

High Human Papillomavirus Oncogene mRNA Expression and Not Viral DNA Load Is Associated with Poor Prognosis in Cervical Cancer Patients

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Abstract Purpose: Cervical cancer is now known to be caused by infection with an oncogenic type of the human papillomavirus (HPV). However, little is known about the continued role of HPV once cancer has been established. Here, we describe the quantitative relation between HPV DNA copy number and mRNA expression of the viral oncogenes (E6 and E7) and the prognostic value of both measures in cervical cancer patients.

Experimental Design: We studied the number of viral DNA copies and the level of HPV E6/E7 mRNA expression in 75 HPV 16–positive or HPV 18–positive International Federation of Gynecology and Obstetrics stage Ib and IIa cervical cancer patients. Measurements were done with quantitative PCR. DNA copy number analysis was done on pure tumor cell samples enriched with flow sorting. mRNA expression data were compensated for the percentage of tumor cells included.

Results: The number of viral DNA copies was not predictive of survival in cervical cancer patients. In contrast, high HPV E6/E7 mRNA expression was strongly related to an unfavorable prognosis ($P = 0.006$). In a multivariate Cox model for overall survival, including all known prognostic variables and stratified for HPV type, the level of E6/E7 mRNA expression was an independent prognostic indicator, second only to lymph node status. No correlation was observed between DNA copy number and the level of HPV E6/E7 mRNA expression, which reflects that not all DNA copies are equally transcriptionally active.

Conclusions: Cervical cancer patients with high HPV E6/E7 oncogene mRNA expression have a worse survival independently from established prognostic factors.

Infection with human papillomavirus (HPV) has now been established as an essential cause of cervical cancer, with HPV 16 and HPV 18 as the most prevalent high-risk types (1). HPV DNA copy number and levels of viral transcripts have been suggested to be markers for progression in precancerous lesions, yet their prognostic significance in cervical cancer is unknown (2).

HPV expresses two viral oncoproteins: E6 and E7. These proteins bind to and inactivate the tumor suppressor proteins p53 and pRb, respectively, causing deregulation of the cell cycle (3). The E6 and E7 proteins are transcribed from one shared

transcript. In addition to the full-length E6/E7 mRNA, high-risk HPV types generate spliced transcripts referred to as E6*. For HPV 16, a second spliced transcript called E6*II is also generated. The function of the spliced transcripts E6*I and E6*II is not well understood. Previously, it was thought that the E7 protein could only be transcribed from the spliced mRNA; however, more recent studies provided evidence that E7 is synthesized from both the spliced and the full-length transcripts (4, 5).

The number of HPV DNA copies per cell, viral load, was suggested to correlate with disease stage, showing increasing copy numbers from mild dysplasia to cervical cancer (6–10). However, results are conflicting because of the variation in sampling techniques and different methods used to calculate viral load (2). Similarly, the presence of transcripts of the E6 and E7 genes was claimed to correlate with severity of cervical intraepithelial neoplasia (4, 11–13). Most of these studies were done nonquantitatively, and most importantly, they did not adjust for the percentage of HPV-infected cells in the total cell mass examined, including normal epithelial cells and stromal cells as well. To overcome this final problem, we designed a method to study tumor cells exclusively. Samples were analyzed by flow cytometry to determine the percentage of tumor cells used in mRNA expression analysis. Subsequently, HPV DNA copy number measurements were done on flow-sorted tumor cells as described previously (14). Both DNA and

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Table 1. Primer and probe sequences for HPV DNA quantification

Gene	Forward primer	Reverse primer	Probe
HPV 16-E7	ccggacagagcccattacaat	acgtgtgtgctttgtacgcac	tgttgcaagtgtgactctacgcttcggt
HPV 18-E7	gactcagaggaagaaaacgatgaaa	gtgacgtgtgtggctcggct	tggagttaatcatcaacatttaccac
β -globin	gacaggtacggctgtcatca	tagatggctctgcccctgact	ctagggttgccaatctactcccag
DCC	ggaacgagcattgaatacaaca	aggctgtgtggccacct	tggggaccggagagagattc

mRNA measurements were done with quantitative PCR, and results were normalized to the expression of housekeeping genes. We assess the prognostic value of DNA copy number and the level of full-length and spliced E6/E7 mRNA expression in HPV 16-infected or HPV 18-infected cervical cancer patients.

