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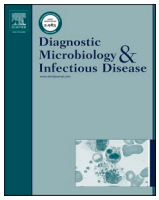

Original Article

Comparative efficacy of HPV 16/18 DNA and E6/E7 mRNA testing in detecting high-grade cervical lesions (CIN2+) in women with cervical biopsies

Sepideh Benvari [a], Masoumeh Aslanimehr [b,*], Fatemeh Samiee-Rad [c], Taghi Naserpour-Farivar [b], Hamid Sadeghi [b,d]

[a] Infection and Global Health Research Division, School of Medicine, University of St Andrews
[b] Medical Microbiology Research Center, Qazvin University of Medical Sciences, Iran
[c] Cellular and Molecular Research Center, Institute for Prevention of Non-Communicable Diseases, Qazvin University of Medical Sciences, Qazvin, Iran
[d] Student Research Committee, Qazvin University of Medical Sciences, Qazvin, Iran

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ABSTRACT

The study evaluated the efficacy of HPV 16/18 E6/E7 mRNA detection in women with abnormal cervical histology. A total of 99 cervical biopsy samples were analyzed, including 49 benign, 16 with cervical intraepithelial neoplasia grade 1 (CIN1), 9 with CIN2/3, and 25 with cervical cancers. Samples were tested for HPV 16/18 using both DNA and mRNA RT-PCR methods. The findings revealed a sensitivity of 85.3 % (29/34) for the HPV DNA test and 76.5 % (26/34) for the mRNA test in detecting CIN2+ lesions. Notably, the E6/E7 mRNA test demonstrated greater specificity for CIN2+ at 75.4 % (49/65), compared to 52.3 % (34/65) for the DNA test. The prevalence of positive results for both tests increased with the severity of squamous cell abnormalities. However, the HPV 16/18 E6/E7 mRNA test provided superior specificity, making it a more effective method for cervical cancer screening in this region, offering more precise results than DNA testing alone.

1. Background

Cervical cancer remains a significant public health challenge, particularly in developing countries where it is one of the leading causes of cancer mortality among women [1–3]. Human papillomavirus (HPV) is critically linked to the etiology of cervical cancer, with specific types being associated with the majority of cases. Epidemiological studies have shown that HPV contributes to approximately 5 % of all cancers globally, affecting a variety of body sites [4]. The strong association between HPV and cervical malignancies is underscored by the detection of HPV in about 90 % of cervical squamous cell carcinoma (SCC) cases [5]. Recent research, including findings by Arbyn et al., highlights that 20 HPV types are significantly more prevalent in cervical cancer cases compared to women with normal cervical cytology [6]. Furthermore, a Swedish study demonstrated that a substantial majority (85.3 %) of screen-detected cervical cancers could be attributed to HPV types 16, 18, 31, 33, 45, or 52. The inclusion of eight additional HPV types in most screening tests increased the detection prevalence by only an additional 1.5 % [7].

Other types classified as Low-Risk HPV (LR-HPV) which include >200 well-known human papillomaviruses are self-limiting, and are not the more prevalent cause of malignancy among the population. It should be noted that, among sensitive societies, different types of LR-HPV can be resistant to treatment and assist the development of cancers. [8–11].

Among High-Risk HPVs (HR-HPVs), 16 and 18 genotypes are the most important, which can cause about 70 % of cervical cancers [12–14]. Squamous intraepithelial lesions with progressive atypical grade, moderate to severe, or squamous in situ carcinoma may develop long before cervical cancer [15]. Therefore, cervical SCC has a long latency period [16]. The patient is asymptomatic during this time [15]. Currently, due to the optimization of screening techniques and the availability of effective therapeutic approaches for various types of squamous intraepithelial lesions, the progression of squamous intraepithelial lesions to squamous cell carcinoma can be prevented [17]. Currently, the most commonly used cervical cancer screening methods include the colposcopy, Pap test (Pap smear), and HPV test [18,19]. Here in, HPV testing is an applied method in screening and longitudinal follow-up studies [20]. In the next step, colposcopy is recommended to

* Corresponding author.
E-mail address: dr.aslanimehr@gmail.com (M. Aslanimehr).

