The diagnostic accuracy of E6 & 7 mRNA detection as a primary screening test for the detection of severe cervical lesions

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Summary

Purpose: This prospective accuracy study aimed to assess the diagnostic accuracy of nucleic acid sequence amplification (NASBA) and flow cytometry for E6/7 human papillomavirus (HPV) mRNA detection as a primary screening test compared to cytology in the triage of severe cervical intraepithelial neoplasia (CIN) lesions.

Methods: 1083 women referred to our outpatient gynecology clinics for a routine Pap test were recruited. Residual material of the Pap smears was tested by NASBA and by flow cytometry for E6/7 mRNA expression. Biopsy results were used as reference standards. The accuracy indices of both techniques and of NASBA type-16 HPV were assessed for the detection of CIN2+ lesions and were compared to cytology.

Results: An increased lesion severity was associated with increased positivity rates of both NASBA and flow cytometry tests ($\chi^2$, $p<0.001$). A positive correlation between NASBA and flow cytometry was identified when these methods were examined with the Phi coefficient (value 0.369, 95% confidence interval [95%CI] : 0.307-0.426). Furthermore, NASBA (89.7 vs 57.7%, $p<0.0005$) and flow cytometry (77.3 vs 57.7%, $p<0.0005$) exhibited higher specificity rates than cytology. However, their sensitivity rates did not exceed those of cytology (NASBA:69.8 vs 84.6%, $p=0.051$; flow cytometry: 69.12 vs 84.6%, $p=0.043$).

Conclusions: Both NASBA and flow cytometry exhibited increased specificity for the triage of CIN2+ lesions. However, their relatively lower sensitivity and higher positivity rates when compared to cytology do not make them ideal for a primary screening test. Hence, the role of mRNA detection in the screening for severe cervical lesions remains to be clarified.

Key words: cervical intraepithelial neoplasia, flow cytometry, human papillomavirus, mRNA detection, nucleic acid sequence amplification (NASBA), screening test

Introduction

HPV infection represents the most common sexually transmitted infection (STI) among women [1]. It is estimated that almost 300 million women worldwide are infected [2] and that without a secondary prevention 1% of them will finally develop cervical cancer [3] constituting thus a significant burden both for families and for societies. However, systematic screening via Pap tests has been more or less (depending on the region) established worldwide over the last decades leading to a substantial decrease in the morbidity and to an increase in the survival from cervical cancer [4]. Under the current US and UK guidelines [5-7], an abnormal cervical cytology leads a woman to referral for colposcopy in order to define further treatment, whereas a negative Pap test leads to repeated routine screening in various intervals (depending on the country) throughout life. On the other hand, despite its well recognized benefits, cytology has several limitations: it has low sensitivity and specificity, it is a subjective test warranting constant quality/control assurance, it
has a poor reproducibility and it is labor-intensive [3,8,9].

Based on the abovementioned observations, clinical and laboratory research has been focused on finding tests that will be both objective and accurate, in order not to overtreat false positives and to avoid repetitive unnecessary tests, but what is most important, to identify those women who are in true risk for developing cervical cancer. HPV-DNA testing has been thought to be a promising alternative in this area. However, despite the fact that it has exhibited higher sensitivity when compared to cytology in several studies [10,11], it cannot be used as a primary screening test for identifying high-risk patients because of its lower specificity in identifying the absence of high-grade cervical intraepithelial lesion (CIN) compared to cytology screening [12].

The deregulatory function of E6 and E7 HPV oncoproteins is one of the primordial steps for HPV-induced cervical carcinogenesis [13]. It is already known that these oncoproteins are not only highly detected in cervical cancer tissues, but are also related to the degree of histological severity [14,15]. Hence, E6/7 mRNA detection may be an indicator both of infection and of progression towards cancer. Furthermore, there are also indications that HPV E6/7 mRNA testing exhibits improved specificity when compared to DNA testing [16,17]. Therefore, the aim of the present study was to assess the accuracy indices of E6/7 mRNA testing when compared to cytology in the general population and hence whether E6/7 mRNA testing may be used as a primary screening test replacing cytology.

Methods

Following ethical approval from the Scientific Committee of ‘Attikon’ University Hospital, all women referred to the outpatient gynecology clinics for a routine Pap test from April 2009 to June 2011 were included in the study after providing written informed consent for participation in a prospective protocol on the use of HPV genome biomarkers in cervical cancer prevention. Women with a history of prior CIN treatment or prior hysterectomy were excluded from the study.

A liquid-based cytology specimen placed in Thin-Prep medium was obtained from each woman enrolled in the study. A Pap test was performed and reported according to the revised (2001) Bethesda classification system [18] and the residual material was analyzed by the following techniques:

i) NASBA to amplify RNA sequences: molecular probes against E6/E7 mRNA for 5 high-risk HPV types: 16, 18, 31, 33 and 45.

ii) Flow cytometric evaluation of E6/E7 mRNA of high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) with HPV OncoTect (Invirion Diagnostics, Oak Brook IL, USA). The test was considered positive if the result was >1.5%.