Materials and Methods

Study population. All patients with cervical cancer who underwent radical hysterectomy with lymphadenectomy in our hospital from 1989 to 1999, had enough archival formalin-fixed, paraffin-embedded tissue available, were inhabitants of the Netherlands, had not received radiotherapy or chemotherapy before treatment, and had either HPV 16 or HPV 18 infection were included in the study. Follow-up of patients until 2004 gave information about state at last follow-up. All clinical data were collected prospectively in a databank. Tumors were HPV typed by PCR and sequencing as described previously (15). The use of clinical material was approved by the institutional review board according to the guidelines of the Dutch Federation of Medical Research Associations.

Sample preparation. H&E-stained slides were reviewed, and formalin-fixed, paraffin-embedded tissue blocks were macrodissected to remove normal and precancerous epithelial tissue. Subsequently, 60- μ m sections were cut for DNA copy number and mRNA expression analysis. Flow cytometry was done using triple staining with keratin (to identify epithelial tumor cells), vimentin (to identify stromal cells), and propidium iodide (for DNA staining; ref. 14). The percentage of tumor cells was calculated followed by separate flow sorting of keratin- and vimentin-positive cell populations.

HPV DNA copy number. Keratin-positive, exclusively tumor cells were used for HPV DNA measurement. Vimentin-positive cells were used as a negative control. Cells were centrifuged and resuspended at a concentration of 1,000/ μ L. DNA was extracted by incubation with proteinase K (3 mg/mL) overnight at 56°C.

A HPV type-specific quantitative Taqman PCR was done to assess the number of HPV DNA copies using primers for the E7 gene (16). In

addition, we amplified *HBG2* (hemoglobin β chain, Genbank accession no. U01317) and *DCC* (deleted in colon cancer, Genbank accession no. NM005215) genes to allow an accurate compensation for the total DNA input and quality. Amplicons were detected with fluorescent Taqman probes (Eurogentec, Seraing, Belgium). Primer and probe sequences are listed in Table 1. We added DNA from ~200 cells to the PCR mix (qPCR Core kit, Eurogentec) and did reactions in a final volume of 25 μ L. The following thermocycler profile was used: 95°C for 2 min; 40 cycles of 15 s at 95°C, 60 s at 60°C. To calculate the viral DNA copy number, we included cell lines established from cervical cancer with known viral copy numbers (SiHa, 2 copies of HPV 16; HeLa, 40 copies of HPV 18; ref. 17) that were fixed in formalin, embedded in paraffin, and prepared identically to patient samples. Standard curves were prepared from cell line DNA. The absolute viral load was calculated as follows: viral load = $F \times (SQ_{HPV} / SQ_{control\ genes})$, where SQ_{HPV} is the starting quantity of HPV DNA, $SQ_{control\ genes}$ is the starting quantity of the two control genes (geometric mean of *HBG2* and *DCC* starting quantity), and F is the replication factor. The replication factor was calculated from the known viral copy number of the cell lines.

mRNA expression. After removing paraffin, tissue sections were incubated twice overnight at 56°C in 1 mg/mL proteinase K. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Contaminating DNA was removed using RQ1 RNase-free DNase according to the manufacturer's protocol (Promega, Madison, WI). Reverse transcription was done in a 20 μ L reaction volume containing 10 μ L RNA solution, 1 mmol/L of each deoxynucleotide triphosphate, 0.02 μ g/ μ L random hexamers, 20 ng/ μ L oligo(dT), 0.2 units of avian myeloblastosis virus reverse transcriptase, 40 units of RNase inhibitor, and 1 \times reverse transcriptase buffer (Roche, Mannheim, Germany). cDNA synthesis was done at 42°C for 90 min.

