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ORIGINAL PAPER

Diagnostic and prognostic validity of the human papillomavirus E6/E7 mRNA test in cervical cytological samples of HC2-positive patients

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Abstract The study aimed to assess the clinical utility in identifying CIN2 or worse (CIN2+), of the Pretect HPV-Proofer test for E6/E7 mRNA detection in Hybrid Capture 2 (HC2)-positive patients, who underwent colposcopy. In particular, the study analyzed the mRNA test performance as the third test in a subgroup of HC2+ patients with less severe than high-grade squamous intraepithelial lesions (HSIL−). We analyzed 464 cervico-vaginal samples by liquid-based cytology (LBC) and PreTect HPV-Proofer. Moreover 231 patients also had a biopsy at baseline and 75, with HSIL−, were followed up within 2 years by LBC, colposcopy, and histology when indicated. The highest sensitivity for CIN2+ belonged to the mRNA compared to LBC, at the HSIL+ threshold (72% vs. 58%), whereas the LBC showed the highest specificity and positive predictive value (PPV) (99 and 93% vs. 73 and 39%, respectively). Focusing on the 408 HSIL− patients, the mRNA positivity was significantly more associated with CIN2+ than CIN2− lesions ($p < 0.0001$). Moreover, among the 75 HSIL− followed up patients, the mRNA displayed high longitudinal Specificity (89%), even if the sensitivity and the PPV were low (50 and 20%, respectively). The present data suggest that the mRNA test may have a diagnostic and a potentially prognostic role in HC2+/HSIL− patients.

Keywords Cervical lesions · Diagnostic biomarker · HPV E6/E7 mRNA · Human papillomavirus · Prognostic biomarker

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Introduction

High risk (HR) human papillomavirus (HPV), the most common sexually transmitted infection, has been identified as the major cause of cervical cancer [1]. Scientific evidences exist which recommend HPV DNA testing in the triage of women with equivocal cytology [2] or in the primary cervical screening program of women aged 30 and above [3]. The combination of cytology and HPV DNA testing significantly increases the sensitivity of screening programs [4, 5]. However, at least for women <30, a number of HPV infections are usually diagnosed in the healthy population [6]. Furthermore, in patients with abnormal cervical smears, HPV infections may easily clear and cellular abnormalities may disappear [7]. Only a few HPV-infected women, namely those with HR HPV persistent infection, will give rise to high-grade cervical lesions with an enhanced risk of developing cervical carcinoma [8].

Several ongoing studies are developing screening algorithms, using the HPV DNA testing for the primary screening, followed by cytology as triage test [4, 9]. Moreover, one of the major issues concerning this algorithm is the clinical management of HPV-positive/cytology-negative women.

In this scenario, there is a need to introduce novel additional biomarkers in cervical screening programs, capable of enhancing the overall diagnostic accuracy and to predict the risk of progression of low-grade lesions with greater accuracy. It has been widely reported that the detection of viral gene expression could potentially be useful in the identification of clinically relevant infections [10, 11]. When progression to a precancerous state occurs, a fact that has been amply demonstrated in several studies [12, 13], the E6 and E7 oncogene transcripts, which are usually expressed at low levels during the normal productive HPV infections, are overexpressed throughout the full epithelial thickness of the precancerous high-grade lesions. In particular, E6 and E7 mRNA have recently been found to increase together with severity of the cervical disease [14] and might be useful as marker for potentially progressive HR HPV infections [13, 15]. In this context, robust commercially available assays have been developed and some heterogeneous studies have been conducted to determine the sensitivity and specificity of this novel biomarker [12, 13, 16].

In this cross-sectional study, we analyzed the E6/E7 mRNA expression of the carcinogenic HPV types 16, 18, 31, 33, and 45 in the cervico-vaginal samples of a series of 464 HPV DNA-positive patients. In particular, we focused on patients with less severe than high-grade squamous intraepithelial lesion (HSIL) cytology, in order to investigate the utility of the mRNA test in a putative double triage protocol of DNA HPV-positive patients. The first triage step was performed by means of cytology and the second triage step by means of the mRNA test. Moreover, we assessed the mRNA test prognostic value in women with less severe than cervical intraepithelial neoplasia (CIN) 2, in order to predict the onset of histological high-grade lesions during a 2-year follow-up.