Reference standard

Colposcopy was performed to all women with biopsy if indicated, either immediately after cervical sampling (upon availability of a colposcopist) or approximately one month later by appointment. Colposcopies were performed only by one of the two British Society for Colposcopy and Cervical Pathology certified gynecologists. Given the fact that colposcopy alone without biopsies is weak as a reference standard [19], histology (cone or punch) was obtained in all cases where a CIN lesion (acetowhite epithelium) was suspected. In those cases that colposcopy was performed without knowledge of the cytology results and no biopsy was taken because of normal colposcopical impression, if the cytology had later revealed any CIN lesion, women were re-invited for colposcopy and subsequent biopsy. Since however previous studies [20] have used colposcopy alone as a reference standard, women with an entirely normal colposcopic examination and a normal Pap test were also included, even without histology, and were classified as negative. Despite the fact that only negative histology can absolutely classify these cases as negative, the reported risk of CIN2+ in these situations is extremely small [21], allowing thus the researchers to include them in the calculation of the accuracy indices of mRNA testing in the identification of high-risk patients.

Statistics

The correlation between NASBA and flow cytometry results was assessed using the phi coefficient. The accuracy indices (sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios and positivity rates) were calculated with 95% CI for cytology, flow cytometry and NASBA for the detection of CIN2+ and CIN3+. In addition to the above, accuracy indices for NASBA for the HPV-16 subtype were also included in the study. Women with invalid results were excluded from this calculation. Comparisons between categorical data were performed using the Fisher’s exact test. Continuous data are presented as means ± standard deviation (SD). All tests were two-sided. The level of statistical significance for all analyses was set as p<0.05. MED-CALC software (version 11.3.0.0, Belgium) was used for the statistical analysis.

Results

A total of 1083 women (mean age 40.1±12.8 years,
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Figure 1. STARD flow diagram for flow cytometry results. Flow: flow cytometry, CIN: cervical intraepithelial neoplasia, STARD: STAndards for the Reporting of Diagnostic accuracy studies. n: number of patients

Figure 2. STARD flow diagram for NASBA results. NASBA: nucleic acid sequence based amplification, CIN: cervical intraepithelial neoplasia, STARD: STAndards for the Reporting of Diagnostic accuracy studies. n: number of patients
Table 1. Association of NASBA and flow cytometry tests to lesion severity as assessed by chi-squared test for trend

<table>
<thead>
<tr>
<th></th>
<th>NASBA+ N</th>
<th>NASBA- N</th>
<th>Flow cytometry+ N</th>
<th>Flow cytometry- N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>30</td>
<td>418</td>
<td>134</td>
<td>554</td>
</tr>
<tr>
<td>CIN 1</td>
<td>31</td>
<td>112</td>
<td>55</td>
<td>111</td>
</tr>
<tr>
<td>CIN 2</td>
<td>21</td>
<td>8</td>
<td>24</td>
<td>13</td>
</tr>
<tr>
<td>CIN 3</td>
<td>8</td>
<td>2</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>Cancer</td>
<td>8</td>
<td>6</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N: number of patients

Table 2. Association of positive NASBA-16 test and high grade lesions. Comparison with the other tests of the study

<table>
<thead>
<tr>
<th></th>
<th>NASBA 16+ N</th>
<th>Other N</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>CIN 2-3</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>p-value</td>
<td>0.028</td>
<td></td>
</tr>
</tbody>
</table>

N: number of patients

Table 3. Test accuracy indices with 95% confidence intervals for the tests assessed

