Comparison of Human Papillomavirus Messenger RNA and DNA Detection: A Cross-sectional Study of 4,136 Women >30 Years of Age with a 2-Year Follow-up of High-Grade Squamous Intraepithelial Lesion

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Abstract

The purpose of this study was to compare the detection of human papillomavirus (HPV) DNA with detection of mRNA. The study included 4,136 women >30 years of age. E6/E7 mRNA expression from the carcinogenic HPV types 16, 18, 31, 33, and 45 was detected by the PreTect HPV-Proofer assay, whereas the presence of HPV DNA was detected by Gp5+/6+ consensus PCR followed by type-specific PCR. A total of 4.0% had an abnormal cytologic diagnosis, 3.0% were positive by PreTect HPV-Proofer, 4.4% by type-specific PCR, and 10.4% by consensus PCR. For detection of HPV in high-grade squamous intraepithelial lesion (HSIL), no significant difference was observed between PreTect HPV-Proofer and consensus PCR. For women with a cytologic normal, atypical squamous cell of uncertain significance, and low-grade SIL diagnosis, the detection rate of HPV was significantly higher by Gp5+/6+ consensus PCR (P < 0.005) than by PreTect HPV-Proofer. Histology confirmed 14 of 23 cytologic HSIL as cervical intraepithelial neoplasia grade >2. Of these women, PreTect HPV-Proofer and type-specific PCR detected 12, whereas consensus PCR detected 13. In conclusion, for HSIL, detection of E6/E7 transcripts from HPV types 16, 18, 31, 33, and 45 are present to the same degree as DNA detected by consensus PCR. Equally important, only a small proportion of the HPV DNA–positive women with a normal, atypical squamous cell of uncertain significance or low-grade SIL diagnosis had a detectable mRNA expression. HPV E6/E7 mRNA detection by PreTect HPV-Proofer represents a new promising test as an adjunct to cytology.

Introduction

In some developed countries cytologic screening programs using Papanicolaou (Pap) smears have reduced cervical cancer incidence and mortality. Yet, cervical cancer is one of the most common malignant diseases and leading causes of morbidity and mortality among women worldwide (1). Single Pap tests suffer from suboptimal sensitivity, limited reproducibility, and many equivocal results (2, 3) and to compensate for these deficiencies a screening program with repeated testing combined with follow-up of positive cases is necessary. Furthermore, difficulties predicting which cervical lesion will progress, and the high rate of regression of low-grade lesion detected in cytologic screening, emphasize the need for additional prognostic and diagnostic markers for detection of cervical cancer precursors.

Infection with high-risk HPV is the main cause of cervical intraepithelial and invasive neoplasias (4, 5) and HPV DNA has been detected in >90% of cervical carcinomas, with the most common HPV types identified as HPV 16, 18, 31, 33, and 45 (6-9). HPV is a common virus among women, particularly in younger age groups, and most infections are transient and asymptomatic (10, 11). Patients with persistent infection with these HPV types have a clearly enhanced risk of developing cervical carcinoma (12).

Large-scale screening studies have shown HPV testing as more sensitive than cytology for the detection of high-grade cervical lesions (13-16). However, low specificity of current assays and commercial kits hampers the use of HPV testing in screening. The combination of HPV DNA detection and cytology is more suitable for risk assessment of progression to cervical intraepithelial neoplasia (CIN) grade 3 and carcinoma than cytology alone (17, 18).

Viral infection is targeted at the parabasal keratinocytes, but with a relatively low level of viral E6/E7 mRNA expression (19). However, in the upper spinous and granular layers of the epithelium of squamous intraepithelial lesion (SIL) and cervical carcinoma, an increasing expression of the viral oncoproteins E6 and E7 occurs. This expression is necessary for conversion to and maintenance of malignancy (20-22), and hence the detection of the E6/E7 transcripts of high-risk HPV types might serve as a better risk evaluation factor than mere DNA detection for the development of HSIL. The same would be true for the progression to cervical carcinoma (23).

The aim of the present study was to compare the detection of HPV mRNA from the carcinogenic HPV types 16, 18, 31, 33, and 45 with the detection of HPV DNA in an outpatient screening population in Norway with a 2-year follow-up of HSIL.