Patients and methods

Study design

We analyzed a consecutive series of cervico-vaginal liquid-based scrapings, collected from February 2006 to June 2009 from a large series of outpatient women at the Oncologic Gynecology Department of the Regina Elena Cancer Institute. The patients were enrolled on the basis of a Hybrid Capture 2 (HC2)-positive test performed in the Regina Elena Pathology Department and on the basis of a colposcopic report, obtained within 2 months of the cervico-vaginal sampling. All enrolled patients had an mRNA test and liquid-based cytology (LBC) on the same sample. Overall 464 patients (median age 32 years, range 17–78 years) with a valid mRNA test were included in this study.

We excluded patients who were previously treated for CIN, with a previous hysterectomy, who underwent chemo and/or radiotherapy for cervical carcinoma. Of the 464 patients, 231 underwent biopsies on colposcopically suspicious areas. It was assumed that the remaining 233 women who had undergone a colposcopy but no biopsy had less than CIN2.

Moreover, 75 women with less severe than HSIL were followed up from a minimum of 6 months to a maximum of 25 months (mean 11.7 $\pm$ SD 4.9; median 11 months), undergoing cytologic and colposcopic tests. A biopsy on suspicious areas was performed in 20 patients.

The study was reviewed by the Ethics Committee of the Regina Elena Cancer Institute, and a written informed consent was obtained from all patients.

Hybrid Capture 2

Cervico-vaginal samples were taken by cytobrushes (Hologic, Italy) and plastic Ayre's spatulas (Hologic), according to the manufacturer's instructions, and stored in the PreservCyt solution (Hologic) at 4°C until use.

Testing for HR HPV DNA was performed by means of the HC2 technique (Qiagen, Italy), a semi-quantitative assay for the detection of 13 HR HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), as described by the manufacturer. Before the HC2 test, 4 ml of PreservCyt solution was processed by means of the HC2 Sample Conversion Kit (Qiagen). The positive cut-off (CO) value was considered the mean of the positive control samples. The results were considered positive when the ratio between the relative-light units of the sample (RLU) and the positive CO (RLU/CO) was 1 or greater.

Morphological tests: cytology and histology

The LBC was performed using the Thin Prep 2000 System (Hologic).

All cytological slides were evaluated independently of the results of the other assays and blindly by two experienced cytopathologists (AV and MB). Cytological slides with contrasting interpretations were reviewed, and a consensus diagnosis was obtained. The smears were classified according to the 2001 Bethesda System Criteria [17]. The reports of negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of unknown significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL) are herein referred to as less than HSIL (HSIL−); the reports of HSIL and squamous cervical carcinoma (SCC) are herein referred to as HSIL+.

The histological slides were diagnosed according to the WHO classification. All histological slides were evaluated independently of the results of the other assays and blindly by two experienced pathologists (FM and MC). Histological slides with contrasting interpretations were reviewed, and a consensus diagnosis was obtained. The benign cases and mild dysplasia/CIN1 diagnoses are herein referred to as less than CIN2 (CIN2−). The diagnoses of moderate dysplasia/CIN2 ($n = 49$), severe dysplasia/CIN3/carcinoma in situ ($n = 31$), and SCC ($n = 9$) are herein referred to as CIN2+. Histology was regarded as the "gold standard."

