Diagnostic validity of human papillomavirus E6/E7 mRNA test in cervical cytological samples

Tong-Yu Liu, Rong Xie, Li Luo, Kathleen H. Reilly, Cheng He, Yu-Zhen Lin, Gang Chen, Xiong-Wei Zheng, Lu-Lu Zhang, Hai-Bo Wang

Abstract

Human papillomavirus (HPV) DNA tests tend to show high sensitivity, but poor specificity in detecting high-grade cervical lesions. This study aimed to explore the clinical performance of QuantiVirus® HPV E6/E7 mRNA in identifying ≥ Grade 2 cervical intraepithelial neoplasia. Thin-prep® liquid based cytology test (LBC) samples were collected from October 2009 to October 2011 from women who underwent outpatient hospital-based gynecological screening. LBC samples were processed for E6/E7 mRNA detection and HPV DNA detection. Of 335 patients, 133 (40.5%) were HPV E6/E7 mRNA positive for high-risk HPV subtypes. The positivity rate of HPV E6/E7 mRNA increased with the severity of cytological and histological evaluation. An optimal cut-off value of ≥567 copies/ml was determined using receiver operating characteristic (ROC) curve, and positive predictive value and negative predictive value of cut-off value (≥567 copies/ml) were higher than those of E6/E7 mRNA positivity only, but not significant. QuantiVirus® HPV E6/E7 mRNA testing may be a valuable tool in triage for identifying ≥Grade 2 cervical intraepithelial neoplasia. A high specificity and a low positivity rate of E6/E7 mRNA testing as a triage test in HPV DNA-positive women can be translated into a low referral for colposcopy. Studies composed of large population-based samples of women and with rigorous disease ascertainment, are needed to establish the optimal cut-off point based on ROC curve analysis.

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step toward the development of cervical cancer (Castle et al., 2007; Cuschieri and Wentzensen, 2008). In addition, it has been reported widely that HPV E6/E7 mRNA is significantly correlated with the severity of cervical lesions (von Knebel Doeberitz, 2002; Ho et al., 2010), and may be useful as a marker for potentially progressive HR HPV infections. Therefore, E6/E7 mRNA quantitation has great potential for cervical screening, especially in women <30 years of age in whom HPV infection is common but transformation and Grade 2 cervical intraepithelial neoplasia are relatively infrequent (Zhao et al., 2011).

Commercially available robust assays for HPV mRNA detection can be performed in reflex following liquid based cytology. In this cross-sectional study, E6/E7 mRNA expression of 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) were analyzed from cervical samples and compared with cytology. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) in identifying >Grade 2 cervical intraepithelial neoplasia lesions were used as indicators of accuracy to evaluate the clinical utility of HPV E6/E7 mRNA. A receiver operating characteristic (ROC) curve was used to demonstrate the diagnostic performance of E6/E7 mRNA for detecting >Grade 2 cervical intraepithelial neoplasia.

2. Methods

2.1. Study design

Thin-prep liquid based cytology test (LBC) samples were collected from October 2009 to October 2011 from women who underwent outpatient gynecological screening at the oncolgic gynecology department of Fujian Cancer Hospital. Women with abnormal cytology, namely atypical squamous cells of unknown significance or higher, or a positive HPV DNA test were included in this study. Those participants who were treated for cervical lesions in the previous 5 years, had a previous hysterectomy, or who underwent chemo and/or radiotherapy for cervical carcinoma, were excluded from this study. The study was reviewed and approved by the Ethics Committee of Fujian Cancer Hospital. All women received information pertaining to the study and written informed consent was obtained from all participants.

2.2. Cytological and histological diagnoses

Cervical samples were collected using a cytobrush and stored in PreservCytTM solution at 4°C. Slides were prepared using a liquid-based cytology method, the Thinprep system. Cytological specimens were blinded and evaluated independently of the results of the other assays by two experienced cytopathologists. If the diagnosis differed between two cytopathologists, cervical samples were reviewed again and a consensus diagnosis was obtained. The cytological specimens were reported using the 2001 Bethesda Reporting System Criteria (Solomon et al., 2002). Samples negative for intraepithelial lesion or malignancy, atypical squamous cells of unknown significance and low-grade squamous intraepithelial lesion were herein referred to as less severe than high-grade squamous intraepithelial lesion; while the reports of atypical squamous cells incapable of excluding high-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion and cancer were herein referred to as more severe than high-grade squamous intraepithelial lesion.