Materials and Methods

Study Design. The present study was a cross-sectional outpatient population–based screening study conducted in
Detection of HPV DNA and mRNA

2001, supplemented with follow-up data from the Cancer Registry of Norway. Cytology was compared with mRNA detection (PreTect HPV-Proofer) and DNA detection by type-specific and consensus PCR. Women with cytologic HSIL were subjected to follow-up for histologic confirmation according to guidelines for the Norwegian cervical cancer screening program (NCCSP). Results were compared with mRNA and DNA detection. The sensitivity, specificity, and positive and negative predictive values of HPV testing for detecting HSIL and of HPV testing in detecting CIN2+ in cytologic HSIL were calculated. McNemar's test was used for comparison of PCR and nucleic acid sequence–based amplification (NASBA). Student's t test was used for comparison of the differences in the mean follow-up time of women with HSIL and confirmed CIN2+ in contrast to those women with an unconfirmed CIN2+. The PCR and NASBA reactions were done independently. Cytology, NASBA, and PCR results were compared after all tests were done.

Study Subjects. In the NCCSP, women are encouraged to take a Pap smear every third year if the preceding Pap test is normal. Women with HSIL should be followed by immediate colposcopy and biopsy unless other medical reasons would indicate otherwise (e.g. pregnancy in which case close, cytologic surveillance might be preferred if there are no indication of invasiveness). A detailed description of the NCCSP is published elsewhere (24). In this study, women age 30 to 69 years (n = 4,154) were included, attending selected specialist gynecological clinics in Oslo in the period from February to May 2001. Of 4,154 women tested, 118 women were found negative for the U1A mRNA sample control. These samples were tested again and this time only 18 were negative for U1A, which were excluded from the study. The total number of women included in the study was 4,136. Gynecologists at 12 different clinics in Oslo performed the Pap smears, which were then screened by experienced cytootechnologists at two different laboratories. In the NCCSP, a hybrid classification with both dysplasia (WHO's classification) and CIN are used. In this article, the cytologic diagnoses HPV condyloma, CIN1, and mild dysplasia are defined as low-grade SIL (LSIL), whereas CIN2, CIN3, atypical glandular cell of uncertain significance, atypical squamous cell—cannot rule out high-grade lesion (ASC-H), moderate dysplasia, severe dysplasia, carcinoma in situ, and cervical cancer are defined as HSIL. Patients diagnosed with HSIL are referred to biopsy. For histopathologic evaluation, the WHO nomenclature and criteria are used.

Collection of Sample Material. The cervical samples were collected with a Cervex Brush (Rovers Medical Devices, Oss, the Netherlands) that was immersed in 9 mL of Nuclisens lysis buffer [5 mol/L guanidine thiocyanate, 0.1 mol/L Tris (pH 6.4), 20 mmol/L EDTA, and 1.2% (w/v) Triton X-100; BioMérieux, Boxtel, the Netherlands] directly after a cytologic smear was done. The samples in lysis buffer were stored at -70°C. DNA/RNA was isolated from 1 mL of this premix were distributed to each 0.8-μL reaction mixture containing these HPV types. Water was used as negative control. Visualization of the PCR products was done on a DNA chip according to the manufacturer's protocol (Agilent Technologies, Palo Alto, CA). The DNA chip uses a microscale gel electrophoresis with an optimal detection limit of 0.5 to 50 ng/μL. The results were interpreted using the Bioanalyzer 2100 software.

PreTect HPV-Proofer. The PreTect HPV-Proofer assay uses real-time multiplex NASBA, which amplifies mRNA in a DNA background with real-time detection of the products by molecular beacon probes. The PreTect HPV-Proofer assay was done according to the manufacturers instructions (NorChip) and automatically using RoboRead (MWG). Briefly, three premixes were made by reconstitution of reagent sphere (nucleotides, diithiothreitol and MgCl2) in reagent sphere diluent (Tris-HCl, 45% DMSO, followed by addition of either U1A/HPV 16, HPV 33/45, or HPV 18/31 primer/molecular beacon mixes and KCl stock solution; 10 μL of this premix were distributed to each well in a reaction plate, followed by addition of 5 μL RNA (diluted 1:5) before incubation for 4 minutes at 65°C to destabilize secondary structures of RNA, followed by cooling down to 41°C. The reaction was started by addition of enzymes (AMV-RT, RNase H, T7 RNA polymerase, and bovine serum albumin) and measured in real-time using the Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT) at 41°C for 2 hours and 30 minutes. Total reaction volume was 20 μL.

The RNA was stored for ~1 year at -70°C before they were tested by NASBA. RNA isolated from CaSkI cells was used as positive control for HPV 16. Artificial and standardized oligo's, corresponding to the viral sequence, were used as positive control for HPV 18, 31, 33, and 45. Water was used as negative control. The U1A serve as mRNA quality control.
A newly developed software package (PreTect Analysis Software, NorChip) was used for analysis of the experimental data. The excitation $\lambda$ (nm) filters for FAM and Texas Red are 485/20 and 590/20, and the emission $\lambda$ (nm) filters are 530/25 and 645/40, respectively.

