



Evaluation of HPV E6/E7 mRNA Detection in Clinically Suspected Cases of Cervical Cancer with Abnormal Cytology: Time to Upgrade the Screening Protocols

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Abstract

Background Human papillomavirus (HPV) E6/E7 mRNA tests determine the oncogenic activity of the virus and represent a good clinical biomarker for predicting the risk of cervical cancer. So, the present study was conducted to know the role of HPV E6/E7 mRNA as a predictive biomarker for cervical carcinoma.

Methodology The present study was conducted on 55 clinical samples of cervical scrapings and biopsy from the clinically suspected cases (based on signs and symptoms) of cervical cancer having abnormal PAP smear. The samples were processed in three steps—(1) HPV DNA detection, (2) HPV E6/E7 mRNA detection, and (3) histopathological analysis.

Results Out of a total of 55 patients, 16 (29.09%) were positive for both HPV E6/E7 mRNA and HPV DNA and six were positive for only HPV DNA. So, a total of 22 (40%) patients were positive for HPV DNA. Out of these 22 samples, 10 (45.5%) were of HPV-16, six (27.3%) were of HPV-18, four (18.2%) were of HPV-31, and two (9.1%) were of HPV-45. Out of total 16 patients positive for HPV E6/E7 mRNA, 10 (62.5%) were of genotype 16 and six (37.5%) were of genotype 18. The patients who were found positive for HPV 31 and 45 genotypes did not have E6/E7 mRNA expression. On colposcopic-guided biopsy, among these 16 samples, eight (50%) were diagnosed with invasive squamous cell carcinoma, six (37.5%) with cervical intraepithelial neoplasia grade 3 (CIN3), and two (12.5%) with CIN2. Out of those six patients in whom only HPV DNA was positive, five had normal biopsy findings and one had CIN1.

Conclusion The present study suggests that HPV E6/E7 mRNA detection could be more reliable than DNA testing for predicting the risk of progression of HPV-induced cervical lesions to cervical carcinoma and it can be used as a non-invasive tool for triage and patient follow-up.

Keywords

- ▶ human papillomavirus
- ▶ E6/E7 mRNA
- ▶ cervical cancer

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Introduction

Invasive cervical cancer is the second most common cancer in women worldwide and the most common cause of cancer-related mortality among women in India.¹ The etiology and pathogenesis of cervical cancer have been clearly linked to persistent infection with human papillomavirus (HPV).² Approximately, 30 distinct HPV types preferentially infect the anogenital mucosa, resulting in lesions that range from condyloma to cervical intraepithelial neoplasia (CIN)/squamous intraepithelial lesion (SIL) and invasive carcinoma.³ The most common oncogenic HPV type in preinvasive and invasive cervical cancer is HPV 16 and is detectable in more than 50% of women with CIN3/cervical cancer followed by HPV 18 which accounts for 10 to 15% of cervical cancers.⁴ HPV DNA detection is one of the most important screening triage available for the detection of cervical cancer nowadays along with PAP smear. There are many clinical applications of HPV DNA testing like triage of females with atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesions (LSIL) findings on PAP smear; as a primary screening test; as a prognostic tool for a therapeutic outcome after the treatment of an intraepithelial lesion; and for determining the prevalence of certain genotypes of HPV at regional, national, or global levels.⁵ However, the major limitation of this modality is inability to differentiate between progressive and regressive lesions as the former can lead to carcinogenesis in the future. HPV E6/E7 mRNA messenger ribonucleic acid tests determine the oncogenic activity of the virus and represent a good clinical biomarker for predicting the risk of developing cervical cancer.⁶ On the molecular level, the conversion of an acute HPV infection into a transforming HPV infection is characterized by the substantially increased expression of viral oncogenes E6/E7 in the basal cells that retain the capacity to replicate.⁷ The deregulated expression of these oncogenes induces chromosomal instability that is the predominant hallmark of cervical carcinogenesis.⁸ So, E6/E7 mRNA expression is a powerful indicator for the progression of cervical carcinoma. The major advantages of HPV E6/E7 mRNA test over HPV DNA test are is a more specific and non-invasive test, has a greater positive predictive value (greater prognostic significance),⁹ correlates better with cytology and histology,¹⁰ and directly detects the expression level of oncogenic HPV E6 and E7 (mRNA).¹¹ However, currently available HPV DNA test generally targets the conserved L1 region and it cannot discriminate between regressive and progressive HPV infections.¹² However, since most women have self-resolving HPV infections that will not develop into cancer, a positive HPV test result provides little actionable information and causes a psychological burden on the patient and also may cause over referral to colposcopy. So, in the given study, we tried to compare both HPV DNA and HPV E6/E7 mRNA with histopathological findings in females with clinical suspicion (based on signs and symptoms) of cervical carcinoma.

