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Expression of Human Papillomavirus Type 16 E6-E7 Open Reading Frame Varies Quantitatively in Biopsy Tissue from Different Grades of Cervical Intraepithelial Neoplasia

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The proteins encoded by the human papillomavirus type 16 E6-E7 open reading frame are essential for transformation of the host cell. Two mRNA species, E6*I and E6*II, generated by alternative splicing of a polycistronic pre-mRNA, encode truncated E6 proteins and the E7 protein. Our investigation assessed whether or not the level of expression of E6*I and E6*II varies quantitatively in relation to the grade of cervical intraepithelial neoplasia (CIN). We used a quantitative reverse transcription PCR assay to quantify these transcripts in concurrently collected biopsy tissue and exfoliated cervical cells from 22 women with a normal cervix or various grades of CIN. We evaluated transcription profiles in relation to CIN grade and specimen type. The expression levels of E6*I and E6*II in exfoliated cervical cells did not vary significantly in relation to the grade of CIN. However, expression of E6*II was significantly diminished or absent in biopsy tissue obtained from CIN grade II and III lesions ($P = 0.014$). Our findings suggest that quantification of E6*I and E6*II expression in biopsy tissue may be more clinically relevant than analysis of exfoliated cells. The identification of distinct patterns of expression in association with low- and high-grade CIN suggests that quantification of E6*I and E6*II expression in biopsy tissue may have prognostic value as an indicator of CIN progression.

A role for human papillomavirus (HPV) in the etiology of cervical cancer is strongly supported by epidemiological evidence and laboratory-based studies. The DNA genome of specific HPV genotypes is detected consistently in malignant genital lesions (6) in conjunction with expression of specific viral genes (1, 3, 19, 23). The E6-E7 open reading frame (ORF) is expressed in the majority of cervical lesions and in continuous cell lines derived from cervical carcinomas, supporting a role for these transcripts in host cell transformation (5, 13). Indeed, HPV type 18 (HPV 18) E6-E7 expression increases the proliferation and delays the differentiation of keratinocytes in vitro (24), thus mimicking the natural course of cervical dysplasia. The extent of these effects correlates directly with E7 protein levels.

HPV 16 generates three polycistronic transcripts from the E6-E7 ORF (22). A minor, nonspliced transcript, encompassing the full-length reading frames of the E6 and E7 proteins, is detected in some cervical carcinoma cell lines (22, 23). However, the existence of this transcript in cervical premalignant lesions and carcinomas remains controversial, since contamination of RNA preparations with viral genomic DNA can result in production of an amplification product identical to that derived from the full-length transcript (5, 9, 17). Moreover, the close proximity of the E6 protein translation termination codon to the initiation codon for E7 in the full-length transcript makes it unlikely that the E7 protein is efficiently translated (14).

Two additional transcripts, E6*I and E6*II, are generated by alternative splicing. The transcripts use the same splice donor site at nucleotide (nt) 226 but different splice acceptor sites at nt 409 and nt 526, respectively. There is general agreement, with the exception of one study (4), that E6*I is the most abundant mRNA species in clinical specimens and cervical cell lines. E6*I potentially encodes a truncated E6 protein and the E7 protein. E6*II encodes a second truncated E6 protein, but the E7 ORF is unlikely to be translated. An E6*III species has been identified, but it appears to be unique to an HPV 16-containing keratinocyte cell line (7).

Qualitative changes in HPV 16 E6-E7 expression are not observed in relation to changes in the grade of cervical intraepithelial neoplasia (CIN) (5, 13). The splicing patterns for the transcripts are conserved. While it has been suggested that quantitative changes may occur (5, 13, 20), few studies have addressed this important issue.