Results

Cytologic Findings. The age distribution of women having a normal smear, HSIL smear, and HPV test positive results is given in Table 1. The age distribution was 24.7% in the group age 30 to 39 years, 29.3% in the group age 40 to 49 years, 29.2% in the group age 50 to 59 years, and 16.8% in the group age >60 years. The mean age was 48.9 years (range = 30-91 years). A total of 98.2% of the 4,136 women included had taken at least one Pap smear with an average number of 5.3 during the previous 10-year period. The average number of Pap smears per woman was 5.3 during the previous 10-year period. The average number of Pap smears per woman was 5.3 during the previous 10-year period.

Table 1. Age distribution of women with normal smear, HSIL smear, and HPV-positive test results

<table>
<thead>
<tr>
<th>Age group</th>
<th>Women (%)</th>
<th>Normal index smear (%)</th>
<th>HSIL index smear (%)</th>
<th>PreTect HPV-Proofer (%)</th>
<th>Type-specific PCR (%)</th>
<th>Consensus PCR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-39</td>
<td>24.7 (n = 1,023)</td>
<td>94.8</td>
<td>1.4</td>
<td>5.9</td>
<td>8.8</td>
<td>15.3</td>
</tr>
<tr>
<td>40-49</td>
<td>29.3 (n = 1,211)</td>
<td>96.1</td>
<td>0.5</td>
<td>2.6</td>
<td>3.6</td>
<td>9.4</td>
</tr>
<tr>
<td>50-59</td>
<td>29.2 (n = 1,208)</td>
<td>96.4</td>
<td>0.3</td>
<td>1.9</td>
<td>2.6</td>
<td>8.2</td>
</tr>
<tr>
<td>&gt;60</td>
<td>16.8 (n = 694)</td>
<td>96.8</td>
<td>0.1</td>
<td>1.7</td>
<td>2.6</td>
<td>8.7</td>
</tr>
<tr>
<td>Total</td>
<td>100 (n = 4,136)</td>
<td>96.0</td>
<td>0.6</td>
<td>3.1</td>
<td>4.4</td>
<td>10.4</td>
</tr>
</tbody>
</table>

NOTE: PreTect HPV-Proofer and type-specific PCR detect HPV types 16, 18, 31, 33, and 45. Type-specific PCR has been performed on Gp5+/6+ consensus PCR-positive samples.

The detection of HPV in cytologically normal and LSIL women was significantly higher than type-specific PCR (McNemar’s $P < 0.05$) than for PreTect HPV-Proofer. For the cytologically normal, ASCUS, and LSIL women, the frequency of HPV DNA positive women detected by Gp5+/6+ consensus PCR was significantly higher than by mRNA detection (McNemar’s $P < 0.005$). Furthermore, for the women having an unsatisfactory smear, no mRNA expression was observed, whereas type-specific PCR and consensus PCR detected 3.1% and 4.7%, respectively.

Table 2. Number of HPV positive samples detected by PreTect HPV-Proofer and PCR

<table>
<thead>
<tr>
<th>Cytology</th>
<th>PreTect HPV-Proofer</th>
<th>PreTect HPV-Proofer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Normal ($n = 3,970$)</td>
<td>$P &lt; 0.000$</td>
<td>$P &lt; 0.000$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>27</td>
</tr>
<tr>
<td>Unsatisfactory ($n = 64$)</td>
<td>$P = 0.083$</td>
<td>$P = 0.017$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>ASCUS ($n = 57$)</td>
<td>$P = 0.317$</td>
<td>$P = 0.317$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>LSIL ($n = 20$)</td>
<td>$P = 0.003$</td>
<td>$P = 0.006$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>HSIL ($n = 25$)</td>
<td>$P = 0.083$</td>
<td>$P = 0.083$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Total ($n = 4,136$)</td>
<td>$P &lt; 0.000$</td>
<td>$P &lt; 0.000$</td>
</tr>
<tr>
<td>Gp5+/6+ PCR</td>
<td>Positive</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>28</td>
</tr>
</tbody>
</table>

NOTE: PreTect HPV-Proofer and type-specific PCR detects HPV types 16, 18, 31, 33, and 45. Type-specific PCR was done on Gp5+/6+ consensus PCR-positive samples. The probability calculus was done using McNemar’s test.

