DNA versus RNA methods for human papillomavirus detection in cervical samples

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Scurta prezentare
The significance of HPV E6/E7 mRNA testing versus DNA testing was established for cervical specimens harvested from 108 women with different cytological grading.

Abstract

Human papillomavirus DNA testing can not discriminate between transient or persistent infections. Detection of E6/E7 viral oncogenes expression might represent a better tool for monitoring women at risk. ***Objective:*** *The aim of the present study was to assess the significance of HPV E6/E7 mRNA presence versus DNA testing in order to identify women with HPV persistent infections.* ***Method:*** *Cervical specimens from 108 women (median age 32.2 years) were analyzed for HPV DNA (Linear Array HPV Genotyping, Roche) and for E6/E7 RNA transcripts (using PreTect HPV-Proofer). As we know, this is the first study regarding E6/E7 RNA testing in Romania.* ***Results:*** *Results were compared with cytological data. The DNA tested positive and mRNA tested negative in 46.8% of cases. The rate of detection increased with the grade of cytological lesions (9.5% in NILM cases- 72.7% for HSIL grading).* ***Conclusion:*** *Our results support the idea of HPV E6/E7 transcripts testing as a useful tool for patient management.*

Keywords: *HPV, genotyping, E6/E7 RNA*

Introduction

Cervical carcinoma is the second neoplasm among women worldwide. Epidemiological data sustained by molecular investigations, clearly indicate that certain HPV genotypes are the principal cause of invasive cervical cancer. From more than 100 human papillomaviruses (HPV) types that have been identified[1], about 40 mucosotropic types infect genital tract. It is now recognized that virtually all cervical cancers (squamous and adenocarcinoma histological types) are related to cervical infections by 14 oncogenic human papillomavirus (hrHPV) genotypes[2]. Most of the HPV genital infections clear in one or two years after debut but persistent infections with carcinogenic genotypes may cause cervical

cancer[2]. Due to the role of persistent HPV infection in cervical neoplasia development, HPV testing for hrHPV was introduced into cervical cancer screening. HPV testing in primary screening is recommended for women older than 30 years because these women are past the age of self-limited infections. Due to its sensitivity, HPV testing is now used in the United States for equivocal cytological triage and for colposcopic referral. One advantage of screening using HPV testing is the ability to identify HPV-positive women with persistent infection[3]. But HPV testing can not discriminate between transient and persistent infection unless consecutive investigation (12 month interval) is performed. Although HPV DNA testing is a more accurate screening test than cytology, it suffers from lower specificity. Due to a low clinical specificity and positive predictive value, HPV testing will result in an increase in referral for unnecessary follow-up[4].

Steps that are leading from initial infection to the development of cancer include overcoming host immune defense, possible integration of HPV DNA into the host genome and accumulation of mutations within the infected host cell[5]. The host-cell cycle is altered by expression of hr-HPV oncogenes (E6 and E7). While in low-grade lesions, E6 and E7 genes are expressed at low levels in the basal cells and higher levels in the upper layers of the epithelium, in high-grade lesions viral oncogenes are expressed at high levels throughout the epithelium[6]. HPV DNA integration into the host-cell chromosome (a characteristic of high grade lesions and cancer disease) abrogates the viral E2 control on E6 and E7 oncogenes expression, leading to proliferation and malignancy[7]. Thus, the expression of viral oncogenes is considered a marker of persistent infection[8]. E6/E7 mRNA testing for hr HPV types, compared with HPV DNA testing, seems to better correlate with the severity of the lesion. E6/E7 mRNAs presence is a potential marker for the identification of women with persistent infections and subsequently, at risk of developing cervical carcinoma. It has been shown that the risk of persistence and progression to cancer differs markedly by HPV type[9]. Therefore, the E6 and E7 proteins of some HPV oncogenic types are more frequently associated with the majority of cervical carcinomas[9]. While HPV tests show accuracy, reproducibility and clinical utility in screening programs, E6E7mRNA testing, if implemented, might improve patient management.