When HPV 16-infected premalignant lesions and cervical cell lines were surveyed for E6*I and E6*II expression by S1 nuclease protection and PCR (21), some tissues showed E6*I expression in the absence of E6*II. Falcinelli et al. (9) did not detect either HPV 16 E6*I or E6*II in cytologically normal cervical cells by nonquantitative reverse transcription PCR (RT-PCR); however, E6*I was observed more often than E6*II in cytologically abnormal cells (8). These data are similar to our own (12). By using a quantitative PCR assay coupled to RT (QRT-PCR), we found that the relative quantities of E6*I and E6*II varied in exfoliated cells collected from women with different grades of CIN. In order to determine whether a distinct pattern of HPV 16 E6*I and E6*II expression could be identified for low- and high-grade CIN, we obtained biopsy tissue and exfoliated cervical cells from 22 women with normal cervical histology or different grades of CIN. The QRT-PCR

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TABLE 1. Primers used to amplify HPV 16 E6-E7 and GAPDH mRNAs

DNA and primer name	Sequence (5′ to 3′)	Genome location (nt's [DNA])	QRT-PCR product (size [bp])
HPV 16 transcripts			
Primer 1	CAAGCAACAGTTACTGCGACGTG	202–224 (E6)	E6*I (321)
Primer 2	TCCGGTTCTGCTTGTCCAGCTGG	682–704 (E6)	E6*II (204)
GAPDH cDNA			
Primer 1	CATCTCTGCCCCCTCTGCTGA	420–440[a]	
Primer 2	GGATGACCTTGCCCACAGCCT	704–725	(305)

[a] Relative to cloned cDNA sequence.

assay was modified to include coamplification of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (2) as an internal standard, facilitating comparisons of E6*I and E6*II levels between different types of specimens from the same patient and expression levels between patients.

MATERIALS AND METHODS

Specimen collection and HPV genotyping. The study cohort was recruited from women attending the colposcopy clinic at the Health Sciences Centre, Winnipeg, Manitoba, Canada, because of a previously abnormal Papanicolaou smear. A colposcopically directed biopsy specimen was obtained and was divided for histological and molecular studies. An exfoliated cervical cell specimen was obtained concurrently, and DNA was isolated from an aliquot for HPV genotyping by PCR (16). The biopsy tissue and the remainder of the cervical cell specimen from 22 HPV 16-positive patients were held at −70°C until QRT-PCR analysis as outlined below.

Isolation and RT of mRNA. The mRNA from exfoliated cells and biopsy tissue was isolated by using the Micro Fast Track mRNA Isolation Kit (Invitrogen, San Diego, Calif.). This system facilitates the isolation of polyadenylated RNA to the exclusion of contaminating DNA and proteins. Further purification of mRNA was not required. The mRNA was quantified spectrophotometrically, and a 400-ng quantity was reverse transcribed in a reaction mixture containing, as final concentrations, 4 mM $MgCl_2$, 0.015% gelatin, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 100 ng of random hexamer primer (Pharmacia Biotech, Alameda, Calif.), 20 U of RNAguard RNase inhibitor (Pharmacia Biotech), 2.5 U of avian myelobastosis virus reverse transcriptase (Pharmacia Biotech), and 200 mM (each) dGTP, dATP, dCTP, and TTP (Pharmacia Biotech) in a total volume of 20 μl. The reaction mixture was incubated at 23°C for 10 min and then at 42°C for 45 min. The samples were denatured at 95°C to inactivate the reverse transcriptase, and then the entire reaction mixture was added to the PCR cocktail.

PCR amplification. The PCR procedure (18) was used to amplify E6*I, E6*II, and GAPDH cDNA sequences by using specific primers (Table 1) generating amplification products of 321, 204, and 305 bp, respectively. The PCR mixture contained, as final concentrations, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin, 2 U of *Taq* polymerase (Promega Corp., Madison, Wis.), and 1 μM primers. Fifty picomoles of each primer pair was 5′-end-labeled with [γ-^{32}P]ATP with a 5′ DNA labeling kit (Boehringer Mannheim Canada, Laval, Quebec, Canada). A cycle of PCR consisted of primer annealing at 60°C for 45 s, primer extension at 72°C for 45 s, and denaturation at 94°C for 1 min. The amplification products were precipitated and resolved by 8% polyacrylamide gel electrophoresis. The mRNA isolated from CaSki cells served as a positive control for HPV 16 E6*I and E6*II transcripts (23). The negative controls consisted of a reaction containing all reagents except mRNA and a paired reaction for each specimen including mRNA and all reagents but no reverse transcriptase.

