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Role of E6/E7 mRNA in discriminating patients with high-risk *human papilloma* virus-positive associated with cytology-negative and atypical squamous cells of undetermined significance.

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Abstract

The aim of this study was to investigate the role of E6/E7 mRNA in discriminating patients who were high-risk *human papilloma* virus-positive associated with cytology-negative and Atypical Squamous Cells of Undetermined Significance (ASCUS). This study comprised of 380 women (age: >30 years) who were associated with high risk of cervical virus infection and they underwent simultaneous examinations of cytology, Human Papilloma Virus (HPV)-DNA, E6/E7 mRNA, and colposcopic pathological biopsy. The end-point of the study was set as the histological confirmation of high-grade Cervical Intraepithelial Neoplasia (CIN) II or higher (II+). In group HPV16/18-positive patients Negative for Intraepithelial Lesion or Malignancy (NILM), after E6/E7 mRNA discrimination, the positive predictive value (PPV) of CINII+ was increased from 21.62% to 40.54%, and the difference was statistically significant (χ^2=4.40, P<0.05). Meanwhile, the Negative Predictive Value (NPV) of E6/E7 mRNA was as high as 97.30%. In other high-risk (HR)-HPV-positive patients with ASCUS, after E6/E7 mRNA discrimination, the PPV of CINII+ was increased from 16.18% to 23.81%, and although the difference was obvious, it was not statistically significant (χ^2=0.98, P>0.05). Meanwhile, the NPV of E6/E7 mRNA detection was as high as 96.15%. E6/E7 mRNA can better discriminate the HPV16/18-positive patients with NILM from other HR-HPV-positive patients with ASCUS.

Keywords: Uterine cervical neoplasms, Screen, E6/E7mRNA, *Human papilloma* virus.

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Introduction

Effective cervical cancer screening can significantly reduce the incidence of cervical cancer. For women over 30 years of age, once *Human Papilloma* Virus (HPV) 16/18 is found positive, colposcopy is recommended even though the cytology appears negative [1,2]. The ATHENA (Addressing the Need for Advanced HPV Diagnostics) trial has found that the risk of high-grade cervical lesions (CINII+) occurring in women with HPV16-positive and cytology-negative is 9.5%, that in women with HPV18-positive and cytology-negative is 5.9% [3], and in women with atypical squamous cells (ASCUS-atypical squamous cells of undetermined significance, which has unknown cytological significance) and other high-risk HPV (HR-HPV)-positive is 8.6% [4]. If these populations had immediately undergone colposcopy, a higher ratio of negative results would have occurred. This, in turn, would result in unnecessary invasive procedures in large populations and increase the psychological burden of patients. Therefore, the aim of our study was to find an effective discriminative method to identify the populations with potential CINII+ from those with No Intraepithelial Lesions/Malignant lesions (NILM) or ASCUS and HR-HPV-positive. Previous studies have confirmed that HPV-DNA has no practical significance for predicting the risk of malignant transformation of cervical lesions [5]. The continuous high expression level of E6/E7 protein is the necessary condition of cervical carcinogenesis caused by HPV infection [6]. This also has an important significance in discriminating high-risk cervical viral infection [7,8]. Thus, we investigated the discriminative effects of E6/E7 mRNA by comparing and analysing the Positive Predictive Values (PPVs) and Negative Predictive Values (NPVs) of E6/E7 mRNA of CINII+ in patients with HR-HPV-positive and different cytological results.

Patients and Methods

Subjects

In this study, 380 women (age: >30 years) underwent simultaneous examinations of cytology, HPV-DNA, E6/E7 mRNA, and colposcopic pathological biopsy from April 2014 to October 2015 in the People's Hospital of Wenzhou City. They were all associated with high-risk cervical virus infection-157 women with HPV16/18 infection and 223 women with other HR-HPV infection. None of these women had history of Cervical Intraepithelial Neoplasia (CIN), cervical cancer, pelvic radiation therapy, total hysterectomy,

nor were currently pregnant. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the People's Hospital of Wenzhou City. Written informed consent was obtained from all participants.

Method for cervical cytology

One cervical brush was rotated 3-5 laps at the cervical squamous columnar junction area, which was then fully rinsed using the ThinPrep solution to maximally collect the cells that fell off. The cells were then sent to the Cell Department for programmed systematic processing, and professional gynaecological pathologists were responsible for the cytological diagnosis. The results were determined referring to the The Bethesda (TBS) classification system revised in 2001.