The aim of the present study was to assess the significance of HPV E6/E7 mRNA presence versus DNA testing for certain HPV genotypes, in order to identify women at risk to develop cervical cancer.

Materials and Methods

Sample collection - From a cohort of 490 women enrolled between 2007-2009 in a study of HPV prevalence and incidence, 108 selected subjects (median age of 33.8 years, range 18 to 56 years) were monitored in three Gynaecology Clinics involved in this study (*Cuza Voda* Clinic from Iassy, Gynecology Clinic from Craiova and *Filantropia* Clinic from Bucharest). The women were subjected to gynecological and viral investigations. The study protocol was approved by the Ethics Committee of each Clinic Hospital. Cervical scrape specimens were collected by gynecologists and conventional cytological screening of the samples was performed. Slides were read according to Bethesda system, independently for each clinic. For viral investigations, cervical swabs were collected in a liquid medium (Copan), transported to Stefan S. Nicolau Institute of Virology and underwent nucleic acids extraction.

DNA isolation. DNA was isolated using the QIAmp Kit protocol (Qiagen) within 2 days from sample collection. Briefly, after 1 ml cell suspension centrifugation (10 minutes, 12000 rpm) the lysis buffer and the proteinase K were added on the pelleted cells and incubated for 1 hour at 56°C. An equal volume of 100% ethanol was added to each sample and the mix was applied to QIAmp columns for DNA isolation. The DNA was eluted with 100 µl deionized distilled water. Samples were tested immediately or stored at -20°C.

RNA isolation was done with High Pure RNA Isolation Kit (Roche) within two days from sample collection. The pelleted cervical cells from 1 ml suspension were lysed with the Roche reagent which contains a chaotropic salt and detergent. After cell debris was removed by centrifugation, the lysate was applied to Spin Filter Tube. Under the buffer conditions nucleic acids bound to the glass fleece in the High Pure tube, while contaminats (salts, proteins and other cellular contaminants) were eluted. Contaminating DNA from sample was digested with DNase I and the purified RNA was eluted in 50 µl of low salt buffer.

HPV DNA genotyping. The commercially available Linear Array HPV Genotyping (Roche Diagnostics) kit based on the reverse hybridization principle was used for HPV DNA genotyping. PCR amplification of HPV DNA uses biotinylated PGMY primers which targets a 450-bp fragment within the L1 region of the viral genome. Amplicons incorporate dUTP allowing the use of AmpErase enzyme (uracil *N*-glycosylase), which is included in the master mixture in order to prevent PCR carryover contamination. An additional primer pair targets the human β-globin gene (268-bp amplicon) and provide a control for adequate DNA extraction and amplification. PCR was performed in a final reaction volume of 100 µl (50 µl HPV master mixture, 40 µl RNase free water and 10 µl isolated DNA). The PCR was done according to manufacturer's instructions. HPV-positive and -negative controls were run in each experiment. The denatured biotinylated amplicons were hybridised with specific oligonucleotide probes immobilised on the strip. After hybridisation and stringent washing, streptavidin-conjugated alkaline phosphatase binds to the formed hybrid. Incubation with chromogen develops a dark blue precipitate for HPV positive cases. The strips were interpreted according to Linear Array HPV reference guide, by reading the individual types.

HPV E6/E7 mRNA expression was estimated in a NASBA qualitative assay (PreTect HPV-Proofer)

that allows the detection of E6/E7 mRNA for HPV 16, 18, 31, 33 and 45 only. The technique was performed according to the manufacturer (NorChip, Norway). The kit provided oligonucleotides corresponding to the viral sequence for positive controls. As a control for RNA integrity, a primer set and probe targeting mRNA of human U1A was also provided. Negative controls consisting of all reagents except target RNA were included in each run. The reaction was performed at 41°C and consisted in the annealing of one primer to the target RNA followed by synthesis of a RNA/DNA hybrid and RNA strand degradation by RNase H. The other primer anneals to the newly synthesized DNA strand which is converted in dsDNAs, which, in turn are targets for T7 RNA polymerase. Molecular beacons bind to complementary RNA transcripts and the probe undergoes conformational change which is followed by a fluorescence signal. Ten µl of premix and 5 µl of RNA were used for each sample. The excitation filters for 485/20nm and 590/20nm, and the emission filters 530/25nm and 645/40nm, respectively were used.