Abbreviation: TS-PCR, type-specific PCR.
Comparison of HPV Type-Specific mRNA and DNA Detection Related to Cytology. In women with a cytologically normal diagnosis \((n = 3,970)\), E6/E7 mRNA was expressed in only 30% of the HPV 16, 56% of the HPV 18 and 75% of the HPV 31 DNA–positive women (Table 3). This difference was significant (McNemar’s \(P < 0.05\)). For HPV 33 and 45, no significant differences were found. In the cytologic ASCUS, LSIL, and HSIL women, no significant differences between DNA and mRNA detection were observed for HPV types 16, 18, 31, 33, and 45. An overall higher prevalence of HPV was detected in the women with cervical cell abnormalities, with HPV 16 as the most common type.

Women positive for HPV mRNA, yet negative for DNA, were retested with Gp5+/6+ consensus PCR and type-specific PCR. Seven cytologically normal women, positive for HPV 31 mRNA, were negative by Gp5+/6+ consensus PCR but positive by type-specific PCR.

**Table 3. Distribution of HPV types among HPV-positive samples**

<table>
<thead>
<tr>
<th>Cytology</th>
<th>HPV 16</th>
<th>HPV 18</th>
<th>HPV 31</th>
<th>HPV 33</th>
<th>HPV 45</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PreTect (P)</td>
<td>PCR (%)</td>
<td>PreTect (P)</td>
<td>PCR (%)</td>
<td>PreTect (P)</td>
</tr>
<tr>
<td>Normal ((n = 3,970))</td>
<td>16 (0.4)</td>
<td>53 (1.3) (\dagger)</td>
<td>18 (0.5)</td>
<td>32 (0.8) (\dagger)</td>
<td>27 (0.7)</td>
</tr>
<tr>
<td>Unsatisfactory ((n = 64))</td>
<td>0</td>
<td>1 (0.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASCUS ((n = 57))</td>
<td>7 (12.3)</td>
<td>9 (15.8)</td>
<td>2 (3.5)</td>
<td>2 (3.5)</td>
<td>1 (1.8)</td>
</tr>
<tr>
<td>LSIL ((n = 20))</td>
<td>3 (15.0)</td>
<td>5 (25.0)</td>
<td>1 (5.0)</td>
<td>1 (5.0)</td>
<td>0</td>
</tr>
<tr>
<td>HSIL ((n = 25))</td>
<td>6 (24.0)</td>
<td>7 (28.0)</td>
<td>3 (12.0)</td>
<td>3 (12.0)</td>
<td>1 (4.0)</td>
</tr>
<tr>
<td>Total ((n = 4,136))</td>
<td>32 (0.8)</td>
<td>75 (1.8)</td>
<td>24 (0.6)</td>
<td>38 (0.9)</td>
<td>29 (0.7)</td>
</tr>
</tbody>
</table>

**NOTE:** PreTect HPV-Proofer and Gp5+/6+ consensus PCR were performed on all samples. PreTect HPV-Proofer and type-specific PCR detects HPV types 16, 18, 31, 33, and 45. Type-specific PCR was done on Gp5+/6+ consensus PCR–positive samples. The probability calculus was done using McNemar’s test.

*PreTect = PreTect HPV-Proofer.

\(\dagger\) McNemar’s \(P < 0.05\), significant different from PreTect HPV-Proofer.

**Discussion**

This study is a comparison of DNA and RNA detection techniques, applied on a cytologic screening material. E6/E7 transcripts from the high-risk HPV types 16, 18, 31, 33, and 45 were detected in 4,136 women, by using the real-time multiplex NASBA based detection method PreTect HPV-Proofer (NorChip). HPV DNA was detected by consensus and type-specific PCR from the same five high-risk types.

Women with cytologic HSIL were subjected to follow-up for histological confirmation according to guidelines for the NCCSP. Cytologic and histologic data were supplemented by the Cancer Registry of Norway.

The women recruited for this study, older than 30 years of age, had several previous Pap tests. This may explain the low percentage of women with a cytologic HSIL diagnosis and therefore the low prevalence of histologic CIN2+. In addition, some women with a CIN2+ lesion may not have been detected due to a negative cytology result or due to an ASCUS or LSIL diagnosis, and hence not tested by histology.

For women with a cytologic HSIL diagnosis, the prevalence of HPV detected by PreTect HPV-Proofer and PCR was not significantly different. When excluding women not confirmed as CIN2+ on the histologic follow-up within 2 years, the detection rate for PreTect HPV-Proofer and type-specific PCR was 86%, whereas consensus PCR detected 93%. This difference is due to one HPV 35 infection, which is not detected by the PreTect HPV-Proofer assay. A high detection rate for E6 and E7 transcripts in CIN and cervical carcinomas has also been observed by Nakagawa et al. (28). In studies of histologically proven CIN3 and invasive cervical carcinoma,
it has been shown that DNA and mRNA detection is practically identical (29). The low prevalence of cytologic HSIL explains the relatively low positive predictive values, since predictive values are dependent on the prevalence. Among the cytologic HSIL women, however, the prevalence of histologic CIN2+ was much higher and therefore also the positive predictive values.