The histological slides were blinded and evaluated independently of the results of the other assays by two experienced pathologists. If the diagnosis differed between two pathologists, histological slides were reviewed again and a consensus diagnosis was obtained. All histological slides were diagnosed according to current World Health Organization classification. The benign cases and mild cervical intraepithelial neoplasia (Grade 1 cervical intraepithelial neoplasia) diagnoses are referred to as less than Grade 2 cervical intraepithelial neoplasia; the diagnoses of moderate cervical intraepithelial neoplasia (Grade 2 cervical intraepithelial neoplasia), severe cervical intraepithelial neoplasia (Grade 3 cervical intraepithelial neoplasia) and carcinoma are referred to as >Grade 2 cervical intraepithelial neoplasia. Histology was regarded as the “gold standard.”

2.3. Lysis working reagent

The LBC sample was shaken for 1 min and 1 ml was transferred to a centrifuge tube. The tube was centrifuged at 1600 x g for 5 min and the supernatant was discarded. Distilled deionized water (ddH2O) was added and then centrifuged again with the supernatant discarded. Then 200 μl lysis mixture, ddH2O 400 μl, and proteinase K5 μl were added to the centrifuge tube and incubated at 65°C for 1 h. The sample tube was shaken 2–3 times during the incubation at regular intervals.

2.4. E6/E7 mRNA detection

LBC samples were used for the detection of E6/E7 mRNA of 14 HR HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 by Quantivir® HPV E6/E7 mRNA detection (referred to as the mRNA test) (DiaCarta, CA, USA) according to the manufacturer’s instructions. Quantivir® HPV E6/E7 mRNA detection is based on branched DNA (bDNA) technology (DiaCarta, CA, USA), which is a sandwich nucleic acid hybridization procedure for the direct quantitation of HPV mRNA from residual LBC samples without RNA purification or RT-PCR.

A quantity of 50 μl Lysis working reagent was added to each sample well and combined with bifunctional oligonucleotide probe sets designed to hybridize to the target E6 and E7 mRNA and to capture oligonucleotides covalently attached to 96-well plates. After 3.5 h incubation at 55°C, samples were washed and then 100 μl pre-amplifier probe working reagent was added to each well of the capture plate. The capture plate was sealed and incubated at 55°C for 40 min. Samples were washed and then 100 μl label probe working reagent was added to each well of the capture plate. The capture plate was sealed and incubated at 50°C for 40 min. Samples were washed and then 100 μl substrate working reagent was added to each well of the capture plate. The capture plate was sealed and incubated at 46°C for 20 min. The plate was cooled to room temperature for 10 min and then read immediately (within 1 min) on the System Kodia Quantiviirs® Luminometer. Light emission was related directly to the amount of HPV mRNA present in each sample and results were recorded as relative light units by the System Kodia Quantiviirs® Luminometer. A cut-off of 1.50-fold over blank was used to determine HPV positive samples.

