CELLULAR AND VIRAL MARKERS IN HUMAN PAPILLOMAVIRUS RELATED CERVICAL LESIONS PROGRESSION

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The etiological role of high-risk human papillomavirus (HPV) in invasive cervical cancer has been demonstrated by epidemiological and molecular studies. In HPV-associated cervical carcinomas, the expression/function of p16 is impaired by the action of viral E6 and E7 oncoproteins. In order to evaluate the potential of p16INK4a and E4 HPV16 mRNA as clinical markers for progression and development of cervical malignant lesions we analyzed cervical specimens from patients with different cytology (collected in liquid cytology medium from 89 women, (22-72 years old) which were used to isolate genomic DNA (High Pure PCR Template, Roche Diagnostics) and RNA (RNAeasy kit- Qiagen). E4 HPV16 mRNA gene expression was performed using E4 HPV16 PCR and the results obtained were confirmed by Southern Blot Hybridization. We evaluated p16INK4a expression using TaqMan Real Time PCR (Applied Biosystems). The molecular detection of HPV DNA and E4 HPV16 expression were corroborated with p16INK4a expression as a positive predictive value for disease progression.

Key words: Cervical cancer; HPV; p16; Biomarkers.

INTRODUCTION

Cervical cancer is one of the most important causes of death in women worldwide.

High risk human papillomaviruses (HR-HPV) are known to play an important etiological role in the cervical cancer, but only a very small part of infected women develop invasive cervical cancer. Persistent infection with HR-HPV, especially type 16, is regarded as a significant risk factor in the development of squamous cervical lesions or squamous cervical cancer. However, most infections with HPV regress spontaneously, and for the cases that do progress to cancer, a long period of latency is normally observed. Persistence of a high-risk HPV-infection is a prerequisite for the development of HGSIL (high-grade intraepithelial lesion) and cancer. The detailed molecular analysis of these cellular changes in HGSIL identified an overexpression of the cyclin-dependent kinase inhibitor- p16INK4a as a marker of an active expression of the viral oncogene E7 of all HR-HPV types. p16, a tumor suppressor protein and also a cyclindependent kinase inhibitor, whose overexpression has repeatedly been reported to be typical for dysplastic and neoplastic epithelium of cervix, slows down the cell cycle by inactivating the cyclin-dependent kinasies that phosphorylate retinoblastoma protein (pRb). The viral oncogenes E6 and E7 of HPV, whose expression is associated with malignant transformation of the cervical epithelial cells, have the ability to bind and inactivate pRb. The pRb status has are remarkable influence on the expression of p16. p16 expression in cervical lesions is hypothesized to be caused by the functional inactivation of pRb by HPVE6 and E7 proteins. Many studies have proposed that p16 is a useful biomarker especially
for HR-HPV type related cervical neoplasia, and also for predicting SIL (squamous intraepithelial lesion) progression\(^4\)\(^6\).

The life cycle of human papillomaviruses (HPVs) is tightly coupled to the differentiation program of their host epithelial cells. HPV E4 gene expression is first observed in the parabasal layers of squamous epithelia, suggesting that the E4 gene product contributes to the mechanism of differentiation-dependent virus replication, although its biological function remains unclear\(^7\). The functional region of E4 necessary for the growth arrest activity was located in the central portion of the molecule, and this activity was independent of the E4-mediated collapse of cytokeratin intermediate filament structures.

The aim of this study was to analyze p16INK4a expression in cervical preneoplastic and neoplastic lesions and to correlate it with lesion grade and with E4 gene expression. As the increased levels of p16INK4a could reveal high risk cervical lesions, p16INK4a could be a valuable marker in patient management. Due to the fact that biopsy is not recommended in all cases, we evaluated p16INK4a expression in cervical smears as a transformation biomarker that might be used as a screening marker.

**MATERIALS AND METHODS**

**Biological samples:** 89 women (22–72 years old) addressing the Bucur Gynecological Hospital Bucharest for periodical cytological investigation, were included in the study. Consent was obtained from each participant. Cervical specimens collected in Copan transport medium were obtained during colposcopic examination.

**DNA extraction:** DNA extraction was performed with High Pure PCR Template (Roche Diagnostics) according to the manufacturer’s procedures. DNA concentration and purity was determined using Nanodrop spectrophotometer.

**HPV detection/genotyping:** viral testing was done with Linear Array HPV Genotyping Test (Roche), according to manufacturer instructions. Briefly, the test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 base pairs long. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 HPV genotypes, including 13 high risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Capture probe sequences are located in polymorphic regions of L1 bound by these primers. An additional primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification.

**RNA extraction:** A 500 µL aliquot of the cervical specimen was used for RNA extraction using RNAeasy kit (Qiagen) according to the manufacturer’s protocol. The quality of isolated RNA was assessed by determining the concentration and purity using Nanodrop spectrophotometer.