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manage women with HR-HPV positive to provide key clues for CIN2 identification [21]. Although colposcopy is easy, its precision is related to human factors [18,19]. A Pap smear is noninvasive, simple, cost-effective, and easily detect precancerous lesions [22]. Nearly 80 % of false-negative Pap smears are related to the sampling technique and misinterpretation [23]. To overcome this problem, it has been suggested that cervical cancer screening could be improved by combining the cytological assay with testing for HPV DNA [12,24]. According to the low positive predictive value of such testing, a more reliable viral marker is required for indicating HPV activity and initiation of cellular transformation [25–27]. E6/E7 oncoproteins are expressed from HPV DNA after integration into the host genome. These two proteins are known to inactivate the major tumor suppressors, p53 and retinoblastoma protein (pRB). Constant overexpression of E6 and E7 leads to the development of cervical intraepithelial neoplasia (CIN) [19,28,29]. Previous studies report that E6/E7 mRNA detection has a higher clinical specificity for detecting high-grade lesions compared to DNA-based tests but a lower sensitivity [30,31]. E6/E7 mRNA test results have a significant clinical predictive value [32,33], and are more consistent with cytology and histology findings compared to DNA-based tests [34]. Having accurate information will lead to a more complete implementation of national guidelines and the appropriate application of regional revisions. Therefore, this study aimed to evaluate the significance of HPV 16/18 E6/E7 mRNA detection in women with abnormal histology in Qazvin province, Iran.

2. Methods

2.1. Patients and sampling

Current study was conducted at Qazvin University of Medical Sciences, Qazvin, Iran. Cervical specimens were taken from women with abnormal histology who were referred to Qazvin Kowsar Referral Gynecology Hospital from 2007 to 2019 for a further histological examination.

2.2. Nucleic acid isolation

FFPE cervical biopsy blocks were cut (2-4 five-micron sections) by a microtome (Hacker instrument-USA). The cuts were collected and numbered in RNase and DNase free sterile microtubes. DNA was extracted using High Pure FFPE Micro DNA Kit (Roche Diagnostics, Indianapolis, IN, USA). The quality of extracted DNA samples was evaluated by β-globin gene detection. Total RNA was extracted by High Pure FFPE Micro RNA Kit (Roche Diagnostics, Indianapolis, IN, USA) and treated with DNase to eliminate DNA contamination according to the manufacturer's instructions.

2.3. HPV 16/18 DNA and E6/E7mRNA detection

Identification of HPV 16/18 and E6/E7 mRNA was performed by Real-Time PCR method in the laboratory of Qazvin University of Medical Sciences. StepOnePlus Real-Time System (Applied Biosystems, CA, USA) and TAKARA Sybr Premix Ex Taq Master (Takara, Otsu, Japan) were used. The primers for HPV 16/18 detection were designed using (Integrated DNA Technologies platform (IDT, Lowa, USA) (Table 1). PCR program included Initial denaturation 95°C/ 60 s, denaturation 95° C/ 30 s, annealing 60°C / 30 s, and extension 72° C/ 30 s. Reverse transcription of RNA to cDNA was performed using Bioneer AccuPower Cyclescript RT premix (dN6) kit (Bioneer, Korea) according to the manufacturer`s instructions. E6/E7 mRNAs of HPV types 16 and 18 were detected by primers chosen as described by Paola Cattani et al. [35]. Thermal cycling conditions were 30 s at 95°C followed by 45 cycles of 30 s at 95°C, 30 s 60°C, and 45 s 72°C. The quality of the synthesized cDNA was checked by running on 2 % agarose. We perform a SYBR green-based real-time PCR, and β-actin (ACTB) was used as an internal control.

Table 1
Sequences of primers used for HPV 16/18 DNA detection.

Primers	Sequence (5′ to 3)	Product size
HPV 16 (F)	TGCTAGTGCTTATGCAGCA	149 bp
HPV 16 (R)	TTACTGCAACATTGGTACATGG	149 bp
HPV 18 (F)	CGCCACGTCTAATGTTTCTG	146 bp
HPV 18 (R)	CCTGTGATAAAGGACGCGA	146 bp

2.4. Statistical analysis

After collecting data, the findings were presented in the form of tables and numerical indices. Statistical analysis was carried out using SPSS for Windows 21.0 (IMM, US). Chi-square test was used to analyze the data. P value <0.05 was considered significant.

3. Results

3.1. Nucleic acid isolation

A total of 103 samples were entered into the extraction stage. All samples were positive for beta-globin DNA, confirming the quality of the DNA preparations. Four samples were negative for ACTB gene expression and omitted from the study population due to the low quality of extracted RNA. The mean age of cervical cancer patients was 46.34 years (range 21–78 years). By dividing the patients into equal groups (<40 and >40), E6 and E7 gene expression increases with age ($P = 0.04$).

3.2. HPV16/18 DNA and E6/E7 mRNA detection

HPV 16/18 genotypes were identified in 60 samples (60/99, 60.6 %), consisting of HPV 16 in 57 (57.6 %) cases and mixed HPV 16 and 18 in 3 cancerous samples (3 %). The results of HPV 16/18 DNA in samples with different histological reports demonstrated a statistically significant difference (Table 2). HPV E6/E7 mRNA was observed in 42/99 (42.4 %) samples. The mRNA test showed a higher positive rate of E6/E7 mRNA in CIN2+ cases than in CIN1- cases (Table 3). E6 mRNA was detected in 7/34 (20.6 %) CIN2+ and 1/65 (1.5 %) CIN1- cases, whereas E7 mRNA was observed in 27/34 (79.4 %) CIN2+ and 15/65 (23.8 %) CIN1- samples.