<table>
<thead>
<tr>
<th></th>
<th>NASBA</th>
<th>NASBA-16</th>
<th>Flow cytometry</th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
<td>% (95% CI)</td>
</tr>
<tr>
<td>Sensitivity for CIN2+</td>
<td>69.8 (55.6-81.7)</td>
<td>47.17 (33.3-61.56)</td>
<td>69.12 (56.74-79.6)</td>
<td>84.6 (75.8-90.6)</td>
</tr>
<tr>
<td>Sensitivity for CIN3+</td>
<td>66.7 (45.4-83.3)</td>
<td>54.2 (32.8-74.5)</td>
<td>74.19 (55.39-88.14)</td>
<td>87 (74.3-93.9)</td>
</tr>
<tr>
<td>Specificity for CIN2+</td>
<td>89.7 (86.9-92)</td>
<td>95.94 (94.02-97.38)</td>
<td>77.34 (74.34-80.14)</td>
<td>57.7</td>
</tr>
<tr>
<td>Specificity for CIN3+</td>
<td>86.8 (86.8-87.4)</td>
<td>94.2 (92.1-95.9)</td>
<td>75.55 (72.55-78.37)</td>
<td>55.9</td>
</tr>
<tr>
<td>PPV for CIN2+</td>
<td>37.8 (28.2-48.1)</td>
<td>51.02 (36.54-65.58)</td>
<td>19.92 (15.02-25.59)</td>
<td>16 (13-19.5)</td>
</tr>
<tr>
<td>PPV for CIN3+</td>
<td>16.3 (11.1-20.4)</td>
<td>26.5 (14.9-41.1)</td>
<td>9.75 (6.28-14.26)</td>
<td>8.3 (6.2-11.1)</td>
</tr>
<tr>
<td>NPV for CIN2+</td>
<td>97.1 (95.3-98.3)</td>
<td>95.29 (95.27-96.85)</td>
<td>96.85 (95.22-98.04)</td>
<td>97.5 (95.9-98.5)</td>
</tr>
<tr>
<td>NPV for CIN3+</td>
<td>98.5 (97.6-99.3)</td>
<td>98.2 (96.7-99.1)</td>
<td>98.8 (96.65-99.8)</td>
<td>98.9 (97.7-99.5)</td>
</tr>
<tr>
<td>PLR for CIN2+</td>
<td>6.76 (5.03-9.6)</td>
<td>11.62 (7.16-18.86)</td>
<td>3.05 (2.49-3.73)</td>
<td>1.999 (1.783-2.242)</td>
</tr>
<tr>
<td>PLR for CIN3+</td>
<td>5.04 (3.56-7.15)</td>
<td>9.33 (5.74-15.16)</td>
<td>3.03 (2.39-3.85)</td>
<td>1.971 (1.728-2.249)</td>
</tr>
<tr>
<td>NLR for CIN2+</td>
<td>0.34 (0.22-0.51)</td>
<td>0.55 (0.43-0.71)</td>
<td>0.4 (0.28-0.57)</td>
<td>0.267 (0.164-0.433)</td>
</tr>
<tr>
<td>NLR for CIN3+</td>
<td>0.38 (0.22-0.68)</td>
<td>0.49 (0.31-0.75)</td>
<td>0.34 (0.19-0.62)</td>
<td>0.233 (0.11-0.493)</td>
</tr>
</tbody>
</table>

NASBA: nucleic acid sequence based amplification, CIN: cervical intraepithelial neoplasia, PPV: positive predictive value, NPV: negative predictive value, PLR: positive likelihood ratio, NLR: negative likelihood ratio

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Two hundred thirty six women (21.8%) had a positive flow cytometry test whereas 666 (61.5%) had a negative flow test. In 144 women data were not available and in 37 women data were invalid. Regarding NASBA, 98 (9.05%) women were found to be positive, whereas 546 (50.4%) were negative. Data were not available in 401 women and invalid results were found in 38 of them. Figures 1 and 2 depict these results according to the STAndards for the Reporting of Diagnostic accuracy studies (STARD) criteria.

Increased positivity rates were associated with increased lesion severity for both NASBA and flow cytometry tests ($x^2$, p<0.001) (Table 1). Furthermore, in women with a positive NASBA test, those found to be positive for type 16 mRNA range 16-73 years) were included in the study. Of the 1083 women assessed, 758 were classified as negative after colposcopy and/or biopsy was performed (negative colposcopy and normal Pap smear 677 women and negative biopsy 81 women, respectively). The remaining 325 women were diagnosed as follows: 231 with CIN1 (21.3%), 46 with CIN2 (4.2%), 27 with CIN3 (2.5%) and 21 with cervical cancer (1.9%).
were significantly more likely to exhibit high-grade lesions (CIN 2-3) than those with a positive NASBA test for any of the other types tested (Fisher’s exact test, p=0.028) (Table 2). A positive correlation between NASBA and flow cytometry was identified when these methods were examined with the Phi coefficient (value 0.369, 95% CI:0.507-0.426). Table 3 depicts the accuracy indices of NASBA, flow cytometry and cytology for the histological diagnoses of CIN2+ and CIN3+. Neither NASBA nor flow cytometry exhibited high sensitivity rates, whereas cytology did (NASBA 69.8 vs 84.6%, p=0.051; flow cytometry 69.12 vs 84.6%, p=0.043). On the other hand, flow cytometry (77.3 vs 57.7%, p<0.0005) and especially NASBA (89.7 vs 57.7%, p<0.0005) exhibited significantly higher specificity rates when testing for CIN2+ lesions when compared to cytology. As a matter of fact, the positive predictive value (PPV) for CIN2+ with a positive NASBA test were found to be 2-fold higher for that of a positive cytology (0.378 vs 0.16). Furthermore, women with positive NASBA for HPV 16 mRNA were found to have more than 50% possibility to be diagnosed with a CIN2+ lesion.