**Reverse-transcription:** Total isolated RNA was reverse-transcribed with Promega reagents. Briefly, 2.5 µg of RNA, 1µl oligo(dT) and 1µl dNTPs were mixed and incubated 5 minutes at 65°C. After cooling on ice, 4 µl RT buffer, 2 µl DTT and 1µl RNase inhibitor were added to the mix. After 2 minutes incubation at 37°C, 1 µl of M-MLV reverse transcriptase was added and the incubation proceeded for 60 minutes at 37°C and 15 minutes at 70°C. The cDNAs were stored at −20°C until use.

In order to determine cDNA quality, PCR assay for β-2-microglobulin (B2M) house-keeping gene was performed on all samples and afterwards the PCR products obtained were analyzed using agarose gel electrophoresis (2%).

**E4 HPV16 PCR:** 2µl cDNAs were amplified for 35 cycles in a 50 µl final volume containing buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl\(_2\), and 50 mM KCl), 200 µM of each dNTPs, 2.5 units of Taq DNA polymerase (Promega), and 0.5 µM HPV16 E4 primers, designed and synthesised by Invitrogen F 5’-AACGAAGTATCCTCTGAA-3′, R 5’-GACAGTGCTCAATCTCTGCTACT-3′). Each cycle consisted of denaturation at 95°C for 1 minute, annealing at 60°C for 2 minute and extension at 72°C for 1 minute. The PCR products were visualized by electrophoresis in 2% agarose gel.

**Southern blot method:** The Southern blotting was performed in order to verify the results obtained in PCR for E4HPV16. PCR products were probed using E4HPV 16 biotin labeled probe (5′- biotin-ACA GACG ACTAT CCAGCGA-

**Quantitative Real-time PCR Assays:** The expression of p16INK4a was evaluated using TaqMan Real Time PCR System (Applied Biosystems). 100 ng total cDNA was amplified using Real-Time amplification and detected in the Applied Biosystems 7300 Real Time PCR System.

**Statistical analysis:** The Student t test was performed to compare differences between groups for quantitative variables. Double-sided P values of ≤0.05 were considered significant.

**2^{-ΔΔCt} Method**

We used this method for relative quantification of p16 target gene expression related to the house - keeping beta – actin gene. This method is appropriate considering the PCR efficiency 100% for TaqMan assay.
RESULTS AND DISCUSSIONS

HPV DNA presence was performed and the correlation between HPV infection and cytological results (ranging from NILM, LSIL, HSIL to cancer) are showned in Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Distribution of HPV positive samples (high risk - hrHPV, low risk - lrHPV) related to the cytological exam (PAP smears)</td>
</tr>
<tr>
<td>AGE</td>
</tr>
<tr>
<td>&lt;30</td>
</tr>
<tr>
<td>&gt;30</td>
</tr>
<tr>
<td>HPV</td>
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<tr>
<td>+</td>
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<tr>
<td>hrHPV</td>
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<td>lrHPV</td>
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Further in our study, from 89 patients we analyzed only the 33 patients (37.1%) that tested positive for HPV-16 genotype (Fig. 1).

To establish p16INK4a potential as a dysplastic lesions biomarker, we analyzed p16INK4a expression at mRNA level. p16INK4a expression level was correlated with cytological grade:
According to the literature the highest level of p16 gene expression was found in high grade lesions and in cancer tumor. Our study demonstrates an increasing level of p16 gene expression in LSIL, HSIL lesions the highest gene expression value was obtained in SCC which is two times greater than the reference.

In NILM cases we found the lower status of p16 gene expression, also the ASCUS lesions present a decreased level this gene expression (Table 2).

Table 2

<table>
<thead>
<tr>
<th>Cytology</th>
<th>p16 levels of expression</th>
<th>Hipereexpression</th>
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<tbody>
<tr>
<td>NILM (n=5)</td>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>ASCUS (n=2)</td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>LSIL (n=10)</td>
<td></td>
<td>30%</td>
</tr>
<tr>
<td>HSIL (n=10)</td>
<td></td>
<td>60%</td>
</tr>
<tr>
<td>SCC (n=6)</td>
<td></td>
<td>100%</td>
</tr>
</tbody>
</table>

We found that E4 HPV16 gene expression presents the highest value in LSIL, while in ASCUS the expression pattern was moderate and high in the same percent 3.03%. In advanced stages HSIL the level of E4 HPV gene expression decreased considerably (6.06% high expression, 24.24% lack of expression) till in SCC where we didn’t find any expression (Fig. 5).

To verify the results obtained in E4 cDNA PCR we performed a Southern Blot Hybridization which confirmed the E4 gene status expression.

Our results showed a moderate E4 gene expression in 9.09% of cases and a high expression in 6.06% of cases (NILM cytology, and HPV 16 positive samples).

**CONCLUSIONS**

The E4 viral gene expression is a possible biomarker for incipient infection (especially for LSIL), a productive infection with the release of new viral particles.

The E4 expression is tightly linked to host cell differentiation; it likely plays an important role in the viral life cycle, while p16 gene expression may indicates a later stage of HPV infection. p16 gene expression level may indicate the transition from a low risk to high risk lesions, as we observed in our study and the hyperexpression of this gene characterized the squamous cell carcinoma.

**REFERENCES**


