Detection of Human Papillomavirus Type 16 Early-Gene Transcription by Reverse Transcription-PCR Is Associated with Abnormal Cervical Cytology

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Human papillomavirus type 16 (HPV-16) is associated with abnormal Papanicolaou smears, indicative of cervical intraepithelial neoplasia. HPV-16 is the most common genital HPV and is found in up to 40% of young women with normal cervical cytology. In order to investigate whether transcriptionally active HPV-16 infection is associated with abnormal cervical smears, a reverse transcription-nested PCR assay with primers from the E5 open reading frame was developed to detect all HPV-16 early-region mRNA (E-mRNA) transcripts. It was used to study HPV-16-infected women with normal and abnormal cervical cytologies to obtain evidence of active infection. Among HPV-16 DNA-positive women, HPV-16 E-mRNA was detected in 15 of 37 (40.5%) women with abnormal cervical cytology but in only 4 of 35 (11.4%) women with normal cytology (P = 0.007). Thus, HPV-16 E-mRNA transcription is associated with abnormal cervical smears and may have value as a prognostic marker of progressive disease.

Oncogenic human papillomaviruses (HPVs), such as HPV type 16 (HPV-16) and HPV-18, are strongly associated with cervical cancer and its precursor, cervical intraepithelial neoplasia (CIN) (43), with HPV-16 detected in at least 50% of cervical cancers (4, 43). High-grade CIN lesions (CIN 2 and 3) have malignant potential, with up to 36% of CIN 3 lesions progressing to cancer (29). The detection of high levels of HPV-16 DNA is predictive of concurrent high-grade CIN (3, 11). Although in a large proportion of women low-grade CIN regress to normal, there is no test available to distinguish women whose lesions will regress from women who will develop higher-grade CIN lesions (reviewed in reference 36).

Transcription of the major oncoproteins of HPV-16, E6 and E7, is associated with abnormal cervical cytology (14, 20, 30). However, these mRNA transcripts are expressed at high levels in high-grade CIN lesions as a consequence of integration (21; reviewed in reference 15) and the E6 and E7 open reading frames (ORFs) are not present in all early-region mRNA (E-mRNA) transcripts (37). E4 and E5 are the most abundant RNA transcripts in low-grade neoplasias and may be the first transcripts to be expressed during infection (39). Furthermore, HPV-16 E5 may play a role in the transformation of cells at an early stage of cervical neoplasia (24, 25, 32, 35, 40). Thus, we have developed a reverse transcription-nested PCR (RT-nested PCR) to detect the E5 ORF, which is common to most transcribed E-mRNAs (13a, 37, 38). This was evaluated with HPV-containing cell lines and used to determine whether HPV-16-infected women with detectable E5-containing E-mRNA transcription are more likely to have abnormal cytology than are those in whom viral transcription cannot be detected.

Materials and Methods

Cell lines. The K1/16 cell line, a human keratinocyte cell line transfected with full-length HPV-16 DNA and known to express E-mRNA transcripts (28), was used as a positive control. In addition, the following three HPV-16-positive cell lines were investigated for E-mRNA transcription: CaSki (ATCC CRL 1550; American Type Culture Collection, Rockville, Md.), SiHa (ATCC HTB 35), and XH1 (16). Cell lines HeLa (ATCC CCL 2), an HPV-18-positive cell line, and A431 (ATCC CRL 1555), an HPV-negative cell line, were used as negative controls.

Patient samples. Cervical brush smears were collected with Axibrushes (Colgate Medical Ltd.) from 80 women (mean age, 37 years; range, 18 to 69 years) attending community well-woman clinics (WWCs) in the Inner London Borough of Lambeth for routine smears and 97 women (mean age, 36.9 years; range, 18 to 69 years) with abnormal cervical smears who were referred to St. Thomas’ Hospital for colposcopy. Confirmatory cervical smears were taken at the colposcopy clinic, and colposcopic directed punch biopsies were taken from 72 women with cervical squamous lesions and examined histologically. Permission for the collection of these specimens was provided by the Research Ethics Committee of St. Thomas’ Hospital. Cytological and histological assessments of cervical smears and biopsies were carried out by using standard United Kingdom criteria (1).