Materials and Methods

The given study was conducted from 1st January 2016 to 30th June 2017. The study comprised of 55 samples of cervical scrapings from the suspected cases of carcinoma

cervix, attending obstetrics and gynecology outpatient department /inpatient department of the institute. The detailed history in relation to demographic features, symptoms such as abnormal vaginal discharge, post-coital bleeding, post-menopausal bleeding, menstrual and obstetric history, personal history, contraception used, and any significant past medical/surgical history was enquired as a routine protocol and all these details were entered in a pretested proforma. Written informed consent was obtained from all subjects. Ethical clearance was obtained from the institute's ethical committee with reference number IEC/GMC/Th 00134. Female patients with age more than 18 years who were sexually active with clinical signs and symptoms of carcinoma cervix along with abnormal PAP smear were included in the study. Pregnant female patients and females having a prior history of cervical carcinoma were excluded.

Collection and Transportation of Samples

The specimen was collected by introducing a bivalve speculum into the vagina for a complete visualization of the external os and ectocervix. The samples were collected in the specimen collection tube containing phosphate-buffered saline. The specimen was sent to the microbiology laboratory for further processing. The samples were processed on the same day, and in case of delay, samples were stored at -80°C . A colposcopic-guided biopsy sample was taken for histopathological analysis.

Processing of Samples

The samples were proceeded in two—(1) HPV DNA detection and (2) HPV E6/E7 mRNA detection.

- HPV DNA detection:

- DNA extraction

Each sample was processed for DNA extraction according to the instruction manual given along the QIAamp DNA by Qiagen (Hilden, Germany).

- DNA amplification

The sequence of primers is given in ► **Table 1**. Details are provided in **Supplementary Material**.

Polymerase chain reaction (PCR) products, thus, obtained were subjected to gel electrophoresis for HPV detection.

- HPV E6/E7 mRNA detection

- RNA extraction by a spin column technique

Each sample was processed according to the instructions manual given along the RNeasy mini kit by Qiagen.

- Reverse transcription and amplification

Both reverse transcription and amplification of cDNA occurred in a single step by Qiagen one-step reverse

Table 1 Sequence of primer GP5+ and GP6+

HPV genotype	Amplicon (bp)	Sequence (5'-3')
GP5+	140	FP- TTT GTT ACT GTG GTA GAT AC
GP6+	140	RP- GAA AAA TAA ACT GTA AAT CA

Abbreviation: HPV, human papillomavirus.