Primers were designed using Beacon Designer 3 software (Premier Biosoft International, Palo Alto, CA) with amplicons as small as possible (80-120 bp). Intron-spanning primers were used for the spliced transcripts, and known polymorphic positions were avoided. PCR products were sequenced to confirm that the target gene was amplified. HPV primer sequences are described in Table 2. The primer sequences of the normalization genes were described previously (18).

Table 2. Primer sequences for mRNA quantification

Name	Genomic position	Sequence
HPV 16 full length (forward)	211-234	gttactcgcagctgaggtatag
HPV 16 full length (reverse)	278-305	catttatcacatacagcatatggattc
HPV 16 E6* I (forward)	214-237	actcgcagctgagggtatataac
HPV 16 E6* I (reverse)	460-481	tggaaatctttgcttttggctcc
HPV 16 E6* II (forward)	211-232	gttactcgcagctgagatcat
HPV 16 E6* II (reverse)	566-589	tcagtcaatgtagggtatctcc
HPV 18 full length (forward)	229-252	cagaggtatttgaatttgcattt
HPV 18 full length (reverse)	292-314	aatctatacatttatggcatgcag
HPV 18 E6* (forward)	223-243	aacttacagaggtgcctgcg
HPV 18 E6* (reverse)	486-506	tagtgcacagctatgttgg

NOTE: Genomic positions based on Genbank sequences K02718 and X05015 for HPV 16 and HPV 18, respectively.

We used 0.2 μ L cDNA template in a total volume of 25 μ L containing 0.3 μ mol/L of each forward and reverse primer and the components of the qPCR Core kit for SYBR Green I (Eurogentec). The same cycle profile as in the DNA analysis was used. SYBR Green fluorescence was measured at the end of the elongation phase, and a melting curve was generated after the last amplification cycle.

Each reaction included a standard curve of cDNA derived from formalin-fixed, paraffin-embedded cell lines SiHa and HeLa. cDNA from paraffin-embedded Caski, a cervical cancer cell line containing ~600 DNA copies of HPV 16, was also done. To ensure contaminating DNA had been removed, a control PCR was done before reverse transcription (no-reverse transcriptase control) using a nonintron-spanning primer set. We measured the expression of four normalization genes (*UBC*, *GAPD*, *CFL1*, and *ACTB*; ref. 18). The most stably expressed genes were identified by using "NormFinder." *UBC* and *ACTB* proved most stable and were used for the normalization of expression data (18). The following equation was used to calculate the expression of the target genes: $Exp_{HPV} = SQ_{HPV} / \text{mean}(SQ_{ACTB}, SQ_{UBC}) \times \% \text{ tumor cells}$, where Exp_{HPV} is the relative expression of the HPV target transcript and SQ is the starting quantities of a transcript as calculated from the standard curves. The percentage of tumor cells were calculated from flow cytometry analysis. The equation was based on the assumptions that HPV is expressed only in tumor cells and that the expression of normalization genes is similar in tumor and nontumor cells. Quantitative real-time PCRs were done with an iCycler iQ and analyzed using Optical System Software version 3.0a (Bio-Rad, Richmond, CA). Each measurement was done in duplicate.

Table 3. Patient characteristics

	<i>n</i>
Total no. patients	75
Age	
Mean	47
Range	24-81
FIGO stage	
Ib1	40
IIb2	21
II	14
Histopathology	
Squamous cell carcinoma	56
Adenocarcinoma and adenosquamous carcinoma	19
Lymph nodes	
Negative	54
Positive	20
Number recovered, mean	18
Number recovered, range	6-41
No lymph node dissection done	1
HPV type	
HPV 16	55
HPV 18	20
Postoperative therapy	
Yes	43
No	32
Length of follow-up (mo)	
Mean	49
Median	43
Follow-up (3 years)	
Alive	45
Recurrence	4
Dead from other cause	2
Dead from disease	11
Lost to follow-up	13

Abbreviation: FIGO, International Federation of Gynecology and Obstetrics.

