High-Throughput Cervical Cancer Screening Using Intracellular Human Papillomavirus E6 and E7 mRNA Quantification by Flow Cytometry

Roberto Narimatsu, MD, and Bruce K. Patterson, MD

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Abstract

The Papanicolaou (Pap) test, based solely on the morphologic examination of exfoliated cells from the cervix, has reduced deaths due to cervical cancer by 74% in the United States during the past 40 years. During that time, the molecular mechanisms of cervical cancer have largely been elucidated. Taken together, these observations have identified a need for a high-throughput cervical cancer screening assay. We report the development of a high-throughput assay consisting of simultaneous immunophenotyping and ultrasensitive in situ hybridization for human papillomavirus (HPV) E6 and E7 messenger RNA (mRNA). This assay can be performed in less than 3 hours directly from liquid-based cervical cytology specimens. Overall, HPV fluorescence in situ hybridization (FISH) for E6 and E7 mRNA demonstrated 83.3% sensitivity and 91.3% specificity for high-grade squamous intraepithelial lesions compared with the Pap test in 231 liquid-based cytology samples from 2 cohorts. In a subset of these samples, HPV FISH demonstrated higher sensitivity and specificity than Hybrid Capture (Digene, Gaithersburg, MD) for high-risk genotypes.

Cervical cancer affects approximately 13,000 women per year in the United States and more than 400,000 women worldwide. In developing countries, cervical cancer screening programs reach only a small proportion of women owing, at least in part, to a shortage of trained cytotechnologists and cytopathologists. Although the Papanicolaou (Pap) smear has been the standard of care in the United States for more than 40 years, resulting in a 74% decline in deaths due to cervical cancer during this period, errors in cervical sampling and interpretation contribute to a sensitivity of only 58% as reported in a meta-analysis. Even though the recent shift toward liquid-based cytology (LBC) with automated monolayer slide production has resulted in a significant increase in adequate specimens and in the detection of squamous intraepithelial lesions (SILs), LBC samples have a sensitivity of only 80%. More than 99% of cervical squamous cell carcinomas are due to infection and subsequent transformation of squamous cells by oncogenic genotypes of human papillomavirus (HPV). Studies also have implicated HPV in the pathogenesis of adenocarcinoma of the uterine cervix. Most HPV assays such as type-specific polymerase chain reaction (PCR) and Hybrid Capture II (Digene, Gaithersburg, MD) detect the presence of HPV L1 DNA from oncogenic types despite the fact that in only a minority of women infected with oncogenic types of HPV will the infection progress to cancer. In HPV-associated lesions, transcriptional activity is always present. The expression of the HPV E6 gene promotes premature degradation of the tumor suppressor gene p53, and the HPV E7 gene associates with the tumor suppressor gene pRB. This association results in the up-regulation of an E2F-like transcription factor, promoting progression of the cell cycle through the G1/S phase. In addition, the RB
gene product represses expression of p16 so the effects of E7 expression on RB de-represses p16 expression, a marker strongly associated with cancer and its precursors.14 The pattern of E6 and E7 gene expression also changes with the severity of the lesion; the level of E6 and E7 gene expression is increased in high-grade lesions compared with low-grade lesions,10 making the level of E6 and E7 gene expression potentially a functional discriminator between high-risk and low-risk HPV infections.

We used a modification of a previously described technique that uses simultaneous immunofluorescence and ultrasensitive fluorescence in situ hybridization (HPV FISH).15 Herein we describe the sensitive and specific screening of cervical cytology samples for cervical cancer precursor lesions using the high-throughput capabilities of flow cytometry. We report the usefulness of this system in detecting abnormal cervical cytology ranging from atypical cells of undetermined significance (ASCUS) to invasive cervical cancer.

Materials and Methods

Subjects

Women undergoing routine cervical cytology at multiple sites were enrolled in this study following informed consent. Cervical cytology specimens were obtained using a cytobrush and preserved using PreserCyt (Cytyc, Boxborough, MA) or SurePath (TriPath Imaging, Burlington, NC) liquid-based cytology fixatives. The smears were classified using the Bethesda System criteria.

