HPV types and E6/E7 mRNA expression in cervical samples from Turkish women with abnormal cytology in Ankara, Turkey

İpek TÜNEY1, Aylin ALTAY2, Köyar ERGÜNAY1, Seyven Çelik ÖNDER3, Alp USUBÜTÜN4, Mehmet Coşkun SALMAN5, Gülendam BOZDAYI5, Erdem KARABULUT1, Osman Selim BADUR5, Kunter YÜCE7, Ahmet PINAR1,*

1Department of Medical Microbiology, Virology Unit, Faculty of Medicine, Hacettepe University, Ankara, Turkey
2Department of Medical Microbiology, Faculty of Medicine, Gazi University, Ankara, Turkey
3Department of Pathology, Cytology Unit, Faculty of Medicine, Hacettepe University, Ankara, Turkey
4Department of Pathology, Faculty of Medicine, Hacettepe University, Ankara, Turkey
5Department of Obstetrics and Gynecology, Faculty of Medicine, Hacettepe University, Ankara, Turkey
6Department of Biostatistics, Faculty of Medicine, Hacettepe University, Ankara, Turkey
7Department of Medical Microbiology, Faculty of Medicine, İstanbul University, İstanbul, Turkey

* Correspondence: apinar@hacettepe.edu.tr

1. Introduction

Human papillomaviruses (HPVs) are a large and heterogeneous group of DNA viruses belonging to the family Papillomaviridae, comprising about 100 well-characterized genotypes and more than 100 putative new types (1,2). Infections with HPVs have been established as a risk factor for invasive carcinoma of the uterine cervix by epidemiological and laboratory data (1,3). Based on the potential risk to induce invasive cancer, HPV types that infect the anogenital tract are currently grouped into high, potentially high, and low risk types, with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 regarded as high risk; 26, 53, and 66 as potentially high risk; and 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 as low risk (4).

The majority of HPV infections are transient and approximately 90% of infections will normally be cleared within 2 years (5,6). However, persistence of high-risk HPV types is associated with the development of high-grade squamous intraepithelial lesions and progression to cervical cancer (6,7). Cytology testing for detection of intraepithelial lesions has low sensitivity (8). HPV DNA testing has been suggested for screening of women over 30 or women with equivocal cytology results (9,10). However, since the overall life-long risk for HPV infection is 80%, only a fraction of these women with detectable DNA are likely to progress to malignant transformation (5,11). Thus, a more reliable viral marker for predicting HPV persistence and initiation of cellular transformation is required to predict progression to cervical neoplasia.
E6 and E7 oncoproteins of HPV contribute significantly to the malignant transformation of persistently infected cells, by inactivation of the p53 and pRb tumor suppressor proteins (12). Viral E6/E7 proteins are consistently expressed in malignant tissues and upregulated expression of E6/E7 is considered necessary for the initiation and progression of cervical neoplasia. The detection of E6/E7 mRNA transcripts may therefore be superior to HPV DNA detection as an indicator of HPV infection associated with increased risk of progression to neoplasia (13,14). Recently, standardized commercial assays based on nucleic acid sequence-based amplification (NASBA) technology have been available for detecting HPV E6/E7 mRNA expression from the five most prevalent HPV types in cervical cancer. Previous reports on the performance of HPV E6/E7 mRNA detection demonstrated a higher clinical specificity for detecting high-grade histological lesions compared to DNA-based tests, but a lower clinical sensitivity (15). The aim of the present study was to evaluate HPV mRNA and DNA detection in samples with abnormal cytology.

2. Materials and methods

2.1. Study design and cervical sampling

The study was conducted at Hacettepe University Hospital, a tertiary care hospital and a major reference center in Ankara Province, Turkey. The study protocol was approved by the local ethics board (13.07.11/0426).

Liquid-based (SurePath) cervical specimens were obtained at the outpatient clinics of the Department of Obstetrics and Gynecology via cervical brushes during January–October 2011 after informed consent was obtained. The brushes were immediately placed into a BD SurePath Preservative Fluid (TriPath Imaging, Burlington, NC, USA) vial and sent to the Department of Pathology, Cytology division. The specimens were processed for thin layer cytology slides in the laboratory within 1 to 3 days of the date of collection. After slide preparation, all residual samples were transported to Hacettepe University Department of Medical Microbiology, Virology Unit and kept frozen at −70 °C until further analyses. The slides were evaluated by experienced cytotecnologists and pathologists under light microscopy. Cytological evaluations were reported according to the 2001 Bethesda System (16). Repeat samples from the same patient were excluded. If a biopsy was performed during the follow-up of the patient, the specimens were fixed routinely in formalin, embedded in paraffin, and stained with hematoxylin–eosin for histopathological diagnosis. The microbiologists from Hacettepe and Gazi Universities were blinded to the cytology results of the patients.

