

HUMAN PAPILLOMA VIRUS E6/E7 MESSENGER RNA AS A BIOMARKER FOR DETECTING THE RISK EVALUATION OF CERVICAL CANCER PROGRESSION: OVERVIEW OF RECENT CLINICAL TRIALS

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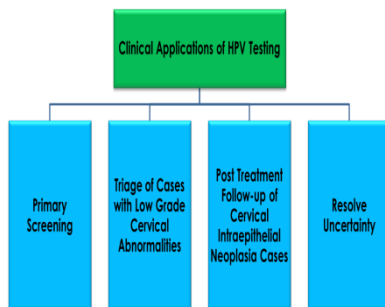
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Graphical abstract



Abstract

Cervical cancer is one of the major women health problems, which is considered to be responsible for significant percentage of cancer related deaths in low and middle income countries. While Human Papilloma virus (HPV) DNA testing has an established role in cervical cancer prevention, there is a need to use other biomarkers with higher specificity and prognostic value to recognize patients at risk of progressive illness. There are evidences suggest that, direct detection of HPV messenger RNA transcripts may establish a more specific method for defining clinically important infection than viral DNA detection. Our objective was to provide an overview of the literature on specificity of HPV mRNA testing compared to DNA testing for detecting the risk evaluation of cervical intraepithelial neoplasia and invasive cervical cancer. We focused on recent clinical studies that support the application of HPV E6/E7 mRNA as a marker in advanced cervical cancer screening program. We provide overview of sample size, recruitment setting, age, HPV mRNA and HPV DNA assays for researches included in our review. The pooled review of clinical studies provided evidence that HPV mRNA might be a relevant diagnostic biomarker but additional studies need to be developed in order to make strong conclusion.

Keywords: Cervical cancer detection, HPV mRNA, biomarker

Abstrak

Kanser serviks merupakan salah satu masalah utama kesihatan wanita yang bertanggungjawab dalam peratusan kematian disebabkan kanser yang signifikan di negara-negara berpendapatan rendah dan sederhana. Walaupun ujian DNA virus papiloma manusia (HPV) terbukti berperanan dalam pencegahan kanser serviks, terdapat keperluan untuk menggunakan penanda bio lain dengan kespesifikan dan nilai prognostik yang lebih tinggi untuk mengesan pesakit-pesakit berisiko penyakit progresif. Terdapat bukti yang mencadangkan bahawa pengesanan secara langsung transkrip RNA pengutus (mRNA) HPV memungkinkan pewujudan kaedah yang lebih spesifik untuk pentakrifan infeksi klinikal yang penting berbanding dengan pengesanan DNA virus. Objektif kami adalah untuk memberikan gambaran keseluruhan literatur berkaitan dengan kespesifikan pengujian mRNA HPV berbanding dengan pengujian DNA untuk mengesan risiko neoplasia intraepitelium serviks dan kanser serviks invasif. Kami fokus pada kajian klinikal terkini yang menyokong aplikasi mRNA HPV E6/E7 sebagai penanda dalam program penyaringan kanser serviks lanjutan. Kami

memberikan di sini suatu gambaran keseluruhan saiz sampel, penetapan perekrutan, umur, asai-asai mRNA HPV dan DNA HPV untuk penyelidikan-penyelidikan yang termasuk dalam ulasan kami. Ulasan kajian-kajian klinikal yang terkumpul membuktikan bahawa mRNA HPV berkemungkinan menjadi penanda bio diagnostik yang relevan tetapi kajian tambahan perlu dihasilkan untuk memberikan kesimpulan yang kukuh.