Cell Lines

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to the instructions. Normal human ectocervical cells (Clonetics, Baltimore, MD) were grown in media supplied by the company.

Simultaneous Immunophenotyping/Ultrasensitive Fluorescence In Situ Hybridization and Flow Cytometry

A 1-mL aliquot was removed from the liquid-based cervical cytology specimen. The cells were pelleted by centrifugation at 400g, washed again in 2× standard saline citrate (SSC), and pelleted by centrifugation. HPV FISH for E6 and E7 mRNA was performed by resuspending the cells in a hybridization mix consisting of 5× SSC, 30% formamide, and 100 µg/mL of sheared salmon sperm DNA and a cocktail of 5’- and 3’-labeled oligonucleotide probes (HPV OncoTect, Invirion). Hybridization was performed at 37°C for 30 minutes and was followed by a 5-minute wash in 2× SSC and 0.1% Triton X-100 (ViroTest Hybridization Reagent Kit, Invirion) and a 15-minute wash in 0.1% SSC and 0.1% Triton X-100. The cells were resuspended in PBS, pH 7.4, with 2% fetal calf serum for flow cytometric analysis.

Slide-Based In Situ HPV E6 and E7 mRNA Detection

We then transferred 100 µL of cells (1 × 10⁶/mL) from a liquid-based cervical cytology specimen into a cytocentrifuge or liquid-based slide system. The slides were centrifuged at 800g for 2 minutes at room temperature. Following a wash in 1× PBS, pH 7.4, the slides were incubated in 1× PermiFlow fixation/permeabilization reagent (Invirion) in a Coplin jar at room temperature for 1 hour. The slides then were washed once in PBS and once in 2× SSC. The cells were hybridized to a cocktail of 3’, 5’-fluorescein labeled oligonucleotide probes (Invirion) specific for E6 and E7 mRNA in a hybridization oven at 37°C ± 3°C for 30 to 120 minutes. The slides then were washed for 5 minutes in a Coplin jar containing 50 mL of preheated 2× SSC and 0.1% Triton X-100. The slides then were incubated for 15 minutes in a Coplin jar containing 50 mL of preheated 0.1× SSC and 0.1% Triton X-100. Following a brief rinse in PBS, pH 7.4, the slides were coveredslipped using Fluorsave mounting medium (CalBioChem, San Diego, CA).

Type-Specific PCR and Hybrid Capture II

Type-specific PCR was performed as previously described for the detection of oncogenic types of HPV. Hybrid Capture II was performed according to the manufacturer’s instructions using the high-risk panel of probes.

Cytometry

Flow cytometry was performed using 3-color analysis on a FACScan cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA). Analysis was restricted to ectocervical cells defined by light scatter characteristics16 and the lack of expression of the low-molecular-weight cytokeratin CAM 5.2. Polymorphonuclear neutrophils (PMNs) were excluded from the analysis by gating on cells positive for the PMN marker CD16.

Image Analysis

Image analysis was performed using an Olympus laser confocal microscope (Olympus, Melville, NJ). Cells were
include the analysis if they exhibited green fluorescence (fluorescein, HPV E6 and E7 mRNA+) and blue fluorescence (4’-6-diamidino-2-phenylindole dihydrochloride, all cells) but lacked red fluorescence (PMNs, endocervical cells).

Statistical Analysis

Statistical analyses were performed using a t test or a Mann-Whitney rank sum test. P values less than .05 were considered statistically significant.