2.5. HPV E6/E7 DNA testing

Testing for HR HPV E6/E7 DNA was performed using Quantiviirs® HPV DNA Diagnostic Kit (DiaCarta, CA, USA) for the detection of the 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) according to the manufacturer’s instructions. Lysis mixture for HPV DNA testing was made the same as E6/E7 mRNA detection. The following were added to the centrifuge tube in turn: 50 μl Lysed sample, 1 μl test probe, 5 μl denatured buffer (2.5 M NaOH), and 12 μl neutralizing solution. The tube was incubated at 95°C for 5 min then added to each sample well. HPV DNA was tested by the same sequential procedures as the mRNA test.
Table 1
Cytological or histological diagnosis and positivity rate for HPV E6/E7 mRNA test.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of women</th>
<th>No. of positive for HPV RNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative for intraepithelial lesion or malignancy</td>
<td>31</td>
<td>9 (29.0)</td>
</tr>
<tr>
<td>Atypical squamous cells of unknown significance</td>
<td>144</td>
<td>31 (21.5)</td>
</tr>
<tr>
<td>Low-grade squamous intraepithelial lesion</td>
<td>33</td>
<td>19 (57.6)</td>
</tr>
<tr>
<td>Atypical squamous cells incapable of excluding high-grade squamous intraepithelial lesion</td>
<td>54</td>
<td>23 (42.6)</td>
</tr>
<tr>
<td>High-grade squamous intraepithelial lesion</td>
<td>46</td>
<td>35 (76.1)</td>
</tr>
<tr>
<td>Cancer</td>
<td>27</td>
<td>18 (66.7)</td>
</tr>
<tr>
<td>Total</td>
<td>335</td>
<td>135 (40.3)</td>
</tr>
</tbody>
</table>

| More severe than high-grade squamous intraepithelial lesion$^a$ | 208          | 59 (28.4)                       |

| Histology                                                  |              |                                 |
| Negative for intraepithelial lesion or malignancy          | 30           | 7 (23.3)                        |
| Grade 1 cervical intraepithelial neoplasia                 | 5            | 2 (40.0)                        |
| Grade 2 cervical intraepithelial neoplasia                 | 1            | 1 (100.0)                       |
| Grade 3 cervical intraepithelial neoplasia                 | 15           | 12 (80.0)                       |
| Cancer                                                     | 41           | 28 (68.3)                       |
| Total                                                      | 92           | 50 (54.4)                       |
| >Grade 2 cervical intraepithelial neoplasia$^a$            | 35           | 9 (25.7)                        |
| >Grade 2 cervical intraepithelial neoplasia$^b$            | 57           | 41 (71.9)                       |

$^a$ Less severe than high-grade squamous intraepithelial lesion, include negative for intraepithelial lesion or malignancy, atypical squamous cells of unknown significance and low-grade squamous intraepithelial lesion.

$^b$ More severe than high-grade squamous intraepithelial lesion, include atypical squamous cells incapable of excluding high-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, and cancer.

A total of 135 (40.3%) patients were positive for HPV E6/E7 mRNA. The positivity of HPV E6/E7 mRNA increased with the severity of cytological and histological evaluation. The positive rate of mRNA rose from 29.0% (9/31) for those who were negative for intraepithelial lesion or malignancy, 21.5% (31/144) for atypical squamous cells of unknown significance, and 57.6% (19/33) for low-grade squamous intraepithelial lesion, to 59.8% (76/127) for more severe than high-grade squamous intraepithelial lesion (Table 1). There was a significant difference in the mRNA test positivity between cases of less severe than high-grade squamous intraepithelial lesions and cases of more severe than high-grade squamous intraepithelial lesions ($p < 0.001$). A similar linear trend was observed between mRNA test positivity and the severity of dysplasia by histological diagnoses.

2.6. Statistical analysis

Statistical tests were performed using SAS 9.1 software (Cary, NC, USA). Chi-square tests were used to compare differences between groups. Sensitivity, specificity, PPV, NPV, and 95% confidence intervals (CI) for detecting ≥Grade 2 cervical intraepithelial neoplasia of mRNA, cytology, and DNA were calculated. A ROC curve was used to assess the diagnostic accuracy (sensitivity and specificity) of E6/E7 mRNA. All tests were two-sided and $p$-value $< 0.05$ was considered the cut-off level for statistical significance for all analysis.