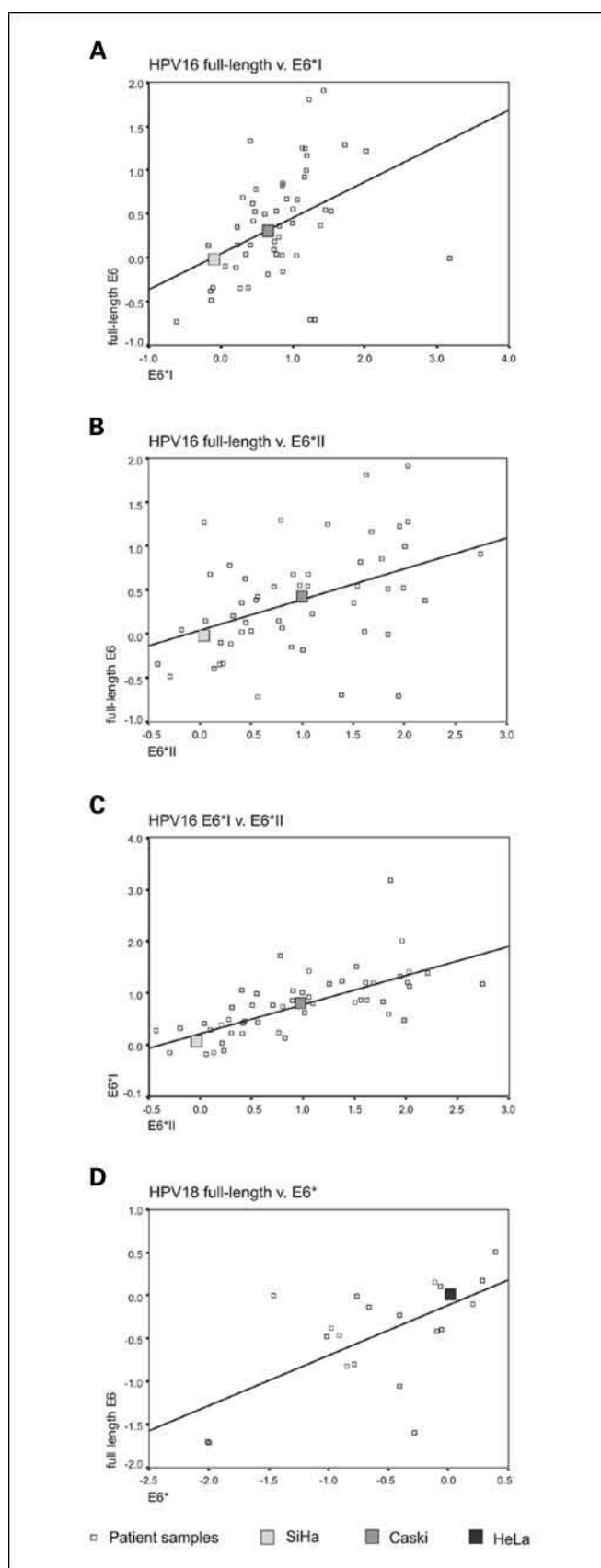


Fig. 1. Correlation between mRNA expression of different transcripts. Scatter plots of the \log_{10} transformed values for all samples.