E6/E7 mRNA detection

A total of 5 ml of PreservCyt solution was used for the detection of E6/E7 mRNA of HPV types 16, 18, 31, 33, and 45 by means of the PreTect HPV-Proofer Kit (referred to as mRNA test) (Biomérieux, Italy) within 14 days of sample collection, according to the insert instructions. mRNA was extracted from cervical samples using NucliSENS miniMAG Magnetic Extraction (Biomérieux). The PreTect HPV-Proofer technology utilizes an isothermal nucleic acid sequence–based amplification (NASBA) that amplifies mRNA in a DNA background, detecting and genotyping HPV transcripts in the same reaction. The amplified products are detected in real time using fluorescent-labeled molecular beacon probes directed against full-length E6/E7 mRNA. Accumulated mRNA fluorescent profiles are analyzed and ascribed with positive/negative status by means of the supplied PreTect analysis software. Human U1 small ribonucleoprotein (U1A mRNA) is used as RNA integrity/adequacy control.

Statistical analyses

Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the relative 95% confidence intervals (CI) for detecting CIN2+ of mRNA and LBC were calculated.

The Pearson's Chi-square test was applied for all comparisons between proportions.

In order to assess the association between the histological diagnosis of CIN2+ and the expression of mRNA, we used unconditional logistic regression.

Odds ratios (OR) and their relative 95% CIs were reported.

Furthermore, we calculated the performance indicators for mRNA test in detecting CIN2+ in patients with at least 6 months of follow-up.

All tests were two–sided, and p values <0.05 were considered to be statistically significant.

All statistical analyses were performed with SPSS statistical software version 17.0 (SPSS inc., Chicago IL, USA).

Results

mRNA test results and LBC cervico-vaginal samples

Table 1 displays the distribution of mRNA positivity according to the cytological indexes. Of the 464 samples, 56 were classified as HSIL+ (48 HSIL and 8 SCC), whereas 408 were classified as HSIL− (191 NILM, 110 ASC-US, and 107 LSIL). Overall, 165 out of 464 samples (36%) were mRNA positive. Of these, 46 out of the 56 HSIL+ cases (82%) displayed a positive mRNA test, whereas only 119 among the 408 HSIL− cases (29%) were positive at the mRNA test. The statistical analysis evidenced a significant difference in the mRNA test positivity between the two index categories: P (X^2) < 0.0001, with an OR = 11.2, 95% CI [3.2–19.2] $p < 0.0001$.

Frequency of HPV genotypes detected by PreTect HPV-Proofer test

The frequency of each genotype detected by the PreTect HPV-Proofer in the 165 patients who had a positive test is represented in Table 2. Briefly, genotype 16 showed the highest frequency (68%), followed by genotypes 18 (9%), 45 and 33 (each 8%) and by genotype 31 (5%). In addition, the table displays the genotype distribution in CIN2−, CIN2, CIN3, and SCC.

Four cases of coinfections were observed: 3 patients harbored two genotypes and only one patient harbored three genotypes.

mRNA test and cytological indexes relationship with histological diagnoses

As summarized in Table 3, the histological diagnoses recorded 89 CIN2+ cases (9 SCC, 31 CIN3 and 49 CIN2)

Table 1 Association between mRNA assay and cytological indexes in 464 LBC cervico-vaginal samples

LBC			
	HSIL+	HSIL−	Total
mRNA			
Positive	46 (82%)	119 (29%)	165 (36%)
Negative	10 (18%)	289 (71%)	299 (64%)
Total	56 (100%)	408 (100%)	464 (100%)

P (X^2) < 0.0001; OR = 11.2 95% CI [3.2–19.2] $p < 0.0001$

Table 2 Frequency of HPV genotypes, as single and multiple infections, detected by mRNA test in the 165 patients with a positive mRNA test, stratified by histological diagnosis

Genotype	Overall (%)*	CIN2− (%)	CIN2 (%)	CIN3 (%)	SCC (%)
16	111 (68)	69 (69)	19 (56)	19 (83)	4 (50)
18	15 (9)	7 (7)	5 (15)	2 (8.5)	1 (12.5)
45	14 (8)	9 (9)	4 (12)	2 (8.5)	0 (0)
33	14 (8)	9 (9)	4 (12)	0 (0)	1 (12.5)
31	7 (5)	5 (5)	2 (6)	0 (0)	0 (0)
16, 33	2 (1)	1 (1)	0 (0)	0 (0)	1 (12.5)
16, 45	1 (0.5)	0 (0)	0 (0)	0 (0)	1 (12.5)
18, 31, 45	1 (0.5)	1 (1)	0 (0)	0 (0)	0 (0)
Total	165 (100)	101 (100)	34 (100)	23 (100)	8 (100)

and 375 CIN2−. Among the 89 CIN2+ cases, 64 (72%) displayed a positive mRNA test. In contrast, among the 375 CIN2− cases, only 101 (27%) displayed a positive mRNA test.

