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ORIGINAL ARTICLE

Evaluation of Cervical Cancer Screening Tools; INNO-LiPA® HPV Genotyping Extra-II Assay versus E7/E6 oncoproteins, How is reliable and practical?

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ABSTRACT

Background: High-Risk Human papillomavirus (HR-HPV) has been well established as the cervical cancer (CC) risk factor. In recent years, various diagnostic methods of human papillomaviruses (HPV) have been developed to promote sensitivity and specificity of CC screening which leads to a low mortality rate. This study aimed to compare diagnostic test metrics of two HPV diagnostic techniques, including Western blot and INNO-LiPA HPV Genotyping Extra II assay methods in asymptomatic or subclinical patients, among the South-Eastern Iranian women.

Methods: 323 women were referred to the Pathology and Stem Cell Research Center, from February 2018 to January 2020. HPV-DNA with the INNO-LiPA HPV Genotyping Extra-II Assay kit and the western blot assays for HPV E7 and E6 assessment were employed.

Results: Overall, 163 (50.4%) samples were dysplastic pap smear, the specificity of the HPV DNA test by INNO-LiPA HPV Genotyping Extra-II Assay test was significantly higher than the E7/E6 oncoproteins finding (67.3 vs. 49.9%), and the sensitivity was lower (96.6 vs. 74.8%), respectively.

Conclusions: HR-HPV E7/E6 oncoproteins expression was evaluated as a possible novel biomarker for CC screening in pap smear as the preliminary test with satisfactory diagnostic values for HR-HPV types 16 and 18. The corresponding diagnostic values may be further improved by combining HPV DNA tests with the INNO-LiPA HPV Genotyping Extra-II test. Also, they may prove helpful for HR-HPV infection diagnosis in cases that the patients are asymptomatic or subclinical.

Keywords: Cervical Cancer; Human Papillomavirus (HPV); Diagnostic Screening Programs; Oncogene Proteins

INTRODUCTION

Human papillomavirus (HPV) can be classified into numerous categories based on localization in cervical tissue and clinical manifestations. The most distinct categories comprise high-risk HPV (HR-HPV) and low-risk HPV (LR-HPV) genotypes (1). HR-HPV is a conclusive risk factor of cervical malignancies (2). HR-HPV types infection is found to correlate positively with the incidence of precancerous and cancerous cervical lesions, HR genotypes contain HPV types 68, 66, 59, 58, 56, 52, 51, 45, 39, 35, 33, 31, 18 and 16 (3, 4). However, a substantial number of HR-HPV infections have disappeared spontaneously, the chronic infections of HR-HPV genotypes could directly create CC (4, 5). In Iran, the increasing HR-HPV trend is a significant public health challenge, especially where we are in epidemiological transition. Also, Iran faces an unbridgeable generation gap due to wide access to the internet, the closer relationships between genders, and social interactions, despite Islamic culture. Moreover, some Iranian studies indicated that running screening programs over time could considerably decrease the mortality rate of CC (6-8).

Virus genes expressed by HR-HPV are integrated into the genome of host cells, They dramatically impact on infection cells such as the cervical cells, For example, the E7 and E6 genes that are inserted into the genome of the host cell are more likely to produce viral E7 and E6 oncoproteins E7 and E6 oncoproteins might be considered as crucial markers for the distinction of HR-HPV and new biomarkers for CC diagnosis (9, 10). Moreover, with the assist of other molecular tests and cytology diagnosis, CC screening programs could be enhanced, The confirmed procedures for evaluating, HPV-DNA designation by INNO-LiPA HPV Genotyping Extra II, HPV messenger RNA (mRNA) diagnosis, and colposcopy. Nevertheless, because of the low sensitivity of some of the mentioned procedures and the high morbidity of CC, regular testing is recommended (11-13).