Preparation of samples for DNA PCR. Cells from brush smears were suspended in 5 ml of sterile phosphate-buffered saline; a 1-ml aliquot was digested overnight with proteinase K (Boehringer Mannheim U.K. Ltd.) (7). One negative distilled-water (molecular biology grade; Merck Ltd.) control was included for every five specimens.

HPV-16 E5 DNA PCR. Samples were tested for HPV-16 E5 DNA with primers from the E5 ORF (7).

HPV consensus-primer PCR. PCR was carried out by using consensus primers MY09 and MY11 (27) with samples negative by HPV-16 E5 DNA PCR.

HPV type-specific PCR. Samples found to contain HPV DNA by consensus-primer PCR but negative by HPV-16 E5 DNA PCR were typed for HPV-6, -11, -18, -31, and -33 with the type-specific primers of van den Brule and colleagues (41).

Human β-globin DNA PCR. Human β-globin DNA PCR was carried out with all samples to ensure that sufficient amounts of DNA of adequate quality were available for PCR amplification and that no nonspecific PCR inhibitors were present (33).

RT-PCR to detect E-mRNA. As there are no splice sites within the E5 ORF (37), the primers selected for this RT-PCR were complementary to untranslated mRNA adjacent to the poly(A) tail of HPV-16 transcripts and to the E5 exon. This RT-PCR was thus designed to amplify polyadenylated RNA, minimizing amplification of contaminating genomic DNA.

Isolation of RNA. One million cultured cells or cells pelleted from a 1-ml aliquot of each clinical sample were stored in 400 μl of RNAzol B (BIOTECX Inc.) at −70°C prior to total RNA extraction according to the manufacturer’s protocol. The RNA pellet was washed once in 70% (vol/vol) aqueous ethanol, air dried, and resuspended in 50 μl of Tris-EDTA buffer. Samples were incubated

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† Deceased.
overnight at 37°C with 5 U of DNase I (Amersham) in 10 μl of RNase-free water. One negative distilled-water (molecular biology grade) control was processed along side every five test samples.

RT. RT was primed by 2.5 μM 25- to 30-mer oligo(dT) (Pharmacia Biotech Ltd.) with Moloney murine leukemia virus (MMLV) reverse transcriptase (Life Technologies Ltd.). The final volume of 20 μl contained 200 U of MMLV reverse transcriptase, 0.7 U of InhibitAce, 1 mM (each) dNTPs, 50 μl of 1× first-strand buffer (Life Technologies Ltd.), and 3 μl of RNA sample. Assays were performed with and without reverse transcriptase to confirm that mRNA, not contaminating DNA, was being amplified. Samples were incubated at 37°C for 90 min, and reverse transcriptase was then inactivated at 99°C for 5 min.

First-round PCR. Primers P1 and P2 were used in the first-round PCR to produce a 421-bp amplicon (Table 1). False priming was minimized by a hot-start method (13). The 100-μl PCR mixture included the 20-μl reverse transcription mixture, 2.5 U of Taq polymerase (Promega U.K.) in a final concentration of 4.0 mM MgCl₂, 0.5 μM primer P1, and 0.5 μM primer P2. Samples were incubated at 94°C for 5 min, 49°C for 15 s, and 72°C for 1 min (cycle). 100°C for 30 s, 49°C for 15 s, and 72°C for 1 min (29 cycles); and finally for 1 cycle at 72°C for 5 min.

Nested PCR. Nested PCR employed primers P3 and P4 to produce an amplicon of 202 bp (Table 1). PCR mixtures included 10 μl of the first-round PCR product, 2.5 mM MgCl₂, 0.5 μM (each) primer, and 2.5 U of Taq DNA polymerase. Samples were heated at 94°C for 5 min (1 cycle), followed by 25 cycles at 94°C for 15 s, 50°C for 15 s, and 72°C for 10 s, and a final cycle at 72°C for 5 min.