Kata kunci: Pengesanan kanser serviks; mRNA HPV; penanda bio

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1.0 INTRODUCTION

According to World Health Organization (WHO) report, cervical cancer is the second most diagnosed cancer in women and one of the major causes of morbidity and mortality worldwide, with approximately 530000 new cases each year [1]. More than 85% of death cases occur in developing countries with lower income and poorer hygiene. Human papillomavirus (HPV) is recognized to be leading cause of cervical cancer. It belongs to a group of more than 150 non-enveloped and double-stranded DNA viruses, with 15 high risk (hrHPV) (Types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) and 12 low risk (lrHPV) genotypes. Types 16, 18, 31 and 45 are account for majority of cervical cancers. Among these, types 16 and 18 are collectively associated with majority of cervical squamous cell carcinomas and type 18 is mainly detected in adenocarcinoma of the cervix [2 -4]. HPV infection does not necessarily cause cervical cancer as most of the infections are transient with an average period of 6–14 months and will be cleared rapidly by immune system [4]. Only small number of cases can be progressed to cancer due to presence of persistent infection which is essential for cervical cancer development [5]. Thus, recognition of persistent hrHPV-caused infections is the key target of HPV DNA testing [6]. Cervical cytology techniques including Papanicolaou smear and liquid based cytology have been used as standard methods of cervical cancer screening for around five decades [7]. While Cytology-based tests were able to reduce the mortality rate of cervical cancer by 80%, other techniques with higher sensitivity were still needed since cytological tests were limited with low sensitivity and various sampling methods. Therefore, alternative techniques such as HPV DNA testing have been proposed [8]. hrHPV infection is one of the main risk factors of cervical cancer progression due to inactivation of tumor suppressor genes like RB and p53 genes and allowing the tumors to grow unnoticed and blocking the spontaneous treatment by immune system [9].

Owing to inability of HPV to grow in culture, serological, molecular biology and immunological assays should be used to detect these viruses.

Polymerase chain reaction-based tests are most commonly used methods of HPV DNA detection followed by Hybrid Capture 2 (HC2) [10]. PCR-based assays often based on target amplification with the help of multiplex or consensus primers to amplify a broad range of HPV types that allows differentiation between multiple infections. The Hybrid Capture test is a batch test which detects 13 genital high-risk HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) using a mixture of long synthetic RNA probes. HPV DNA tests detect the presence of the viral genome in the samples taken from patients while RNA tests detect the messenger RNA expression of cancer-related viral genes. Prediction of the risk of cervical cancer would be more accurate by applying HPV E6/E7 messenger RNA techniques compare with HPV DNA testing as mRNA expression profile shows better correlation with the harshness of the lesions. HPV DNA detection methods have no ability to distinguish between persistent and regressive infections therefore these tests are not specific enough for identification of the patients at risk of developing cervical cancer [11]. Overexpression and deregulation of E6 and E7 viral oncogenes is an essential factor for malignant transformation of infected cells following HPV integration in the host genome [12] [6].

For our review, PubMed and Medline libraries were interrogated with language restricted to English and search terms: human papilloma virus, HPV DNA, HPV Ribonucleic acid, HPV mRNA, HPV and cervical cancer, HPV detection methods, HPV mRNA and CIN, sensitivity, specificity and histology. Abstracts and titles were independently screened and the studies which were completely based on technical features rather than sensitivity and specificity were excluded. Final inclusions were based on full articles. Our purpose was to compare the specificity of HPV mRNA versus DNA testing for detecting the risk evaluation of cervical cancer progression. We also described the general clinical applications of HPV testing to gain better insight into the importance of HPV testing in cervical cancer care, followed by overview of the studies which carried out a comparison of HPV DNA and mRNA for their specificity and sensitivity.

2.0 POTENTIAL CLINICAL APPLICATIONS OF HPV TESTING

Astounding progress has been made towards understanding pathology and development of cervical cancer in recent years. Several molecular viral detection tools have been developed due to the important role of HPV as an essential cause of cervical carcinogenesis [13-14]. These methods are basically relying on detection of viral nucleic acids in infected samples taken from patients as HPV cannot be cultured (Table1) [14-15]. Potential clinical applications of HPV tests classified into four categories: primary screening [16-17], triage of cases with low grade cervical abnormalities [17-18] follow up after treatment of CIN [17] [19] and resolution of uncertainty [20] [18].

