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MANAGEMENT AND TRIAGE OF WOMEN WITH HUMAN PAPILLOMAVIRUS INFECTION IN FOLLOW-UP FOR LOW-GRADE CERVICAL DISEASE: ASSOCIATION OF HPV-DNA AND RNA-BASED METHODS

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Type-specific persistent infection with Human Papillomavirus (HPV) is a significant risk factor for the development of cervical diseases. Persistent infection could be further refined by a sequencing approach to detect early cervical lesions that are at high risk of developing an invasive squamous cervical cancer. The aim of the present study is to investigate the clinical utility of detecting mRNA transcripts of HPV oncogenes E6/E7 by using a Real-time NASBA technology (mRNA test) and to identify women with low-grade cytological disease but with an increased risk of developing high-grade cervical abnormalities or invasive squamous cervical cancer. Our preliminary results show that E6/E7 is detected in only a subset of HR-HPV-positive cases. Since viral persistence is considered to be the true precursor of neoplastic progression, only the detection of E6/E7 mRNA can identify the infection which is more likely to persist and induce neoplasia in future. For these reasons we believe that this test would be useful for the characterization of women with HR-HPV DNA positivity who should be effectively treated because at high-risk of developing a high grade cervical lesion or an invasive squamous cervical cancer.

The prevalence and incidence of cervical cancer has decreased worldwide with the introduction and widespread diffusion of cervical cancer screening programmes based on regular Papanicolaou test (Pap) tests. However, cervicocarcinoma is still the third most common cancer worldwide with about 250,000 deaths annually (1).

Based on epidemiological evidence, there is no doubt that HPV represent a prerequisite for almost all cervical cancers (1). Diagnosis of Human Papillomavirus (HPV) infection can present difficulties since HPV cannot be grown in conventional cell cultures (2). Serological assays are inaccurate in determining whether an infection is present or past because HPV infection is followed by a humoral immune response against the major capsid protein (2) and antibodies are detectable for several years. Consequently, an accurate diagnosis

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of HPV infection relies on the detection of viral nucleic acid.

The Pap test is a subjective method with several limits regarding its accuracy: the estimated true specificity and sensitivity of the conventional Pap test is on the order of 80-85% and 30-87%, respectively (3). Therefore, false-negatives and false-positives continue to haunt cytopathologists, resulting in difficulties which range from potentially deleterious implications for patients and possible unnecessary invasive clinical investigations up to overtreatment, with an associated increase in cost and psychological distress (4-6).

Pap smear diagnostic disagreement has been found especially in the cytological diagnosis of Low-grade (Low Grade Squamous Intraepithelial Lesion - LSIL) and Borderline Cervical Abnormalites (Atypical Squamous Cells - ASC-). Therefore, the management of women with such cytological lesions remains controversial (7).

These considerations further underline the need to identify new approaches and biomarkers with a better positive predictive value in identifying women with low-grade/borderline cervical lesions but at high risk of developing cervical cancer.

Recently, a significant increase in the understanding of the natural history and the molecular pathogenesis of cervical cancer has been achieved. High Risk Human Papillomavirus (HR-HPV) subtypes 16, 18, 31, 33 and 45 are among the main culprits implicated in cervical carcinogenesis; in particular, HR-HPV DNA was found in 99.7% of squamous cell carcinomas (8). Interest in molecular techniques capable of identifying carcinogenic HPV in cervical samples has recently increased: the most used molecular test is Hybrid Capture II Assay (HCII) that detects 13 high-risk types of HPV DNA (9-11). However, due to the high prevalence of infection among women (approximately 5-30% of women harbour the virus), the mere detection of HR-HPV infection permits the identification of the first step in carcinogenesis but not the tendency to induce a neoplasia in future. Presently, the vast majority of women with HR-HPV infection will spontaneously clear the virus without any relevant pathological effects and only a few infected patients will develop clinically relevant lesions (12).

Epidemiological surveys of HPV prevalence, the natural history of HPV infection and disease and studies of HPV positivity have lead to the following conclusions: persistent infection with high risk types of HR-HPV is a necessary condition for the development of H-SIL and invasive squamous cancer (8). Therefore, the single best predictor of risk of cervical cancer is viral persistence (13).