Keratin RT-PCR. An RT-nested PCR for human keratin mRNA (5) was performed with all samples to ensure that clinical samples contained sufficient amounts of RNA for amplification in the RT-PCR.

RESULTS

Cervical brush smears. HPV-16 E5 DNA was detected in 40 of 97 (41.2%) smears from women attending the colposcopy clinic and 32 of 80 (40%) smears from women attending WWCs (P = 0.878) (Table 2). When the results for these two groups were analyzed according to cytological gradings at the time of sample collection, HPV-16 E5 DNA was detected in 35 of 97 (36.1%) normal cervical smears and 37 of 80 (46.25%) abnormal cervical smears (P = 0.218), with the prevalence increasing with advancing abnormality, although the increase was not significant (P = 0.177) (Table 2). All specimens were positive for human β-globin DNA.

When the HPV-16 E5-positive smears were further assayed for HPV-16 E-mRNA, 13 of 40 (32.5%) and 6 of 32 (18.8%) smears were positive from the colposcopy clinic and WWCs, respectively (P = 0.287) (Table 3). By analyzing the results according to cytological gradings, HPV-16 E-mRNA was detected in 4 of 35 (11.4%) normal cervical smears and 15 of 37 (40.5%) abnormal smears (P = 0.007) (Fig. 1), with the prevalence increasing with advancing abnormality (P = 0.007) (Table 3). Representative results with HPV-16 E5 RT-nested PCR products are shown in Fig. 2. All specimens were positive for keratin mRNA.

Histological diagnoses were available for 72 women with cervical squamous lesions who attended the colposcopy clinic; 35 (48.6%) of them were positive for HPV-16 DNA. Eleven (31.4%) of these 35 women were positive for HPV-16 E5 mRNA. However, there was no statistically significant association between the presence of E-mRNA and histological grading (P = 0.25) (Table 4).

Controls. No positive signals were detected from negative water controls in DNA PCRs or RT-PCRs. No contaminating DNA was detected in RT-PCR controls lacking MMLV reverse transcriptase, indicating that all positive bands resulted from mRNA amplification (Fig. 2). Sensitivity. In a log dilution series (4.1 × 10⁻¹ to 4.1 × 10⁻⁸ ng/μl) of synthetic E5 mRNA, the RT-nested PCR detected as little as 2 × 10⁻⁷ ng of synthetic RNA. This equates to approximately 2,000 copies of E5-containing mRNA.

### Table 1. Primers used for HPV-16 E-mRNA RT-PCR

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Nucleotide sequence</th>
<th>Nucleotide localization</th>
<th>PCR product size (bp)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 (+)</td>
<td>5' TTI TTT TTI TTI TTI TTI TTA AGT 3'</td>
<td>HPV-16 4227-4330</td>
<td>421</td>
</tr>
<tr>
<td>P2 (--)</td>
<td>5' ATT TAG ATC TAT ATG ACA AAT CTT GAT ACT GC 3'</td>
<td>E5 3837-3868</td>
<td></td>
</tr>
<tr>
<td>P3 (+)</td>
<td>5' TAC AGC ATC TTT ATG TAA TTA AAA AGC GTG CAT 3'</td>
<td>E5 4078-4110</td>
<td>202</td>
</tr>
<tr>
<td>P4 (--)</td>
<td>5' GTG CTT TTI TGG TGT GTC GTA TTA ATA C 3'</td>
<td>E5 3910-3936</td>
<td></td>
</tr>
</tbody>
</table>

* +, primer for positive-strand DNA; --, primer for negative-strand DNA.
* All amplicons were detected by gel electrophoresis on ethidium bromide-stained 2% agarose gels.
* Taken from reference 35.