Among molecular tests, protein-based methods have more advantages, For example, they are essential for the phenotypic diagnosis of a disease, and some genes present mutations affecting different domains of the related protein causing remarkably different phenotypes, and common proteins can be simply detected by immunohistochemistry and Western blot. Thus, the evaluation of HR-HPV E7 and E6 oncoproteins may be more potent than other agents (14-17). Besides, scant data is available for HR-HPV oncoproteins and to the best of our

knowledge no special comparative studies of oncogene proteins and other molecular methods have been performed in Middle Eastern countries yet. This study aimed to compare the diagnostic metrics of human papillomaviruses detection techniques and also, asymptomatic or subclinical patients detection, by HR-HPV E7/E6 oncoproteins (Western blot), and HPV DNA testing (INNO-LiPA HPV Genotyping Extra II assay) performed among the South-Eastern Iranian women Papulation using cervical fluid cytology samples.

MATERIALS AND METHODS

Study participants and design: The cases were collected for CC screening in South-Eastern Iranian Women. 323 cervical fluid cytology samples, from women aged 15-65 years were collected by gynecologists during routine cervical physical examination, then samples were delivered to the Pathology and Stem Cell Research Center for tests. Exclusion criteria were as follows: previous hysterectomy, vaginal bleeding, symptomatic cervical or vaginal infection, a history of CC, pregnancy, radiation therapy, and using immunosuppressive drugs (18, 19). This project was approved by the ethical board of the Kerman University of Medical Sciences (IR.KMU.REC.1398.552). All the participants filled out the written informed consent before collecting the samples.

Preparation of samples: The liquid-based cytology (LBC), liquid-based Pap test (Ilia Tak Kimia Sahand, Iran), was employed for cytological specimen collection. The gynecologists prepared endo and ectocervical cells with a cytobrush. The cytobrush was placed in the vial, including preservative fluid. The samples were transported at room temperature for analysis at the research center. The cells were separated by shaking the vial containing cytobrush and solution, and then it was centrifuged at 6000 rpm for 5 min. The supernatant was removed and sediment cells were used for cytological examination, DNA and protein were extracted and the smears were prepared on the slide, all slides were stained and examined by two pathologists (20).

LBC test results were recorded based on the Bethesda Gynecologic Cytology Guideline, Bethesda System: 1- Atypical squamous cells: of undetermined significance (ASC-US), cannot exclude HSIL (ASC-H), 2- Low-grade squamous intraepithelial lesion (LSIL) including, HPV/mild dysplasia/CIN 1, 3- High-grade squamous intraepithelial lesion (HSIL) including, moderate and severe dysplasia, CIS; CIN 2 and CIN 3, with appearance doubtful for invasion, 4- Squamous cell carcinoma (SCC) (21).

DNA Extraction and PCR condition: Women's specimens were collected and taken from the cervix using the cytobrush and poured into vials containing 15 ml of the cellular preservative solution by the Gynecologist. The HPV tests are performed by experts. For DNA extraction, DNA purification and amplification MN kits (MACHEREY-NAGEL GmbH, Germany) were used in accordance with the manufacturer's instructions, and the application of DNA extract condensation was measured with NanoDrop 2000c spectrophotometer. (Isogen Life Science, Veldzigt, Netherlands).

HPV-DNA PCR was accomplished applying forward primer 5′-CGTCCMAARGGAWACTGATC-3′ and reverse primer 5′-GCMCAGGGWCATAAYAATGG-3′. The PCR duplication steps included denaturation stage 10 min at 94 ºC, 40 cycles at 94 ºC for 1 min, at 55 ºC for 30 sec, and 72 ºC for 1 min, and final step at 72 ºC for 10 min. In this project, β-actin was used as control to validate the PCR condition. During the PCR test, for every 15 samples, negative (distilled water) and positive control were used to prevent possible contamination and to validate accuracy. For further validation, the PCR products were run on the 2% agarose gel and were visualized by ethidium bromide. The PCR products were visualized by a UV transilluminator (Gel Doc ChemiDoc XRS+ System, BIO-RAD, CA). Additionally, the positive samples were selected for further analysis (22, 23).

