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Human papillomavirus (HPV) E6/E7 mRNA detection in cervical exfoliated cells: a potential triage for HPV-positive women*

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Abstract: Cytology triage has been generally recommended for human papillomavirus (HPV)-positive women, but is highly dependent on well-trained cytologists. The present study was designed to explore whether HPV E6/E7 mRNA detection in cervical exfoliated cells can be a potential triage for HPV-positive women from a clinic-based population. Both the primary HPV testing and Papanicolaou (Pap) test were performed on all eligible HPV-positive women. HPV E6/E7 mRNA was detected by QuantiVirus® HPV E6/E7 mRNA assay in cervical exfoliated cells. All HPV-positive women underwent colposcopy and further biopsy if indicated. The data were assessed by Pearson's Chi-squared test and the receiver operating characteristic curve. A total of 404 eligible HPV-positive women were enrolled. Positive rate of E6/E7 mRNA in high-grade squamous intraepithelial lesion (HSIL) cases was higher than that in low-grade squamous intraepithelial lesion (LSIL) or normal cases. There was no statistical difference found between mRNA and cytological testing with sensitivity (89.52% vs. 86.67%, P=0.671), specificity (48.96% vs. 48.96%, P=1.000), positive predictive value (39.00% vs. 38.24%, P=1.000), and negative predictive value (92.76% vs. 90.97%, P=0.678) for detecting ≥HSIL. HPV E6/E7 mRNA detection in cervical exfoliated cells shows the same performance as Pap triage for HSIL identification for HPV-positive women. Detection of HPV E6/E7 mRNA may be used as a new triage option for HPV-positive women.

Key words: Human papillomavirus (HPV); HPV E6/E7 mRNA; High-grade squamous intraepithelial lesion (HSIL)
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1 Introduction

Human cervical cancer is the fourth most common cancer in women worldwide, and most cervical cancer cases arise in less developed countries (Torre *et al.*, 2015). Oncogenic human papillomavirus (HPV) infection is a prerequisite for the development of cervical cancer and its precursor lesions (Walboomers *et al.*, 1999; Clifford *et al.*, 2003). More than 150 serotypes of HPV have been identified to date, of which about 40 types can infect the cervix. These HPV types are divided into high- and low-risk groups (Schiffman *et al.*, 2011). Persistent high-risk HPV (HR-HPV) infection is a major cause of cervical cancer. Supported by clinical trials (Condel *et al.*, 2002; Wright and Schiffman, 2003; Kitchener *et al.*, 2009; Siebers *et al.*, 2009), and taking the advantage of high sensitivity while identifying high-grade squamous intraepithelial lesion and worse lesions (HSIL+), HPV testing has been recommended as a primary screening for cervical cancer in many countries (Zappacosta *et al.*, 2013). The introduction of cytological screening has remarkably reduced the incidence and mortality of cervical cancer, and the cytology test has been a most widely accepted triage for patients with positive primary HPV test (Zappacosta

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et al., 2013). However, many developing countries, including China, are very lacking in well-trained cytologists, and the cytology test cannot be carried out effectively. Therefore, other triage options should be considered in these countries.

The oncogenic potential of the HR-HPV depends on the increased expressions of the E6 and E7 genes (Sotlar *et al.*, 2004; Cuschieri and Wentzensen, 2008). The E6/E7 oncogene transcripts are usually expressed at low levels during transient HPV infection (Benevolo *et al.*, 2011). When the viral genome has integrated into the host genome, the oncogene transcripts are over-expressed, leading to the development of cervical cancer (Castle *et al.*, 2007; Cuschieri and Wentzensen, 2008). E6 and E7 proteins bind to the tumor suppressor proteins p53 and pRb, respectively (Scheffner *et al.*, 1990; Chellappan *et al.*, 1992), and drive cervical cell proliferation and transformation (Benevolo *et al.*, 2011). It has been reported that HPV E6/E7 mRNA expression level is highly correlated with the severity of cervical lesions (Ho *et al.*, 2010). HPV E6/E7 mRNA may be useful as a marker for potentially progressive HR-HPV infections and may constitute a useful tool for screening and/or patient management (Jeantet *et al.*, 2009; Sorbye *et al.*, 2011; Giorgi Ross *et al.*, 2013).