Quantification of transcription products. The primers must have similar amplification efficiencies and ranges of exponential amplification for their respective target sequences in order for the QRT-PCR assay to be valid (11). The efficiencies of the primers for each target sequence were determined by measuring the slope of a standard curve relating product accumulation to cycle number (10).

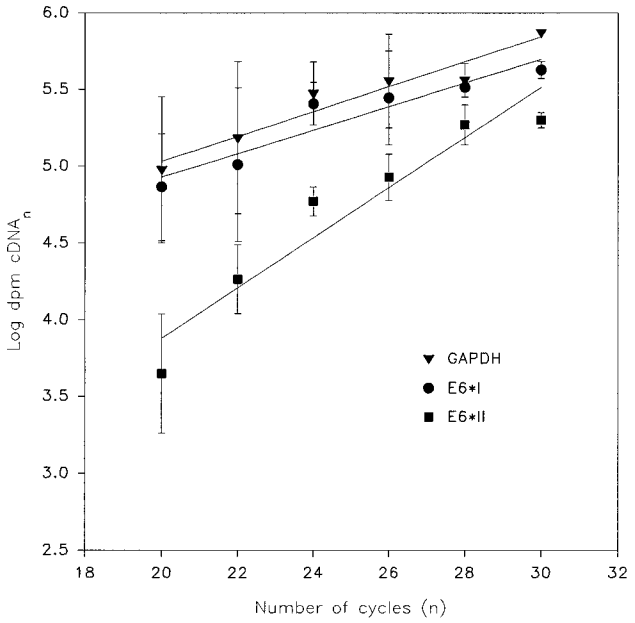

FIG. 1. Log-linear relationship between cycles of amplification and the formation of the PCR-amplified product. The regression lines for amplification of E6*I, E6*II, and GAPDH cDNAs were generated from the mean values obtained from three experiments.

TABLE 2. Expression of E6*I and E6*II in biopsy tissue and exfoliated cervical cells

Patient no.	CIN grade	Log dpm cDNA$_o$ (10^4)			
		Biopsy tissue		Cervical cells	
		E6*I	E6*II	E6*I	E6*II
1	0	0	2.10	0	0
2	0	4.98	1.62	ND[a]	ND
3	0	5.02	1.75	0.55	1.53
4	0	5.09	1.47	5.10	1.80
5	0	5.32	2.46	5.66	1.80
6	I	5.04	2.26	5.20	2.10
7	I	5.12	0.79	4.83	0
8	I	5.13	0	5.34	1.62
9	I	5.20	2.10	5.31	2.12
10	I	5.26	2.24	4.44	0
11	I	5.29	2.00	5.17	1.90
12	I	5.59	2.48	5.38	1.97
13	II	4.22	0	5.55	0
14	II	4.90	1.49	5.24	2.51
15	II	5.26	1.68	ND	ND
16	II	7.05	0	7.31	2.25
17	III	4.42	1.81	4.91	1.71
18	III	4.49	0	6.27	2.28
19	III	4.80	0.62	4.83	1.65
20	III	5.62	0.94	6.98	2.14
21	III	5.89	0	ND	ND
22	III	6.26	1.07	6.70	1.33

[a] ND, specimens not included because of a lack of GAPDH cDNA amplification.