2.1 Primary Screening

The main goal of cervical cancer screening is to reduce the morbidity and mortality rate of the cancer. Since most cases of the HPV-related benign lesions are transient, it is necessary to detect the exact lesions that are at the greater risk of progressing to invasive cervical cancer and avoid unnecessary treatment and pointless discomfort of the patients. The newest version of various international primary cervical screening cancer guidelines were released at year 2012 [21-22]. Based on latest recommendations, adding HPV testing enhance the sensitivity of screening especially for CIN3+ women and may help to increase the length of intervals. The guidelines are agreed in the point that HPV co-testing is preferred for the women over age of 30 with interval of every 5 years.

Table 1 commercial techniques available for Human Papilloma Virus screening

Techniques	Methodology	Target nucleic acid	Manufacturer
PreTect HPV Proofer	NASBA amplifications via molecular beacons	mRNA	Norchip
NucliSENS Easy Q	NASBA amplifications via molecular beacons	mRNA	BioMérieux
APTIMA HPV Assay	Transcription-Mediated Amplification (TMA),Dual Kinetic Assay (DKA) technology and chemiluminescent	mRNA	Gen-Probe
Hybrid Capture 2	Signal amplification using microplate chemiluminescence	DNA	Qiagen
CareHPV	Signal amplification using microplate chemiluminescence (spin-off of HC2)	DNA	Qiagen
Roche amplicor HPV	PCR-based test with micro-well plate detection	DNA	Roche
Abott real-time HPV	PCR-based with Taqman probe detection	DNA	Abbott
Cervista HPV HR	Signal amplification by invader chemistry	DNA	Hologic
Cervista HPV 16/18	Signal amplification by invader chemistry	DNA	Hologic
MALDI-TOF	Mass spectroscopy with multiplex primary PCR	Type specific DNA	BioMérieux
Cobas 4800 HPV Test	Nucleic acid amplification based on RT-PCR technology	DNA	Roche
HR-HPV 16/18/45 Probe Set Test	Signal amplification using hybrid capture technology	DNA	Qiagen

2.2 Triage of Cases with Low Grade Cervical Abnormalities

Several countries apply HPV testing as one of the triage options for low grade cervical lesions. HPV triage is suggested to show higher sensitivity than

repeated cytology and have an approximate same sensitivity with immediate colposcopy in case of CIN2+ detection. The referral of patients to colposcopy will also be reduced by using this test for patients with ASCUS. On the other hand, because more than 80% of LSIL patients are HPV positive, HPV

testing is not able to reduce their referral to colposcopy. In a recent meta-analysis paper [23], the authors stated that mRNA testing is significantly more specific than HC2 testing for detection of CIN2+ in women with ASCUS and LSIL while the sensitivity is lower and therefore negative mRNA result is not reliable enough to prove the absence of CIN2. Another investigation [24] was set to study the performance of HPV mRNA assay in primary screening and triage of women. Endocervical samples of subject aged 30 to 50 were tested for APTIMA HPV mRNA assay, HC2 and cytology. The sensitivity of HC2 and APTIMA assay for detection of CIN3+ were higher while in case of specificity ThinPrep and APTIMA were significantly better.

2.3 Post Treatment Follow-up of Cervical Intraepithelial Neoplasia Cases

Patients who went through local excision or conization treatments for their cervical lesions should be monitored regularly as these methods do not necessarily eradicate HPV and patients remain at risk of reappearance of the lesions. In meta-analysis of 11 studies, Zielinski *et al.* reported the failure of up to 25% of CIN3 treatments [25]. Therefore early detection of residual or recurrent CIN after treatment of high grade cervical neoplasia is necessary. HPV testing is proven to have similar specificity and higher sensitivity than other follow-up methods. 520 women with negative or low grade biopsies were studied [26] to compare the specificity of E6/E7 mRNA test and cytology for follow-up application. Results reported that mRNA test showed significant higher specificity while the sensitivity was quite same. Persson *et al.* [27] designed an experiment for using L1 DNA and high risk E6/E7 mRNA as biomarkers for detection of residual or recurrent CIN in women treated by conization. HPV linear array and APTIMA assays were used in this study. HPV DNA assay reported to be more sensitive while less specific than APTIMA assay.