Results

Validation of Flow Cytometric E6 and E7 mRNA Detection

To validate our flow cytometric E6 and E7 mRNA assay, we compared the mean fluorescence intensity of E6 and E7 mRNA detected by our HPV FISH E6 and E7 mRNA assay and flow cytometry with the number of copies of E6 mRNA in the HPV-containing cell lines SiHa and HeLa and in commercially available HPV– ectocervical cells. SiHa, HeLa, and HPV– ectocervical cells grown in culture were split into multiple samples and run by flow cytometry or real-time reverse transcriptase–polymerase chain reaction (RT-PCR) for HPV E6 mRNA with a detection limit of 10 copies per cell. The mean fluorescence intensity was plotted against the number of copies of E6 mRNA per cell in 25 replicates for each cell line. Linear regression analysis was performed, and the correlation between the flow cytometric assay intracellular E6 and E7 mRNA and E6 mRNA quantified by real-time RT-PCR was 0.88 with a P value of .001. We calculated the theoretical sensitivity of the flow cytometry–based assay to be 10 to 20 copies of HPV E6 and E7 mRNA per cell (data not shown). In all subsequent analyses in patient samples, cells were considered positive for E6 and E7 mRNA if they exhibited a mean fluorescence intensity greater than 200 copies per cell.

Linearity of HPV FISH to Detect HPV E6 and E7 mRNA+ Cells in a Mixed Population

To validate the sensitivity and specificity of the HPV FISH E6 and E7 mRNA assay at the single cell level, we performed cell-mixing experiments using normal ectocervical cells and the HeLa cervical cancer cell line. HeLa cells previously have been shown to express approximately 100 copies of HPV DNA per cell.16 In cell-mixing experiments using HeLa cells and HPV– ectocervical cells, there was a signal/noise ratio of 1.5 to 2.0 logs. There was a linear range between 0.3% and 100% for HPV E6 and E7 mRNA+ cells with a lower limit of detection in a cell mixture of 0.3%.

Morphologic Assessment of E6 and E7 mRNA+ Cells

To further confirm the specificity of HPV FISH E6 and E7 mRNA detection for abnormal cells and to adapt our HPV FISH assay for use on slide-based platforms, we hybridized HPV+ HeLa cells and HPV– normal ectocervical cells with our HPV

\[
\text{MFI (Linear Channel Number)}
\]

\[
\text{HPV E6 mRNA (Copies)}
\]

\[
\text{% HPV+ Cells (Dilution)}
\]

\[
\text{% HPV+ Cells (Flow Cytometry)}
\]

\[
\text{FIGURE 1} \quad \text{Linear relationship between serial dilutions of human papillomavirus (HPV) E6 and E7 messenger RNA (mRNA)+ cells (HeLa) with HPV E6 and E7 mRNA− cells (normal ectocervical cells) and quantification of HPV E6 and E7 mRNA+ cells using flow cytometry. MFI, mean fluorescence intensity.}
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\text{FIGURE 2} \quad \text{Relationship between human papillomavirus (HPV) E6 and E7 messenger RNA (mRNA) quantification by flow cytometry and HPV E6 mRNA quantification by real-time (Tagman, Applied Biosystems, Foster City, CA) reverse transcriptase–polymerase chain reaction.}
\]
FISH E6 and E7 mRNA probe and examined the morphologic features of cells expressing detectable levels of E6 and E7 message. The HPV FISH E6 and E7 mRNA assay produced a characteristic cytoplasmic staining pattern in HeLa cells [Image 1A] without staining the HPV– cell line C33A [Image 1B] or normal ectocervical cells (data not shown). By using the same probe cocktail as used in flow cytometry, we were able to detect atypical cells with a characteristic high nuclear/cytoplasmic ratio (arrows) [Image 1C, Image 1D], and Image 1E in a field of many normal squamous cells (arrowheads, Images 1C-1E). The atypical cells staining with the E6-E7 probe have large nuclei with a high nuclear/cytoplasmic ratio. An overlay image of the Nomarski phase contrast image with the 488-nm fluorescence confocal image confirms the localization of the hybridization signal over the abnormal cells (Image 1E).