Table 2 Primers designed for HPV 16, 18, 31, and 45 E6/E7 mRNA detection

HPV	Region	Size	Sequence
16	E6	± 659 pb	5' TAAACTAAGGGCGTAACCG 3' 5' TCTATTTTCATCCTCCTCTG 3'
16	E7	± 491 pb	5' ACTGTGTCTGAAGAAAAGCAA 3' 5' AACCATCCATTACATCCCGT 3'
18	E6	± 608 pb	5' TAGGTTGGGCAGCACATACT 3' 5' ATACTTGTGTTTCTCTGCGTGC 3'
18	E7	± 358 pb	5' CGACGCAGAGAAACACAAGTAT 3' 5' ATTGTTGCTTACTGCTGGGAT 3'
31	E6	± 606 pb	5' AAGTAGGGAGTGACCGAAAGT 3' 5' ACAGTGGAGGTCAGTTGCC 3'
31	E7	± 437 pb	5' AGGCACGGCAAGAAAGACT 3' 5' AAAGAACCAGCCATTACACC 3'
45	E6	± 659 pb	5' ATACTACATAAAAAAGGGTG 3' 5' TCGTAACACAACAGGTCAACA 3'
45	E7	± 439 pb	5' AGGCACGGCAAGAAAGACT 3' 5' AAAGAACCAGCCATTACACC 3'

Abbreviation: HPV, human papillomavirus.

transcription (RT)-PCR Kit 210212. Details are given in **Supplementary Material**.

- *Type-specific PCR for HPV 16, 18, 31, and 45 oncogenes E6 and E7 mRNA*

Next PCR reactions were performed to ascertain the presence of E6/E7 mRNA of the following HPV types: 16, 18, 31, and/or 45. The reaction was performed separately for each HPV genotype using specifically designed primers (–**Table 2**). For details of cycling condition, refer **Supplementary Material**.

- *Analysis of amplified products*

Amplified products were subjected to gel electrophoresis and analyzed on a gel documentation system.

Cytopathological Analysis

All samples for cytology were taken using the Thin Prep PAP smear cytology and were analyzed in the pathology laboratory of the institute. Cytological results were classified according to the revised Bethesda classification,^{13,14} such as ASC-US; ASC, cannot exclude a high-grade squamous intraepithelial lesion (HSIL); LSIL (CIN grade 1[CIN1]); HSIL (CIN2; CIN3; carcinoma in situ) and invasive squamous cell carcinoma.

Histopathological Analysis

Colposcopic-guided biopsy samples for histopathological analysis were taken and were sent to the histopathological department of the institute, and diagnoses were made according to the CIN reporting system.¹⁵

Statistical Analysis

Different parameters like age and parity were presented as mean ± standard deviation (SD). A *p*-value <0.05 was considered statistically significant. Fisher's exact test was used

when the number of observations in a cell was less than 5, and to compare between the continuous variables like age at presentation and marital age, independent *t*-test was used. In others, the chi-square test was used to study the associations between various risk factors and HPV E6/E7 mRNA expression. The minimum sample size was calculated as 45 using a formula: $n = Z^2P(1 - P)/d^2$, where *n* is the sample size, *Z* is the statistic corresponding to the level of confidence (taken as 95%), *P* is expected prevalence, and *d* is precision after taking the prevalence of cervical cancer as 3% in Punjab region.¹⁶ Data were analyzed using Microsoft Excel version 16 (Microsoft Corp., Richmond, CA, United States), and statistical analysis was performed using GraphPad Prism V.6.0 (GraphPad Software, La Jolla, CA, United States). Diagnostic performance in terms of sensitivity, specificity, positive, and negative predictive values (confidence interval was taken as 95%) were calculated using MedCalc version 12.1.4 (MedCalc Software bvba, Mariakerke, Belgium).

Results

• Demographic details and risk factors

The mean age of the females in the given study was 34 ± 10 years. Mean parity in the patients was 2.1 ± 0.7 . The mean age of the patients at the time of marriage was 25 ± 8 years. The majority (96.36%) of the patients had a single sexual partner, and only 3.64% gave the history of multiple sexual partners. Out of total 55 patients, partners of majority patients, i.e., 26 (54.17%) were using male condoms. Oral contraceptive pills (OCPs) were used by 16 (33.33%) patients, two (4.17%) patients were using a copper intrauterine device, one (2.08%) practiced coitus interruptus, and three (6.25%) patients gave a history of tubectomy done in the past. Seven patients had no history of use of contraception since they were in the post-menopausal state.