Human Papilloma Virus Screening: The HPV-DNA examination was done by the INNO-LiPA HPV Genotyping EXTRA II Assay kit from Fujirebio Europe N.V. Company (Belgium). The laboratory kit's principles were on the essential reverse hybridization. In short, a part of the L1 region called the SPF10, or biotinylated primers, a 65 base pair region of the HPV DNA was amplified by special primers. First, the biotinylated amplimers were denatured and then hybridized with individual probes. Besides, to check the quality of the extracted DNA, the human HLA-DPB1 gene-specific primers were added. Subsequently, streptavidin-conjugated alkaline phosphatase was used then incubated with BCIP/NBT and confirmed by vision (Figure 1) (22).

Western blot assay: The cervical cells were obtained from centrifuged liquid-based cytology. Protein extraction was performed from cells frozen at -80 ºC by adding RIPA buffer cells lysates (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0), which included Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Inc.) [27]. The cells with RIPA buffer were maintained on ice for 30 min and centrifuged at 14,000 g for 15 min at 4 ºC. The protein concentration was measured with the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). For electrophoresis, regarding the molecular weight of E7/E6 proteins (E6: 16.5 kDa band is HPV-18, E6: 17 kDa band is HPV-16, and HPV16 E7, 16 kDa), a total of 25 µg protein was loaded in the wells (10-70 kDa) 12.5% polyacrylamide gels [11]. Anti β-Actin (Sigma-Aldrich, Cat. No. A5441) was applied as an internal control to confirm the identical loading of cell lysates. Immediately after electrophoresis, specimens were moved to PVDF membranes and then blocked with 3-5% skimmed milk diluted in TBS-T buffer. Subsequently, primary antibodies (C1P5; Cat. No. AB70) against the HPV16/18 E6 (HPV16/18 E6 dilution, 1:1000), and antibodies (HyTest, Cat. No. 3HP16) against the E7 proteins of HPV16) HPV16 E7 dilution, 1:1,000) were added and kept overnight at 4 ºC, then by the addition of HRP-labeled secondary antibodies (dilution 1:1,0000), the solution was kept for 1 h at room temperature. Afterward, specimens were identified using chemiluminescence detection kit (Bio-Rad, USA). The ultimate examination was accomplished with boosted chemiluminescence Western blotting detection kit (ClarityTM Western ECL Substrate, Bio-Rad Laboratories), and the images were acquired using the Image Lab

Software version 5.2.1, ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories), (Figure 2) (10, 24).

Statistical analysis: All statistical analyses were conducted using Stata, version 14 (Stata Corp, College Station, TX). Frequencies and percentages were calculated for categorical variables. Comparison among groups was based on percentage values and assessed for statistical significance. A P-value less than 0.05 was considered as statistically significant.

RESULTS

Characteristics of participants are shown in Table 1 by Pap smear status. Pap smear categorization was done by HR-HPV DNA status, if the sample included HR-HPV DNA, it was considered as Dysplasia Pap smear and others as Normal Pap smear. The number of combined detection of E7/E6 protein-positive patients with Dysplasia Pap smear was approximately twenty times more than that of Normal Pap smear patients (121 vs. 6, P<0.0001). By HR-HPV genotype, no participant was positive for E7 protein HPV-18 while 39 women were positive for E7 protein HPV-16 (37 Dysplasia Pap smear and 3 Normal Pap smear, P = 0.008). In addition, E6 protein HPV-16 was higher in Dysplasia Pap smear than Normal Pap smear (32 vs 3, P < 0.0001).

The analogy of diagnostic value of E7/E6 proteins HPV with HPV DNA test (INNO-LiPA HPV Genotyping Extra II assay) about sensitivity and specificity for the diagnosis of Dysplasia Pap smear samples cases is shown in Table 2. The specificity of HPV DNA test was significantly upgraded compared with that of the E7/E6 oncoproteins finding (67.3 vs. 49.9%), however, the sensitivity was lower (96.6% vs. 74.8%).

