

E6/E7 mRNA Expression of High-risk HPV Types in 849 Greek Women

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Abstract. *Aim: The goal of this study was to investigate human papillomavirus (HPV) geno typing and high-risk HPV E6/E7 mRNA expression in 849 women. Patients and Methods: 849 Cervical samples were obtained from patients attending an outpatient clinic to have their annual gynaecological check-up. All patients underwent a conventional Pap test. The patients were also offered HPV test with the knowledge that it is not part of the screening. Results: Overall prevalence of HPV DNA was 41.3%, while E6/E7 mRNA expression was found in 20.7% of the patients. HPV DNA and E6/E7 mRNA expression were detected in 21.1% and 9.1% of normal cytological samples, respectively, 38.1% and 23.8% of atypical squamous cells of unknown significance, 84.8% and 40.7% of low-grade squamous intraepithelial lesions, and 96.4% and 89.3% of high-grade squamous intraepithelial lesions, respectively. HPV 16 was the most frequent genotype identified. Conclusion: E6/E7mRNA detection might be useful as a screening marker for the early prediction of active infections and subsequent progression to severe dysplasia.*

Human papillomavirus (HPV) is recognized as the leading factor that causes cervical neoplasia in women (1, 2). The major cause of these cancer types is infection with high-risk (hr) types (3). Although not all hrHPV infections progress to cancer, persistent infection can lead to cervical lesions due to the oncogenic activity of the hrHPV E6/E7 proteins (4).

E6 and E7 oncoproteins are responsible for the immortalization and malignant transformation of cervical squamous cells by interfering with the cell cycle.

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Deregulation in the expression of the oncogenic proteins E6 and E7 is the key in the progression to high-grade neoplasia as it leads to an increase in cell proliferation and loss of the ability to repair secondary mutations in the host cell DNA (5, 6). During integration, the viral genome usually breaks in the E1/E2 site. E2 protein regulates E6 and E7 expression during viral replication, thus loss of E2 results in uncontrolled increase of E6 and E7 (7).

Cervical intraepithelial neoplasia encompasses a range of intraepithelial lesions, starting from minimun cellular atypia that can result in squamous and cylindrical invasive carcinoma (8).

HPV DNA testing contributes not only to detecting women with cervical lesions but also to follow-up after treatment. The mRNA test that detects E6 and E7 oncoproteins of hrHPV types 16, 18, 31, 33 and 45 enables us to detect integration, which is the most crucial factor in transformation (9, 10).

The aim of our study was to detect hr and low-risk (lr) HPV types with an HPV DNA test and E6/E7 oncoproteins of hrHPV types with the RNA test and correlate these findings with cytology.

Patients and Methods

Study population and specimen collection. Cervical smears were obtained from a consecutive sample of 857 patients, who attended the Outpatient Gynaecological Clinic of the St. Savvas Regional Anticancer Oncology Hospital of Athens, in order to have their annual gynaecological check-up. The majority of our patients were Caucasian with an age range of 18-60 years (mean age=34.7 years). The patients were also offered HPV test with the knowledge that it is not part of the screening. Ethical approval was granted by the Ethics Committee of St. Savvas Regional Anticancer Oncology Hospital of Athens and all participants provided written, informed consent.

The classification of cytological findings was performed according to the criteria of the Bethesda 2001 system and were categorized into normal, atypical squamous cells of unknown significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL).

Nucleic acid isolation. Cervical cell scrapings were collected and preserved in Thin Prep (PreservCyt Solution Corporate Headquarters: Hologic, Inc. Ltd., UK). Five millilitres were used for DNA and RNA extraction. They were centrifuged and diluted into lysis buffer (NucliSens lysis buffer, BioMérieux Hellas S.A). The samples were then subjected to the NucliSens easyMAG platform (BioMérieux Hellas S.A) for total nucleic acid extraction, according to the manufacturer's instructions. Nucleic acids were eluted in 55 µl of elution buffer. Aliquots were stored appropriately for further processing. A DNA quality test was carried out using Human Globin, Beta, Primer set kit (Maxim Biotech, Inc., South San Francisco, CA, USA) according to the manufacturer's instructions. To assess RNA integrity, 5 µg of RNA per sample were separated on 1% formaldehyde-agarose gel.