E6/E7 mRNA detection

The Quanti Virus HPV E6/E7 mRNA diagnostic kit (Henan Kodia Biotechnology Co.) was used, together with the branched DNA technology, to detect for 14 kinds of HR-HPV (HPVl6, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).

Pathological inspection

Cervical biopsy was performed in patients with colposcopic abnormalities, and the pathological results were diagnosed by two professional pathologists referring to the classification criteria issued by World Health Organization (WHO).

Statistical analysis

SPSS16.0 statistical software was used, and the count data were analysed using the χ^2 test, with $P<0.05$ considered as statistically significant. The sensitivity, specificity, PPV, and NPV were calculated using traditional contingency tables.

Results

Relationships of E6/E7 mRNA and pathological test

The test results of E6/E7 mRNA in various pathological conditions are shown in Table 1. With increased levels of cervical lesions, the E6/E7 mRNA positive rate also increased gradually. A comparison of the normal or inflammation group with CINI showed no statistically significant differences ($P>0.05$). The differences among CINII, CINIII, and cervical cancer group had no statistical significance ($P>0.05$). A comparison of the E6/E7 mRNA positive rate between CINII- and CINII+ groups was statistically significant ($P<0.05$, Tables 1 and 2).

Table 1. *Relationship of E6/E7mRNA positive rate with pathological results.*

Index	n	E6/E7 mRNA positive rate (%)
Normal or inflammation	159	90 (56.60%)
CINI	68	45 (66.18%)
CINII	74	67 (95.52%)*#
CINIII	68	62 (91.18%)*#
Cervical cancer	11	11 (100%)*#

Note: *Compared with the normal or inflammation group, $P<0.05$; #Compared with CINI, $P<0.05$.

Table 2. *Relationship of E6/E7mRNA positive rate with pathological results.*

Index	n	E6/E7mRNA positive rate (%)
CINII^{-a}	227	135 (59.47%)
CINII^{+b}	153	140 (91.50%)
χ^2		46.91
P		0

Note: CIN II^{-a}, including normal or inflammatory cervix and CIN I; CIN II^{+b}, including CINII, CIN III, and cervical cancer.

Expression levels of E6/E7 mRNA in HPV16/18-positive patients associated with different cytological results

In group NILM, 37 patients were E6/E7 mRNA-positive, and 15 patients showed CINII+, but among the 37 patients who were E6/E7 mRNA-negative, only 1 patient showed CINII+. In group ASCUS, 30 patients were E6/E7 mRNA-positive, and 23 patients showed CINII+, but among the 9 patients who were E6/E7 mRNA-negative, 3 patients showed CINII+. In group>ASCUS, 40 patients showed E6/E7 mRNA-positive, and 36 patients showed CINII+, but among the 4 patients who were E6/E7 mRNA-negative, 3 patients showed CINII+ (Table 3).

Table 3. *Expressions of E6/E7mRNA in HPV16/18-positive associated with different cytological results.*

Cytology	Pathology	n	E6/E7mRNA (+)	E6/E7mRNA (-)
NILM	CINII+	16	15	1
	CINII-	58	22	36
ASCUS	CINII+	26	23	3
	CINII-	13	7	6
>ASCUS	CINII+	39	36	3
	CINII-	5	4	1
Sum		157	107	50

Expression levels of E6/E7 mRNA in other HR-HPV-positive patients associated with different cytological results

In group NILM, 62 patients exhibited E6/E7 mRNA-positive, and 12 patients showed CINII+, but among the 13 patients who were E6/E7 mRNA-negative, only 1 patient showed CINII+. In group ASCUS, 42 patients were E6/E7 mRNA-positive, and 10

patients showed CINII+, but among the 26 patients who were E6/E7 mRNA-negative, only 1 patient showed CINII+. In group>ASCUS, 64 patients exhibited E6/E7 mRNA-positive, and 44 patients showed CINII+, but among the 16 patients who were E6/E7 mRNA-negative, 4 patients showed CINII+ (Table 4).