The aim of our study is to compare the prevalence of HPV DNA positive cases to the prevalence of E6/E7 oncogenic expression and then investigate the clinical utility of adding the detection of m-RNA transcripts of HPV oncogenes E6/E7 by using Real-Time NASBA technology to the triage of women with low-grade/borderline cervical disease or previously treated for cervical intraepithelial neoplasia.

MATERIALS AND METHODS

Patients

The present is a cross-sectional outpatients-population-based follow-up study. A cohort of 104 women with mean age 39 years (range 16-82) was selected on the basis of having a follow-up over two years (2.3 median) for LSIL/ASC- cervical disease, from January, 2002 to June, 2005 (80 patients), or of having a previous treatment for cervical intraepithelial neoplasia (24 patients). For our intentions, an LBC and colposcopy were performed for each woman. All scrapes were analyzed morphologically in thin layer preparations and tested for the presence of HPV-DNA (HCII) and for HR-E6/E7 oncogenic expression (PreTect HPV Proofer Assay). HPV DNA and RNA tests were performed on the same cytological sample by the same person who was blinded versus morphology.

Collection of Sample Material and Slide Preparation

Cervico-vaginal secretions were collected from the squamous-columnar junction, placed in PreservCyt (Cytyc Corporation, Boxborough, MA) liquid cytology medium and transferred to cytopathology laboratory; Thin Prep 2000 processor (Cytyc Corporation, Boxborough, MA) was used to prepare cytological slides which were stained with Papanicolaou procedure. Thin Prep cytological slides were then evaluated by a single cytopathologist. Cytological reports were performed according to the "Bethesda System 2001".

Colposcopy

For each patient, after the collection of the Pap smear, a colposcopic examination was performed. Colposcopy was considered positive in cases where any abnormal

vessel, leukoplakia or acetowhite lesions were observed.

HPV-DNA Testing

After cytological slide preparation, an aliquot (4 mL) of each LBC sample, stored at 4°C, was removed for HCII assay (Digene, Beltsville, MD).

HCII assay is a semi-automated hybridization test of HPV target DNA, FDA approved. It was performed as described in the manufacturer's instructions. The kit contains two probes: one for detecting High Risk (HR) DNA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and the other for Low Risk (LR) DNA of HPV genotypes. (14-15).

mRNA testing

A second aliquot (5 mL) of each residual LBC specimen was removed within 14 days after collection (16) and mRNA was isolated from cervical samples using Rneasy Mini Kit (Qiagen, Hilden, Germany). mRNA test (PreTect HPV-Proofer, NorChip AS, Kokkarstua, Norway) is based on the use of NASBA technology which amplifies mRNA in a DNA background and detects the products of amplification in Real-Time through molecular beacon probes. The HPV-Proofer Assay contains molecular markers for detection of full-length E6/E7 mRNA from five high risk carcinogenic HPV types (16, 18, 31, 33, and 45). The RNA amplification was performed at an isothermal temperature of 41°C (17). A fluorescent analyzer measured in real-time the emitted fluorescence from molecular beacon hybridized with amplified mRNA and labelled with FAM and Texas Red fluorochromes.

Statistical Analyses

Statistical analysis was performed utilizing the SPSS software (version 11.5). McNemar test was used to compare the prevalence of HPV E6/E7 mRNA to the prevalence of HPV DNA.

RESULTS

Cytologic findings and colposcopic results

Cytological diagnosis of follow-up LBC samples are shown in Table I; in particular, 17 of the 104 women were negative (16.3%), 33 ASC- (31.7%), 46 LSIL (44.2%) and 8 H-SIL (7.7%). Colposcopic investigation, emphasized in Fig. 1, show normal colposcopical findings in 58 cases; colposcopic abnormal results were found in 46 patients.