2.2. Detection of HPV E6/E7 mRNA

All nucleic acid extraction and E6/E7 mRNA detections were performed in Hacettepe University Department of Medical Microbiology, Virology Unit. The residual specimens that had abnormal cytology results were centrifuged for 5 min at 6000 rpm, washed three times, resuspended in 500 µL of Tris-EDTA, and subjected to total nucleic acid extraction via a magnetic silica-based automated commercial system (NucliSensa easyMAG, bioMérieux, France). E6/E7 mRNA detection was performed using a commercial NASBA assay, the NucliSensa EasyQ HPV v1.1 (bioMérieux, France), according to the manufacturer's instructions. The system incorporates 5 molecular beacons to identify and differentiate E6/E7 mRNA expression in HPV types 16, 18, 31, 33, and 45. Concurrent amplification and detection of small nuclear ribonucleoprotein specific protein A (U1A) are also used as an internal control in the system.

2.3. Detection and typing of HPV DNA

HPV detection and typing were performed in Gazi University Department of Medical Microbiology. A real-time PCR-based commercial system (Heliosis Human Papilloma Virus LC PCR Kit, Metis Biotechnology, Turkey) was employed for HPV DNA detection and HPV-16 typing via melting curve analysis. The amplifications were carried out in PTC-200 Thermal Cycler (MJ Research, USA) and LightCycler 2.0 (Roche Diagnostics, Germany) instruments. Amplification of the internal control was visualized at a melting temperature (Tm) of 90 °C (±3.5 °C), whereas amplifications of HPV-16 and other HPV types were interpreted by melting curves at 69 °C (±1.0 °C) and 82 °C (±3.5 °C), respectively. The analytical sensitivity of the system is given as 20 copies/µL for HPV types 16 and 18.

2.4. Statistical analysis

Statistical analysis of collected data was performed in Hacettepe University Department of Biostatistics. Descriptive parameters of the study group, HPV types, and the presence E6/E7 mRNA expression were assessed via statistical tests. Student's t and extension of Fisher's exact test to rxc tables were employed wherever appropriate. Statistical significance was considered as P < 0.05 level. All statistical analyses were performed by SPSS version 15.0 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Cytological and histological diagnoses in cervical samples

A total of 81 women (age: 22–89 years, mean: 42.49, median: 40, standard deviation: 13.37) with suitable specimen conditions and reactive internal control signals were evaluated for HPV DNA and mRNA expression after informed consent was obtained. The cytological diagnoses were atypical squamous cells of undetermined significance (ASC-US) in 25 samples (30.9%), atypical
squamous cells - cannot rule out high-grade squamous intraepithelial lesion (ASC-H) in 3 samples (3.7%), atypical glandular cells (AGC) in 4 samples (4.9%), atrophic cells in 2 samples (2.5%), low-grade squamous intraepithelial lesion (LSIL) in 35 samples (43.2%), high-grade squamous intraepithelial lesion (HSIL) in 9 samples (11.1%), and adenocarcinoma in 3 samples (3.7%) (Table 1). Biopsy results were available for 25 individuals (25/81, 30.9%) and they revealed the presence of chronic cervicitis in 7 (28%), cervical intraepithelial neoplasia grade I (CIN I) in 8 (32%), cervical intraepithelial neoplasia grade III (CIN III) in 7 (28%), and adenocarcinoma/anaplastic carcinoma or carcinosarcoma in 3 samples (12%).

3.2. HPV DNA and E6/E7 mRNA expression in cervical samples

HPV DNA was identified in 73 samples (73/81, 90.1%), consisting of HPV-16 in 46 samples (63.1%), HPV other than 16 in 15 samples (20.5%), and mixed HPV infections in 12 samples (16.4%) (Table 2). HPV DNA positivity was not associated with patient age (P = 0.939) and did not demonstrate a statistically significant difference according to the cytological diagnosis (P = 0.35). Variations in HPV type distribution in samples with different cytological lesions, as determined via DNA assay, were also not statistically significant (P = 0.464) (Table 1).