Results

The study encompassed 108 women with a median age of 32.2 years selected from a cohort of 490 women. All women were tested for HPV DNA presence/genotyping. Since PreTect HPV-Proofer allows the detection of E6/E7 mRNA for 5 HPV genotypes only (16, 18, 31, 33 and 45), subjects presenting the above mentioned genotypes but also women infected with other HPV types or HPV DNA negative subjects were selected for this study. The patients were grouped according to cytological features: women with normal PAP smears classified as NILM (negative for intraepithelial lesion or malignancy) and women with cytological abnormalities, some of them being referred to biopsy/conisation. The overview of the investigated group at enrollment, according to cytological and histological investigations is shown in figure 1.

Regarding the HPV DNA detection, we found that 70.8% of the women were HPV positive, 41.1% presenting co-infections with two to five different genotypes. HPV prevalence in women with high-grade histology was 100%. The type distribution showed that HPV16 was the most common hrHPV type found in our study (44.8% in single or co-infections). This is not surprising since HPV16 is the prevalent hrHPV genotype in Romania. Among other high risk genotypes, we found, in single or co-infections, HPV 18 (22.9%), HPV 31 (18.7%), HPV 33 (8.3%), HPV45 (9.4%), HPV 51 (5%) HPV66 (8.9%). We mention that in eight cases HPV16 and 18 were found as co-infections.

All 108 samples were tested for E6 and E7 mRNA included in PreTect HPV-Proofer kit. High risk oncogenes expression was noted in 32.4% of cases. The DNA tested positive and mRNA tested negative in 46.8% cases. Viral messengers for HPV 16 were present in the highest percent (17.7% of the patients) followed by HPV18 and HPV31 (3.8% each). HPV33 and HPV 45 were present in the same percent (2.8% each). Two cases with expression for two oncogenic types were found (16+31, 16+33). The rate