In this study, E6/E7 mRNAs of 14 HR-HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) were detected in cervical exfoliated cell samples, and the performance of HPV E6/E7 mRNA detection as an optional triage for HPV-positive women was evaluated.

2 Materials and methods

2.1 Patient recruitment and sample collection

Female patients who visited the Women's Hospital, School of Medicine, Zhejiang University (Hangzhou, China), between July 2014 and December 2014, with positive HPV test results were prospectively included.

Women were recruited in the study based on the following criteria: (1) aged 24 years or older, (2) without previous cervical cancer or precancerous lesions, (3) no history of therapeutic procedure of cervix, (4) HPV DNA positive by Hybrid Capture 2 assay, and (5) non-pregnant.

Women were excluded from the study according to the following criteria: (1) younger than 24 years, (2) previously confirmed cervical cancer, (3) therapeutic procedure to cervix, and (4) pregnancy. Cervical exfoliated cell samples, used for both cytology test and HPV E6/E7 mRNA detection, were collected by sample brush from the cervical surface. This study was approved by the Ethics Committee of the Women's Hospital, School of Medicine, Zhejiang University. All patients signed the consent forms and were informed regarding the purpose of the proposed study.

2.2 HPV testing

HR-HPV DNA was detected by Hybrid Capture 2 assay (HC2, Digene, Gaithersburg, MD, USA), according to the manufacturer's instructions. At least 1 pg/ml HPV DNA or more was required to be identified as positive.

2.3 Cytology test

The technology of liquid-based cytology (LBC) was used for the cytology test. Thin-layer slides were prepared using the Thin Prep 2000 Processor (Cytyc Corporation, Marlborough, MA, USA) according to the manufacturer's instructions. The prepared slides were stained by the Papanicolaou (Pap) method and assessed by cytologists of the hospital according to the criteria set out in the Bethesda System 2001 guidelines. A diagnosis was assigned to each case as being negative for intraepithelial lesion or malignancy (NILM) or having any epithelial cell abnormalities, such as atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells, cannot exclude high-grade squamous intraepithelial lesion (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC) (Liu *et al.*, 2014).

2.4 HPV E6/E7 mRNA detection

E6/E7 mRNAs of 14 HR-HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) were detected in cervical exfoliated cell samples by QuantiVirus® HPV E6/E7 mRNA assay (Kodia, Xinxiang, China) according to the manufacturer's instructions. The QuantiVirus® HPV E6/E7 mRNA assay is a sandwich nucleic acid hybridization

procedure, based on branched DNA (bDNA) technology (DiaCarta, CA, USA).

Briefly, each sample was incubated with 600 μl lysis mixture and 5 μl proteinase at 65 °C for 1 h to release viral RNA. A total of 50 μl specimen and 50 μl working probe solution were mixed in 96-well plates and incubated at 55 °C for 3.5 h to capture target RNA (Discacciati *et al.*, 2014). After the samples were washed, 100 μl of ThinPrep-amplifier probe working reagent was added to the samples. Each well of the capture plate was incubated at 55 °C for 40 min. Samples were then washed and 100 μl label probe working reagent was added to each well; the capture plate was then incubated at 50 °C for 40 min. Samples were washed again and 100 μl substrate working reagent was added to each well. The capture plate was incubated at 46 °C for 20 min. After reaction with substrate working reagent, the plate was cooled to room temperature for 10 min and read immediately (within 1 min) with the Kodia QuantiVirus® Benchtop 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 luminometer system. If the signal was greater than or equal to 1.0, the QuantiVirus® HPV assay result for the patient was positive. Otherwise, the result was negative (Liu *et al.*, 2014).

2.5 Colposcopy and histological diagnosis

All HPV-positive women were examined by colposcopy and underwent cervical biopsy. Histological diagnosis was made by pathologists of the hospital according to previous descriptions (Tavassoéli and Devilee, 2003).