HPV E6/E7 mRNA expression was observed in 45 samples (45/81, 55.6%) (Table 1). Expression from HPV-16 was identified in 26 samples (57.8%), followed by HPV-18 (2/45, 4.4%) and HPV-31 (1/45, 2.2%), whereas mRNA from multiple HPV types was revealed in 16 (35.6%) (Table 2). A significant age difference was observed in patients with and without E6/E7 expression, wherein individuals with positive mRNA results were younger (mean age: 39.56 versus 46.17, P = 0.026). Moreover, a statistically significant difference in E6/E7 expression among the various cytological diagnosis groups was noted (P = 0.025).

Distribution of HPV DNA types and mRNA expression according to the cytological evaluation results is provided in Table 2.

3.3. Comparison of HPV DNA and mRNA expression

Overall, the detection rates of HPV DNA and E6/E7 mRNA in samples were statistically significant (90.1% versus 55.6%, P = 0.002). However, no significant difference in mRNA expression among HPV types (as interpreted as type 16, other than 16, and mixed infections) via DNA assay was noted (P = 0.95).

A total of 8 samples (8/81, 9.9%) were negative in both assays, which comprised 2 cervical atrophy, 3 ASCUS, and 3 LSIL specimens. All samples with detectable mRNA were positive for HPV DNA. However, expression of E6/E7 mRNA was absent in 28 samples (28/73, 38.4%) with detectable DNA (Table 3). Expression of mRNA was identified in 28 samples (28/46, 60.9%) with positive HPV-16 DNA, in 7 samples (7/46, 15.2%) with HPV types other than 16, and in 10 samples (10/12, 83.3%) with mixed HPV types (Table 3). Expression patterns inconsistent with DNA typing were obtained in 3 samples (3/45, 6.7%), which included HPV-16 mRNA detected in 2 non-HPV-16 DNA samples and HPV-31 expression in a sample with HPV-16 DNA (Table 3). In 4 samples with only HPV-16 DNA (4/46, 8.7%), expression of other HPV types (18 and 45) was also detected. Expression from HPV-16 was observed in 3 samples (3/15, 20%), interpreted as non-HPV-16 according to the DNA assay. Expression from HPV-16 or HPV-18 was detected in 2 samples (2/12, 16.7%) with mixed HPV types (Table 3).

In patients with available biopsy results, HPV DNA was detected in all cytology samples. HPV E6/E7 expression was present in all individuals with a diagnosis of CIN I–III (n: 17), while it was only detected in 2 out of 7 individuals with chronic cervicitis (Table 4).

Table 1. Detection of HPV DNA and E6/E7 mRNA expression according to the cytological diagnosis.

<table>
<thead>
<tr>
<th></th>
<th>HPV DNA</th>
<th></th>
<th>HPV mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive (#/%)</td>
<td>Negative (#/%)</td>
<td>Positive (#/%)</td>
</tr>
<tr>
<td>ASCUS (n: 25)</td>
<td>22 (88)</td>
<td>3 (12)</td>
<td>12 (48)</td>
</tr>
<tr>
<td>ASC-H (n: 3)</td>
<td>3 (100)</td>
<td>0 (0)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>AGC (n: 4)</td>
<td>4 (100)</td>
<td>0 (0)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Atrophy (n: 2)</td>
<td>0 (0)</td>
<td>2 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>LSIL (n: 35)</td>
<td>32 (91.4)</td>
<td>3 (8.6)</td>
<td>18 (51.4)</td>
</tr>
<tr>
<td>HSIL (n: 9)</td>
<td>9 (100)</td>
<td>0 (0)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>Adenocarcinoma (n: 3)</td>
<td>3 (100)</td>
<td>0 (0)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>Total</td>
<td>73 (90.1)</td>
<td>8 (0.9)</td>
<td>45 (55.6)</td>
</tr>
</tbody>
</table>
4. Discussion
Persistent infections with high-risk HPV genotypes constitute a preventable risk factor for the development of cervical intraepithelial lesions and cervical cancer (1,3). HPV DNA detection, along with Pap test and colposcopy, provides an efficient method of screening to facilitate identification of high-risk HPV infections, which may provide adequate follow-up and treatment on an individual basis, and thus reduced incidence and mortality due to cervical carcinoma (17). Recently, HPV E6/E7 oncogene expression emerged as a promising biomarker to determine the risk for the progression to high-grade cervical lesions, since unregulated and prolonged expression of these viral oncogenes is better correlated with progression of cervical lesions to cancer (18,19).