Molecular Testing

Cytological diagnosis results related to DNA and RNA testing are also represented in Table I. Of 104 recruited women, a total of 61 patients (58.6%) were positive for HR-HPV-DNA, 5 were Low-Risk HPV-DNA (LR-HPV DNA) positive (4.8%) and 38 (36.5%) were HPV-DNA negative; an important difference between detection of HR-HPV DNA and E6/E7 mRNA was observed (Fig. 2): exactly, E6/E7 mRNA occurred in only 23 of 104 (22.1%) cases. RNA-positivity was associated with the presence of HPV high-risk type in only 20 of 61 (32.8%) cases. These differences were statistically significant (McNemar test: $p<0.001$).Two samples (LSIL and HSIL) being negative for HPV-DNA were found positive for mRNA test; one sample mRNA positive was associated with only the presence of LR-HPV DNA.

Moreover, of the 46 women who resulted negative with colposcopy, 21 were HR-HPV DNA test positive, 5 of those resulting mRNA positive (Fig. 2); of the 58 women who resulted positive with colposcopy, 36 were HR-HPV DNA positive, only 17 of those resulting mRNA positive (Fig. 1).

DISCUSSION

Cytological evaluation (Pap test) is unanimously accepted as the most cost-effective screening tool for detecting precancerous cervical disease. However, for ASC- and LSIL lesions the appropriate management guidelines remain unclear, and follow-up recommendations for these women range from repetition cytology at a shorter interval to referring immediately for colposcopy and biopsy. This guideline causes an increase in over-diagnosis and over-treatment. The ALTS study attested that HPV-DNA triage using HPV-DNA test represents the best approach to this problem (18-19). Each of the numerous methods currently in use for research and virus typing has different characteristics (cost, applicability on various biological material and level of automation) but all have a common factor: identification of the viral DNA within the host cells. Actually, HC2 is the only test that has received FDA approval. Previous reported studies indicate that this test has a good diagnostic accuracy due to its high negative predictive value (15). None the less, HC2 test is only indicative of an generic possibility of developing cervical carcinoma. In fact, approximately two-thirds of the women with

Table I. *Cytological diagnosis results related to DNA and mRNA tests.*

Cytological diagnosis	DNA				E6/E7 mRNA	
	Negative	LR-HPV	HR-HPV		negative	positive
BCC/negative	10	0	7		15	2
(n=17)	59%	0%	41%		88%	12%
ASC-	19	1	13		28	5
(n=33)	58%	3%	39%		85%	15%
LSIL	8	3	35		34	12
(n=46)	17%	7%	76%		74%	26%
HSIL	1	1	6		4	4
(n=8)	13%	13%	74%		50%	50%

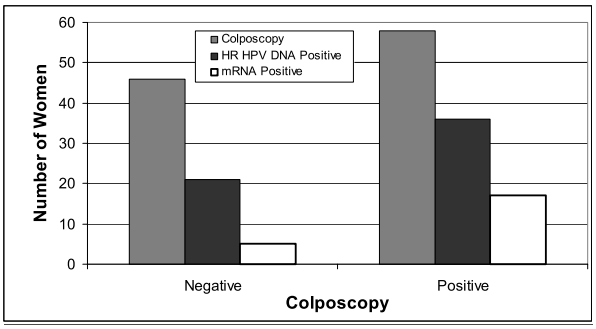

Fig. 1. *Colposcopic outcome correlated to HR-HPV DNA and mRNA positivity.*

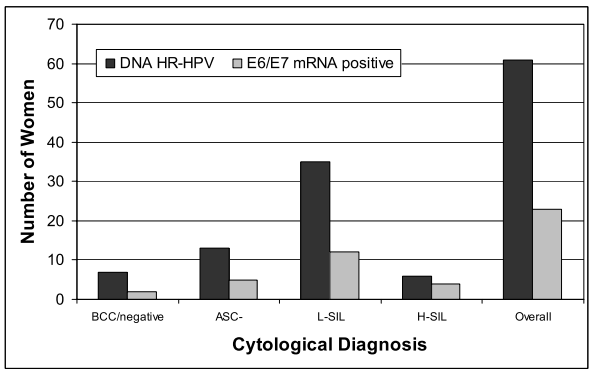

Fig. 2. *Number of women DNA HR-HPV positive and E6/E7 mRNA positive according to the four cytological diagnosis groups and overall.*

low grade cytological abnormalities after additional analysis resulted as not being oncogenetically active. This result is in agreement with results reported in literature: HR-HPV DNA was identified in 80% of women with LSIL who underwent regression (20). Then, despite the advantages offered by the DNA test, it is not capable of distinguishing a clinically transient infection, which is common in sexually active women, from a clinically active infection with a high risk for cancenogenic transformation (21-22).