2.4 Resolve Uncertainty

Sometimes colposcopy results show patients to be in low risk but with some uncertainty. Since HPV testing has a high negative predictive value it can be useful to resolve the uncertainty of colposcopy results of patients entered in colposcopy referral process. Bowring *et al.* [20] studied 755 women over 12 months, of them two categories showed 90.3% of the resolution of uncertainties. HC2 technique was applied for HPV testing and their findings proved its ability to recognize the patients at higher risks.

3.0 HPV E6/E7 mRNA EXPRESSION AND PROGRESSION OF CERVICAL CANCER

Cell transforming ability of Human papilloma virus relies on over-expression of E6/E7 oncogenes. HPV E6

proteins are able to associate with the cellular tumor suppressor (p53), resulted in degradation and inhibition of its transcriptional regulatory actions. On the other hand, E7 proteins possess the ability to endorse the degradation of pRb (cellular tumor suppressor) through a proteasome-mediated pathway and prevent the gene to bind to the cellular E2F transcription factors, thus inactivate it. Series of immunological responses caused by these actions, lead to formation of malignant phenotype and destruction of cell cycle regulation. Thus, targeting E6/E7 mRNA may result in more trusted outcomes than detecting the presence of viral DNA.

4.0 OVERVIEW OF RECENT CLINICAL TRIALS

4.1 Cross-sectional Studies

Researchers conducted an investigation on 400 women, previously found to be infected with high risk types of HPV, using their cervical specimens for detecting HPV DNA along with RNA [28]. hrHPV DNA detection was performed by liquid hybridization assay. E6 and E7 transcripts of hrHPV RNA were also identified using nucleic acid sequence-based amplification assay (NASBA) and real-time reverse transcription PCR. Findings of this study confirmed that monitoring E6 and E7 expression would be a better way to identify the risk evaluation of patients infected with HPV as the rate of E6 and E7 detection were progressively increased by raising the grade of patient's experiential lesions. In another study, Rijkaart *et al.* [29] applied PreTect HPV-proofer for detecting E6/E7 HPV mRNA and estimating the risk of high grade intraepithelial neoplasia among patients infected with hrHPV DNA. hrHPV RNA found to be higher in cases with more severe cytological abnormalities, as around 63% of women with CIN2 and above have been diagnosed with positive results in compare with 32% in other patients.

In recent years, biomarkers that highlight the occurrence of transformation of HPV infected cells attracted the interest of many scientists around the world. In one experiment, researchers studied the performance of HPV mRNA, HPV DNA and p16INK4a-cytology to detect high grades of cervical dysplasia in patients diagnosed with abnormal cytology [30]. Liquid based cytology specimens of totally 275 women with median age of 36 years were taken. CINtec p16INK4a-cytology assay has been applied for p16INK4a-cytology followed by APTIMA HPV mRNA assay and Hybrid Capture 2 (HC2). Results of the study revealed that there were no great differences in sensitivity of the tests for identification of CIN3+ and CIN2+ patients as all of the tests showed high sensitivity, however specificity were higher in case of APTIMA and CINtec assays. Therefore HPV mRNA and p16INK4a-cytology tests would give more accurate results in compare with HPV DNA detection.

A total of 105 HPV DNA-infected women with abnormal Pap smear test were undergone a study by Salimović-Bešić *et al.* [31]. This research aimed to compare type specific DNA and E6/E7 mRNA based assays for detection of HPV-16, 18, 31, 33, and 45. Human papilloma virus typing was carried out using multiplex real-time PCR. Type-specific real-time NASBA assay was also performed for determination of HPV messenger RNA. Severity of cervical cytology has been found to be in direct relation with increasing hrHPV-caused infections. Lower identification rate of mRNA assay were lower in patients with atypical squamous cells, however the authors recommended to study the role of hrHPV E6/E7 mRNAs in future studies. In another study, Cattani *et al.* [32] tried the possibility of using E6 and E7 transcripts of HPV genome as reliable markers for detection of cervical cancer progression. Total of 180 patients with median age of 35 years underwent cytology and colposcopy tests. Patients diagnosed with suspicious lesions were also subjected to biopsy test. Hybrid Capture 2 system was executed to detect HPV DNA followed by real-time multiplex nucleic acid sequence-based amplification to detect HPV E6 and E7 mRNA. Transcripts of HR HPV types were also detected by NucliSens EasyQ HPV assay. Results of this study indicated the strong association between the level of HPV DNA and RNA detection and grade of the lesion. The sensitivity of HPV RNA and DNA tests were approximately same while RNA test presented higher specificity and positive predictive value which makes it stronger marker for prediction of cervical cancer development than HPV DNA test.