### Table 2. Smear and HPV-16 E5 DNA PCR results for women attending the colposcopy clinic and WWCs

<table>
<thead>
<tr>
<th>Smear result</th>
<th>No. of women positive for DNA/no. of women tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colposcopy clinic</td>
<td>WWCs</td>
</tr>
<tr>
<td>Normal</td>
<td>9/25 (36)</td>
</tr>
<tr>
<td>Borderline* or mild dyskaryosis</td>
<td>12/36 (33.3)</td>
</tr>
<tr>
<td>Moderate or severe dyskaryosis</td>
<td>19/36 (52.8)</td>
</tr>
<tr>
<td>Total</td>
<td>40/97 (41.2)</td>
</tr>
</tbody>
</table>

* ASC-US.
* No. of abnormal smears (borderline, mild, moderate, or severe dyskaryosis) positive for HPV-16 E5 DNA/no. of abnormal smears tested = 73/80 (46.25%).
Specificity. HPV-16 DNA and E-mRNA were detected in the HPV-16-positive cell lines, K1/16, CaSki, SiHa, and XH1, but not in the HeLa, which contains HPV-18, or A431, which contains no HPV DNA, cell line. In addition, to confirm that cervical-smear samples negative for HPV-16 E5 DNA were also negative for HPV-16 E5-containing mRNA, 10 DNA-negative specimens from both the colposcopy clinic and WWC groups were randomly chosen for further testing. Other HPV types were detected in 6 of 10 (60%) patients from the colposcopy clinic (4 with HPV-6, 1 with dual infection with HPV-6 and -33, and 1 with HPV-31); however, none of the 10 patients from WWCs were positive for other HPV types. All 20 specimens were HPV-16 E-mRNA negative.

DISCUSSION

An RT-nested PCR which detects HPV-16 E-mRNA transcripts containing the E5 ORF in HPV-16 DNA-positive cervical cell lines and cells from cervical brush smears has been developed.

When cervical brush smears collected from both the colposcopy clinic and WWCs were analyzed for HPV-16 E5 DNA, there was no statistically significant difference in prevalence. Similarly, when results were analyzed according to abnormal and normal cytologies, no difference in HPV prevalence was observed between the two groups. These figures reflect the prevalence of HPV-16 in the inner London population, as detected by a sensitive PCR that detects <10 copies of HPV-16 E5 DNA (7), highlighting the need for additional PCR-based assays to aid in the diagnosis of CIN lesions. There was no statistically significant increase in the prevalence of HPV-16 E5 DNA with advancing abnormality.

In cervical brush smears from HPV-16 DNA-positive women, HPV-16 E-mRNA was detected more frequently in samples from the colposcopy clinic than in samples from WWCs, although this was not statistically significant. By analyzing the results according to cytological grading, however, E-mRNA was detected significantly more frequently in cells from abnormal cervixes than in cells from normal cervixes (P = 0.007) and the prevalence was found to increase with advancing cervical abnormality (P = 0.007). These results indicate that transcriptionally active HPV-16 infection may be required to induce cytological abnormalities.

In other studies, RT-nested PCRs have been designed to analyze HPV-16 transcripts by detecting E6 and E7 transcripts in cytologically abnormal cell scrapes (14, 16, 30). Indeed, the prevalence of E6 and E7 transcripts has been shown to be related to the degree of cytologic abnormality (16). However, these methods may fail to identify transcriptionally active infection that occurs prior to virus genome integration, after which E6 and E7 ORFs are overexpressed (21). We propose that our method allows earlier detection of active HPV infection, as it detects transcripts which are not dependent on integration of the virus genome.

In this study, the prevalence of E-mRNA among HPV-16 E5-positive women with histologically proven high-grade lesions was greater than that among women with low-grade lesions. This difference was not statistically significant, but the number for whom histological diagnoses were available was small. Hsu and colleagues also found no statistical association between early-region transcription and histological diagnosis (20). Further studies employing larger numbers are required to clarify these findings.