A significant difference between E6/E7 mRNA positivity rate among younger and older women was detected (≤ 40 and >40); the presence of E6/E7 mRNA raised from 26.3 % to 51.9 % in the older group ($p = 0.02$). The results of HPV E6/E7 mRNA detection in HPV 16/18 positive cases are provided in Table 4.

3.3. Correlation of nucleic acid tests with histology results

The sensitivity, specificity, positive predictive value, and negative predictive value of the HPV 16/18 DNA and E6/E7 mRNA tests for detecting CIN2+ were calculated by considering histology results as the gold standard (Table 5). The sensitivity, PPV, and NPV among HPV DNA assay and HPV E6/E7 mRNA testing showed no statistical difference (p

Table 2
HPV 16/18 DNA detection results in different histological groups.

Group	HPV 16/18 DNA		Positive rate (%)	P-value
	Positive	Negative		
Benign	21	28	42.9	
CIN1	10	6	62.5	
CIN2,3	6	3	66.7	0.0007
Cancer	23	2	92.0	

Table 3
E6/E7 mRNA detection results in different histological groups.

Group	HPV E6/E7 mRNA		Positive rate (%)	P-value
	Positive	Negative		
Benign	10	39	20.4	
CIN1	6	10	37.5	
CIN2,3	5	4	55.6	0.00001
Cancer	21	4	84.0	

Table 4
E6/E7 mRNA detection results in HPV 16/18 DNA positive cases based on different histological groups.

Group	Total	HPV E6/E7 mRNA	P-value
Benign	21	10 (47.6 %)	
CIN1	10	6 (60 %)	
CIN2,3	6	5 (83.3 %)	0.01
Cancer	23	21 (91.3 %)	
Total	60	42 (70.0 %)	

= 0.355), but a statistical difference was shown among HPV E6/E7 mRNA testing specificity ($p = 0.006$).

4. Discussion

In this study, we evaluated the role of HPV 16/18 E6/E7 mRNA detection in patients with abnormal histology in Qazvin province, Iran. Based on our results, HPV 16/18 DNA was detected in 60.6 % of the patients included. The positivity rate of the test increased by increasing the degree of squamous intraepithelial dysplasia from 42.9 % in benign to 92 % in cancerous samples. A study by Farahmand *et al.* [36] found that in cancer patients from Tehran and Mashhad, cities in Iran, HPV DNA was in 78.8 %, including 43.4 % HPV16, 8 % HPV18, and 27.4 % an unknown HPV type. In a meta-analysis of HPV prevalence and types among Iranian women by Salavatiha et al. [37], the overall HPV prevalence was found to be 55 % in atypical squamous cells of undetermined significance (ASCUS), 58 % and 69 % in women with low and high grade squamous intraepithelial lesions, respectively, and 81 % among women with invasive cervical cancer. In all of the studied groups, HPV 16 was the most common HPV type, followed by HPV 18. However, in our study, HPV 18 was detected in only 3 % of samples, also mixed with genotype 16. Nonetheless, the results of the HPV 16/18 DNA examination in our study are consistent with studies in other regions, which showed an increase in HR-HPV detection, along with an increase in the histopathological grade of the cervical lesion [38,39]. In this study, the RNA assay produced fewer positive results compared to the HPV DNA test, and E6/E7 mRNA was observed in 42.4 % (42 of 99) of the patients. Also, among HPV 16/18 positive cases, E6/E7 mRNA was detected in 70 %. In a study by Cattani et al. [35] and TÜNEY et al. [40], the presence of E6/E7 mRNA in HR-HPV positive samples was 52.9 % and 60 %, respectively. These fewer positive findings can be representative of an excessive chance of HPV regression [25]. According to our results, 84 % of the cancerous samples were positive for HPV 16/18 E6 and E7 mRNA. In studies by Kraus et al. [41] and Yao et al. [42], the expression rates were 92 % and 100 % in cancerous samples, respectively. This high frequency confirms that the expression of E6 and E7 are essential conditions for the development of cervical malignancy. Our findings indicate that E6/E7 mRNA from HPV 16/18 was detected in 55.6 % of women with CIN2/3. In studies by Castle et al. [43] and Valença et al. [44], E6/E7 mRNA was detected in 84 % and 70 % of cases with a high-grade squamous intraepithelial lesion (HSIL), respectively. Negative results in CIN2,3 cases can be because of the low level or lack of viral transcriptional activity. In our study, 37.5 % of CIN1 samples had E6/E7 mRNA. In studies by Bruno et al. [45], Sotlar et *al.* [46], and Castle et al. [43], E6/E7 mRNA was detected in 23.6 %, 58 %, and 70 % of CIN1 cases, respectively. Moreover, in studies by Casagrande et al. [47] and Yao et al. [42], which evaluated abnormal cytology cases, E6/E7 mRNA was detected in 42.7 % and 77 % of HSIL cases, respectively. These dissimilar rates in different studies might be due to different techniques used for detecting RNA, samples of study (FFPE or cytology specimens), or incomplete coverage of relevant HPV types. Recently, the APTIMA test (Hologic, San Diego, CA, USA), which detects E6/E7 mRNA of 14 HPV types, is suggested to be more efficient in cervical cancer screening [48]. The presence of E6/E7 mRNA in 20.4 % of our cases without dysplastic changes is clinically important since the HPV virus can be oncogenically active even before it produces detectable cytomorphologic changes. A study by Ren et al. [49] showed that high expression of HPV E6/E7 mRNA could be a good diagnostic marker to triage ASCUS superseding HPV DNA test. Moreover, the results of a study by Giorgi Rossi et al. [50] showed that a negative E6/E7 mRNA has a good prognostic value for clearance and CIN2+ regression. Long-term studies conducted by Rad [51], Shibli [52], Sørbye [53], and Forslund [54] have confirmed the value of the E6/E7 mRNA test. In our study, E6 mRNA was detected in 7/34 (20.6 %) CIN2+ and 1/65 (1.5 %) CIN1- cases, while E7 mRNA was observed in 27/34 (79.4 %) CIN2+ and 15/65 (23.8 %) CIN1- samples. The fewer detection of E6 mRNA could be a result of its role in the secondary stage of disease progression. The results of the study by Wang-Johanning *et al.* [55] also showed early expression of E7 and increased E6 expression in the later stages of tumor progression.