In our study, HPV DNA and E6/E7 mRNA expression were investigated in a cohort of 81 individuals with cytological diagnoses of ASC-US (30.9%), LSIL (43.2%), and HSIL (11.1%). The biopsy results of 25 patients with were classified as chronic cervicitis (28%), CIN I (32%), CIN III (28%), and adenocarcinoma/anaplastic carcinoma/carcinosarcoma according to their histological diagnosis. However, due to the lack of patients with a diagnosis of CIN II, HPV DNA and E6/E7 mRNA results of that group of patients could not be compared.

In women with abnormal cytology, HPV DNA was detected at a rate of 90.1%. Global HPV prevalence in invasive cervical cancer is reported to be about 92.4% (20). Based on a study in Greece, the rate of HPV DNA and high-risk HPV DNA in patients with abnormal cytology or colposcopy was identified as 55.5% and 50.6%, respectively (21). In Brazil, 93% HPV DNA positivity in women with CIN II biopsy was demonstrated and 90% of them were high-risk HPV genotypes (19). In the present study, high-risk HPV genotypes could only be differentiated as type 16 and non-16 due to the detection methods employed. Therefore, 90.1% HPV reactivity reflects all HPV types in the study group, and is slightly higher compared to the

| Table 2. Distribution of HPV DNA types and mRNA expression according to the cytological diagnosis. |
| HPV DNA | HPV mRNA |
| Type 16 | OT-16 | Mixed | Type 16 | Type 18 | Type 31 | 16, 18 | 16, 45 | 18, 31 | 16, 18, 31 | 16, 18, 45 |
| ASC-US | 15 | 4 | 3 | 8 | 0 | 1 | 0 | 0 | 0 | 2 | 1 |
| ASC-H | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| AGC | 2 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| LSIL | 20 | 7 | 5 | 8 | 1 | 0 | 4 | 1 | 0 | 3 | 1 |
| HSIL | 6 | 2 | 1 | 7 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| AC  | 2 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| Total | 46 | 15 | 12 | 26 | 2 | 1 | 4 | 1 | 1 | 8 | 2 |

*Expression from undetected HPV types and/or combinations are not depicted in the table

| Table 3. HPV E6/E7 mRNA expression according to DNA typing. |
| HPV mRNA |
| Negative | Type 16 | Type 18 | Type 31 | 16, 18 | 16, 45 | 18, 31 | 16, 18, 31 | 16, 18, 45 |
| HPV DNA | |
| Negative 8 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| HPV-16 | 18 | 23 | 0 | 1 | 2 | 0 | 0 | 0 | 2 | 46 |
| OT-16 | 8 | 2 | 1 | 0 | 2 | 0 | 1 | 1 | 0 | 15 |
| Mixed | 2 | 1 | 1 | 0 | 0 | 1 | 0 | 7 | 0 | 12 |

*Expression from undetected HPV types and/or combinations are not depicted in the table

*Other than 16
high-risk HPV prevalence around the world. Nevertheless, HPV type 16 emerged as the most frequently detected genotype among all, which is in concordance with various previous reports (13,20).

HPV DNA other than type 16 as well as multiple HPV infections were determined as 20.5% and 16.4%, respectively, in the present study. In another study performed in Turkey, type 16 HPV DNA was detected in 20.8% of the samples and non-16 HPV constituted 79.2% (22). These variations are likely to reflect the difference in study groups where the latter also included samples with normal cytology.

Expression of HPV E6/E7 mRNA was identified in 55.6% of the samples where HPV type 16 constituted 57.8%, followed by type 18 (4.4%) and type 31 (2.2%). Furthermore, expression from multiple HPV types was revealed in 35.6%. A study by Broccolo et al. in Italy reported HPV E6/E7 mRNA expression was present in 7% of women with normal cytology, in 20.3% with ASC-US, in 37% with LSIL, and in 70.9% with HSIL (23). Discacciati et al. found mRNA expression in 49% of patients with CIN2 grade lesions and with HPV DNA 16/18/31/33/45 (19). Sixty percent of them were type 16, 30% of them were type 33, 15% of them were type 31, and 5% of them were types 18 and 45. Castro et al. detected 55.7% E6/E7 mRNA in patients with ASC-US/LSIL and 77.5% in patients with HSIL (24).

Although there was not a statistically significant difference between HPV DNA positivity and patient age,
an association was observed between E6/E7 oncogene expression and age where E6/E7 mRNA expression was correlated with younger age. This is likely to represent active and transient infections in young adults. In a study by Frega et al., frequency of HSIL/LSIL in mRNA positive patients older than 35 was significantly higher than in mRNA negative patients (25). However, this difference was not detected in patients younger than 35.