HPV detection and genotyping. The PapilloCheck® HPV genotyping assay (Greiner Bio-One GmbH, Germany) was used. This technology is based on a DNA chip for the type-specific identification of 24 types of HPV (hr: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 70, 73, 82 probable hr: 53 and 66; and lr: 6, 11, 40, 42, 43, 44). Nucleic acids were extracted from the cervical scrape preserved in ThinPrep. E1-based polymerase chain reaction (PCR) was performed according to the manufacturer's guidelines. For each sample, 19.8 µl PapilloCheck® MasterMix, 0.2 µl HotStarTaq DNA polymerase (5 U/µl) and 5 µl DNA from the cervical sample were mixed. Hybridization followed by mixing 30 µl of the PapilloCheck® Hybridization buffer in a fresh reaction tube with 5 µl of the PCR product at room temperature and transferring 25 µl of the hybridization mix into each compartment of the chip. The chip was incubated for 15 min at room temperature in a humid atmosphere, then washed in three washing solutions (I, II and III for 30 sec, 1 minute and 30 sec, respectively), centrifuged and scanned on a CheckScanner™ (Greiner Bio-One GmbH, Germany).

HPV E6 E7 mRNA detection. Real time nucleic acid sequence-based amplification (NASBA) and detection assay NucliSENS® EasyQ® HPV (BioMérieux) was performed for the qualitative detection of E6/E7 oncoproteins of five hr HPV types (16, 18, 31, 33 and 45).

The NucliSENS EasyQ HPV assay was performed according to the manufacturer's instructions (BioMérieux). Firstly, three pre-mixes were prepared by adding reagent sphere diluent [Tris-HCl, 45% dimethyl sulfoxide (DMSO)] to reagent spheres (nucleotides, dithiothreitol and MgCl₂). To each pre-mix U1A/HPV 16, HPV 33/45, or HPV 18/31 primer and molecular beacon mixes, KCl stock solution and NASBA water were added. Secondly, 10 µl of this pre-mix were distributed to each well in a reaction plate and the addition of 5 µL RNA followed.

The plates were incubated for 4 min at 65°C to destabilize secondary structures of RNA, followed by cooling to 41°C. The reaction was started by addition of enzymes (AMV-RT, RNase H, T7 RNA polymerase, and bovine serum albumin) and measured in real time using a Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT, USA) at 41°C for 2 h and 30 min.

Statistical analysis. Data were analyzed using SAS v9.0 (SAS® Greece). Absolute and relative frequencies were used to present the HPV positivity according to the test performed and cytology. The HPV prevalence and 95% confidence intervals (CI) were calculated using binomial methods stratified by cytology report and HPV

status. Two by two contingency tables with Fisher's exact test were used to assess statistical significance of any differences in prevalence and *p*-values of <0.05 were considered statistically significant.

Results

We collected 857 samples, eight of which were unsatisfactory for molecular analysis due to insufficient quantity of DNA and RNA, thus 849 samples were finally analyzed. All patients underwent a conventional Pap test, all of which were of satisfactory quality. The majority of women, 67% (n=569), had normal results, whereas 33% (n=280) showed different kinds of abnormalities. A total of 2.5% (n=21) of the examined women had ASCUS; in 27.2% (n=231) LSILs were found, whereas HSILs were detected in 3.3% (n=28).

The DNA and RNA tests were performed before having knowledge of the cytological results. HPV infection was detected in 41.3% (95% CI=38.0% to 44.7%) (n=351) of the patients, while the hrHPV types 16, 18, 31, 33 and 45 were detected in 16.7% (95% CI 14.2% to 19.2%) (n=142). Lr HPV types were found in 7.4% (95% CI=5.7% to 9.2%) (n=63).

The mRNA of the E6/E7 oncoproteins expressed by the hrHPV types 16, 18, 31, 33 and 45 were found in 20.7% (95% CI=18.0% to 23.5%) (n=176) of women. This assay includes an internal RNA control (U1A) to avoid false-negative results and all samples were U1A-positive.