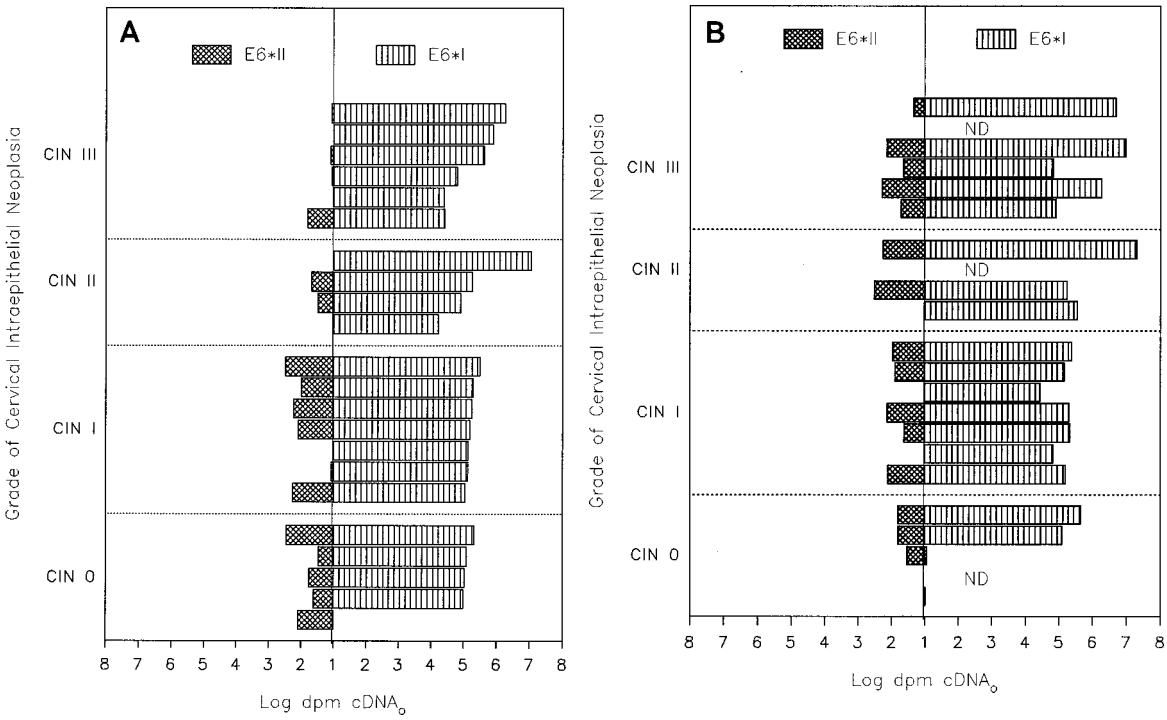

FIG. 2. Expression of E6*I and E6*II relative to expression of GAPDH in biopsy tissue and exfoliated cervical cells obtained from women with a normal cervix or different grades of CIN. The results are plotted relative to patient number; the values for patient 1 are plotted at the bottom of the charts, while the values for patient 22 are plotted at the top. (A) Expression of E6*I and E6*II observed in biopsy specimens. (B) Expression of E6*I and E6*II in exfoliated cervical cells obtained concurrently with the biopsy specimens. ND, the mRNA was not adequate for analysis.

Our standard curve was generated by coamplifying E6*I, E6*II, and GAPDH cDNAs, generated from CaSki cell mRNA, for 30 cycles (Fig. 1).

The equation $cDNA_n = cDNA_o + (1 + R)^n$ relates the number of cycles (n), primer efficiency (R), and quantity of each amplicon ($cDNA_n$) after n amplification cycles to the amount of target mRNA in the RT reaction ($cDNA_o$). For our analysis, we used 30 cycles of amplification. The values of R for E6*I, E6*II, and GAPDH were determined from the standard curve (Fig. 1) to be 0.244, 0.621, and 0.288, respectively. The quantity of product after PCR amplification ($cDNA_n$) was determined by scintillation counting of the radiolabeled PCR products excised from the polyacrylamide gel and then correcting for background by subtracting the disintegrations per minute of the paired reverse transcriptase-negative control. Once all of the other parameters were known, the quantity ($cDNA_o$) of each mRNA species was calculated by using the equation given above.