When the pathology results of biopsy material is accepted as the gold standard, chronic cervicitis was detected in 7 patients, CIN I was detected in 8 patients, and CIN III was detected in 7 patients. Regarding the 7 chronic cervicitis patients, 3 of them were diagnosed with ASC-US, 2 of them with ASC-H, and 2 of them with LSIL cytologically. Only two of the chronic cervicitis patients were positive for E6/E7 mRNA, while all of them positive for HPV DNA. One of the mRNA positive patients was diagnosed with ASC-H and the other with LSIL by cytology. For CIN I and III patients both mRNA and DNA tests were positive. Thus, mRNA test results seem more correlated with pathology results than cytology and DNA tests especially for nonprecancerous lesions for this study group. The significant difference in E6/E7 expression detected among various cytological diagnosis groups definitely requires further investigation in larger cohorts.

In the present study, a statistically significant difference was observed between HPV DNA (90.1%) and E6/E7 mRNA (55.6%) results. Similar to our study, Tezcan et al. found a significant difference between HPV DNA 16/18/31/33/45 (7.9%) and E6/E7 mRNA (2.5%) (22). Although integration of some HR-HPV types is not always necessary for progression of squamous intraepithelial lesions, mRNA testing is useful to predict the progression of these lesions (26). mRNA expression could not be detected in 28 samples (38.4%) with HPV DNA. It can be hypothesized that mRNA testing reflects the active replication of HR HPV. The risk of development of CIN2 and higher grades is five times higher in mRNA positive women than in mRNA negative women (18). Rossi et al. found 74.1% HPV DNA and 23.6% mRNA and only 3 HR-HPV DNA negatives were mRNA positive (18). In the study by Spathis et al., HPV DNA and mRNA were detected as 55.5% and 29.7%, respectively, and consistency between the 2 methods was 71.6% (21). Persson et al. stated that mRNA testing has the highest sensibility to predict CIN2 and higher pathologies in ASC-US and LSIL groups; however, they indicated that its specificity is insufficient (<50%) and HPV DNA testing and repeat cytology are more specific than mRNA testing (27).

Sixty percent of the patients with HPV DNA also demonstrated mRNA expression. Expression of mRNA was detected in 15% of other than type 16 HPV positives and 83% of multiple HPV types. There was no significant difference between HPV DNA types and their mRNA expressions. In the study by Discacciati et al., E6/E7 mRNA was detected at 49% rate for the HPV DNA types 16/18/31/33/45 (19). Two cervical atrophy, 3 ASC-US, and 3 LSIL (totally 8) samples were found negative by the two methods. However, false negativity or latent infection possibility should not be ignored. There were 3 samples whose HPV DNA and mRNA typing results were inconsistent. Although HPV 16 DNA was detected in one of them, HPV type 31 was detected by mRNA testing. The other 2 samples were HPV 16 DNA negative, but HPV 16 mRNA expression was detected in these samples. It is possible that some problems in PCR could cause this situation. Castle et al. found negative 3% of mRNA positive samples by PCR (13).

When HPV DNA and E6/E7 mRNA results are compared according to cytology reports, it is seen that DNA positivity increases a little with lesion grades. Rates of mRNA distinctly increase towards ASC-US, LSIL, and HSIL; however, mRNA could not be detected in one of the three DNA positive samples with carcinoma (Table 1). Moreover, in all of the patients with CIN I and CIN III pathology (n: 17), mRNA testing was positive. Castle et al. detected E6/E7 mRNA positivity in 43% of patients with ASC-US, in 71% of patients with LSIL, and in 87% of patients with HSIL (13). Thus, our data reveal mRNA testing to be more relevant to lesion grade than DNA testing and more useful for the diagnosis and follow-up of women with the risk of progressive cervical disease.

Acknowledgments
This study was supported by Hacettepe University Scientific Research Unit Grant No: 5849. The authors would like to thank Mr Levent Öztepe and Mr Can Aydoğan from BioMérieux, Turkey, for technical assistance and support. Preliminary findings of this study were presented as a poster presentation at the 4th National Virology Congress held in Istanbul, Turkey, during 23–26 July 2011, and appear in the abstract book (p. 142).

References


14. Coquillard G, Palao B, Patterson BK. Quantification of intracellular HPV E6/E7 mRNA expression increases the specificity and positive predictive value of cervical cancer screening compared to HPV DNA. Gynecol Oncol 2011; 120: 89-93.