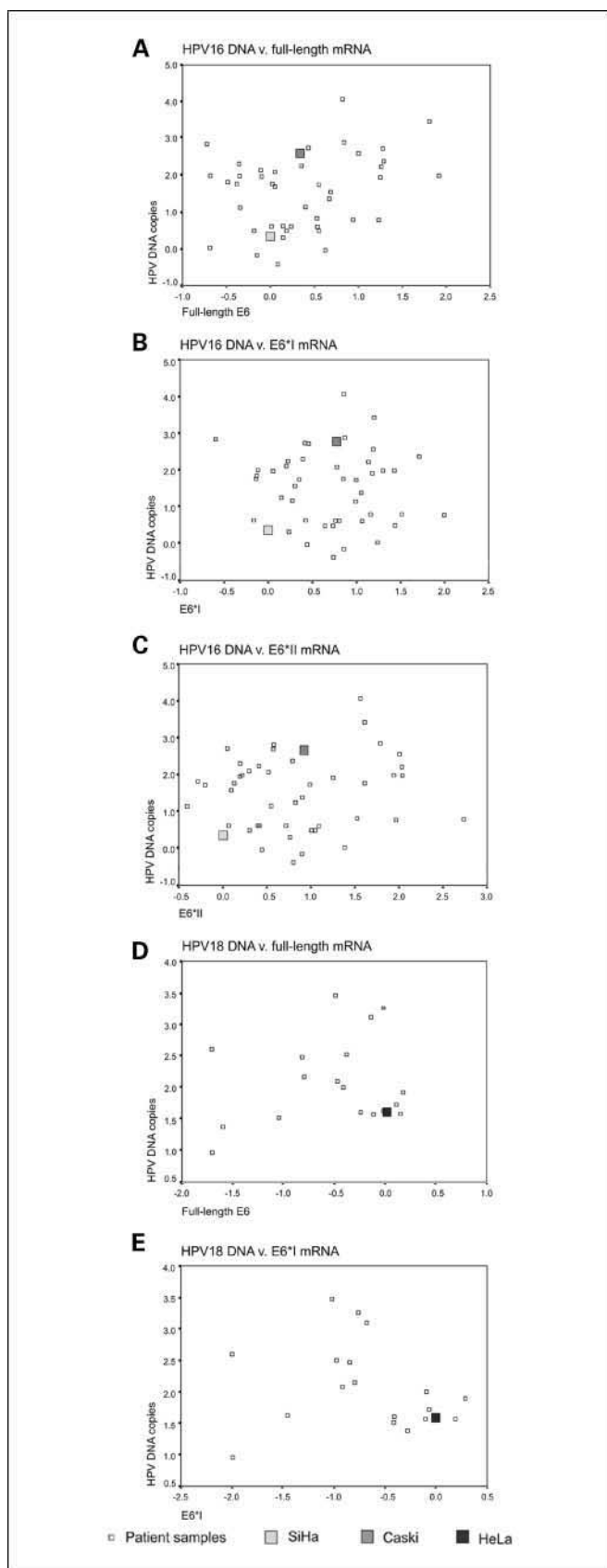


Fig. 2. Scatter plots of viral load versus HPV E6/E7 mRNA expression. Scatter plots of the \log_{10} transformed HPV DNA copy number versus mRNA expression data of all samples.

Statistical analysis. Statistical analysis was done using the Statistical Package for the Social Sciences 10.0.7 software package. Real-time data were log transformed before applying statistics. Differences between groups were evaluated with the Student's *t* test. Correlation analysis was done using Pearson's correlation. Univariate analysis of overall 3-year survival was done using the Kaplan-Meier method and the log-rank test. The Cox proportional hazard model was used for multivariate survival analysis. All tests were two sided and the significance level was set to 5%, corresponding to 95% confidence intervals.

Results

Patients. A total of 75 patients diagnosed with early-stage cervical cancer was enrolled: 55 being infected with HPV 16 and 20 with HPV 18. Patient characteristics are summarized in Table 3.

HPV DNA copy number. HPV DNA measurements were done on keratin- and vimentin-positive cell fractions. The percentage of tumor cells in the samples used in flow cytometry analysis varied from 2% to 80% (mean, 30.2 ± 19.9). We excluded one sample in which all measurements, including control genes, were negative and four samples that were HPV DNA negative. In 39 of the 70 remaining samples, we did not detect any HPV DNA in the vimentin-positive fraction. In 27 of 70 others, we detected an amount of HPV DNA that was <4% of the amount in the keratin-positive fraction; we regarded this low level of contamination as acceptable for further analysis. In four samples, we detected an amount of HPV DNA in the vimentin-positive cells >4% that in the keratin-positive cells and excluded these from further analysis. Thus, 48 HPV 16-positive and 18 HPV 18-positive tumors were used for HPV DNA analysis. Levels of *HGB2* and *DCC* were strongly correlated (Pearson's correlation: $R = 0.813$; $P < 0.0001$). The HPV 16 viral load ranged from <1 to 11,400, and the HPV 18 viral load ranged from 9 to 2,995 viral DNA copies per cell. The mean viral load of HPV 18 was significantly higher than that of HPV 16 ($P = 0.004$). Viral load was not associated with the age of the samples, histopathology, lymph node status, or International Federation of Gynecology and Obstetrics (FIGO) stage (data not shown).