Concerning cytological indexes, 52 out of the 89 CIN2+ (58%) were HSIL+ and only four out of the 375 CIN2− (1%) were recorded as HSIL+. The accuracy indicators are calculated according to dichotomous histological classification CIN2+ vs CIN2−, and comparing the accuracy of the mRNA test with that of LBC, we observed that the mRNA sensitivity in detecting CIN2+ was significantly higher than that obtained by cytology (72% vs. 58%; $p < 0.0001$). Whereas cytology appeared to be the most specific test (99%). Nevertheless, the mRNA test displayed a good specificity (73%). mRNA PPV was significantly lower than cytology (39% vs. 93%; $p < 0.0001$), and NPV was quite similar for mRNA and cytology (92% vs. 91%; $p = 0.585$).

Table 4 mRNA assay results and histological diagnoses in patients with HSIL− index

Histology					
	CIN2+			CIN2−	Total
	SCC	CIN3	CIN2		
mRNA					
Positive	0	6 (55%)	15 (58%)	98 (26%)	119
Negative	0	5 (45%)	11 (42%)	273 (74%)	289
Total	0	11 (100%)	26 (100%)	371 (100%)	408

P (X^2) < 0.0001 is calculated according to dichotomous histological classification CIN2+ vs CIN2−; OR = 3.6; 95% CI [1.8–7.3]

mRNA findings in 408 HSIL− patients according to histological diagnosis

To better investigate the performance of the mRNA assay and its possible clinical application, we analyzed the data concerning the group of 408 HSIL− patients. As shown in Table 4, the Pretect HPV-Proofer assay identified E6/E7 mRNA in 21 out of the 37 CIN2+ women (57%). In contrast, in the group of 371 CIN2− patients, the mRNA was positive only in 98 cases (26%). Therefore, in HSIL− patients, mRNA test positivity was significantly higher in the CIN2+ than in the CIN2− category (P (X^2) < 0.0001, calculated according to dichotomous histological classification CIN2+ vs CIN2−).

Moreover, an unconditional logistic regression model evidenced that a woman with a positive mRNA test was 3.6

Table 3 mRNA assay and cytological indexes: relationship with histological diagnoses

Histology§					
	CIN2+			CIN2−	Total
	SCC	CIN3	CIN2		
mRNA					
Positive	8 (90%)	22 (71%)	34 (69%)	101 (27%)	165 (36%)
Negative	1 (10%)	9 (29%)	15 (31%)	274 (73%)	299 (64%)
Total	9 (100%)	31 (100%)	49 (100%)	375 (100%)	464 (100%)
LBC					
HSIL+	9 (100%)	20 (65%)	23 (47%)	4 (1%)	56 (12%)
HSIL−	0 (0%)	11 (35%)	26 (53%)	371 (99%)	408 (88%)
Total	9 (100%)	31 (100%)	49 (100%)	375 (100%)	464 (100%)

§ The accuracy indicators are calculated according to dichotomous histological classification CIN2+ vs CIN2−

Sensitivity mRNA: 72% [63–81]*, LBC: 58% [48–69]*, $p < 0.0001$

Specificity mRNA: 73% [69–78]*, LBC: 99% [98–100]*, $p < 0.0001$

Positive predictive value mRNA: 39% [31–46]*, LBC: 93% [86–100]*, $p < 0.0001$

Negative predictive value mRNA: 92% [87–96]*, LBC: 91% [83–99]*, $p = 0.585$; *95% confidence interval