3. Results

3.1. Positive rate of HPV E6/E7 mRNA test by cytological and histological diagnosis

All enrolled patients ($n = 335$) were tested for HR HPV E6/E7 mRNA and LBC on the same sample. For 92 (27.5%) of the 335 patients, histological evaluations from biopsies taken at the same time of enrollment were available: 30 participants were diagnosed negative for intraepithelial lesion or malignancy, 5 with Grade 1 cervical intraepithelial neoplasia, 1 with Grade 2 cervical intraepithelial neoplasia, 15 with Grade 3 cervical intraepithelial neoplasia and 41 with cancer. Among the 92 patients with available histological data, no participants were negative for intraepithelial lesion or malignancy in cytology, 4 (15.4%) participants had atypical squamous cells of unknown significance, 6 (60.0%) participants had low-grade squamous intraepithelial lesion, 5 (50.0%) participants had atypical squamous cells incapable of excluding high-grade squamous intraepithelial lesions, 23 (88.5%) participants had high-grade squamous intraepithelial lesion, and 19 (100.0%) participants with cancer were diagnosed as ≥Grade 2 cervical intraepithelial neoplasia by histology. Overall, histological examination results positively correlated with severity of dysplasia by cytological diagnoses.

A ROC curve was used to demonstrate the diagnostic performance of E6/E7 mRNA for detecting ≥Grade 2 cervical intraepithelial neoplasia (Fig. 1). The figure included the sensitivity and the specificity of the assays. The clinically relevant portion of the area under the curve was the 0.78 (95% CI, 0.69–0.87). An optimal cut-off value ($>$567 copies/ml) was determined using the ROC curve to predict diagnostic performance.

3.3. Correlation of E6/E7 mRNA and cytology with histological diagnosis

The E6/E7 mRNA assay and cytology tests were analyzed in relation to histology status. There were 92 women with cytology, histology and E6/E7 mRNA. The rates of the E6/E7 mRNA positivity, E6/E7 mRNA >567 copies/ml, and more severe than high-grade squamous intraepithelial lesion by cytology were 54.4% (50/92), 45.7% (42/92) and 59.8% (55/92), respectively (Table 2).

The sensitivity of E6/E7 mRNA for detecting ≥Grade 2 cervical intraepithelial neoplasia was 71.9% (41/57) (95% CI, 58.5–83.0) and the specificity was 74.3% (26/35) (95% CI, 56.7–87.5). The PPV, NPV, PLR and NLR were 82.0% (41/50) (95% CI, 68.6–91.4), 61.9% (26/42) (95% CI, 45.6–76.4), 2.80 (95% CI, 1.56–5.03) and 0.38 protocol, and results were analyzed by System Kodia DiaCarta QuantViurs® Luminometer.
Table 2. Correlation of E6/E7 mRNA and cytology with histological diagnoses.

<table>
<thead>
<tr>
<th></th>
<th>E6/E7 mRNA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\geq$Grade 2 cervical intraepithelial neoplasia$^a$</td>
<td>$&lt;\text{Grade 2 cervical intraepithelial neoplasia}$</td>
<td>$\geq$Grade 3 cervical intraepithelial neoplasia</td>
<td>$&lt;\text{Grade 3 cervical intraepithelial neoplasia}$</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>50 (54.4)</td>
<td>22 (24.7)</td>
<td>26 (28.3)</td>
<td>10 (27.8)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>42 (45.6)</td>
<td>34 (37.3)</td>
<td>38 (40.2)</td>
<td>20 (52.2)</td>
<td></td>
</tr>
<tr>
<td>$\geq$567 copies/ml</td>
<td>42 (45.7)</td>
<td>36 (39.5)</td>
<td>38 (40.2)</td>
<td>20 (52.2)</td>
<td></td>
</tr>
<tr>
<td>$&lt;$567 copies/ml</td>
<td>50 (54.4)</td>
<td>22 (24.7)</td>
<td>18 (19.8)</td>
<td>10 (27.8)</td>
<td></td>
</tr>
<tr>
<td>Cytology only</td>
<td>55 (59.8)</td>
<td>43 (47.2)</td>
<td>46 (52.8)</td>
<td>22 (57.5)</td>
<td></td>
</tr>
<tr>
<td>More severe than</td>
<td>37 (40.2)</td>
<td>10 (11.1)</td>
<td>27 (30.7)</td>
<td>9 (24.3)</td>
<td></td>
</tr>
<tr>
<td>high-grade squamous</td>
<td>26 (28.3)</td>
<td>16 (17.2)</td>
<td>22 (24.7)</td>
<td>6 (16.2)</td>
<td></td>
</tr>
<tr>
<td>intraepithelial lesion$^d$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV E6/E7 DNA</td>
<td>28 (73.7)</td>
<td>26 (72.2)</td>
<td>10 (27.8)</td>
<td>12 (32.2)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10 (26.3)</td>
<td>4 (11.1)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>92</td>
<td>57 (62.0)</td>
<td>56 (60.0)</td>
<td>36 (39.1)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Grade 2 cervical intraepithelial neoplasia, include benign cases and Grade 1 cervical intraepithelial neoplasia.