Discussion

The main cause of cervical cancer is infection with high-risk HPV, with its DNA being discovered in the vast majority of the cases [22]. However, despite the fact that HPV-DNA detection techniques exhibit increased sensitivity for detecting CIN2+ lesions when compared to cytology, they do not exhibit high specificity as a solely screening test, leading thus possibly to increased unnecessary referrals for colposcopy [10,12,23]. Furthermore, there is evidence that many HPV infections, even with CIN2 lesions [23,24], regress spontaneously or remain in latency for a long time before progressing, explaining in part the aforementioned results. On the other hand, E6/E7 oncogene expression indicates active viral transcription and is a prerequisite for progression to malignancy in HPV infection in the cervical tissue [25]. Hence, detection of E6/E7 mRNA of high-risk HPV types may be a better primary screening test than simply detecting HPV-DNA presence, in order to identify high-risk patients.

In the present study, the rate of detectable mRNA transcripts appeared to progressively increase with the severity of the lesions observed. This result was in accordance with those reported by previous studies [17,26]. Furthermore, both NASBA and flow cytometry exhibited higher specificity but lower sensitivity than cytology in this study. Whereas these findings agreed with those reported by Cattani et al. [17], the study by Monsonego et al. [27] reported inverse results (higher sensitivity and similar specificity for mRNA testing vs cytology). However, several previous studies report higher specificity of the RNA assays [28-30].

In addition to the above, the positive predictive value (PPV) of both NASBA and flow cytometry exceeded that of cytology. As a matter of fact, NASBA exhibited a PPV more than double of that of cytology (37.8 vs 16%) for a CIN2+ lesion. These latter results concur with those of the FASE study [26] and of that by Cattani et al. [17] who reported a significantly higher probability of mRNA testing in detecting a CIN2+ lesion when compared to cytology or DNA testing. This was also true for the detection of CIN3+ lesions for both our and the FASE study. At that point, it has to be stressed that the aforementioned finding, that is a high PPV, is of primordial importance for a screening test as this means that this test has higher possibilities to detect true lesions obviating thus the need for further tests in order to establish the diagnosis.

Based on the evidence that an important percentage of CIN2 lesions may regress over the years [23,24], but HPV 16 positive CIN2 lesions are probably less likely to regress than other lesions due to different high-risk genotypes [24] we included a separate assessment of the value of HPV 16 mRNA detection. A NASBA HPV 16 positive test had a possibility of more than 50% for a CIN2+ lesion detection. Despite its low sensitivity when compared to cytology, this test may be of clinical importance in the further management of pathological Pap smears. For example, it may be used for the referral of women with minor cytological lesions for a cone excision based on a NASBA HPV 16 positive test.

Our study proved that among women infected with HPV high risk types, E6/E7 detection further increases the risk of a high grade lesion, a fact that underlines the effect of E6/E7 gene expression which is in agreement with previous studies [13,14,17]. In the present study we tried to assess the accuracy of 2 HPV-mRNA tests (NASBA, flow cytometry) as primary screening tests for the detection of CIN2+ lesions. Their sensitivity was at best moderate and in no case better than that of cytology which has a reported sensitivity in the range of 80% [20]. In practical terms however, NASBA will detect 5 out of 7 CIN2+ cases and 2
out of 3 CIN3+ cases. In the same sense, flow cytometry will detect 5 out of 7 CIN2+ cases and 3 out of 4 CIN3+ cases respectively.

This study has some limitations. The number of patients, although higher than that of other studies, is relatively small, precluding thus definitive conclusions. Another potential problem that could lead to bias is that we did not have histological verification in all patients included in the study as women with an entirely normal colposcopic examination and a normal Pap test were also included and were classified as negative. However, as abovementioned, the reported risk of CIN2+ in these situations is extremely small [21], allowing us thus to include them in the study.

**Conclusion**

An ideal single primary screening test for cervical cancer has to exhibit both increased sensitivity and specificity in order to avoid unnecessary and costly referrals for colposcopy. However, the results of the present study indicate that HPV mRNA detection cannot yet be used as a sole test for primary screening but rather to be used as an adjunct to cytology. It has to be noted though, that the differences observed in the specificity and sensitivity rates of cytology among different studies coming from different centers, indicate the subjectivity of this particular test, constituting thus a significant obstacle in the safe interpretation and comparison of the results of these reports. The present study adds some evidence in the existing literature of HPV mRNA testing in cervical cancer screening that may help to clarify its future role. However, further large-sample prospective studies with colposcopy follow-up will probably elucidate with certainty the exact validity of mRNA testing in cervical cancer screening.

**Acknowledgements**

This work was supported in part by the Hellenic Ministry of Development, Project AKAKOS (code ATT95) and by the Hellenic Ministry of Health, Project “A study and evaluation of the methods for the success of HPV vaccination”

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