Table 5 mRNA test at baseline and diagnosis at 6–25 months of follow-up in 75 HSIL− patients

mRNA Baseline	Histological diagnoses at follow-up		
	CIN2+	CIN2−	Total
Baseline			
Positive (%)	2 (50%)	8 (11%)	10
Negative (%)	2 (50%)	63 (89%)	65
Total	4 (100%)	71 (100%)	75

Specificity: 89 [81–96]*; Sensitivity: 50 [10–99]*; PPV: 20 [4–56]*; NPV: 97 [86–100]*; *95% confidence interval

times more likely to be diagnosed as CIN2+ at histological diagnosis than a mRNA-negative woman (OR = 3.6 95% CI [1.8–7.3]

Prognostic value of mRNA test in HSIL− patients

Among the 371 HSIL−/CIN2− patients, 75 were submitted to a follow-up (6–25 months). As shown in Table 5, four patients developed a histologically confirmed CIN2+ during the follow-up (after 10, 12, 17, and 24 months, respectively). Two out of the 4 CIN2+ patients (50%) presented a positive mRNA test (genotypes 16 and 18, respectively) at baseline. Among the other 71 patients who did not progress to high-grade lesions (55 with a cytologic and 16 with a histologic record), only 8 (11%) had a positive mRNA test at baseline. When we calculated the mRNA longitudinal sensitivity, specificity, and negative and positive predictive values, we found a high specificity and NPV (89 and 97%, respectively), even if the sensitivity and the PPV are only 50 and 20%, respectively. However, due to the low number of patients in the CIN2+ subgroup, these findings should be interpreted with caution (Table 5).

Discussion

Our study presents the assessment of the mRNA test's role in cervico-vaginal scrapings, in order to evaluate its clinical usefulness in identifying patients with or prone to develop a CIN2+ lesion, among HPV DNA-positive women. The PreTect HPV-Proofer, for the detection of E6/E7 mRNA of HPV types 16, 18, 31, 33, and 45, was utilized to analyze 464 HC2-positive patients, independently of cytological findings. The ratio between HC2 and mRNA test positivity is in line with the current literature [18, 19] as is the percentage of mRNA test positivity in high-grade cytological lesions [19, 20]. In our series, we firstly compared the performance of the PreTect HPV-Proofer assay with that of the LBC, in identifying high-grade histological lesions. Although, in this context (i.e., considering the cytology results at the HSIL threshold), the mRNA test evidenced a higher sensitivity than LBC, the latter appeared to be the more specific tool with a higher PPV. The diagnostic role of mRNA test has, to date, been evaluated in only a limited number of studies that are often difficult to compare mainly due to methodological differences concerning patient enrollment.

To the best of our knowledge, only three authors have considered the performance of the mRNA test on the HPV DNA-positive samples [19–21]. Varnai analyzed only a limited number of patients ($n = 66$), and Cuschieri and Cattani, differently from our study, did not compare their results with histological findings. Moreover, the Molden study [22], which analyzed a large number of women considering histological CIN2+ as end-point, only determined the diagnostic performance of the mRNA test in the HSIL+ cases. The study found a higher sensitivity, specificity, and PPV of the mRNA test than we detected, even though NPV was lower than ours. These differences are likely due to the different patient populations taken into account.

However, it is noteworthy that the majority of studies enrolled a series of patients with a higher CIN2+/CIN2− ratio than the present study [20, 23, 24]. In contrast, in our series, the CIN2+/CIN2− ratio is more similar to that usually found in a cervical cancer screening population [25, 26]. This patient selection might alter the results obtained, and overall, the studies are difficult to compare. However, a limit of the present study is that our series does not originate from a population-based screening, which means there is still a relevant difference, because the proportion of women with abnormal cytology is higher than in an unselected population of HPV DNA-positive women.

Another partial limit of this study is that, following the local protocol, biopsies were performed only on colposcopically suspicious areas. In fact, as reported by Pretorius et al. [27], the use of a colposcopic-directed biopsy as the gold standard in cervical cancer screening studies may underestimate the prevalence of CIN2 or worse and this may influence the results of the study, both positively and negatively for mRNA test performance.