2.6 Statistical analysis

All statistical analyses were performed using SPSS statistics software Version 13.0 (Chicago, IL, USA). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and their 95% confidence intervals (CIs) of HPV E/E7 mRNA detection and cytology test were calculated for detecting HSIL+. The χ^2 test was used to compare the performance parameters of HPV E/E7 mRNA detection and the cytology test. Statistical significance was defined by a P-value of less than 0.05. The predictability was measured via the area under the receiver operating characteristic (ROC) curve.

3 Results

3.1 Histological and cytological diagnoses

In total, 404 women were enrolled during the study period, and their ages ranged from 25 to 71 years (mean age 42.3 years).

All the 404 women underwent biopsy under colposcopy, of which 11 women were excluded: 10 were diagnosed as Grade 1 vaginal intraepithelial neoplasia, and one was diagnosed as Grade 2 vaginal intraepithelial neoplasia. Cytology test results of the remaining 393 women consisted of, 155 (39.44%) NILM, 80 (20.36%) ASC-US, 89 (22.65%) LSIL, 28 (7.12%) ASC-H, and 41 (10.43%) HSIL (Table 1). Their histological diagnosis consisted of 227 normal, 61 LSIL, 101 HSIL, and 4 cancers.

Table 1 Cytological and histological diagnoses

Group	Histological diagnosis	Cytological diagnosis				
		NILM	ASC-US	LSIL	ASC-H	HSIL
Normal	227	127	51	40	5	4
LSIL	61	14	14	25	5	3
HSIL	101	14	15	24	18	30
Cancer	4	0	0	0	0	4

NILM: negative for intraepithelial lesion or malignancy; ASC-US: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; ASC-H: high-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion

3.2 Positive rate of HPV E6/E7 mRNA assay by histological diagnosis

HPV E6/E7 mRNA was positive in 241 (61.32%) of 393 total cases. The positive rate in each histologic group is shown in Table 2. The mRNA test showed a higher positive rate of E6/E7 mRNA in high-grade lesion cases than in low-grade lesion or normal cases. Positive rates were 100.00%, 89.11%, 77.05%, and 44.05% for cancer, HSIL, LSIL, and normal cases, respectively. The positive rate of HPV E6/E7 mRNA in HSIL+ (HSIL or worse) group was 89.52% (94/105), which was significantly higher than that in LSIL− (LSIL or better) group (51.04%, 147/288) (P=0.000). Increasing severity of histological lesions was associated with higher positive rates of HPV E6/E7 mRNA.

ROC curve analysis was also used to assess the assays for detecting HSIL+. The clinically relevant portion of the area under the curve is 0.721 (95% CI, 0.667–0.775; Fig. 1).

Table 2 HPV E6/E7 mRNA detection results in different histological groups

Group	HPV E6/E7 mRNA		Positive rate (%)
	Positive	Negative	
Normal	100	127	44.05
LSIL	47	14	77.05
HSIL	90	11	89.11
Cancer	4	0	100.00

HPV: human papillomavirus; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion

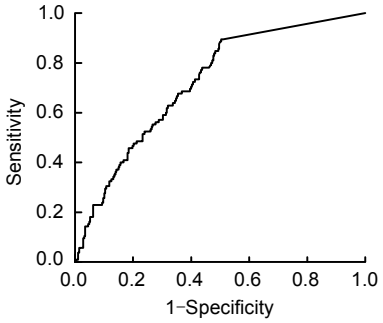

Fig. 1 Receiver operating characteristic (ROC) curve of E6/E7 mRNA for detecting HSIL+

3.3 Concordance between HPV E6/E7 mRNA assay and cytological test

To better investigate the performance of E6/E7 mRNA and LBC for detection of samples, the concordance between these two methods was analyzed. Concordance rates between HPV E6/E7 mRNA assay and the cytology test were 81.90% (86/105) and 61.11% (176/288) in HSIL+ and LSIL− groups, respectively (Table 3).