mRNA expression. HPV E6/E7 transcripts were identified in 72 of 75 HPV 16-positive tumors. In one case where transcripts were not detected, the percentage of tumor cells (5%) were low, and in the other two, a low amount of mRNA was isolated. These three samples were excluded from further analysis of mRNA levels. Importantly, all control reactions lacking reverse transcriptase were negative. In HPV 16-positive tumors, the full-length transcript was identified in 50 of 52 cases, whereas the spliced transcripts E6*I and E6*II were identified in all cases. In HPV 18-positive tumors, we detected the full-length transcripts in all samples and the HPV 18 E6* transcript in 18 of 20 samples.

The expression levels of all three HPV 16 E6/E7 transcripts were significantly correlated: full-length E6 versus E6*I, $R = 0.418$, $P = 0.002$ (Fig. 1A); full-length E6 versus E6*II, $R = 0.438$, $P = 0.001$ (Fig. 1B); and E6*I versus E6*II, $R = 0.652$, $P < 0.0001$ (Fig. 1C). Similarly, the expression levels of HPV 18 full-length transcripts and E6* transcripts were correlated: $R = 0.623$, $P = 0.003$ (Fig. 1D). mRNA expression was not associated with the age of the samples, FIGO stage, histopathology, lymph node status, tumor size, or depth of infiltration.

Table 4. Multivariate Cox proportional analysis for overall survival, including known clinicopathologic factors, HPV DNA copy number, and HPV full-length E6 mRNA expression

Variable	Hazard ratio (95% CI)	P
Lymph node status	14.52 (1.00-210.48)	0.050
Parametrium infiltration	1.87 (0.25-13.86)	0.540
Vaginal wall infiltration	2.39 (0.27-21.47)	0.435
Depth of infiltration	1.05 (0.90-1.23)	0.516
Tumor size	1.05 (0.97-1.13)	0.207
Vasoinvasion	0.68 (0.60-7.81)	0.759
DNA copy number	1.43 (0.53-3.86)	0.481
Full-length E6/E7 mRNA expression	7.99 (1.21-52.78)	0.031

NOTE: Lymph node status, parametrial infiltration, vaginal wall infiltration, and vasoinvasion were binary variables (0 for negative, 1 for positive); depth of infiltration, tumor size, DNA copies, and full-length E6/E7 expression were continuous variables. Viral DNA copy number and mRNA expression were log transformed. Abbreviation: 95% CI, 95% confidence interval.

No correlation was found between the viral DNA copy number and the level of mRNA expression of the full-length or spliced transcripts in either HPV 16-positive or HPV 18-positive tumors: for HPV 16 full length, $R = 0.28$, $P = 0.06$ (Fig. 2A), $E6^*I$, $R = 0.00$, $P = 0.98$ (Fig. 2B), and $E6^*II$, $R = 0.15$, $P = 0.33$ (Fig. 2C); for HPV 18 full length, $R = 0.27$, $P = 0.27$ (Fig. 2D) and $E6^*$, $R = -0.07$, $P = 0.78$ (Fig. 2E).