$^b$Grade 2 cervical intraepithelial neoplasia, include Grade 2 cervical intraepithelial neoplasia, Grade 3 cervical intraepithelial neoplasia, and cancer.

$^c$More severe than high-grade squamous intraepithelial lesion, include atypical squamous cells incapable of excluding high-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, and cancer.

$^d$Less severe than high-grade squamous intraepithelial lesion, include negative for intraepithelial lesion or malignancy, atypical squamous cells of unknown significance and low-grade squamous intraepithelial lesion.

Fig. 1. ROC curve of E6/E7 mRNA for detecting $\geq$Grade 2 cervical intraepithelial neoplasia.

The use of HPV DNA testing for cervical cancer screening is growing globally. However, newer HPV mRNA-based assays are becoming popular because of the increased understanding that mRNA provides increased specificity compared to HPV DNA assays. This study utilized the QuantiVirus® E6/E7 HPV mRNA Diagnostic Kit that can detect 14 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Our study showed that detection of E6/E7 mRNA strongly correlated with the grade of cytological diagnoses and histological diagnoses. One quarter (25.7% (9/35)) of cases with $<$Grade 2 cervical intraepithelial neoplasia had positive E6/E7 mRNA tests, while 71.9% (41/57) $\geq$Grade 2 cervical intraepithelial neoplasia cases had positive E6/E7 mRNA tests. Positive rates of E6/E7 mRNA were 28.4% (59/208) for cases of less severe than high-grade squamous intraepithelial lesion and 59.8% (76/127) for cases of more severe than high-grade squamous intraepithelial lesion, respectively. In addition to E6/E7 mRNA positivity, an optimal cut-off value ($\geq$567 copies/ml) of E6/E7 mRNA was determined using a ROC curve to evaluate its clinical performance. The cut-off value can attain better clinical performance (PPV, 80%; NPV, 62%) compared with only E6/E7 mRNA positivity (PPV, 82%; NPV, 61%), although this difference was not statistically significant.

The presence of HR HPV is necessary, but not sufficient to cause cervical lesions (Bertuccio et al., 2011). The prevalence of HPV DNA in cervical specimens is very high and most infections are transient, especially in women under 30 years of age. For women with...
cervical HPV, E6 and E7 are usually expressed at low levels in the differentiated exfoliating epithelial cells, whereas a dedifferentiated neoplasia would express more E6/E7 in the surface epithelium (Molden et al., 2005a). The present study found that E6/E7 mRNA expression correlated with the severity of cervical lesions, consistent with other studies (Coquillet et al., 2011; Piery et al., 2012). Piery et al. found that 6% of benign cases, 22% of Grade 1 cervical intraepithelial neoplasia, 83% of Grade 2 cervical intraepithelial neoplasia and 93% Grade 3 cervical intraepithelial neoplasia had positive E6/E7 mRNA in women under 30 years of age (Piery et al., 2012). In the current study, 25.7% (9/35) of Grade 2 cervical intraepithelial neoplasia cases and 71.0% (41/57) of Grade 2 cervical intraepithelial neoplasia cases had positive E6/E7 mRNA tests and the positivity rate of HPV E6/E7 mRNA increased with the severity of cytological diagnoses. E6/E7 mRNA may be useful as a biomarker for potentially persistent HR HPV infections that require intervention and treatment (Zhao et al., 2011). High specificity and low positivity rate of HPV E6/E7 mRNA tests can lower the number of women unnecessarily referred to colposcopy, which is advantageous in a triage setting (Piery et al., 2012; Benevolo et al., 2011b; Koliopoulos et al., 2012).