Diagnostic values of both HPV DNA test and HPV E7/E6 oncoproteins showed, that the sensitivity of HPV DNA test indicator was 100% (95% CI: 96.6%, 100%) and HPV E7/E6 oncoproteins indicator was 87.5% (95% CI: 74.8%, 95.3%). In regard to specificity, the values were 73.7% (95% CI: 67.3%, 79.5%) for HR-HPV DNA and 56% (95%CI: 49.9%, 62%) for HPV E7/E6 oncoproteins. Moreover, using the receiver operating characteristic curve **(**ROC curve) it was estimated that the area under the curve of HPV E7/E6 oncoproteins was 0.718 (95% CI: 0.662, 0.773); in other words, the test was acceptable. As we expected, the area under the ROC curve of HR-HPV DNA was excellent, 0.896 (95% CI: 0.839, 0898), as the gold standard.

In table 3, to explore whether the HPV E7/E6 oncoproteins could play a role in subclinical patients with HR-HPV negative test, we sought positive HPV proteins among negative HR-HPV DNA. Six out of 160 (3.75%) Normal Pap smear patients were positive for HPV E7/E6 proteins. Besides, 42 (39.6%) patients were both HR-HPV DNA and HPV E7/E6 positive among Dysplasia Pap smear.

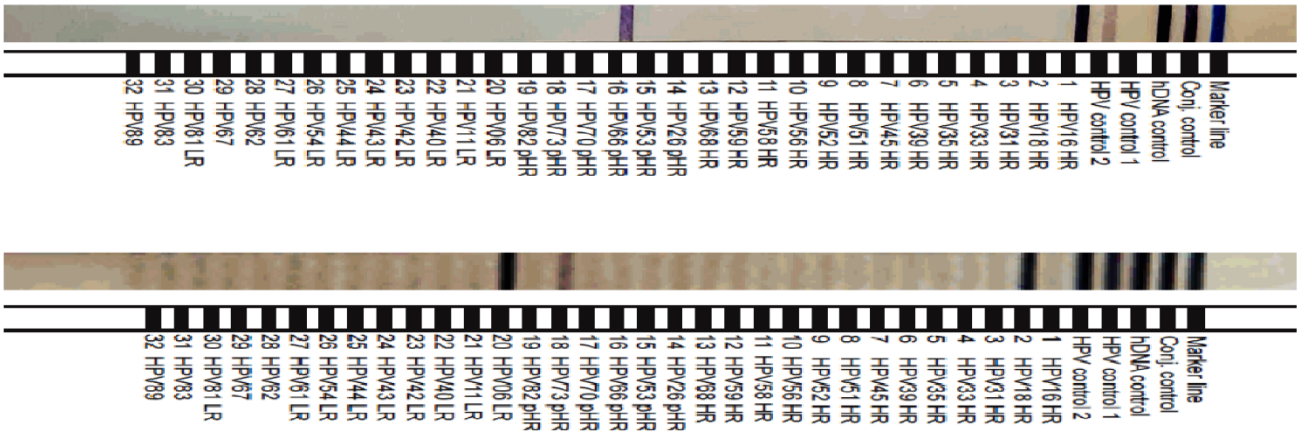

Figure 1. INNO-LIPA HPV PCR genotyping assay strip showing HPV positivity on LiPA strips, displaying separative subtypes that become visible as black bands. black band HPV18, 73 and 06 positive test specimen (on the down strip); black band HPV 16 and 66 positive test specimen (on the above strip). Reading card indicating the location of specific examine on the INNO-LiPA HPV Genotyping Extra II Assay.