• Clinical signs and symptoms

Out of the 55 patients, majority (27) presented with foul-smelling discharge per vaginam. The next common complaint was pain in the lower abdomen (24) followed by irregular bleeding per vaginam (18) and post-coital bleeding (12). Only eight patients presented with post-menopausal bleeding. The majority of the patients had multiple complaints.

On examination, the majority of them (34) had cervical erosions followed by hypertrophied cervix with discharge in 20 patients. Only 10 patients had polypoidal growth. Multiple findings were observed in some of the patients

• Cytopathological (PAP smear) findings

All the patients had abnormal PAP smear findings. Out of 55 patients, 36 patients had ASC-US on their cytology, one had LSIL, eight had HSIL, eight had invasive squamous cell carcinoma, and only two patients had atypical glandular cells of undetermined significance.

• HPV DNA detection and its genotype

Out of the total, 22(40%) were positive and 33(60%) were negative for HPV DNA. Out of these 22 patients, 10 (45.5%)

Table 3 Comparison between HPV E6/E7 mRNA and HPV DNA vis-à-vis the histopathological and cytological finding

	HPV DNA (n)		HPV E6/E7 mRNA (n)	
	Positive (22)	Negative (33)	Positive (16)	Negative (39)
<i>Cytology (n)</i>				
ASC-US (36)	4	32	0	36
AGUS (2)	1	1	0	2
LSIL (1)	1	0	0	1
HSIL (8)	8	0	8	0
Invasive squamous cell carcinoma (8)	8	0	8	0
<i>Histopathology (n)</i>				
Normal (38)	5	33	0	38
CIN 1 (1)	1	0	0	1
CIN 2 (2)	2	0	2	0
CIN 3 (6)	6	0	6	0
Invasive squamous cell carcinoma (8)	8	0	8	0

Abbreviations: AGUS; atypical glandular cells of undetermined significance; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; n, number of the patients.

were of HPV-16, six (27.3%) were of HPV-18, four (18.2%) were of HPV-31, and two (9.1%) were of HPV-45.

- HPV E6/E7 mRNA expression and its specific genotype
Out of total 55 patients, 16 (29.09%) were positive and 39 (70.91%) were negative for HPV E6/E7 mRNA. Out of total 16 patients positive for HPV E6/E7 mRNA, 10 (62.5%) were of genotype 16 and 6 (37.5%) were of genotype 18. The patients who were found positive for HPV 31 and 45 genotypes did not have E6/E7 mRNA expression. So, in total, 16 patients were positive for both HPV-DNA and HPV E6/E7 mRNA, whereas six were positive for only HPV DNA (→ **Table 3**).

- **Histopathological findings**

On histopathological analysis, 38 had normal findings, eight had invasive squamous cell carcinoma, six had CIN3, two had CIN2, and one had CIN1. Out of all HPV DNA-positive cases (22), only 16 were positive for HPV E6/E7mRNA. Among those 16, eight (50%) were diagnosed with invasive squamous cell carcinoma, six (37.5%) with CIN3, two (12.5%) with CIN2. Whereas out of those six patients in whom only HPV DNA was positive, five had normal biopsy findings and one had CIN1. Sensitivity, specificity, positive predictive value, and negative predictive value of HPV DNA and HPV E6/E7 mRNA against gold-standard histopathology method were calculated (→ **Table 4**).

- Association between various risk factors and HPV E6/E7mRNA expression

The positivity rate of HPV E6/E7 mRNA between different age groups was compared along with various other risk factors (→ **Table 5**).

Discussion

The given study highlights the role of detection of E6/E7 mRNA of HPV for predicting the risk of progression of HPV-induced cervical lesions to cervical carcinoma as all the patients in the given study showing expression of HPV E6/E7 mRNA had features of cervical cancer on histopathology. It shows that the detection of HPV E6/E7 mRNA is more specific than HPV DNA detection.