**Cytometric Enrichment for HPV E6- and E7-Expressing Cells in a Heterogeneous Cervical Cytology Sample**

Because cervical cytology specimens contain numerous cell types, including squamous ectocervical cells, columnar endocervical cells, PMNs, lymphocytes, and others, we developed an antibody cocktail to be used in conjunction with detection in intracellular HPV E6 and E7 mRNA that distinguishes the cell types of interest in an LBC specimen. To distinguish between neutrophils, endocervical cells, and ectocervical cells in LBC preparations of study subjects, cells were stained with a combination of CD16, a neutrophil marker, and CAM 5.2, an antibody that detects a 70-kd molecular weight cytokeratin that is expressed in endocervical cells but not ectocervical cells. Cells staining for a particular marker were backgated to identify their presence within forward and orthogonal (90°) light-scatter gates, a method described by Grundhoefer and Patterson to distinguish PMNs, endocervical cells, and ectocervical cells in an LBC. As demonstrated in Figure 3A, a gating strategy was used based on the lack of CD16 and CAM 5.2 expression to unequivocally identify ectocervical cells for analysis of E6 and E7 mRNA. Similarly, expression of E6 and E7 mRNA in endocervical cells can be analyzed using a gating strategy that identifies CD16–, CAM 5.2+ cells (data not shown).

**Comparison of HPV E6 and E7 mRNA Detection With Pap Smears for Detecting SIL**

To determine the ability of our assay to screen for premalignant conditions in LBCs, we obtained LBC specimens from a high-risk cohort consisting of 41 women with normal cervical cytology and 41 women with abnormal cervical cytology (9 ASCUS, 22 low-grade SIL [LSIL], 10 high-grade SIL [HSIL]). The quantitative cutoff for a positive E6 and E7 mRNA result was set at 2 SD from the mean of true negative.

Cervical cells were stained with CD16 and CAM 5.2 and hybridized as described. Ectocervical cells were differentiated from endocervical cells by forward and orthogonal light scatter and by expression of CAM 5.2 on endocervical cells but not on ectocervical cells. Ectocervical cells coated with neutrophils were eliminated from analysis by the presence of CD16 staining.

HPV FISH detected E6 and E7 mRNA in 5 of 9 ASCUS samples, 13 of 22 LSIL samples, and 10 of 10 HSIL samples. HPV FISH detected E6 and E7 mRNA in 3 of 41 normal cervical cytology specimens. Of the 3 normal samples, 2 had E6 and E7 mRNA confirmed by quantitative real-time RT-PCR. The percentage of E6- and E7-mRNA–expressing cells positively correlated with the cytologic diagnosis (Figure 3B) and Table 1. The percentage of E6- and E7-mRNA–expressing cells in HSILs was significantly greater than the percentage of expressing cells in samples diagnosed as within normal limits (WNL) or LSIL (P < .001). Similarly, the percentage of E6- and E7-mRNA–expressing cells in LSILs was significantly greater than the percentage of expressing cells in WNL samples (P < .01). The percentage of expressing cells in HSILs was significantly greater than the percentage of expressing cells in ASCUS (P < .001); however, the percentage of expressing cells in LSILs was not significantly different from the percentage of expressing cells in ASCUS. This would suggest that the molecular alterations in ASCUS more closely resemble LSIL than HSIL. In this high-risk cohort, the overall sensitivity and specificity for high-grade lesions were 100% and 93%, respectively.

**Comparison of Detection of Intracellular E6 and E7 mRNA Expression With Hybrid Capture II in a Low-Risk Cohort**

To compare HPV FISH E6 and E7 mRNA detection with Hybrid Capture II for high-grade cervical lesions, we screened 149 samples from a low-risk US cohort. Of these samples, 109 were diagnosed as WNL, 21 as ASCUS, 5 as LSIL, 12 as HSIL, and 2 as invasive cervical cancer by cytologic examination (Table 1). Using HSIL and invasive cervical cancer as an endpoint, the sensitivity and specificity of these tests to detect cervical abnormalities were 71.4% and 91%, respectively, for HPV FISH E6 and E7 mRNA detection and 64.2% and 88%, respectively, for Hybrid Capture II. HPV FISH E6 and E7 mRNA detection and Hybrid Capture II both detected 8 of 12 HSILs, but HPV FISH E6 and E7 mRNA detection also identified an invasive cervical cancer that was missed by Hybrid Capture II.