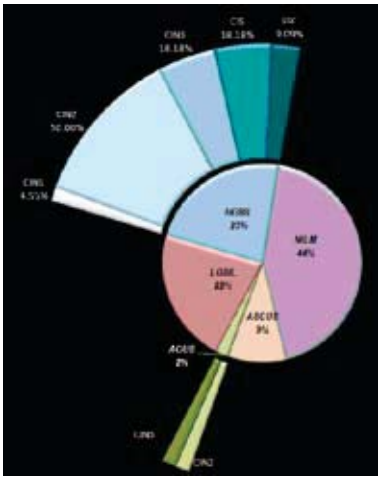

Figure 1. Cytological and histology diagnoses of the investigated group

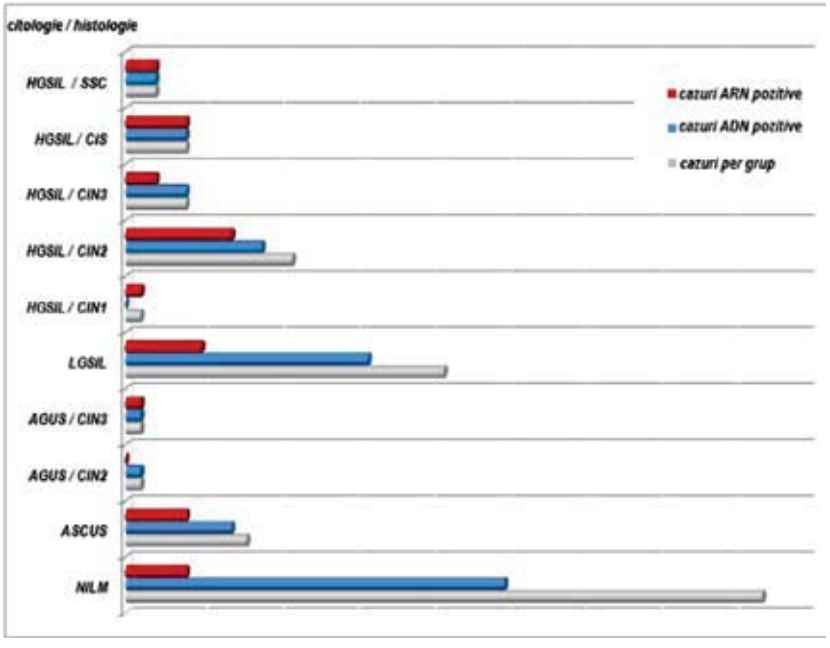

Figure 2. E6/E6mRNA positive cases versus HPV DNA positive viral testing for each cytology group

of detection increased gradually with the grade of the observed lesions, rising from 9.5% for patients with NILM to 72.7% for those with HSIL. The outcome of HPV DNA testing versus mRNA expression for each group is presented in figure 2.

While HPV 16 mRNA predominated in all cytological groups, HPV 18 mRNA was found in normal and moderate dysplasia. Two cases presenting CIN1 hystology tested negative for HPV DNA but tested positive for HPV16 E6E7mRNAs. All carcinomas and CIS samples were positive for mRNA test, HPV 16 being present in 66.6% of these cases (HPV31 an 33 representing the difference). In none of the carcinoma cases, mRNAs for multiple HPV genotypes were found although in 5 patients multiple infections with hrHPV were detected by genotyping. Only in four cases with NILM cytology we found E6E7 mRNAs, in one case for 16+31 co-infection.

As we were interested to correlate the viral mRNAs presence with age, we divided women in three age groups, according to cytology results (figure 3). While the percentage of subjects with normal cytology decreased from women less than 30 years old to those above 40 years (58% to 30% respectively) the percentage of cases with HGSIL cytology increased (from 11% to 31%).

The percentage of E6/E7mRNAs decreases according to age group. We found 23% mRNA positive cases in women under 30 years, 33.3% in women between 29-40 years and 46.1% in those over 40. In the group of young women, viral messengers were detected in AGUS cases (CIN3) as well as in ASCUS and HGSIL (CIN2) lesions. It is no surprising that the highest percentage of mRNAs were noted in the group of women over 40 since it comprised most of HGSIL lesions with severe histology (SCC, CIS and CIN3). Although HPV 16 mRNAs were present in all age groups, we found some differences regarding other mRNAs types: most cases with E6E7mRNA for HPV 18 and 31 were included in group of women between 30-40 years old.

Discussions

Human papillomavirus infection is associated with the development of cervical neoplasia, but not all infected women develop this disease. In 90-95% of cases, high-risk HPV infections regress spontaneously, with only a small percentage progressing to high grade lesions. Many authors highlight that viral persistence is required for neoplastic progression[11]. Viral persistence is accompanied by hrHPV integration into host cell genomes which results in transcription of E6/E7 viral oncogenes[12]. Thus, E6 and E7 oncoproteins have significant roles in malignant transformation, and are consistently expressed in malignant tissue[13]. Many countries already implemented screening programs based on DNA detection. HPV DNA testing is a more accurate screening test than cytology but it suffers from lower specificity. Due to a high percentage of transient infections, an alternative method which could monitor expression of the E6/E7 viral oncogenes seems to be more accurate in identifying women at risk. Therefore the detection of E6/E7 mRNA may be of higher prognostic value and may improve the specificity and positive predictive value compared with HPV DNA testing in screening[14]. Commercial assays for genotyping and detection of E6/E7 mRNA from the five most common high-risk HPV types are now available and require further evaluation for primary screening, triage and follow-up after treatment[15].