3.4 Correlation of E6/E7 mRNA and cytology with histological diagnoses

The sensitivity, specificity, PPV, and NPV of the HPV E6/E7 mRNA test for detecting HSIL+ were 89.52% (94/105; 95% CI, 81.64%–94.40%), 48.96% (141/288; 95% CI, 43.07%–54.88%), 39.00% (94/241; 95% CI, 32.87%–45.50%), and 92.76% (141/152; 95% CI, 87.11%–96.15%), respectively. The sensitivity, specificity, PPV, and NPV of the cytology test for detecting HSIL+ were 86.67% (91/105; 95% CI, 78.31%–92.26%), 48.96% (141/288; 95% CI, 43.07%–54.88%), 38.24% (91/238; 95% CI, 32.09%–44.76%), and 90.97% (141/155; 95% CI, 85.03%–94.70%), respectively. No statistical difference performance parameters were found between the HPV E6/E7 mRNA test and the cytology test (Table 4).

Table 3 Concordance between HPV E6/E7 mRNA assay and cytological test

Group	E6/E7+/ASC-US+	E6/E7−/NILM
LSIL−	91/288	85/288
HSIL+	83/105	3/105

HPV: human papillomavirus; ASC-US: atypical squamous cells of undetermined significance; NILM: negative for intraepithelial lesion or malignancy; LSIL: low-grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion

4 Discussion

The two primary oncogenic transcriptions E6 and E7 mRNA are expressed by HR-HPV in the early stage of the viral life cycle. E6/E7 mRNA expressions are negatively regulated by high E2 levels. When HR-HPV DNA integrates into the host genome by disruption of E2 open reading frame, the expression of E2 decreases, preventing E2 repression on E6/E7 mRNA, and the increased E6/E7 mRNA expression in the host cell promotes cellular proliferation and malignant transformation by inactivating two tumor suppressor proteins, p53 (inactivated by E6) and

Table 4 Correlation of E6/E7 mRNA and cytology with histological diagnoses*

Diagnosis	LSIL−	HSIL+	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
E6/E7 mRNA assay						
Negative	141	11	89.52 (81.64–94.40)	48.96 (43.07–54.88)	39.00 (32.87–45.50)	92.76 (87.11–96.15)
Positive	147	94				
Cytological test						
NILM	141	14	86.67 (78.31–92.26)	48.96 (43.07–54.88)	38.24 (32.09–44.76)	90.97 (85.03–94.70)
≥ASC-US	147	91				
P			0.671	1.000	1.000	0.678

* Data are expressed as percentage (95% CI). LSIL: low grade squamous intraepithelial lesion; HSIL: high-grade squamous intraepithelial lesion; PPV: positive predictive value; NPV: negative predictive value; NILM: negative for intraepithelial lesion or malignancy; ASC-US: atypical squamous cells of undetermined significance

pRb (inactivated by E7) (Scheffner *et al.*, 1990; Scheurer *et al.*, 2005). HPV E6/E7 mRNA was found to be significantly associated with the severity of cervical lesions (Cuschieri and Wentzensen, 2008). According to Liu *et al.* (2014), 25.7% of LSIL− cases had positive E6/E7 mRNA tests, while 71.9% of HSIL+ cases had positive E6/E7 mRNA tests. Pierry *et al.* (2012) found that in women under 30 years of age, 6% of benign cases, 22% of cervical intraepithelial neoplasia grade 1 (CIN1), 83% of CIN2, and 93% of CIN3 had positive E6/E7 mRNA. In our study, the positive rate of HPV E6/E7 mRNA increased as the severity of cervical lesions increased, and it was statistically higher in HSIL+ cases than that in LSIL− cases. Our results were consistent with the findings of other studies (Coquillard *et al.*, 2011; Pierry *et al.*, 2012; Liu *et al.*, 2014), indicating the potential utility of HPV E6/E7 mRNA detection in cervical cancer screening.