In our study, HPV infection was detected in 40% of patients which is quite less than the previous studies.¹⁷ The difference may be attributed to the smaller sample size taken in our study. According to the International Agency for Research on Cancer analysis, the five most common HPV genotypes with decreasing frequency are, HPV-16, -18, -45, -31, and -33, found in 82.9% of the cases.¹⁸ Similarly, according to one study,¹⁷ the most prevalent genotype was HPV 16 (87.28%) followed by HPV 18 (24.56%) and HPV 51(3.46%). Similarly, our study depicted that among HPV DNA-positive patients, HPV 16(45.45%) was the most prevalent type followed by

Table 4 Sensitivity, specificity, positive predictive value, and negative predictive value of HPV DNA and HPV E6/E7 mRNA against histopathology

	Sensitivity (95% CI)	Specificity (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)
HPV DNA	100 (80.5–100)	86.84(71.9–95.6)	19.03((9.4–34.7)	100
HPV E6/E7 mRNA	94.12 (71.3–99.8)	100 (90.7–100)	100	99.82 (98.8–99.8)

Abbreviations: CI, confidence interval; HPV, human papillomavirus.

Table 5 Association of various risk factors with HPV E6/E7 mRNA positivity

Demographic/Risk factors (total number)	HPV E6/E7 mRNA positive (16)	HPV E6/E7 mRNA negative (39)	p-Value
<i>Age at the time of presentation, in years (n)</i>			
18–30 (23)	9	14	0.0023
31–40 (22)	1	21	
> 40 (10)	6	4	
<i>Age at the time of marriage, in years (n)</i>			
18–25 (30)	13	17	< 0.05
26–30 (20)	3	17	
31–35 (5)	0	5	
<i>Parity (n)</i>			
Para 1 (10)	1	9	0.0046
Para 2 (28)	5	10	
Para 3 (17)	10	7	
<i>Number of sexual partners (n)</i>			
Single (53)	14	39	0.0245
Multiple (2)	2	0	
<i>Contraceptives used (n)</i>			
Male condom (26)	1	25	0.0001
OCPs (16)	12	4	
Tubectomy (3)	1	2	
Cu-T (2)	1	1	
Coitus interruptus (1)	1	0	

Abbreviations: Cu-T, copper intrauterine device; HPV, human papillomavirus; *n*, number of the patients; OCPs, oral contraceptive pills. Note: $p = 0.0001$, statistically significant.

HPV 18(27.27%), HPV 31(18.18%), and HPV 45(9.10%). The present study showed that the positivity of HPV E6/E7 mRNA in women with abnormal PAP smear was 29.09%. Orioni et al,¹⁹ Ho et al,²⁰ and Molden et al²¹ demonstrated the positivity of HPV E6/E7 mRNA among women with abnormal cytology as 26, 21, and 22.8%, respectively. However, in a recent study by Dabeski et al²² high-risk HPV E6/E7 mRNA infection was detected in 60.94% of patients with squamous cell abnormalities of the cervix. These differences could be due to different sample sizes in different studies along with different methodologies used. In a study by Cuschieri and Wentzensen²³ HPV E6/E7 mRNA was found to be significantly associated with the severity of cervical lesions. The similar fact was proven by a study of Liu et al,²⁴ which found that 25.7% of LSIL cases and 71.9% of HSIL+ cases had positive E6/E7 mRNA tests, and as the severity of cervical lesions increased, the positive rate of HPV E6/E7 mRNA also increased. The findings were similar in our study also as in the patients who have normal biopsy findings, HPV E6/E7 mRNA is negative and as the severity of cervical lesions increases on biopsy, positivity of HPV E6/E7 mRNA also increases and all the patients who had HSIL+ lesions had HPV E6/E7 mRNA expression also. A study by Salimović-Bešić et al²⁵ on 105 women with abnormal cervical cytological findings showed low HPV E6/E7 mRNA positivity in women with cytological findings of ASC-US on the PAP test. In our study, 36 patients