**Discussion**

Despite the success of the Pap smear in decreasing the incidence and death rates of cervical cancer, Pap testing has a relatively low sensitivity and is skill- and labor-intensive. Many of the molecular events leading to cervical cancer...
Image 1: Detection and morphologic features of human papillomavirus (HPV) E6 and E7 messenger RNA (mRNA)+ cells stained on slides. HPV E6 and E7 mRNA+ (HeLa, A) and HPV E6 and E7 mRNA− (C33A, B) control cells. C-E, Representative abnormal cervical cytology sample following HPV fluorescence in situ hybridization with a Nomarski overlay. Abnormal cells (arrows) have a characteristically high nuclear/cytoplasmic ratio, whereas normal squamous epithelial cells (arrowhead) exhibit a low nuclear/cytoplasmic ratio. C, E6 and E7 mRNA (green). D, Nomarski image. E, Overlay of fluorescence image and the Nomarski image.
Figure 3. Representative flow cytometry histograms from women with high-grade squamous intraepithelial lesion (HSIL) and within normal limits (WNL) cytologic diagnoses. Histograms have been gated by light scatter and on CAM 5.2–, CD16– (ectocervical cells without bound polymorphonuclear neutrophils) immunophenotyping (A). B. Representative histograms from HSIL (top row) and WNL (bottom row) samples. The bars represent cells expressing high levels of E6 and E7 messenger RNA.

Cy5, cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 1
Comparison of HPV FISH E6 and E7 mRNA Detection With Cervical Cytology (Papanicolaou) Results in a High-Risk HPV Cohort

<table>
<thead>
<tr>
<th>Cytologic Diagnosis</th>
<th>High</th>
<th>Low</th>
<th>Median</th>
<th>HPV E6 and E7 mRNA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNL</td>
<td>5.6</td>
<td>0.1</td>
<td>0.5</td>
<td>3/41</td>
</tr>
<tr>
<td>ASCUS</td>
<td>7.3</td>
<td>0.1</td>
<td>1.9*</td>
<td>5/9</td>
</tr>
<tr>
<td>LSIL</td>
<td>12.7</td>
<td>0.1</td>
<td>2.7†</td>
<td>13/22</td>
</tr>
<tr>
<td>HSIL</td>
<td>46.1</td>
<td>2.1</td>
<td>13.9‡</td>
<td>10/10</td>
</tr>
</tbody>
</table>

ASCUS, atypical squamous cells of undetermined significance; FISH, fluorescence in situ hybridization; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; mRNA, messenger RNA; WNL, within normal limits.

* ASCUS > WNL, P < .05.
† LSIL > WNL, P < .01; LSIL > ASCUS, P = .3.
‡ HSIL > WNL, P < .001; HSIL > LSIL, P < .001.
described during the past decade may be adapted to high-throughput detection methods. We report a high-throughput cervical cancer screening assay with high sensitivity and specificity that can be performed on a variety of platforms, especially the widely used, high-throughput flow cytometer.

Current HPV DNA testing is used to detect oncogenic types of HPV in cervical cytology specimens. Within 5 to 10 years, in a minority of women infected with high-risk types of HPV and some women infected with low-risk HPV, the infection will progress to high-grade lesions or even cervical cancer in the absence of treatment.9

The molecular switch for the development of cervical intraepithelial neoplasia and, ultimately, cervical cancer is the continued expression of the E6 and E7 genes of oncogenic HPVs.1,10,16-21 This initiates a cascade of events that results in the loss of tumor suppressor activity and cell cycle dysregulation. Cell proliferation antigens such as Ki-67 and proliferating cell nuclear antigen have been assessed for their ability to detect atypical cervical cells,22 as have the cell cycle (DNA replication)-dependent antigens cdc2, cdc6, and Mcm5.23,24 Because these are cell-cycle markers and commonly are present in nonneoplastic, regenerating cells, the use of these markers for cervical cancer screening is limited. The MN transmembrane glycoprotein antigen has been studied extensively, especially the widely used, high-throughput flow cytometer.