Survival analysis. A multivariate Cox regression analysis for overall survival was done. The analysis was stratified for HPV type and included all known prognostic variables (lymph node status, positive parametrial or vaginal surgical margins, tumor diameter, depth of infiltration, and vasoinvasion; ref. 19), the number of DNA copies, and full-length mRNA expression. Full-length expression was used because it proved a stronger indicator compared with the expression of $E6^*$. This multivariate analysis showed that only lymph node status and full-length E6 expression remained significant prognostic indicators (Table 4). When analyzing lymph node status and full-length expression together, both were significant independent indicators of survival (full length, $P = 0.016$, hazard ratio, 3.56; positive lymph nodes, $P < 0.0001$, hazard ratio, 9.75; Table 4). When the analysis was restricted to lymph node-negative patients, expression of full-length transcript remained significantly predictive of death ($P = 0.0035$; hazard ratio, 4.40; 95% confidence interval, 1.13-17.23). When data were stratified for receiving postoperative radiotherapy, the expression of full-length E6 remained a significant predictor of survival ($P = 0.049$; hazard ratio 4.63; 95% confidence interval, 1.004-10.814).

Kaplan-Meier analyses were done to generate survival plots. Data were stratified for HPV type. For this analysis, patients were divided into groups with high and low copy number of viral DNA or high and low levels of E6/E7 transcript levels. A high DNA copy number, defined as >100 DNA copies per cell, was not prognostic for worse overall survival in Kaplan-Meier survival analysis stratified for HPV type ($P = 0.627$). In HPV 16-infected patients, high mRNA expression was defined as levels >10 times the level expressed in SiHa cells. In HPV 18-infected patients, high expression was defined as levels higher than the expression of HeLa. High mRNA expression of full-

length E6 ($P = 0.006$), $E6^*$ ($P = 0.007$), and $E6^*II$ ($P = 0.038$) transcripts was associated with worse overall survival. In Fig. 3, the Kaplan-Meier curve of HPV 16-positive patients with low versus high $E6^*$ expression is shown. The 3-year survival in HPV 16-positive patients was 93% in patients with high HPV $E6^*I$ mRNA expression and 53% in patients with low HPV $E6^*I$ mRNA expression. In HPV 18-positive patients, the 3-year survival was 76% in patients with high HPV 18 $E6^*$ mRNA expression and 53% in patients with low HPV 18 $E6^*$ mRNA expression. The cutoff values for high and low levels were arbitrary and were used only in the Kaplan-Meier survival plots. In the multivariate analysis, true values were used.

Discussion

This study shows that the level of HPV E6/E7 mRNA is an independent prognostic factor in cervical cancer, second only to lymph node status. HPV DNA copy number had no prognostic value and was not correlated to the level of E6/E7 mRNA. The relation between the quantities of HPV DNA and mRNA was never studied before in pure tumor cell samples. We clearly showed for the first time in clinical samples that HPV DNA and mRNA levels are not associated. A possible explanation is that not all DNA copies are transcriptionally active as has been shown by Van Tine et al. in an *in situ* assay on cervical cancer cell lines. It was argued that too high HPV E6/E7 expression might cause cell death, as shown *in vitro*, because cells are not able to replenish cellular components (20).

In contrast to previous studies, we restricted both DNA and mRNA analyses to tumor cells. DNA copy number analysis was limited to tumor cells by flow sorting, and the quantitative PCR was normalized for total DNA input. In mRNA quantitative PCR analysis, we compensated for the percentage of tumor cells estimated by flow cytometry. Several studies described an association between viral DNA copy number and the level of mRNA expression, but no compensation for the percentage of

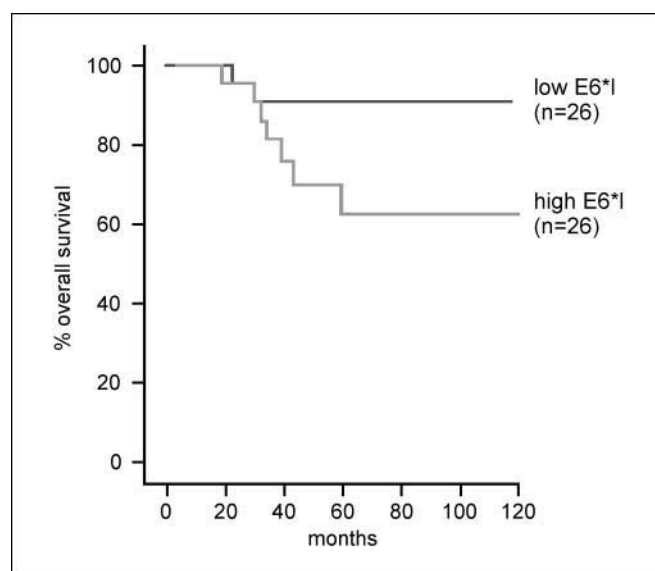


Fig. 3. Survival in patients with low versus high HPV 16 $E6^*I$ mRNA expression. Kaplan-Meier curve of the overall survival in months of patients with low versus high $E6^*I$ mRNA expression. $P = 0.012$, log-rank test.