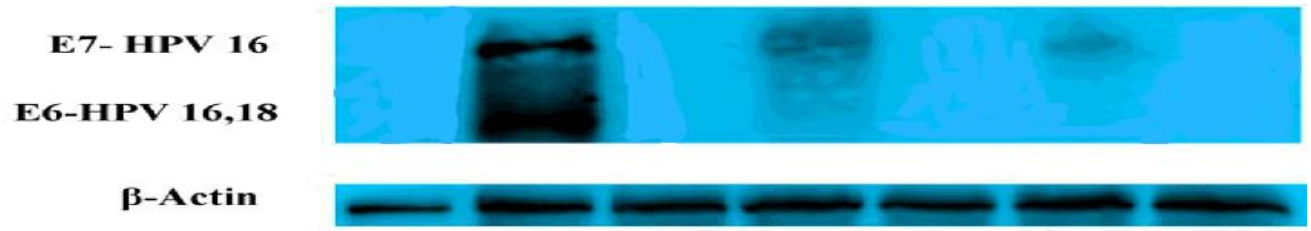

Figure 2. Selective image of HPV16/18 E7/E6 protein detection by western blotting with specific antibodies against E7/E6 proteins and β-Actin. HPV, human papillomavirus. Patients in the Pap test with positive results were detected for western blot analysis.

Table 1. Characteristics of the patients

Characteristic		Dysplasia Pap smear (n=163)	Normal Pap smear (n=160)	P-value
Age (Mean ± SD)		35.49 ± 10.26	32.55 ± 6.61	0.002
Cell abnormalities via Pap-smear, n (%)				
	Normal	-	160 (100)	
Dysplasia Pap smear	ASC	105 (64.42)	-	
	LSIL	53 (32.52)	-	
	HSIL	5 (3.07)	-	
HPV DNA				
Positive		106 (65.03)	0	<0.0001
Negative		57 (34.97)	160 (100)	
HPV16- E6 protein detection				
Positive		35 (21.47)	3 (1.88)	<0.0001
Negative		128 (78.53)	157 (93.13)	
HPV 16-E7 protein detection				
Positive		37 (22.70)	3 (1.88)	<0.0001
Negative		126 (77.30)	157 (98.13)	
HPV 18-E7 protein detection				
Positive		7 (4.29)	0	0.008
Negative		156 (95.71)	160 (100)	
Combined HPV E6/E7 proteins detection				
Positive		121 (74.23)	6 (3.75)	<0.0001
Negative		42 (25.77)	154 (96.25)	

SD, standard deviation; ASC, atypical Squamous Cells; LSIL, Low-grade squamous intraepithelial lesion ; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus

Table 2. Diagnostic value of HPV16-E6/E7, HPV18-E7 protein detection, and HPV DNA detection on Pap-smear samples.

Indicator	HPV type	Triage	Cases	Sensitivity [a],% (95%CI)	Specificity [b],% (95%CI)	[c]PPV [c],% (95%CI)	NPV [d],% (95%CI)
HPV E7/E6 oncoproteins	16 and 18	Western Blotting	323	87.5 (74.8, 95.3)	56 (49.9, 62)	25.8 (19.2, 33.2)	96.3 (92, 98.6)
HPV DNA		INNO-LiPA® HPV Genotyping Extra-II	323	100 (96.6, 100)	73.7 (67.3, 79.5)	65 (57.2, 72.3)	100 (97.7, 100)

[a]Sensitivity=true positive/(true positive + false negative).
[b]Specificity=true negative/(true negative + false positive).
[c]PPV=true positive/(true positive + false positive).
[d]NPV=true negative/(true negative + false negative).
HPV, human papillomavirus; CI, confidence interval

Table 3. Comparison of HR-HPV DNA test and HPV E6/E7 protein detection.