The most important key factor in screening the risk of cervical cancer is to identify the hrHPV positive patients who are at the higher risk of high grade intraepithelial neoplasia (CIN 3) and invasive cervical cancer development. Molecular biomarkers related to HPV can be served as effective tools to improve the success of screening and prediction of predominant cervical lesions. Varnai *et al.* [33] performed a diagnostic and prognostic study on a total of 66 women infected with at least one of HPV 16, 18, 31, 33 and 45 types and cytology results from normal to high-grade squamous intraepithelial lesions. PreTect HPV-proofer used to detect the expression of E6/E7 transcripts from the same HPV types. Cervical situation was also monitored 18 months after the messenger RNA test. The sensitivity of E6/E7 mRNA detection was approximately 100% for prevalent CIN3 lesions screening. Very high negative predictive value was gained for disease development throughout the normal course of HPV infection. Outcomes of this research were indicative of the fact that E6/E7 messenger RNA can be count as a strong predictive marker to increase the efficiency of screening methods.

HPV mRNA and DNA based methods were compared by Lie *et al.* [34], recruiting 383 patients with median age of 35 years and positive cytology

for either squamous or glandular lesions. All patients went through specimen collection with Cervex-Brush. Samples were analyzed by Pap smear and histology tests along with Hybrid Capture II and PreTect HPV-proofer for detection of HPV. Some of the samples were also used for PCR and sequencing techniques. HPV mRNA was detected in all the cases with invasive carcinoma and DNA or mRNA of high-risk Human papilloma virus types were present in 90% of samples. Histologically confirmed high grade lesions (CIN2+) were found to be positive for HPV DNA in 275 and HPV mRNA in 225 out 291 patients. Results of this study suggested that these HPV tests are able to improve the detection rate of high-grade cervical neoplasia while mRNA test is more suitable for cervical cancer risk assessment.

Human papilloma virus DNA and messenger RNA detection were compared by Moldet *et al.* [35] in a cross sectional study of 4136 women. Women were subjected to 2 years follow up for high-grade squamous intraepithelial lesions histologic confirmation during the research and results were compared to results of the study. Samples were collected using Cervex Brush and HPV DNA detection was performed by consensus Gp5+/6+ PCR and HPV type specific PCR for HPV types 16, 18, 31, 33 and 45. Besides, PreTect HPV-proofer test was used for HPV mRNA detection. A total of 166 women were diagnosed with cytological abnormality by Pap smear test. The specificity and positive predictive value of Gp5+/6+ consensus PCR for detection of cytological HSIL was found to be 90% and 3.7% respectively in compare with 97.3% and 10.3% for HPV mRNA detection using PreTect HPV-Proofer. In case of histologic CIN2+ detection sensitivity and positive predictive value of Gp5+/6+ consensus PCR were also found to be 66.7 and 81.3 respectively compared with 88.9% and 92.3% for PreTect HPV-Proofer. The study results concluded that HPV mRNA detection would be a promising method. In a recent study by Liverani *et al.* [36] the authors assessed detection of HPV mRNA transcripts of HPV types 16,18,31,33 and 45 along with frequency of HPV DNA genotypes in 134 patients diagnosed with cervical intraepithelial neoplasia CIN 2+. 85.8% (n=115) of cases were found to be infected with hrHPV types, of which 68 were positive for HPV DNA and 50 were positive for HPV mRNA of five most common carcinogenetic HPV types. Based on their study results, the authors theorized that the replication stage of hrHPV may be revealed by mRNA testing other than its physical rate.