Screening programs play a pivotal role in cervical cancer prevention, for both non-vaccinated and vaccinated women (Arbyn *et al.*, 2012; Moyer, 2012). Accumulated evidence shows that HPV testing has advantages such as higher sensitivity (Mayrand *et al.*, 2007; Kitchener *et al.*, 2009; Ronco *et al.*, 2010), prolonged screening interval (Naucler *et al.*, 2007), and independence of cytologists in cervical cancer screening, and has been recommended as the primary cervical cancer screening in many countries (Franceschi *et al.*, 2011). However, more than 90% women will experience HPV infection in their lifetime, of which most have a transient infection with nearly 90% eliminated spontaneously within two years and will not cause any cervical lesion (de Sanjose *et al.*, 2007). Therefore, HPV testing alone may increase the psychological burden and may cause over referral to colposcopy. A proper triage is indispensable for HPV-positive women, and the cytology test (Pap smear and LBC) has become a widely recommended triage for HPV-positive women (Franceschi *et al.*, 2011). Though some disadvantages of HPV testing can be overcome by cytology triage, cytology triage has some limitations. Its performance is heavily dependent on the availability of trained cytologists, and many less developed countries are lacking well-trained cytologists, and even in developed countries, cytology is flawed by poor interobserver agreement, and the interpretations may differ substantially among personnel (Condel *et al.*, 2002).

It is very important to explore triage options which are cytologist-independent and with fewer subjective man-made factors for HPV-positive women. In our study, the performance of HPV E6/E7 mRNA detection in cervical exfoliated cells as a triage for HPV-positive women was evaluated, and some promising results were found. As a triage option for HPV-positive women, the HPV E6/E7 mRNA test achieved comparable performance with the cytology test. The results of the HPV E6/E7 mRNA test were highly concordant with cytology test results, and both triages resulted in similar sensitivity, specificity, PPV, and NPV while identifying HSIL+ lesions. Compared with cytology triage, the HPV E6/E7 mRNA test is cytologist-independent, and the result may be more objective. The HPV E6/E7 mRNA test may take the place of the cytology test of the triage program in those countries lacking cytologists.

In conclusion, our findings from a clinic-based population demonstrated that, for HPV-positive women, HPV E6/E7 mRNA detection and cytology triages have comparable performance. HPV E6/E7 mRNA detection may be an optional triage for HPV-positive women, especially in areas which lack well-trained cytologists. However, the conclusion may be somewhat limited due to the small sample size and because the present study is a hospital-based one. The conclusion cannot be generalized to the general patient population.

Compliance with ethics guidelines

Ye-li YAO, Qi-fang TIAN, Bei CHENG, Yi-fan CHENG, Jing YE, and Wei-guo LU declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要

题　目：宫颈脱落细胞人乳头瘤病毒（HPV）E6/E7 mRNA 检测在 HPV 阳性女性筛查中的应用

目　的：基于临床样本探讨宫颈脱落细胞 HPV E6/E7 mRNA检测能否作为HPV阳性女性的筛查手段。

创新点：首次比较了宫颈脱落细胞 HPV E6/E7 mRNA 检测与宫颈脱落细胞学检测对于 HPV 阳性女性宫颈活检结果的预测价值。

方　法：所有入组的 HPV 阳性女性均进行宫颈脱落细胞学及 HPV E6/E7 mRNA 检测。比较两种方法对于宫颈活检结果的预测价值。

结　论：对于 HPV 阳性的女性，高级别鳞状上皮内病变（HSIL）HPV E6/E7 mRNA 阳性率高于低级别鳞状上皮内病变（LSIL）及正常病例。HPV E6/E7 mRNA 与细胞学检测相比，HSIL 及以上级别病变的检测敏感性（89.52% vs. 86.67%，P=0.671）、特异性（48.96% vs. 48.96%，P=1.000）、阳性预测值（39.00% vs. 38.24%，P=1.000）及阴性预测值（92.76% vs. 90.97%，P=0.678）均无显著差异。HPV E6/E7 mRNA 检测或能成为 HPV 阳性女性的一种新的筛查手段。

关键词：人乳头瘤病毒（HPV）；HPV E6/E7 mRNA；高级别鳞状上皮内病变（HSIL）