Test status	Dysplasia Pap smear		Normal Pap-smear	
	Positive HPV E6/E7 proteins	Negative HPV E6/E7 proteins	Positive HPV E6/E7 proteins	Negative HPV E6/E7 proteins
Positive HR-HPV DNA	42 (39.62)	64 (60.38)	6 (3.75)	154 (96.25)
Negative HR-HPV DNA	0	57 (100)	0	0

HPV, human papillomavirus

DISCUSSION

Chronic HR-HPV infections are the most common risk factors for the occurrence of cervical cell abnormalities. More than 70% of the sexually active females in the reproduction life span could face one or more HPV genotypes (25). We found that HR-HPV DNA infection was detected in two-thirds of Dysplasia Pap smear patients and less than four percent among Normal Pap smear patients. In detail, oncoproteins (HPV E7/E6 of HPV 16 and 18) could be an acceptable approach to detect HPV infection, although HPV DNA testing was negative. Due to the revealed distribution of HR-HPV strain infection in cervical cancer, most of the potential malignant causes can be detected by the HPV DNA test (INNO-LiPA HPV Genotyping Extra II assay). According to International Agency for Research on Cancer (IARC) analysis, the eight most common HR-HPV DNA genotypes 16, 18, 31, 33, 35, 45, 52, and 58 are detected in 90% of women CC cases (26, 27). The HPV E7/E6 proteins is produced from HPV E7/E6 mRNA, this is because the two stages are very closely related (28). Studies on cervical fluid samples of HPV E7/E6 high-risk oncoproteins remain very scarce, and the load of virus protein cellular cervical fluid sample seems quite low, hence, the findings were mostly based on HPV E7/E6 mRNA (29-31).

In this study, high-risk HPV E7/E6 oncoprotein infections were found in 14.86% of LBC cases, which was a relatively average percentage of high-risk HPV E7/E6 oncoprotein infections. Shi et al. in a study with 450 LBC and biopsy samples from Chinese women patients with suspected cervical intraepithelial neoplasia (CIN) and the histopathologic diagnosis, reported that HPV E7/E6 proteins positive samples were observed in 32.4% of women (10). Specimens from one hundred twenty-eight women in Spain, collected over the course of seven years were tested for E6/E7 mRNA oncogene expression, 68.3% of the samples were HPV E7/E6 mRNA positive (29). In 2013, the test was performed on 554 LBC samples from sexually active females, mRNA of E7/E6 oncoproteins HPV were detected in 55.1% of them (30). In a study to identify HPV E7/E6 mRNA, 400 Italian women age range 20 to 60 years were examined, 18.2% of the results were HR-HPV E7/E6 mRNA positive (32), In a study by Tuney et al. in 2017 females LBC sample with abnormal cervical cytological findings were tested, 55.6% of E7 and E6 HPV mRNA were observed (31). In the study by Doganov et al. in 2012, a complete correlation was detected between the results of the HPV DNA data and HPV E7/E6 mRNA testing (33). According to the sample size and region of study, it seems that our finding was likely different from other's findings, however, LBC samples may well have a lower load of virus oncoproteins, and repeated sampling would be suggested; besides, almost more the samples of other studies mentioned are the combination of LBC and biopsy.

Moreover, the combination of E7 and E6 protein examination could boost the precision of the test. Due to the available evidence, the usage of E7/E6 protein diagnosis in CC screening could overcome the limitations. In addition to these cases, some women will still develop CC despite regular screening, some abnormal cell changes may be missed, and not every unusual cell variation will be detected by other methods such as the Pap test and HPV DNA test (INNO-LiPA HPV Genotyping assay) for the cervical pre-cancerous detection method (34, 35). However, not all types of anti-E7/E6 monoclonal antibodies exhibit high specificity (36, 37); thus, further studies should be performed about the anti-E7/E6 oncoproteins and HPV DNA testing to modify the specificity and sensitivity of CC screening. Clinical studies are needed, with multi-center and more specimens for the efficiency survey of E7/E6 oncoproteins detection as a new indicator for the screening and diagnosis of CC.

CONCLUSIONS

HR-HPVE7/E6 protein expression was evaluated as a possible novel biomarker for CC screening in pap smear as the primary test with satisfactory diagnostic values for HR-HPV types 16 and 18. The corresponding diagnostic values may be further improved by combining HPV DNA tests with the INNO-LiPA HPV Genotyping Extra-II test. Also, they may be helpful for HR-HPV infection diagnosis in cases that the patients are asymptomatic or subclinical.

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