had ASC-US cytological findings on PAP smear, but none of them showed HPV E6/E7 mRNA expression and also these patients had normal biopsy findings. A recent study done by Zhang et al²⁶ showed that HPV DNA, mRNA, increased with the severity of histopathology diagnosis, from 25.5%, 19.1%, in normal to 100.0% in squamous cell carcinoma, respectively. In our study HPV E6/7 mRNA has higher specificity (100%) and positive predictive value (100%) as compared with HPV DNA. Previous literature also showed that HPV E6/E7 mRNA testing has higher specificity and positive predictive than HPV DNA testing.^{22,27} In our study, out of the total patients who had normal biopsy findings (38), five had HPV DNA positive and none of them had the expression of HPV E6/E7 mRNA showing that E6/E7 mRNA expression is more specific for cervical carcinogenesis as compared with the presence of only HPV DNA. Yao et al also concluded in their study that the HPV E6/E7 mRNA test were highly concordant with cytology test results, and both the diagnostic modalities resulted in similar sensitivity, specificity, PPV, and NPV while identifying HSIL+ lesions.²⁸ Various studies showed that HPV E6/E7 mRNA expression can reflect the stage of HPV infections, and its positive pattern can predict the development direction of CINs, so guiding the accurate treatment protocols for these patients.^{29,30} Nowadays various studies are being done on the detection of HPV E6/E7 mRNA expression by different methods including both quantitative and qualitative. A study

done by Wang et al³¹ evaluated the performance of the Optimygene HR-HPV RT-qDx assay, which is an HPV E6/E7 mRNA-based assay, to detect 16 HR-HPV subtypes, concluded that the higher specificity and positive predictive value of this assay are valuable for predicting insignificant HPV DNA infections among patients with a borderline cytological diagnosis. Recently, a study done by Liu et al³² on quantitative HPV E6/E7 mRNA assay using QuantiVirus HPV E6/E7 mRNA assay (Kodia, Henan, China) to compare the expression levels of HPV E6/E7 mRNA in HSIL and LSIL showed that the median E6/E7 mRNA copy number in HSIL (median, 14,684 copies/mL) was far higher than that in LSIL (median, 936.00 copies/mL) ($p < 0.001$). In our study, when we compared the positivity of HPV E6/E7 mRNA with various risk factors, it has been seen that out of 16 patients positive for HPV E6/E7 mRNA, 56.3% patients were of < 30 years of age, 81.2% had marriage in the age of 18 to 25 years, 62.5% were of para 3, and 75% were using OCPs making them all as risk factors for cervical cancer, as shown by previous studies also.^{33–36} Recently, the American Cancer Society recommends cervical cancer screening for females from age of 25 to 65 years with an HPV-DNA test alone every 5 years. If HPV testing alone is not available, females can get screened with an HPV/Pap co-test every 5 years or a Pap test every 3 years.³⁷ Our data suggest that HPV E6/E7 mRNA detection may be a useful non-invasive tool for the screening of HPV-induced cervical neoplasia. The smaller sample size was the major limitation of our study. Hence, further studies on HPV-E6/E7 mRNA with a large sample size are needed for establishing this diagnostic modality as a routine screening test for ruling out cervical cancer in patients with borderline cytology. The smaller sample size was the major limitation of our study, and also the patients in which only HPV DNA was present without the expression of HPV E6/E7 mRNA were not followed-up further.

Conclusion

The present study suggests that HPV E6/E7 mRNA detection could be more reliable than DNA testing for predicting the risk of progression of HPV-induced cervical lesions to cervical carcinoma as the former had 100% specificity as compared with latter (86.8%). Hence, it can be used as a non-invasive tool for triage and patient follow-up. However, large-scale studies with proper cost factor analysis are further needed to substantiate the findings of our study for better management of this growing menace of cervical carcinoma.

Note

Ethical clearance was taken from institute's ethical committee.

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Conflict of Interest

None declared.

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