Table 5. Mean, median, and range of time interval between HSIL diagnosis and histology

<table>
<thead>
<tr>
<th>Histology after end-point histological CIN2+</th>
<th>n</th>
<th>Mean time (months)</th>
<th>Median time (months)</th>
<th>Min/Max (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN2+</td>
<td>14</td>
<td>2.6</td>
<td>1</td>
<td>1/10</td>
</tr>
<tr>
<td><em>Normal</em></td>
<td>9</td>
<td>4.1</td>
<td>3</td>
<td>2/13</td>
</tr>
</tbody>
</table>

NOTE: Two cases without follow-up within 24 months.

*p* = 0.29 (t test for differences in mean follow-up time).

*Normal* histology is defined as less than CIN2 or normal by cytology or histology.

Table 6. Sensitivity, specificity, PPV, and NPV for PreTect HPV-Proofer and consensus PCR on cytological HSIL and histological CIN2+ samples

<table>
<thead>
<tr>
<th></th>
<th>All cytology (<em>n</em> = 4,136), end-point cytological HSIL (<em>n</em> = 25), %</th>
<th>Cytological HSIL (14), %</th>
<th>PreTect HPV-Proofer</th>
<th>Gp5+/6+ PCR</th>
<th>PreTect HPV-Proofer</th>
<th>Gp5+/6+ PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>52.0</td>
<td></td>
<td>85.7</td>
<td>92.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>97.3*</td>
<td></td>
<td>88.9</td>
<td>66.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPV</td>
<td>10.3</td>
<td></td>
<td>92.3</td>
<td>81.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPV</td>
<td>99.7</td>
<td></td>
<td>80.0</td>
<td>85.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Women with cytological HSIL have been followed-up by histology. Abbreviations: PPV, positive predicted value; NPV, negative predicted value.

*Significant different according to nonoverlapping 95% confidential intervals (data not shown).

regarding the development of CIN and its progression to cervical cancer (23). This minimizes unnecessary concern among HPV DNA positive women with a low risk for progression to HSIL. Consequently, a combination of HPV RNA testing and cytology done in a primary screening context may give the most optimal sensitivity and specificity. However, longer follow-up of women with cytologically normal, ASCUS, and LSIL diagnosis is needed.

Based on this study, as well as other studies done on dysplasia and carcinomas, a natural question is the number of different HPV types needed for efficient screening as an adjunct to cytology. Type-specific identification is important for monitoring possible persistent infections, which is a major risk factor for development of severe dysplasia (34). However including additional HPV types makes the testing procedure more complicated and also increases the detection rate for cervical carcinomas merely marginally. Today, 15 HPV types have been classified as high risk and sorted according to decreasing prevalence in cancer samples worldwide (8, 35). Detecting the five high-risk HPV types 16, 18, 31, 33, and 45 will identify most of the carcinoma cases worldwide, but regional differences have to be taken into regard. The choice of test procedure also depends on the local strategy; whether all women at risk for CIN2+ should be identified no matter the cost, or whether some women can be missed by screening to keep the costs down.

A limitation of our study is the testing by type-specific PCR only on the Gp5+/6+ consensus PCR positive samples and hence sensitivity, specificity, and predictive values cannot be calculated for type-specific PCR. However, performing type-specific PCR on all samples was considered too labor intensive in relation to a possible gain in information. In addition, the PCR products were detected by gel electrophoresis, and not by hybridization probes, which would have optimized the procedure and enhanced the sensitivity. Also, according to Norwegian guidelines, confirmation by histology has only been done on cytologic HSIL and consequently, some histologic CIN2+ may have been missed. Hence, the sensitivity and specificity values reported may be inflated due to verification bias.

In conclusion, for HSIL and CIN2+, E6/E7 transcripts from HPV types 16, 18, 31, 33, and 45 are present to the same degree as DNA detected by consensus PCR. Equally important, only a small proportion of the HPV DNA positive women with a cytologically normal, ASCUS, or LSIL diagnosis had a detectable mRNA expression. Introduction of carcinogenic HPV E6/E7 mRNA detection by PreTect HPV-Proofer represents a new promising technology in HPV diagnostics work as adjunct to cytology.
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