Standardization relative to GAPDH expression. The values obtained for E6*I $cDNA_o$ and E6*II $cDNA_o$ were adjusted relative to an equivalent content of GAPDH $cDNA_o$ in each specimen. This correction compensated for differences in efficiency of the RT reaction and for differences in the number of cells per specimen.

Statistical analysis. Spearman rank correlation analysis was used to assess the correlation between HPV 16 E6*I and E6*II expression and the grade of CIN. The findings for CIN grade 0 and CIN grade I lesions were grouped as low-grade lesions, while findings for CIN grade II and CIN grade III lesions were grouped as high-grade lesions for purposes of statistical analysis.

RESULTS

Quantification of gene expression in clinical specimens. The values of HPV 16 E6*I $cDNA_o$ and E6*II $cDNA_o$, standardized relative to the value of human GAPDH $cDNA_o$, were determined for the biopsy tissue and exfoliated cervical cells obtained from 22 HPV 16-positive women. These values are presented in Table 2. The relative levels of expression of E6*I and E6*II for the same specimens are illustrated in Fig. 2A and 2B. The 503-bp amplification product generated from the E6-E7 ORF full-length transcript was not observed in the specimens (Fig. 3). This product was either absent or observed at low levels and was generated by amplification of minute

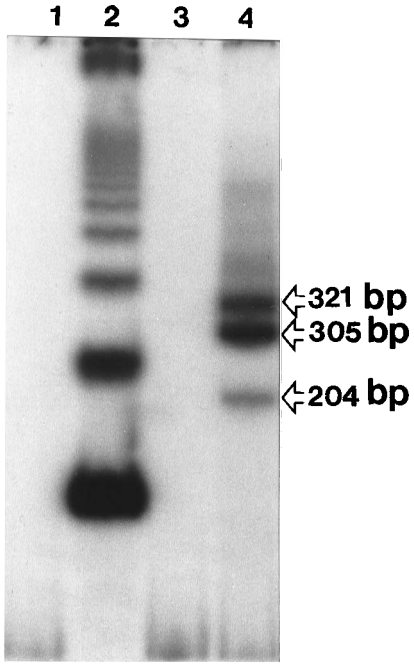

FIG. 3. Autoradiograph of HPV 16 E6-E7 and GAPDH QRT-PCR amplification products resolved by polyacrylamide gel electrophoresis. Lanes: 1, negative control includes all reagents but lacks mRNA; 2, 123-bp molecular mass ladder; 3, reaction lacking reverse transcriptase acts as a control for amplicons arising from viral genomic DNA; 4, amplification products generated from E6*I (321 bp), GAPDH (305 bp), and E6*II (204 bp) mRNAs.

quantities of viral DNA coisolated with the mRNA since the level of this transcript in a QRT-PCR was never greater than that produced in the paired negative control lacking reverse transcriptase.

Histology. The histopathologic assessment of biopsy tissue identified seven women with CIN grade I, four women with CIN grade II, and six women with CIN grade III. Despite previously abnormal cytology, the remaining five women were histopathologically normal, although inflammation and other benign processes may have been present. These specimens were classified as CIN grade 0, indicating the absence of neoplasia.

Expression of E6*I and E6*II in biopsy tissue. The expression of E6*I was observed in all but 1 of the 22 biopsy specimens. The exception was a specimen from a women with a normal cervix (CIN grade 0). E6*II expression was detected for all but one of the biopsy specimens from women with normal cervixes or low-grade CIN (CIN grade I). However, E6*II was detected at diminished levels or was absent from specimens from women with high-grade CIN (CIN grade II and CIN grade III) (Table 2; Fig. 2A). The loss or decreased level of expression of E6*II relative to that of E6*I was significantly associated ($P = 0.014$) with high-grade CIN (Fig. 2A).