The cell cycle–dependent antigen p16 is up-regulated following the association of E7 with RB and the inactivation of this tumor suppressor gene.14 Previous studies have acknowledged the principal role of E7 in the development of cervical atypia, and an antibody to p16 was developed as a surrogate marker for E7 activity. The p16 marker had a sensitivity of 82% and a specificity of 84% for the detection of atypical cervical lesions from LSIL to invasive cervical cancer.14 The staining pattern of this antibody was critical for the reported sensitivity and specificity; however, it minimized the usefulness of this marker in high-throughput platforms.

Although HPV DNA detection is biologically relevant, the detection of high-risk HPV DNA loses power because it detects a molecular event too early in the process. If disease in 100% of the women determined to be infected with a high-oncogenic-risk HPV type progressed to cervical cancer, then type-specific HPV DNA detection would be the “gold standard” of cervical cancer screening. Although more than 99% of cervical cancers contain high-risk HPV,6 only a small proportion of the women infected with a high-oncogenic-risk type will have disease that progresses to cancer.9 Recent studies also have demonstrated that high-grade lesions also could develop in women infected with non–high-risk types.9 Whether these non–high-risk lesions that progressed to high-grade lesions developed as a result of increased expression of E6 and E7 mRNA is a distinct possibility if not a necessity. Further prospective studies will be required to confirm this hypothesis.

In a direct comparison of intracellular E6 and E7 mRNA detection with Hybrid Capture II in low-risk and high-risk HPV cohorts, our analysis demonstrated statistically equivalent results between the 2 assays, yet intracellular E6 and E7 mRNA detection was performed in 2 hours, compared with 8 hours for Hybrid Capture II. Furthermore, intracellular E6 and E7 mRNA detection used only 0.5 mL of the 10-mL LBC preparation, whereas Hybrid Capture II required 4 mL of sample.

Although we present no prospective data, it is possible that the E6 and E7 mRNA assay described herein will be useful in predicting the natural history of patients with HPV-related cervical lesions and could serve as a “secondary marker” for patients who are HPV DNA+. This might be most useful in screening young women, a cohort in which the prevalence of HPV DNA positivity may be as high as 20%. In any case, the E6 and E7 mRNA assay seems to have potential application as an adjunct to the Pap smear in screening programs or even as a primary screening tool. Overall, the sensitivity of this assay in patients with HSIL was 83.3%. HPV FISH also might identify patients with negative Pap smears in whom the process of cellular transformation into precancerous cells is present. The 2 patients who had negative Pap smears from the high-risk cohort but were positive for E6 and E7 mRNA were HPV E6 mRNA+ using real-time RT-PCR and high-risk HPV DNA+ using type-specific PCR. The sensitivity for HSIL in our study using Hybrid Capture II was low compared with some studies but not compared with other studies.26 This might be explained by the fact that our analysis included low-HPV-prevalence and high-HPV-prevalence cohorts, and our analysis was not limited to women older than 30 years. Furthermore, histology was used as the gold standard in studies that report Hybrid Capture II sensitivities of greater than 90%.

### Table 21

<table>
<thead>
<tr>
<th>Papanicolaou Result</th>
<th>Intracellular E6 and E7 mRNA</th>
<th>Hybrid Capture II</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNL (n = 109)</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>ASCUS (n = 21)</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>LSIL (n = 5)</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>HSIL (n = 12)</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Squamous cell carcinoma (n = 2)</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

ASCUS, atypical squamous cells of undetermined significance; FISH, fluorescence in situ hybridization; HCII, Hybrid Capture II; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; mRNA, messenger RNA; WNL, within normal limits.

* For intracellular E6 and E7 mRNA and Hybrid Capture II, respectively, sensitivity, ≥71.4% and 64.2%, and specificity, 91% and 88%. For proprietary information, see the text.
Our assay might satisfy the recommendations of Stoler\textsuperscript{27} who has suggested even greater usefulness of HPV testing as a front-line approach to cervical cancer detection.

\textit{From the Department of Pathology and Medicine, Division of Infectious Diseases and Geographic Medicine, Stanford University Medical School, Stanford, CA.}

Address reprint requests to Dr Patterson: Dept of Pathology, Stanford University Medical School, 300 Pasteur Dr, Stanford, CA 94305-5629.

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