNA (RFU ratio readings) and number of HPV 18/45 copies per cell. HPV 18/45 E6/E7 mRNA expression (RFU ratio readings) were compared with number of HPV 18/45 copies per cell, obtained from the real-time PCR assay.

Viral load and E6/E7 mRNA expression. Since HPV 16 is the most predominant type in this material, as well as in cervical squamous carcinomas, results of HPV 16 E6/E7 mRNA expression (RFU ratio readings) were compared with number of HPV 16 copies per cell, obtained from the real-time PCR assay. As shown in Fig. 1A, there is no obvious correlation between oncogene expression and number of HPV 16 copies per cell. However, all 8 samples which were negative in the E6/E7 mRNA assay had ≤10 copies per cell (Fig. 1A). A similar lack of correlation was found for HPV 18/45, as shown in Fig. 1B.

Table IV. Distribution of percentage p16 antigen expression positivity at different CIN grades.

	CIN grade (%)			
Assay	NA	I	II	III
p16^{ink4a} antigen	2/12 (17)	5/17 (29)	8/13 (62)	13/15 (87)

The p16 positivity represents the sum all samples scored as +, ++ or +++.

Table V. Correlations between results of the p16 antigen, and E6/E7 mRNA expression.

	p16^{INK4a}			
Assay	Pos			Neg
	+	++	+++	
E6/E7 mRNA				
Pos (%)	4/13 (31)	6/11 (55)	4/4 (100)	14/28 (50)
Neg (%)	9/13 (69)	5/11 (45)	0/4 (0)	14/28 (50)

Data out of the paranthesis are expressed as number of cases per total cases in the category. Scoring of immunohistochemistry results as +, ++ or +++ were based on both the staining intensity and percentage of immunoreactive epithelial cells, compared to the total normal or neoplastic cells.

p16 expression in relation to CIN grade and E6/E7 mRNA. In Table IV a summary of the distribution of p16 antigene expression, determined with immunocytochemistry, at different CIN grades is presented. The percentage positivity found for each CIN grade was: CIN I-29, CIN II-62 and CIN III-87. In samples scored as WNL, the positivity was only 17%. The correlation between p16 antigene expression and the expression of E6/E7 mRNA is shown in Table V. The percentage of samples, which were positive in both methods was dependent on the scoring of p16 positivity as +, ++ or +++. Thus, the p16 positivity increased in the E6/E7 mRNA positive cases from 31% in + scored, 55% in ++ scored to 100% in +++ scored samples. Of the samples negative for p16, 76% were also negative for E6/E7 mRNA. Thus, 24% of the samples negative in the p16 analysis, were positive for E6/E7 mRNA expression (Table V). It is noteworthy, that the p16 analysis showed a positivity figure for WNL cases (17%; Table V) in

accordance with that obtained in the E6/E7 mRNA assay (14%; Table III).

Discussion

Human papillomavirus (HPV) infection is associated with the development of cervical neoplasia and is necessary, but not sufficient factor in the aetiology of cervical carcinoma (3,4,35). HPV 16, 18, 45, 31 and 33 are the most frequently identified high-risk HPV (HR-HPV) types found in CIN II-III and cervical squamous cell carcinomas (10). The neoplastic progression to a cervical carcinoma is a process often extending over decades. This process is in part driven by expression of the oncogenic HPV genes.

Viral persistence is required for neoplastic progression (36), and increased risk has been associated with early high viral loads (37,38). HR-HPV is often found integrated into host cell genomes, and this event is regarded as critical for subsequent malignant transformation. However, only a very small proportion of the integrated HPV DNA is non-methylated, and may cause high and stable expression of E6 full-length proteins (39). Integration results in transcription of the human sequences to very long full-length mRNA, giving stable production of full-length E6 proteins. More often it also results in breakage and deletions in the E2 reading frame. As an E2 gene product has a repressing effect on E6 and E7 expression, such integration will result in increasing levels of these oncogenes. Thus, it should be clinically interesting to monitor expression of the E6/E7 genes, although little is known regarding the possible transient nature of such an expression. The E6 and E7 oncoproteins have significant roles in malignant transformation, and are consistently expressed in malignant tissue. Their mechanism of action is centered on inactivation of the p53 and pRb tumor suppressor proteins (40,41). Increasing levels of E6 and E7 expression cause genetic instability, and implies a risk for cellular changes, which results in a selective growth advantage (24,42,43).