tumor cells was done (21–23). The amount of HPV-infected cells included in the analysis might have largely influenced the detected levels of both DNA and mRNA. In addition, many studies on precancerous lesions, which were done on cervical exfoliated cells, described a correlation between the quantity of viral DNA (6–9) or viral mRNA (16, 24) and the severity of cervical dysplasia. Those studies did not compensate for the amount of HPV-infected dysplastic cells, and results could well be the effect of lesion size as suggested previously (25). We showed in the present study that the contamination of stromal and normal epithelial cells can be substantial, with a percentage of tumor cells ranging from 2% to 80%. The same effect might be present in precancerous lesions. Therefore, viral DNA load and viral mRNA measurements should be compensated for the percentage of HPV-infected cells included in the analyzed sample, or results should be interpreted with caution.

Apart from restricting the analysis to tumor cells, several other measures were taken to ensure validity. We treated the mRNA samples with DNase to make sure genomic DNA was not being amplified with the transcripts of interest, and a control reaction lacking reverse transcriptase ensured that contaminating DNA was not present. We tested internal control genes, and the two most stably expressed genes were used for normalization of the data. Outcome measures were related to cell lines that were treated the same as patient samples. We used formalin-fixed, paraffin-embedded tumor tissue in which mRNA is partly fragmented, yet the relative gene expression is equivalent to fresh tissue (26). In previous studies, HPV oncogene transcripts could not be detected in all tumors (27–29). We detected all three E6/E7 transcripts in almost every sample, indicating that previous methods might have been insufficiently sensitive. Some specific patterns of expression of the different transcripts have been described to relate to clinical variables of cervical cancer (27, 28). In our study, the levels of expression of the three different HPV oncogene transcripts were well correlated, and no specific expression patterns were noticed.

The finding that patients with a high level of HPV E6/E7 mRNA expression have a shorter survival can be explained by

the cell cycle deregulating actions of E6 and E7, which bind to and inactivate the tumor suppressor proteins p53 and pRb, respectively, and cause proliferative cell growth (3). Other HPV-induced effects could contribute to the worse survival as well. The process of tumor invasion, which requires changes in epithelial cell-stroma interaction, is influenced by HPV through the induction of vascular endothelial growth factor, permitting the tumor to acquire blood supply (30). In addition, the inhibition of transforming growth factor- β is influenced by HPV, resulting in increased metastatic potential (31).

The fact that not all DNA copies are transcriptionally equally active might also be a way of immune escape by producing as little antigen as possible. Yet, the relation between the quantity of HPV mRNA expression and immune response has never been studied. Still, from the present study, it can be concluded that, even if antitumor immune response is HPV antigen dose dependent, the effect on the immune response is weaker than the proliferative effect of HPV oncogene expression on tumor growth. The association that we found between HPV oncogene expression and prognosis of cervical cancer patients suggests that a therapy directed against E6 and E7 mRNA expression might be effective. Although none of these molecular therapies have been clinically tested, several successful *in vitro* studies have been done (3).

In conclusion, we found that a high level of HPV 16 and HPV 18 E6/E7 mRNA expression was an independent predictor of unfavorable prognosis in cervical cancer, whereas the number of HPV DNA copies per cell was not. Importantly, when the analysis was limited to lymph node-negative patients, HPV E6/E7 mRNA expression was still of significant prognostic value. Our study underscores the importance of HPV E6/E7 mRNA expression and indicates that, apart from an essential role in carcinogenesis, HPV plays a critical role in the progression of cervical cancer.

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