Our study comprised 108 investigated women who were under clinical investigations. Although HPV DNA was detected in 70.8% only in 32.4% of cases E6/ E7 expression was found. HPV E6/E7 mRNA testing for high-risk types seems to correlate better with the severity of the lesion compared with HPV DNA testing and is a potential marker for the identification of women at risk of developing cervical carcinoma. In our study the rate of mRNAs detection increased gradually with the grade of the observed lesions, rising from 9.5% for patients with normal cytology to 72.7% for those with HSIL. The presence of E6/E7 mRNA expression in NILM samples, indicates that the virus can exert an oncogenic activity before cell changes can be cytologically visualized. Our results suggest that testing for HPV E6/E7 transcripts might be useful for patient management and screening, providing more accurate predictions of risk than DNA testing. Compared with other types HPV 16 was, as expected, the most common type found in both the PCR and the mRNA assays. Based on these findings we may confirm that HPV 16 is the major oncogenic genotype involved in cervical carcinogenesis in our country.

In specimens co-infected with HPV-16 and HPV18 and/or HPV31/ HPV33, with one exception, only HPV-16 E6/ E7 mRNAs were detected. This may reflect a higher probability of persistent infection of HPV-16 and implies that infections with other HPV genotypes with no detectable E6 and E7 mRNA may not contribute to carcinoma development. Our data is supported by other authors[10]. We supposed that the expression of a single messenger in these lesions might support the hypothesis of clone selection involved in cervical oncogenesis, idea sustained by other authors[16]. It is known that women who tested positive with PreTect HPV-Proofer are 70-times more likely to be diagnosed with cervical intraepithelial neoplasia (CIN) 2+ than women who tested negative[18]. A low number of women testing positive with the mRNA test represents fewer women to be followed up.

Regarding two cases which tested negative for HPV DNA and positive for HPV16 E6E7mRNAs, we consider that this confirms the new data that HPV DNA might be sequentially detected in cervical smears[19]. Moreover, one of these patients confirmed HPV16 presence when retested after one year.

The prognostic value of PreTect HPV-Proofer test was partially evaluated through HPV DNA retesting of 36 subjects who were not subjected to surgical treatment. Only 12 of them presented mRNAs at enrollment and we found (after one year follow-up) the same HPV genotype in 10 cases (including a multiple infection). The

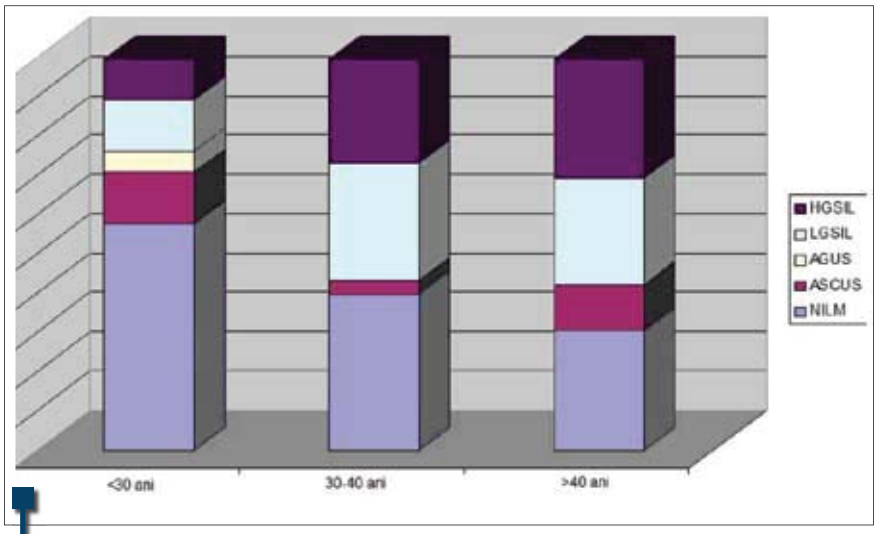

Figure 3. Distribution of investigated women according to age and cytology results

other two cases tested HPV positive for other genotypes. Many authors suggested that it should be clinically interesting to monitor expression of the E6/E7 genes, although little is known about a possible transient nature of such expression[20,21].

Conclusions

Compared with PCR, PreTect HPV-Proofer showed a higher clinical specificity. HPV mRNA testing, allow a better risk stratification of high-risk HPV-positive women and might improve the specificity of the cervical cytology test. Thus, the PreTect HPV-Proofer assay may serve as a tool in monitoring HPV infections producing viral oncoproteins. ■

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