HPV 16 was the commonest HPV genotype, found in 10.2% (95% CI=8.2% to 12.3%) (n=87) of our patients, followed by HPV 42 in 8.2% (95% CI=6.4% to 10.1%) (n=70), HPV 51 in 5.8% (95% CI=4.2% to 7.3%), HPV 53 in 4.9% (95% CI=3.5% to 6.4%) and HPV 56 in 4.5% (95% CI=3.1% to 5.9%). HPV 18 was detected at a rather low rate of 2.4% (95% CI=1.3% to 3.4%).

In 207 cases (24.4%; 95% CI=21.5% to 27.3%) the test detected single-genotype infections and 144 cases (17%; 95% CI=14.4% to 19.5%) represented multiple genotypes. The most common co-infections were those with HPV 16 and HPV 51, found in 3.9% (8/207) of cases with multiple infections.

Overall E6/E7 mRNA from HPV 16 was found in 11.7% (95% CI=9.5% to 13.8%) of our patients, followed by HPV 18 (4.1%; 95% CI=2.8% to 5.5%), HPV 31 in 3.4% (95% CI=2.2% to 4.6%), HPV 33 in 2.8% (95% CI=1.7% to 3.9%) and HPV 45 in 1.6% (95% CI=0.8% to 2.5%).

In 152/849 cases (17.9%; 95% CI=15.3% to 20.5%), E6/E7 mRNA was detected in single-genotype infections and 24/849 cases (2.8%; 95% CI=3.7% to 7.5%) represent multiple genotypes. The most common co-infections were those with HPV 16 and HPV 18 found in 37.5% (95% CI=18.1% to 56.9%) of those with multiple infections.

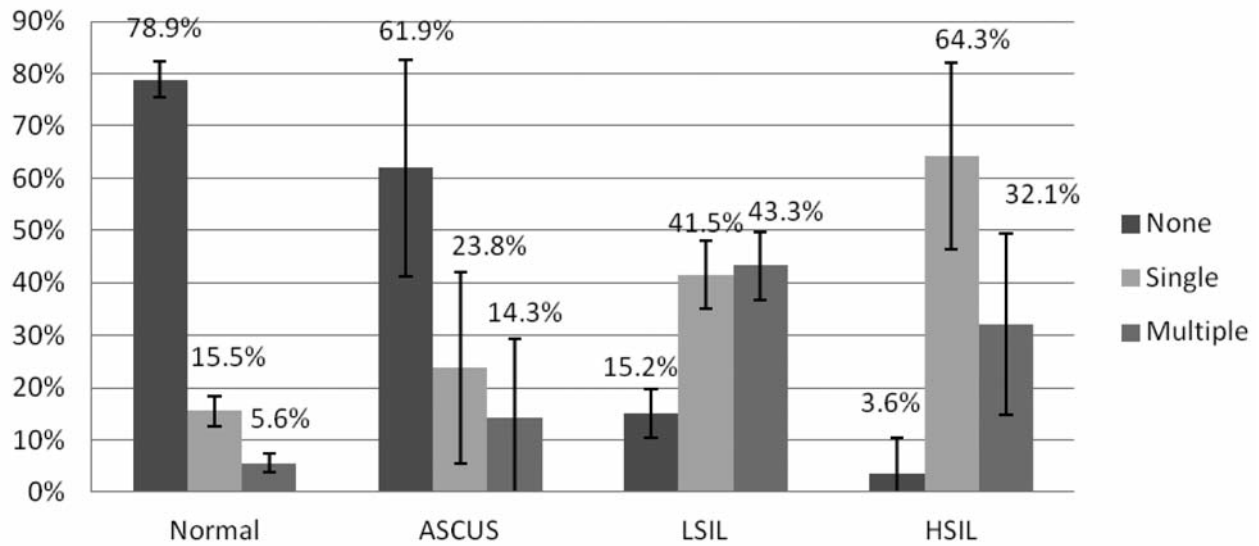


Figure 1. Prevalence (with 95% confidence intervals) of single and multiple human papillomavirus (HPV) infection detected by the DNA assay, according to cytology. ASCUS: atypical squamous cells of unknown significance; LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions.