Other methods of HPV diagnosis have been investigated for the prognosis of CIN. Filter DNA-DNA hybridization and Southern blot hybridization, which have sensitivities of approximately 1 copy of DNA per 100 cells (reviewed in reference 9), have been used to show that women developing high-grade CIN lesions are more likely to have detectable HPV DNA than are women who do not develop high-grade CIN lesions (6, 26). In a study using hybrid capture, which has a sensitivity of approximately 50,000 DNA copies per reaction (7), high levels

![FIG. 1. Detection of HPV-16 DNA and E-mRNA among women with normal and abnormal cervical smears. The numbers above the columns are ratios of the number of positive samples to the number of samples tested. Only samples positive by HPV-16 E5 DNA PCR were included.](http://jcm.asm.org/)

<table>
<thead>
<tr>
<th>Smear result</th>
<th>No. of women positive for RNA/no. of women tested (%)</th>
<th>Colposcopy clinic</th>
<th>WWCs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2/9 (22.2)</td>
<td>2/26 (7.7)</td>
<td>4/35 (11.4)</td>
<td></td>
</tr>
<tr>
<td>Borderline or mild dyskaryosis</td>
<td>2/12 (16.7)</td>
<td>3/5 (60)</td>
<td>5/17 (29.4)</td>
<td></td>
</tr>
<tr>
<td>Moderate or severe dyskaryosis</td>
<td>9/19 (47.4)</td>
<td>1/1 (100)</td>
<td>10/20 (50)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>13/40 (32.5)</td>
<td>6/32 (18.8)</td>
<td>19/72 (26.4)</td>
<td></td>
</tr>
</tbody>
</table>

a ASCUS.

b No. of abnormal smears (borderline, mild, moderate, or severe dyskaryosis) positive for HPV-16 E-mRNA/no. of abnormal smears tested = 15/37 (40.5%).
of HPV DNA were shown to be predictive of concurrent high-grade CIN lesions in young women with atypical squamous cells of undetermined significance (ASCUS) on smears (8). Studies using semiquantitative DNA PCR have shown similar results (3, 10–12). These studies suggest that a high viral load is prognostic for increasing CIN severity. A high viral load is likely to reflect high levels of actively transcribing virus. Further studies are required to determine the relative diagnostic and prognostic significances of assays that detect HPV DNA and mRNA.

The specificity of the RT-PCR for HPV-16 E-mRNA was demonstrated by testing an HPV-18-containing cell line (HeLa), an HPV-negative cell line (A431), and 20 HPV-16 DNA-negative cervical brush smears, 6 of which were positive for HPVs other than HPV-16 (HPV-6, -31, and -33). While a transcribed human DNA sequence on chromosome 7p13-14, related to the HPV-18 E5 gene, has previously been reported (18), the results confirm that this human sequence was not amplified by our primers in either the DNA PCR or RT-PCR.

HPV-16 E-mRNA was detected in K1/16 cells, which are known to express early-region transcripts (28) as well as the E5 protein (22). In addition, E-mRNA was detected in CaSki, XH1, and SiHa cells, all of which contain integrated HPV-16 DNA (2, 19, 42). In CaSki and SiHa cells, most transcripts use a cellular polyadenylation signal flanking the integrated HPV DNA, but a minority of full-length transcripts in CaSki cells use the defined HPV-16 early polyadenylation signal (2, 37, 38). From our present data, it is unclear whether the nested PCR products from these cell lines represent the amplification of transcripts using the HPV-16 early polyadenylation signal or the cellular signal downstream of integrated HPV DNA (35).

Although there has been little previous work on HPV-16 E5 expression, one recent report concerning the translation of mRNAs derived from HPV-16 early-region constructs suggested that HPV-16 E5 is coexpressed with E2 protein from one of two bicistronic mRNAs (23). Our RT-PCR should detect such E2-containing transcripts, and with primers specific to these bicistronic mRNAs, these assays may be used for further studies to investigate E5-producing transcripts of HPV-16.

In summary, our preliminary results show that E-mRNA detection is significantly associated with abnormal cytology in women infected with HPV-16 and that the prevalence of HPV-16 E-mRNA increases with advancing abnormality. At present, the prognostic significance of HPV-16 E-mRNA detection is unknown. However, a prospective study in which HPV-16-positive women with normal cytology or low-grade CIN lesions are being monitored and sequential samples are being tested for E-mRNA by RT-nested PCR, as described above, is in progress. This in turn may lead to improved methods for cervical-cancer screening and may increase our understanding of the role of HPV infection in transformation, oncogenesis, and cervical-disease progression.

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REFERENCES


