ANALYSIS OF P53, P16INK4A, PRB AND CYCLIN D1 EXPRESSION AND HUMAN PAPILLOMAVIRUS IN PRIMARY OVARIAN SEROUS CARCINOMAS

O.O. Bilyk1*, N.T. Pande2, L.G. Buchynska1

1RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine
2Oregon Health & Science University, Portland, Oregon 97239, USA

Aim: To evaluate the prognostic relevance of key cell cycle regulatory proteins p53, p16INK4a, pRb and Cyclin D1 expression, the presence of high risk HPVs and their association with clinicopathological parameters and the clinical follow up in ovarian cancer patients. Methods: 53 cases of primary ovarian serous carcinomas were immunohistochemically examined for the expression of p53, p16INK4a, pRb and Cyclin D1 proteins. Tumor DNA was extracted from paraffin blocks and subjected to HPV 16 and 18 testing. The association between HPV 16 and 18 E6 oncprotein and cell cycle proteins expression in ovarian carcinomas also was evaluated by immunohistochemistry. Results: We demonstrated that a majority of moderately and poorly differentiated ovarian carcinomas are characterized by strong expression of p53 and p16INK4a proteins. In contrast, strong staining with cyclin D1 antibody was observed in well differentiated tumors. The correlation between strong p53, pRb, Cyclin D1 and clinical stages of disease was also observed. We show that patients with high positivity for p53, p16INK4a and Cyclin D1 had a poor prognosis and reduced overall survival. The presence of HPV 16/18 DNA was detected in 17% of ovarian carcinomas. The tumor tissues that reacted positively to HPV E6 antibody in focal and diffuse manners had also significantly low p53 expression profile. Conclusion: These findings suggest that p53, p16INK4a and Cyclin D1 expression and HPV infection may represent a promising tool toward the identification of ovarian cancer patients with poorer prognosis and shorter survival who might therefore need a more aggressive therapy and HPV screening. Key Words: ovarian cancer, cell cycle proteins, prognostic significance, HPV.
Despite the number of reports indicating the presence of HPV in OC tissues there is no evidence linking HPV infection to clinicopathological and molecular features of OC. The function of HPV E6 oncoprotein in primary ovarian cancer cells with respect to cell cycle proteins expression in situ is not clear.

We designed this study to investigate the expression of key cell cycle proteins p53, p16INK4a, pRb, cyclin D1, the presence of HPV, clinicopathological features of OC, and the clinical follow up in a group of 53 ovarian cancer patients.

**MATERIALS AND METHODS**

**Patients and tumor samples.** Formalin-fixed, paraffin-embedded tumor specimens were studied from 53 patients with primary serous ovarian cancer. All patients received the treatment at the department of Oncogynecology at the Kyiv National Cancer Institute, Ukraine. The mean age at diagnosis was 50.7±1.9 (range 16–79) years. The study protocol was approved by the Ethical Committee permission of Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine for studies with human materials.

Sections of 4-μm thickness were cut from the paraffin blocks for hematoxylin-eosin staining and a detailed histopathological classification was assigned according to the criteria of the WHO (1981). Clinical stages were determined according to the International Federation of Gynecology and Obstetrics system (FIGO) which specifies tumor size and the extent to which it has spread.

**Immunohistochemistry.** Immunohistochemical staining was performed using the primary mouse monoclonal antibodies against HPV16 E6+HPV18 E6, dilution 1:100 (clone C1P5, AbCam); p53 (clone DO7), pRb, dilution 1:50 (clone Rb1), cyclin D1, dilution 1:50 (clone DCS-4) and p16INK4a, dilution 1:40 (clone E6H4, DakoCytomation). DO7 antibody reacts both with wild-type and mutant p53 protein, recognizing an epitope between amino acids 21 and 25 [18,19]. Immunopositivity was defined to be distinct nuclear staining for p53 and both nuclear and cytoplasmic immunostaining for p16INK4a, pRb and cyclin D1.

After routine deparaffinization in xylene and rehydration through serial dilutions of alcohol the sections were subjected to heat-mediated antigen retrieval for 15 minutes in citrate buffer (pH 6.0). To minimize nonspecific binding, blocking was performed with 1% BSA at RT for 30 minutes. The primary antibodies were applied overnight at 4 °C followed by Envision visualization mouse system (DakoCytomation). DO7 antibody reacts both with wild-type and mutant p53 protein, recognizing an epitope between amino acids 21 and 25 [18,19]. Immunopositivity was determined to be distinct nuclear staining for p53 and both nuclear and cytoplasmic immunostaining for p16INK4a, pRb and cyclin D1.