It has been concluded that HPV E6/E7 mRNA tests have a slightly lower sensitivity for detecting ≥Grade 2 cervical intraepithelial neoplasia than DNA detection, but that HPV E6/E7 mRNA tests are significantly more specific than DNA detection (Benevolo et al., 2011b; Piery et al., 2012). The advantage of high specificity often has the unavoidable cost of decreasing sensitivity. Among the 41 participants with cancer, only 68.3% (28/41) patients had positive E6/E7 mRNA tests. The large proportion of missed ≥Grade 2 cervical intraepithelial neoplasia may limit the application of E6/E7 mRNA in primary screening, however, in HPV DNA-positive women, mRNA test as a triage test can reduce referral to colposcopy and biopsy (Benevolo et al., 2011a).

In the current study, the HPV E6/E7 mRNA test was negative in 28.1% of histologically confirmed ≥Grade 2 cervical intraepithelial neoplasia cases. It is assumed that some ≥Grade 2 cervical intraepithelial neoplasia cases may clear their infections or cell abnormalities spontaneously restraining E6/E7 mRNA expression (Ronco et al., 2006, 2007; Confortini et al., 2010), but they require time to recover completely. It is therefore possible that ≥Grade 2 cervical intraepithelial neoplasia cases with negative E6/E7 transcripts could be more likely to regress or less likely to progress toward cancer, as HPV infection could have been cleared or viral gene expression is very low. Regression of ≥Grade 2 cervical intraepithelial neoplasia is frequent among younger women referred to colposcopy (Ronco et al., 2008). However, there is no chance to verify the assumption that ≥Grade 2 cervical intraepithelial neoplasia cases with negative E6/E7 mRNA test were associated with higher probability of regression, since women with ≥Grade 2 cervical intraepithelial neoplasia should be referred to treatment as soon as possible to prevent worsening outcomes. Conversely, ≥Grade 2 cervical intraepithelial neoplasia cases with positive E6/E7 mRNA tests may have higher risk of progression. A prospective study over 24 months demonstrated that of low-grade squamous intraepithelial lesion cytology samples, 81% participants who were positive for E6/E7 mRNA progressed, while 85% participants who were negative for E6/E7 mRNA regressed during follow-up (Molden et al., 2005b). It is necessary to verify the role of E6/E7 mRNA as a biomarker in predicting the development of cervical lesions based on large follow-up studies, primarily for ≥Grade 2 cervical intraepithelial neoplasia cases (Giorgi Rossi et al., 2013).

ROC curve analysis was used to choose an optimal cut-off value and to evaluate the diagnostic performance of the E6/E7 mRNA test. The cut-off value can improve diagnostic performance of E6/E7 mRNA tests, as indicated by the PPV and NPV of cut-off value (≥567 copies/mL) were higher than that of E6/E7 mRNA (positivity only), although not significantly different.

The current study was subject to several limitations. The study population was referred to an mRNA test for several reasons (i.e., an abnormal cytology test or a positive HPV DNA test), therefore, those who had an mRNA test may differ from screening populations referred to mRNA testing. Also, the study sample was relatively small (especially the number of participants with both an HPV DNA test and histological results) to compare the clinical performance of HPV DNA and E6/E7 mRNA.

These data suggest that QuantiVirus® HPV E6/E7 mRNA testing may be a valuable tool in triage for identifying ≥Grade 2 cervical intraepithelial neoplasia. A high specificity and a low positivity rate of E6/E7/mRNA testing as a triage test in HPV DNA-positive women can be translated into a low referral for colposcopy. Larger clinical studies are needed to confirm these preliminary results. Specifically, studies composed of large population-based samples of women and women of progressive aggressive disease, are needed to establish the optimal cut-off point based on ROC curve analysis.

Conflict of interest statement

The authors declare no conflict of interest.

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