In a paper by Sotlar *et al.* [37] cervical scrapes sample of patients were examined for detecting oncogene spliced/unspliced E6/E7 transcripts of 14 high risk HPV types by nested RT-polymerase chain reaction. HPV DNA detection was carried out on 1699 samples by nested multiplex PCR and out of these 799 high-risk HPV DNA positive samples used for mRNA detection. E6/E7 detection was visibly increased with severity of lesions, with 18%, 58%, 77%

and 84% from no CIN to CIN3. The sensitivity of using hrHPV E6/E7 mRNA as biomarker for detection of CIN lesions was lower than HPV DNA. In contrast HPV mRNA showed higher specificity rate. The results of previous study was supported by the work of same researcher [38] who stated that, detection of hrHPV E6 and E7 mRNA transcripts, improves the specificity of cervical intraepithelial neoplasia screening. Nested RT-PCR was again used as a tool to detect E6 and E7 oncogenes in cervical samples of 779 hrHPV-DNA-positive patients, diagnosed with all grades of CIN. The results were same to previous study, as HPV E6 and E7 mRNA prevalence were increased by increasing the level of CIN. Specificity and positive predictive values were also higher in case of HPV mRNA than HPV DNA detection.

CIN 2+ detection in women younger than 30 years old mainly depends on cytology due to the fact that transient HPV infections were shown to be higher in this group [39-40]. In order to overcome this issue, Pierry *et al.* [40] tested intracellular HPV E6, E7 mRNA quantification for prediction of high-grade cervical intraepithelial using cytology specimens of 3133 women aged 19-75 years. Their findings demonstrated no huge difference between the sensitivity of E6/E7 mRNA quantification and abnormal cytology for CIN2+ and CIN3+ detection while the specificity was significantly higher by E6/E7 mRNA detection. In a study which set out to use increased levels of HPV16 E6*1 mRNA as a diagnostic biomarker to detect CIN2+, Kosel, *et al.* [41] analyzed cervical samples of 301 HPV16 positive women with normal cytology, ASCUS, mild-to-moderate or severe dysplasia to quantify the levels of HPV16 DNA and HPV16 E6*1 mRNA using real time PCR. Levels of HPV16 E6*1 mRNA transcripts were significantly correlated with severity of the abnormality. Positive predictive value of detecting CIN2+ was also considerably higher with increasing the level of detected transcripts.

A recent study by Liu *et al.* [42] involved applying QuantiVirus®HPVE6/E7 mRNA test on cervical cytological samples of 335 patients to identify CIN2+. Severity of the cytological and histological diagnosis was shown to be positively correlated with prevalence of HPV E6/E7 mRNA. Castle *et al.* [43] reaches same conclusions, finding carcinogenic HPVE6/E7 mRNA as a potential biomarker to detect cervical pre-cancer and cancer as they found an enhancing probability of testing positive for HPVE6/E7mRNA with increasing intensity of cytology and histology. Waldstrom and Ornskov [44] discussed about the clinical performance of linear array HPV genotyping (LA) (HPV DNA detection) and APTIMA HPV assay (HPV mRNA detection) on consecutive PreservCyt samples of women with atypical squamous cells of undetermined significance (ASC-US). The specificity and positive predictive value of mRNA detection was shown to be significantly higher in CIN2+ and CIN3+ whereas DNA detection exhibited higher sensitivity in case of CIN2+. Data

from Kottaridi *et al.* [45] study have identified the clinical performance of flow cytometric detection of HPV mRNA versus HPV DNA array technology for detection of high grade cervical lesions. Liquid based cytology samples of totally 189 women with confirmed histology were screened for presence of HPV mRNA and DNA. The specificity of HPV mRNA detection by OncoTect assay was shown to be significantly higher than HPV DNA detection, thus the authors stated that mRNA detection with flow cytometry can be a powerful tool for detection of high grade lesions which is able to reduce the unnecessary referral of the patients for colposcopy.