Our study showed that by dividing patients' age into two groups of ≤40 and >40, a significant difference can be observed in HPV 16/18 E6/E7 mRNA positivity rate (26.3 % vs 51.9 %, $p = 0.02$). This increase may be indicative of the slow progression of the disease till the formation of cervical cancer, which creates a proper opportunity for early diagnosis and treatment of cervical cancer. By considering the histology results as the gold standard, HPV 16/18 E6/E7 mRNA test specificity for detecting CIN2+ was higher than HPV 16/18 DNA (75.4 % vs 52.3 %, $p = 0.006$). There were no significant differences between the Sensitivity, PPV, and NPV of these two tests. Although both HPV 16/18 DNA and E6/E7 mRNA positive results increased with the severity of the squamous cell abnormalities, the E6/E7 mRNA test correlates better with progressive lesions.

5. Conclusions

The findings of this study provide valuable insights into the ongoing research in this field. In Qazvin province, Iran, a significant proportion of patients diagnosed with CIN2+ were found to have HPV 16/18 DNA. Despite RNA's susceptibility to degradation, our results demonstrate that FFPE samples are viable for E6/E7 mRNA detection using PCR

Table 5
Correlation of HPV 16/18 DNA and E6/E7 mRNA with histological diagnosis.

Test		CIN1-	CIN2+	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
HPV 16/18 DNA	**Negative**	34	5	85.3	52.3	48.3	87.2
	Positive	31	29				
E6/E7 mRNA	**Negative**	49	8	76.5	75.4	61.9	86
	Positive	16	26				
P			0.355	0.006	0.176	0.864	

Data are expressed as percentage (95 % CI). CIN1-: CIN1 and benign; CIN2+: CIN2, CIN3 and cancer; PPV: positive predictive value; NPV: negative predictive value.

methods. The positivity rates for both HPV 16/18 DNA and E6/E7 mRNA tests increased with the progression from normal histology through CIN1, CIN2/3, to cervical cancer, underscoring the superior specificity of the E6/E7 mRNA test for cervical cancer screening. The detection of E6/E7 mRNA in patients with benign diagnoses also suggests its potential as a predictive marker for disease progression. Further studies are necessary to substantiate this potential.

Limitations

Four major limitations in this study could be addressed in future research. First, retrospective observational. Second, a small sample size. Third, FFPE biopsy samples, Fourth, evaluating most common HR-HPV genotypes (16 and 18).

Ethic statement

This study was approved by the ethics committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1394.145) and signed informed consent was obtained from all patients who participated in the study.

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CRediT authorship contribution statement

Sepideh Benvari: Writing – original draft, Investigation. **Masoumeh Aslanimehr:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Fatemeh Samiee-Rad:** Methodology, Investigation, Data curation, Conceptualization. **Taghi Naserpour-Farivar:** Methodology, Investigation, Data curation, Conceptualization. **Hamid Sadeghi:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hamid Sadeghi reports financial support was provided by Qazvin University of Medical Sciences. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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