Table 4. *Expressions of E6/E7mRNA in other HR-HPV-positive associated with different cytological results.*

Cytology	Pathology	n	E6/E7mRNA (+)	E6/E7mRNA (-)
NILM	CINII+	13	12	1
	CINII-	62	50	12
ASCUS	CINII+	11	10	1
	CINII-	57	32	25
>ASCUS	CINII+	48	44	4
	CINII-	32	20	12
Sum		223	168	55

Comparison of PPVs and NPVs of E6/E7 mRNA

In group HPV16/18-positive with NILM, when E6/E7 mRNA was further used for the discrimination, the PPV of CINII+ was increased from 21.62% to 40.54%, and the difference was statistically significant (χ^2=4.40, P<0.05). Meanwhile, the NPV of E6/E7 mRNA was as high as 97.30%. In group HPV16/18-positive with other cytological types, PPVs of E6/E7 mRNA was not significantly increased and NPVs of E6/E7 mRNA was only 66.67% and 25.00%. In group Other HR-HPV-positive with ASCUS, when E6/E7 mRNA was further used for the discrimination, the PPV of CINII+ was increased from 16.18% to 23.81%, and although the difference was obvious, it was not statistically significant (χ^2=0.98, P>0.05). Meanwhile, the NPV of E6/E7 mRNA was as high as 96.15%. In group Other HR-HPV-positive with other cytological types, the PPV of E6/E7 mRNA was not significantly increased and NPVs of E6/E7 mRNA were only 92.31% and 75.00% (Table 5).

Table 5. *Comparison of PPVs and NPVs of E6/E7mRNA in predicting CINII+ in different groups.*

Group		NILM	ASCUS	>ASCUS
		% (95% CI)	% (95% CI)	% (95% CI)
HPV16/18	PPV	21.62% (12.23, 31.01)	66.67% (51.87, 81.47)	88.64% (79.27, 98.01)
E6/E7mRNA	PPV	40.54% (24.72, 56.36)*	76.67% (53.70, 99.63)	90.00% (80.71, 99.29)
E6/E7mRNA	NPV	97.30% (92.10, 100.00)	66.67% (35.88, 97.46)	25.00% (0.00, 67.43)
Other HR-HPV	PPV	17.33% (8.76, 25.90)	16.18% (7.42, 24.94)	60.00% (26.05, 93.95)
E6/E7mRNA	PPV	19.35% (9.51, 29.19)	23.81% (10.93, 36.69)	68.75% (57.40, 80.10)
E6/E7mRNA	NPV	92.31% (77.83, 100.00)	96.15% (88.76, 100.00)	75.00% (53.77, 96.23)

Note: *Compared with HPV16/18 PPV group, P<0.05.

Discussion

Studies have shown that persistent high-risk HPV infection is the main reason of the vast majority of cervical lesions and cervical cancer [9,10]. However, 80% of high-risk viral infection is transient [11], and the majority of new infections can dissipate in two years [12]. Although the HPV-DNA detection has a high sensitivity, its specificity is relatively low [13], thereby limiting its risk prediction ability. So, more specific biological markers for early detection of cervical cancer are needed. It is now widely known that only the high-risk HPV infection with the expression of viral oncogenes E6/E7 mRNA has the real carcinogenic risk. There are presently two mRNA -based tests developed and commercialized, the PreTect HPV-Proofer and the Aptima HPV assay with the indication that the detection of E6/E7 mRNA could improve the specificity of HPV testing [14,15]. At this study The Quanti Virus HPV E6/E7 mRNA diagnostic kit was used, together with the branched DNA technology. This assay was a new method. This study investigated the role of E6/E7 mRNA in discriminating patients with high-risk HPV associated with cytology-negative and ASCUS. The results of our study showed that E6/E7 mRNA had a good discrimination effect for HPV16/18-positive with NILM and other HR-HPV-positive with ASCUS. Therefore, for populations with negative E6/E7 mRNA, immediate colposcopy and clinical intervention were not required, and a close follow-up was sufficient. If there is a persistent infection of high-risk cervical virus or abnormal cytology, then colposcopy should be performed. Benevolo et al. [16] also believed that E6/E7 mRNA detection could be used to discriminate the population with high-risk cervical virus HC2-positive with cytology-negative or population with mild cytological atypia. This is similar to the results of our study.

The results of this study showed that the positive expression rate of E6/E7 mRNA increased with increasing cervical histopathological grades, consistent with that reported by Ratnam et al. and Coquillard et al. [17,18]. Particularly, the expression rate of E6/E7 mRNA in CINII+ was significantly higher than that in CINII-, indicating that the expression of E6/E7 mRNA is closely related to the level of cervical lesions,

and its high expression is likely to promote the occurrence and development of cervical lesions.