4.2 Prospective Cohort Studies

77 women diagnosed with atypical cells of undetermined significance (ASCUS) and low-grade squamous intraepithelial lesion (LSIL), joined to Norwegian cervical cancer screening program (NCCSP) for 2-year follow-up study performed by Molden *et al.* [46]. The aim of this research was to predict cervical intraepithelial neoplasia (CIN) 2+ by detection of HPV mRNA and DNA with Gp5+/6+ consensus PCR and PreTect HPV-Proofer. The sensitivity of both tests for diagnosing CIN2+ were around 85.7% during 2 years of follow up. However PreTect HPV-proofer presented higher specificity. The overall outbreak of HR-HPV (types 16, 18, 31, 33 and 45) mRNA was shown to be 23.4% which was nearly half of HPV DNA (54.6%). Although the overall detection rate of HPV mRNA was lesser, most of the histological CIN2+ cases were detected during the follow-up. The consequences of this research recommend that, women with positive ASCUS/LSIL Pap smear and positive mRNA should go through more follow ups than women with negative mRNA tests. In another investigation [47], mRNA detection was performed on 53 women with normal cytology and positive HPV DNA results. Cytology assessment and HPV genotyping were repeated after 2 or 3 years. The results of study indicate higher possibility of persistent infection for women who were mRNA positive at the baseline.

5.0 IS RNA A SUITABLE BIOMARKER

One of the limitations of working with mRNA is the instability of RNA as a single stranded moiety in compare with DNA, as results of Castle *et al.* [48] study on archived liquid-based cervical specimens of 9205 sexually active women suggested that, HPV DNA in exfoliated cervical cells can be well preserved in particular types of liquid based cytology medium.

Waldstrøm and Ørnskov [49] tested the sensitivity and specificity of HPV mRNA test (APTIMA HPV mRNA assay) on remaining supplies of 3 year old PreservCyt low grade squamous intraepithelial lesion samples of 405 women. They achieved 92.5% and 38.2% of

sensitivity and specificity of messenger RNA assay respectively for identifying CIN2+ followed by 93.9% and 35.5% for CIN3+. Their findings suggested that using mRNA would be an effective method for identifying patients with low grade squamous

intraepithelial lesions. Cuschieri et al. [50] were also performed an investigation on evaluation of HPV mRNA detection in liquid based cytology cervical specimens and checked the stability of RNA over 14 days of sample collection.

Table 2 Summaries of published clinical trials

Authors	Year	Country	Total sample size	Age range (mean)	Recruitment setting	HPV DNA test	HPV mRNA test
Cattani et al.	2009	Italy	400	21-59	hrHPV DNA infection	Liquid hybridization	NASBA and real-time RT-PCR
Rijkaart et al.	2012	Netherlands	375	30-60	Normal cytology, borderline, mild, moderate or worse dyskaryosis	GP5+/6+ PCR	PreTect HPV-Proofer
Reuschenbach et al.	2010	Germany	275	28-44 (36)	Abnormal cytology or follow-up after previous treatment for cervical dysplasia	HC2	APTIMA HPV assay
Salimović-Beršić et al.	2013	Bosnia and Herzegovina	105	19-62	Positive hr HC2 or Abbott Real-Time hrHPV screening, abnormal Pap smear	Multiplex real-time PCR	Type-specific real-time NASBA
Cattani et al.	2009	Italy	180	20-77 (35)	secondary screening	HC2	NucliSens EasyQ HPV assay
Varnai et al.	2008	Hungary-Germany	66	—	Normal to high-grade SIL at baseline, hrHPV DNA positive	MY09/MY11 and GP5+/6+ PCR	PreTect HPV-Proofer
Lie et al.	2005	Norway	83	19-85 (35)	Squamous or glandular lesions	HC2	PreTect HPV-Proofer
Molden et al.	2005	Norway	77	>30	ASCUS and LSIL Pap smear	Gp5+/6+ consensus PCR	PreTect HPV-Proofer
Molden et al.	2005	Norway	4136	30-69	LSIL and HSIL	Gp5+/6+ PCR and HPV type specific PCR	PreTect HPV-Proofer
Liverani et al.	2012	Italy	1113	(36.7)	CIN2 and above	PCR	PreTect HPV-Proofer
Sotlar et al.	2004	Germany	1699	—	All grades of CIN	Nested multiplex PCR	Nested RT-PCR
Sotlar et al.	2005	Germany	779	—	hr-HPV positive exhibiting all grades of CIN	Nested multiplex PCR	Nested RT-PCR
Piery et al.	2012	USA	3133	19-75	LSIL, HSIL, ASCUS and positive for HPV DNA	—————	HPV OncoTect
Kosel et al.	2007	Germany	301	17-57 (31 ± 7.7)	HPV 16 positive	RT-PCR	RT-PCR
Liu et al.	2014	China	335	—	Abnormal cytology, atypical squamous cells of unknown significance or higher, or a positive HPV DNA test	QuantiVirus®HPV DNA diagnostic kit	QuantiVirus® HPV E6/E7 mRNA test
Castle et al.	2007	USA	540	—	Normal, ASC, LSIL and HSIL cytology or worse.	LA, HC2	APTIMA HPV assay
Waldstrom and Ornskov	2011	Denmark	369	>30	Women with cytological diagnosis of ASCUS	LA	APTIMA HPV assay
Kottaridi et al.	2011	Greece	189	21-65	Women with histologically confirmed lesions	HPV DNA array	OncoTect assay