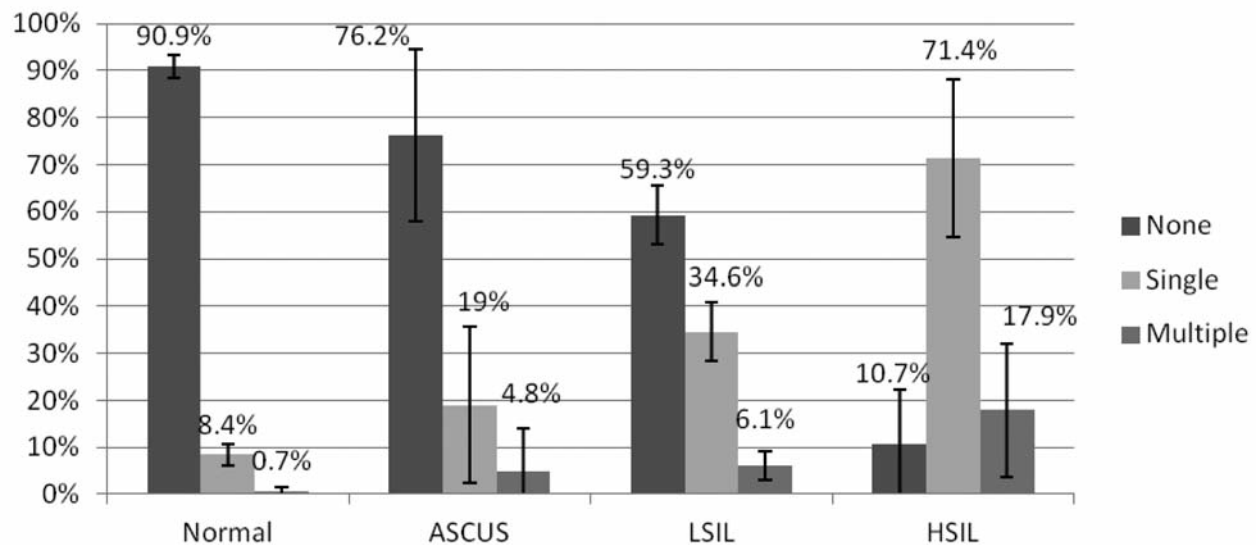


Figure 2. Prevalence (with 95% confidence intervals) of single and multiple HPV infection detected by the RNA assay, according to cytology. ASCUS: atypical squamous cells of unknown significance; LSIL: low-grade squamous intraepithelial lesions; HSIL: high-grade squamous intraepithelial lesions.

Our molecular analysis data were compared with the cytological data. The lowest rates for HPV DNA-positive samples were in women with normal cytology at 21.1% (95% CI=3.7% to 7.5%), followed by women with ASCUS 38.1% (95% CI=17.3% to 58.9%). A total of 196/231 patients (84.8%) (95% CI=80.2% to 89.5%) with LSIL were found to have HPV DNA, while 96.4% (27/28) of those with HSIL were also positive (Figure 1).

E6 and E7 transcripts of the five hrHPV types were detected in 52/569 (9.1%; 95% CI=6.8% to 11.5%) patients with normal cytology. Among the patients with ASCUS, 5/21 (23.8%; 95% CI=5.6% to 42%) were found to have HPV RNA, compared with 94/231 of women with LSIL (40.7%; 95% CI=34.4% to 47%) with the proportion rising to 25/28 (89.3%; 95% CI=71.8% to 97.8%) in women with HSIL (Figure 2).

Discussion

Apart from cytological evaluation, molecular tests are crucial for the management of cervical diseases. In many cases, cytologically-normal women have been detected as having cervical intraepithelial neoplasia grade 2+ (CIN2+) on biopsy (11). This may be due to the fact that cells with latent HPV infection are usually located in basal and parabasal layers and not detected in the cytological sample (12). As a result, hrHPV DNA and mRNA assays play an important role in detecting women who are at risk for cervical cancer. The goal of this study was to investigate HPV typing and hrHPV E6/E7 mRNA expression according to cytology.