The results of our study show that among the 74 patients with HPV16/18-positive and NILM, further discrimination using E6/E7 mRNA can significantly improve the PPV of CINII+. More importantly, the NPV of E6/E7 mRNA was as high as 97.30%, and among the 37 patients with E6/E7 mRNA-negative, only one exhibited the pathological result of CINII; this patient underwent cervical conization, and the postoperative pathological report was chronic inflammation with small focal CINI-II. Among the populations with HPV16/18-positive and NILM, if E6/E7 mRNA was tested as negative, no immediate colposcopy was required, because these populations have very low probability of high-grade cervical lesions, so they need only a close follow up. Therefore, among the original 74 patients who needed colposcopy, only 37 patients underwent colposcopy because of the discrimination by E6/E7 mRNA. Thus, this greatly reduced the rates of colposcopy and biopsy, thereby reducing the psychological burden of the patients. Among the patients with HPV16/18-positive and ASCUS, or>ASCUS, E6/E7 mRNA discrimination showed no obvious advantages. Therefore, detecting E6/E7 mRNA will be an effective discrimination method for populations with HPV16/18-positive and NILM. The results of this study are consistent with that reported by Rijkaart and Perez [7,19].

The results of this study showed that among the 68 patients with other HR-HIV-positive and ASCUS, further E6/E7 mRNA discrimination did not significantly increase the PPV of CINII+, but NPV was as high as 96.15%. Furthermore, among the 26 patients with E6/E7 mRNA-negative, only one person's pathological result was confirmed as CINII; this patient also underwent cervical conization, and the postoperative pathology report was local CINI-II with glands involved. Therefore, among the populations with other HR-HIV-positive and ASCUS, if E6/E7 mRNA is tested as negative, these populations do not need immediate colposcopy, because the probability of high-grade cervical lesions is also very low, and only close follow-up observation is required. Therefore, among the original 68 patients who needed colposcopy, only 42 patients required colposcopy because of the discrimination by E6/E7 mRNA, and this also greatly reduced the rates of colposcopy and biopsy. Among the patients with other HR-HIV-positive and NILM, these populations also do not require colposcopy, and E6/E7 mRNA does not significantly improve the PPV of CINII+ in these populations. Therefore, the E6/E7 mRNA discrimination is not so relevant in this case. Among the patients with other HR-HIV-positive and>ASCUS, E6/E7 mRNA also does not significantly improve the PPV of CINII+ in these populations, and the NPV of E6/E7 mRNA is also only 75%; thus, the effect of E6/E7 mRNA discrimination is also little in this case.

Zappacosta et al. [20] believe that in a population cytologically diagnosed with ASCUS/LSIL combined with high-risk cervical virus infection, if E6/E7 mRNA is negative, the risk of CINII+ is only 4.6%; if the E6/E7 mRNA is positive, the risk of CINII + is as high as 93.9%. They also reported that the NPV of E6/E7 mRNA is very high, which is similar to the results of the present study. However, our study shows that the PPV of E6/E7 mRNA is not significantly higher than that of HPV-DNA, which may be related to the use of branched DNA technology to detect E6/E7 mRNA. Study showed that branched DNA technology has similar sensitivity in prediction of CINII+ with HPV-DNA detection technique when it is used to detect E6/E7mRNA, but it has lower specificity than HPV-DNA [21]. It is possible that after the copy number reaches a certain value, E6/E7 mRNA is significant for prediction of the CINII+ risk. This is also a question that needs to be further explored.

In summary, E6/E7 mRNA has good discrimination effects for patients with HPV16/18-positive and NILM, as well as with Other HR-HPV-positive and ASCUS, that is, if E6/E7 mRNA is tested as negative, these populations do not need immediate colposcopy and clinical interventions, and they just need a close follow-up. If persistent high-risk cervical viral infection or abnormal cytology changes occur, colposcopy and biopsy will be needed.

Limitations of this Study

Firstly, the sample size is relatively small; a larger population needs to be studied in the future. Secondly, this was not a prospective study that further confirms the correlations between the expression of E6/E7 mRNA and CINII+ risk. Therefore, we need to further follow-up the patients with negative colposcopic results to further investigate the roles of E6/E7 mRNA in assessing the risk of CINII+.

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Conflict of Interest

All authors have no conflict of interest regarding this paper.

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