We took this kind of analysis one step further and we showed that mRNA could be a test of clinical usefulness in selecting HC2-positive patients with a negative Pap-test or with mild cytological abnormalities to be referred to colposcopy. Our data showed that mRNA positivity, even though in a small number of CIN2+ cases, would seem to be significantly more associated with CIN2+ than CIN2− (p value < 0.0001). This result indicates that the mRNA test could recover CIN2+ cases that are underestimated by cytology.

We also tried to position the mRNA test as the first triage step and subsequently to triage the negative mRNA

cases by LBC (data not shown). No better efficiency was obtained, because there were the same number of colposcopic examinations and the same number of missed CIN2+.

However, it remains to be established whether or not the cut-off of HSIL+ would, in fact, be cost-effective. This might be considered as a "third-step triage-like" strategy in line with Ronco and Cuzick [26, 28]. These authors suggest, in fact, that the algorithm that utilizes the most sensitive test (i.e., HPV DNA testing) as up-front, followed by a more specific test (i.e., cytology) to triage the HPV-positive women, should be refined utilizing novel biomarkers.

To the best of our knowledge, none of the published studies have verified the association between mRNA test positivity and histologically proven CIN2+ in HSIL−/HC2+ patients. In our opinion, it is noteworthy that the application of the mRNA test may represent a novel diagnostic strategy and a very attractive option, because it might recover the high-grade lesions not identified by cytology and it might lower the number of women uselessly referred to colposcopy and reduce the overmanagement of low-grade abnormalities. Moreover, its application on the residual sample used for HC2 and LBC may overcome the difficulties, as reported by Ronco, concerning the patients who do not easily return for an adjunctive stage testing recall [5].

In our study, the mRNA test was negative in 43% of histologically confirmed high-grade lesions. Taking into account that some CIN2+ lesions may spontaneously regress, mainly when they are CIN2 [5], we could hypothesize that the preinvasive lesions that are negative for E6/E7 transcripts could be more susceptible to regression. Even the Ronco study [26] evidenced that a regression of CIN2+ is frequent mostly in the younger HPV-positive women referred to colposcopy**.** However, we have no chance to verify whether a negative mRNA test in the CIN2+ cases could be associated with a higher probability of regression, since CIN2+ women, according to the local ongoing protocol, usually undergo excisional treatment.

The final part of our study aimed to evaluate the longitudinal risk of developing a CIN2+ lesion by means of the PreTect HPV-Proofer test result. To this purpose, the study followed up, within 2 years (median time 11 months, range 6–25 months), 75 HC2-positive patients who were cytologically HSIL−. Our data showed a high longitudinal specificity and NPV with low sensitivity and PPV of mRNA test. Very few studies have verified the role of mRNA test in predicting the development of a high-grade lesion during follow-up [20, 21, 29]. Differently from our study, the Molden study considered 77 patients with a shorter median time follow-up (7 months, range 1–24 months), obtaining a very high OR, that is "age adjusted," as reported by the Authors. However, the dependance from the age of the mRNA test has yet to be fully elucidated. Varnai et al. followed up a small group of 39 women, but they did not report any histologic confirmation. Cuschieri study followed a completely different approach from ours, focusing on HPV persistence and finding that RNA-based techniques may be more predictive of persistent infection compared to DNA-based test. However, our data, as well as data presented in literature, due to the limited number of patients analyzed and the low number of CIN2+ observed, need to be refined and confirmed by larger follow-up studies.

In conclusion, in the light of our comprehensive results, a positive mRNA test in the selected category of HC2+/HSIL− patients appears to be of diagnostic significance. Concerning the mRNA's prognostic role, a test positive at baseline could have the potential to identify HPV-related lesions at higher risk of progression.

On the basis of these encouraging results, this study could represent a rational basis for a randomized controlled trial to verify whether the PreTect HPV-Proofer test, which appears to be a reliable and not subjective test, is a useful test to be introduced into a new algorithm of the screening protocol, in particular, in the triage of HC2+/HSIL− patients.

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