To our knowledge, this is the first study comparing results from HR-HPV oncogene mRNA and real-time PCR quantitative DNA analysis, using LBC samples. In addition, these results were compared with expression levels of $p16^{INK4a}$ in different grades of cervical dysplasia. We found a clear increase in the incidence of HR-HPV DNA in the samples, with increasing severity of the lesion. The positivity for HR-HPV DNA, using the real-time PCR assay (Table III), in the different CIN grades was in very good agreement with the corresponding figures from our previous publication (36% CIN I; 63% CIN II; 80% CIN III) using the same method with archival samples (34). Only a part of the HR-HPV positive cases (69%; 50/72) were also positive in the mRNA analysis (40%; 29/72). This was to be expected, since not all of the HR-HPV infected cases will be transcriptionally active for E6/E7 expression. In this context it is interesting to note, that the very high positivity (71%) for HR-HPV in the WNL cases is in contrast to the low figures in the E6/E7 mRNA (14%) and p16 (17%) assays. It should be noted, that the patients in this study showed abnormal smears in primary screening. The low oncogene mRNA expression compared to the high figures for HR-HPV DNA detection might be an indication of a higher predictive power for progression of the E6/E7 mRNA and p16 methods, since it is more likely that 14-17% than 71% from this group will progress. Thus, the finding that CIN I and WNL cases demonstrated HR-HPV oncogenic expression, may be a signal that these patients have an increased potential risk for development of high-grade lesions. Furthermore, it is possible, that many of the WNL study secondary smears are still positive in cytology, although no abnormal cells could be detected on careful rescreening of the samples.

We did not observe a clear increase in the incidence of HR-HPV oncogene mRNA expression with increasing severity of the lesion. In samples scored as CIN II we found HR-HPV E6/E7 mRNA only in 18% (3/17) of the cases. The negative samples may represent cell abnormalities prone to regression as a consequence of HR-HPV E6/E7 expression being switched off (5). Kraus *et al* found HPV mRNA in 18% of 'normal' cases, which is in good agreement with our 14% figure for WNL cases (44). However, these authors did not find HPV mRNA in samples classed as CIN I, and the figures of HPV mRNA in CIN II-III cases were higher than in our study.

The findings of HR-HPV E6/E7 mRNA expression in apparently 'normal' cell samples, indicates that the virus can be present in cervix, exerting an oncogenic activity before cell changes can be visualised histologically, supporting the idea that the natural history of HR-HPV includes periods long before the manifestation of cell abnormality (45). With regard to the frequencies of types recorded, HPV 16 was, as expected, the most common type found in both the real-time PCR and the HR-HPV mRNA assays.

The gene for the tumour suppressor protein $p16^{INK4a}$ has been reported to be inactivated in many forms of human cancer, whereas in cervical cancer, a strong nuclear and cytoplasmic overexpression of $p16^{INK4a}$ has been observed (46). This overexpression of $p16^{INK4a}$ in cervical cancer, and its precursor lesions, is supposed to result from the functional inactivation of the pRb protein by the HR-HPV E7 oncoprotein (29). It has been suggested that $p16^{INK4a}$ transcription may be directly induced by the transcription factor E2F, released from pRb after binding to the HR-HPV E7 protein (47). We found a correlation between the general expession of $p16^{INK4a}$ and different grades of CIN. However, such a correlation with E6/E7 mRNA expression (Table V) was only found for the highest score of p16 positivity (+). This indicates, that the lower scores for positivity (+ and ++) in this test should be interpreted with caution, also considering the risk for non-specific binding of antibodies, resulting in a false positive score.

It was somewhat surprising to find that there was no correlation between viral load and expression of E6/E7 mRNA (Fig. 1). In view of the prevailing hypothesis, oncogene expression should increase following integration. Presumably, there are usually fewer copies per cell of integrated, than of episomal virus, which should result in the inverse relation between viral load and oncogene mRNA expression. However, such a correlation could be masked by the mixture of cells harbouring integrated and episomal virus commonly obtained in cervical smear samples.

Taken together, our results seem to support the finding that HR-HPV infections results in progression to cervical cancer in only a small percentage of infected women, after a

long period of latency (6,48). Thus, detection of mRNA transcripts of HPV genes known to be involved in oncogenesis may be more useful than pure HPV DNA tests for detection of active and potentially persistent infection (49). Since HPV-Proofer always detects full-length E6/E7 mRNA, a positive result should correlate very well with integrated HPV, loss of HPV replication and stabilized E6/E7 full-length mRNA expression. Stabilized E6/E7 full-length mRNA caused by integration of HPV expression correlate very well with a high and stable expression of full-length E6 proteins resulting in response both from p16 and p14. This explains why a positive HPV-Proofer result was independent of viral load and correlate only with high expression of p16^{INK4a} in this study. Thus, E6/E7 oncogene expression analysis yielded information, which is consistent with and will complement the results from a real-time PCR method in a clinical prognostic procedure.

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