Almost half of the women tested positively for HPV DNA and one sixth of the women tested were infected by one of the five most common hrHPV types 16, 18, 31, 33 and 45. Studies in Greece report that HPV infection ranges from 20.7% to 50.7% (13), while hrHPV types are detected in one fifth to one third. The rates we present here are lower than what have been reported as we refer to only five hrHPV types. According to the NASBA assay, positivity for these five hrHPV types increases to 20.7%, presenting a difference of 4% between these methods. As we refer to common types, this can be partially explained by differences in the biology of infections and the molecular differences induced by the virus in each woman. HrHPV types 16, 18, 31, 33 and 45 are considered to be implicated in >90% of cervical carcinomas (4, 14). The role of mRNA expression of the oncoproteins E6/E7 by these types is well-established in carcinogenesis. E6/E7 is highly expressed by HPV DNA integrated into the host's genome and thus remains undetectable with HPV DNA tests if totally integrated.

LrHPV types without co-infection with hrHPV types were detected at a low percentage. The frequencies of multiple infections in the present study are significantly higher compared to that reported by our team in a study conducted between 2007-2010 (15).

HPV 16 was detected in 10.2% of the women tested with the HPV DNA test while mRNA expressed by HPV 16 was detected as being only slightly raised (11.7%). On the contrary, HPV 18 was detected at almost double the percentage with the RNA assay. This highlights the role of HPV 18 in many latent transforming infections as it produces a great number of E6/E7 transcripts. It has been shown that E7 induces the E2F5 repressor in infected cells, acting as an activator rather than as a repressor and this is specific for cervical carcinoma associated with HPV 18 (16).

An interesting finding is that in HSILs, single-genotype infections were twice as frequent as multiple genotype infections. This is in contrast to what was observed in LSILs, where single and multiple infections were approximately equal in frequencies. In HSIL, a single genotype may persist, leading to high-grade neoplasia. Regarding the mRNA assay,

multiple infections represent a very small proportion mainly because the test detects only five genotypes. According to this test, women with HSIL were infected with multiple genotypes at a rather higher percentage, confirming that elevated expression of E6/E7 mRNA from HPV 16 and 18 are a major factor that can lead to cervical cancer.

Not surprisingly, all cases of normal cytology and cervical lesions of any grade displayed lower E6/E7 mRNA positivity than HPV DNA detection. The results from the DNA test related well with the grade of lesion. The lowest prevalence rates for hrHPV DNA for at least one of the types 16, 18, 31, 33 and 45 were observed in women with cytologically normal findings. In contrast, the higher rates of the above hrHPV types were seen in women with high-grade lesions, which is in accordance with what is reported by other studies (17, 18). This is expected as disease progression may depend on persistent hrHPV infection.

In agreement with other reports and according to the RNA test, the proportion of women with detectable hrHPV types, 16, 18, 31, 33 and 45, increased progressively as the grade of cervical lesion increased (19-21). Interestingly, in our series, the NucliSens EasyQ HPV assay was positive in 9.1% of women with normal cytology, which is higher than what has been reported by a larger cross-sectional study, where the RNA test was positive in 2.4% of women with the above cytology (22). However, other previously published data indicated the prevalence of E6 and E7 transcripts in a significantly higher proportion of women with normal findings than our study (18.2% and 25%, respectively) (17, 18). The detection of E6 and E7 mRNA expression in some women with normal cytology or low-grade lesions may reflect the oncogenic activity of the above hrHPV types before initiation of transformation of the infected cells. Therefore, E6 and E7 transcripts could be used as screening markers for better surveillance in this subset of women.

This study described the detection rates of hr and lr HPV genotypes, as well as E6/E7 mRNA expression, in 849 Greek women displaying both normal and abnormal cytological results. In summary, single-genotype infections were twice as frequent as multiple genotype infections in HSIL. In our series, E6/E7 mRNA expression was prevalent in 9.1% of women with normal cytology, which is rather high compared to other studies. HPV 18 was detected at almost double the rate with the RNA assay.

Conflicts of Interests

The Authors declare that there are no conflicts of interests.

Acknowledgements

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