LBC samples were taken in PreservCyt® from 7 women with cytological report of moderate or severe dyskaryosis RNA extraction has been done at 6 hours (baseline) and 4, 7 and 14 days after collection of samples. PreTect HPV Proofer used for detection of HPV mRNA with human U1 small ribonucleoprotein (U1A mRNA) as a control. Linear array HPV DNA genotyping was also performed with the aim of detecting HPV types other than 16, 18, 31, 33 and 45 that women may have been infected with. 5 out of 7 samples were mRNA positive from the base line (four samples with HPV type 16 and one sample with HPV type 45) up to 14 days. 2 remaining samples were found to be HPV mRNA negative. Study conclusions stated that RNA can be detected in normally collected LBC specimens for at least 14 days.

In another experiment performed on liquid based cytology specimens, Tarkowski *et al.* [51] tried to improve detection of isolated viral RNA by seeding serial dilutions of a human papillomavirus 16 positive cell lines on PreservCyt medium. Their findings showed that this method is suitable to produce appropriate HPVE6/E7 transcripts after 1 year of storage. Molecular quality of routine exfoliated cervical cytology has been checked for the effect of different sample collection methods and mediums on the yield of RNA in the study by Habis *et al* [52]. Various collection mediums included different tissue culture medium, high salt solutions, cationic detergent-based surfactant, alcohol-based collection fluids and MTM along with 2 different commercial extraction kits (Master Pure Complete DNA and RNA Purification and RNawiz) have been used. The outcome of this examination revealed that collection medium had the highest effect on the quantity and quality of RNA as PAXgene and methanol-based PreservCyt which are widely used in clinical settings showed higher yield of RNA compared with other mediums.

One of the most critical points in detecting the risk evaluation of cervical cancer progression is to grade cervical lesions. Misdiagnosis of the lesions may results in over or under-treatment of the patients which can be life threatening. Evans *et al.* [53], applied CIN/SIL grading by using hrHPV E6/E7mRNA chromogenic in situ hybridization (CISH) signal patterns as biomarkers and RNAscope assay as a new method of detection. Their findings support the ability of the hrHPV E6/E7mRNA CISH as a biomarker for CIN histological grading.

6.0 CONCLUSION

Over the years scientists intend to use their best efforts to find novel relevant biomarkers and improve the efficacy of cervical cancer screening. There are number of recent overviews available [54-58]. The current review tried to describe E6/E7 mRNA

expression as a biomarker of transforming viral infection. It does not required highly specialized stages and can be easily adapted to future operations. There are studies and evidences suggested that HPV mRNA might be a relevant diagnostic biomarker but if the debate is to be moved forward, additional studies and more clinical data needs to be developed in order to make strong conclusion.

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