E6*I and E6*II levels in cervical cells. Three specimens were eliminated from statistical analysis because the lack of GAPDH cDNA amplification indicated that poor-quality mRNA had been isolated. For the remaining 19 specimens, E6*I was observed in all but 1 specimen; this specimen was collected from a woman with a normal cervix. E6*II expression was detected in most of the exfoliated cells, independent of the CIN grade. While there was a slight decrease in the level of E6*II expression relative to that of E6*I for specimens from women with CIN grades II and III (Fig. 2B), the decrease was not statistically significant ($P = 0.137$).

Comparison of E6*I and E6*II expression in paired specimens. The levels of expression of E6*I and E6*II were similar in many of the paired biopsy and cell specimens obtained from women with normal histology or CIN grade I. However, this was not the case for specimens taken from women with CIN grade II or III. While the level of expression of E6*I was similar in biopsy tissue and exfoliated cells, the level of E6*II expression was greatly reduced in the biopsy tissue compared with that in exfoliated cells collected from the same woman.

DISCUSSION

The etiologic association between HPV 16 and uterine cervical cancer prompted us to look for prognostic indicators of HPV 16-associated CIN progression. In the present study, we set out to determine whether or not distinct patterns of viral oncogene expression could be identified for low- and high-grade CIN. We also examined whether HPV 16 transcription levels were similar in biopsy tissue and exfoliated cervical cells collected concurrently from the same patient.

By using a quantitative RT-PCR assay to assess the level of HPV 16 E6*I and E6*II expression in relation to the grade of CIN, we found a significant correlation between the diminishment or loss of E6*II expression and high-grade CIN. Thus, distinct HPV 16 E6*I and E6*II expression profiles are evident for lesions from women with low- and high-grade CIN. However, the source of the specimen is important because of the nature of the QRT-PCR assay.

The level of HPV 16 E6*I and E6*II expression, as determined by the QRT-PCR assay, is an average of gene expression in all cells in the specimen. In women with low-grade CIN, the majority of squamous cells in biopsy tissue and exfoliated cell specimens are normally differentiated and mature. In women with high-grade CIN, the majority of squamous cells in the biopsy tissue are abnormal and are less well differentiated. However, the exfoliated cell specimen collected from women with high-grade CIN is an admixture of cells from normally differentiated tissue surrounding the lesion as well as the poorly differentiated cells from the lesion itself. Thus, HPV expression in the exfoliated cell specimen is measured not only in cells originating from the lesion but in grossly normal tissue as well. It is well documented that the normal tissue surrounding a lesion is also infected with HPV (15). The fact that the biopsy and the cervical scraping sample different sites may account for the discordant results obtained for the paired biopsy and exfoliated cell specimens collected from women with high-grade CIN. Therefore, quantification of HPV mRNA in biopsy tissue rather than cervical cells may identify more clinically relevant viral expression.

The mechanism behind the quantitative changes in E6*I and E6*II expression, observed in relation to CIN grade, has not been investigated. However, there is a direct correlation between E6*II expression and cellular differentiation. A possible explanation relates to increased activation of the 526-nt splice acceptor site in differentiated cells. This would result in the E6-E7 polycistronic pre-mRNA being processed to produce E6*II (22). Other mechanisms including mRNA transport and stability also may be involved in the posttranscriptional regulation of E6*I and E6*II mRNA levels.

The cross-sectional design of the study described here precluded determination of whether progression from low- to high-grade CIN can be predicted on the basis of HPV 16 transcription profiles. This must be determined by prospectively quantifying E6*I and E6*II expression in conjunction with CIN progression. Moreover, consideration should be given to determining whether high levels of E6*II expression can retard CIN progression by protein interactions or simply by concomitant reduction of the level of the alternate transcript E6*I (and E7 protein) below the level required for cellular transformation. These issues are under investigation.

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