During an acute HPV infection, expression of viral genes, specifically E6/E7 oncogenes, is restricted to differentiated epithelial cells which have the capacity to replicate their genome and are at no further risk of acquiring functional mutations. On the other hand, one of the key events of HPV-induced carcinogenesis is the integration of HPV genome into a host chromosome and the continuous and deregulated expression of viral oncogenes in replicating epithelial stem cells (basal cells). Here E6/E7 products submerge control of cell cycle through complex interactions with various cellular protein complexes and induce instability. Two major aspects are involved: high-risk E6 protein supports premature degradation of p53 tumour suppressor gene thus interfering with apoptotic functions of the host cell (23-24); E7 protein induces destabilization of the retinoblastoma protein (pRb) complex, thus allowing the cell to evade cell cycle control through the pRb pathway (25). Then, deregulated and continuous expression of E6/E7 HR-HPV oncogenes in cervical basal compartment is an essential and indispensable prerequisite to maintain the neoplastic growth of dysplastic cells (26-27). Numerous

studies on HPV infection in immunocompromised individuals report that E6/E7 could also play an important role in the inhibition of the host cell immune response (28): in particular, evasion of immune response would contribute to the survival and propagation of HPV-infected cells. E6/E7 genes would have a negative impact on immune response by inhibiting the production of immune mediators (i.e. Interleukin-18); E6/E7 oncoproteins would also have the ability to down-regulate Interleukin-8 (IL-8) expression (IL-8 is a T-cell chemoattractant) (29) and to suppress the expression of Chemochine Monocyte Chemoattractant Protein 1 (MCP-1) in epithelial cells of female genital tract (30) . In addition E6/E7 would favor the escape from the antiviral and antiproliferative properties of Tumor Necrosis Factor Alpha (TNF-α) (31)

The presence of HR-HPV DNA in the host cells is generally accepted as a necessary condition (32), but far from sufficient for the development of a cervical neoplasia. In fact, cervical cancer is an infrequent complication of HR-HPV infections that requires supplementary conditions and events in order to establish itself: in particular, the persistence of viral oncogenic activity, indicated by the expression of oncogenes E6 and E7 in the basal cervical cells. Consequently, detection of E6/E7 transcripts is considered the best biological indicator of neoplastic risk, the more precise indicator for progression towards malignancy (33-35).

With such knowledge, the supplemental test that should be added to the diagnostic algorithm of ASC-/LSIL cervical lesions would give an indication of whether these HPV-induced lesions are in progression or regression. The ideal test would also define the different stages of cellular changes associated with HPV clearance, persistence or progression toward cervical cancer.

This paper, comparing the prevalence of HPV-DNA positivity with the prevalence of E6/E7 mRNA expression in women with low-grade cervical disease or previously treated for intraepithelial neoplasia, investigates the clinical utility of mRNA test in the management of this group of patients.

The results obtained show that HR-HPV DNA prevalence is high (about 59%) while E6/E7 expression is observed in approximately one out three of the HR-HPV DNA positive women. These differences may be explained by the different viral biology: latent, sub-clinical or transient HPV infection display HR-HPV DNA positivity without necessarily the presence of mRNA E6/E7 protein; viceversa, active persistent infection shows persistent oncogenic E6/E7 expression and then an increased risk of progression towards HSIL-plus lesions.

CONCLUSION

The introduction of mRNA test in the clinical work-up of women with ASC-/LSIL cervical lesions would certainly increase the diagnostic accuracy of HPV infections, thereby better identifying the infection which is more likely to persist and induce neoplasia in future, and would also reduce psychological distress and cost for women who have only a transient infection. The addition of mRNA test would improve the results obtained from the diagnostic work-up presently in use with all the advantages that the correct identification of women with a true risk of developing cervical cancer would entail.

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