After routine deparaffinization in xylene and rehydration through serial dilutions of alcohol the sections were subjected to heat-mediated antigen retrieval for 15 minutes in citrate buffer (pH 6.0). To minimize nonspecific binding, blocking was performed with 1% BSA at RT for 30 minutes. The primary antibodies were applied overnight at 4 °C followed by Envision visualization mouse system (DakoCytomation). 3,3-diaminobenzidine (DAB) was used as the chromogen for 5 minutes and haematoxylin, as a counterstain. Stained sections were dehydrated and mounted in xylene.

The percentage of immunopositive cells was evaluated (labeling index — LI). In each sample 600–700 cells were counted. Cervical intraepithelial neoplasia III (CIN III) known to be positive for HPV16 was used as a positive control for HPV 16/18 E6 protein staining. Negative controls were obtained by omitting the primary antibodies.

The immunoreactivity for all cell cycle regulatory proteins investigated in this study was evaluated as strong and weak according to the values of median (Me) of its expression (Table 1).

### Table 1. Evaluation of the immunohistochemistry

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Evaluation criteria</th>
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<tbody>
<tr>
<td></td>
<td>weak expression, Me, %</td>
</tr>
<tr>
<td>p53</td>
<td>≤30.0</td>
</tr>
<tr>
<td>p16INK4a</td>
<td>≤32.0</td>
</tr>
<tr>
<td>pRb</td>
<td>≤1.0</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>≤49.0</td>
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</table>

For E6 HPV 16 and 18 oncoprotein, staining was defined to be negative (no stained cells), focal (10–30%) of stained cells, spreading in one tissue area) and diffuse (30–100% of stained cells spreading in several areas in the tissue).

**DNA extraction.** Formalin-fixed, paraffin-embedded samples cut into 20 μm slices, were deparaffinized in xylene and rehydrated in 96 and 70% ethanol. The samples were processed either with QiAamp DNA Mini kit (Qiagen) according to the instructions of manufacturer. DNA was eluted from the columns in a volume of 50 ul of AE buffer; or by phenol chloroform extraction following Proteinase K (20 ug/ml) digestion in 475 ul of digestion buffer, (100mM Tris-HCl (pH 8.5), 10% SDS) at 55 °C. DNA was precipitated with ethanol and dissolved in AE buffer.

**PCR amplification.** The quantity of DNA from each tumor specimens was confirmed by PCR using primers for b-globin (224 bp): β-globin F: CACTCAGTGTG-GCAAAGGTGCCC; β-globin R: GGACTGACTCTCTCTCT-GCTT.

The HPV type-specific primers sets were used for amplification of E6 gene fragment of HPV types 16 and 18: HPV 16 F: 5’-TTAGAATGTGTGTACTGCAAGC-3’; HPV 16 R: 5’-TTGTCCAGATGTCTTTGCTT-3’; HPV 18 F: 5’-CACTTCACTGCAAGACATAG-3’; HPV 18 R: 5’-CTATGTTGTGAAATCGTCGT-3’.

PCR reactions were performed in a total volume of 50 ul. The reaction mixture contained 1x GoTaq PCR buffer (Promega, USA), 3 mM MgCl2, 0.2 mM each dNTP, 2.0 μM primers and 1.25 u Taq polymerase (Promega, USA) and 300 ng of DNA. Samples were amplified on ABS programmable thermocycler (Applied Biosystems). The amplification was carried out at 92 °C for 5 min followed by 37 cycles: denaturation at 92 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min. The final extension was for 10 min at 72 °C.

The amplified E6 gene fragments were of 281 bp for HPV16, 326 bp for HPV18 and were visualized on 2% agarose gels. As positive control for HPV types 16 and 18 amplification, HeLa and CasKi cell lines DNA were used, and water as template was used as a negative control.

**Statistical analysis.** The association between clinicopathological characteristics of patients and biomolecular markers expression was assessed by the Kruskal — Wallis test for two or more groups. The non-parametric Fisher exact-test was used to compare the
biomolecular markers expression rank between different groups. An $p$-value of $\leq 0.05$ was considered to be statistically significant. The survival analyses were estimated by the Kaplan — Meier method. Survival was calculated from the date of diagnosis until patient's death or until the last date the patient was known to be alive (range from 12 to 84 months). The statistical significance of differences between survival times was determined by the log-rank test in univariate analysis. Statistical analysis was carried out using the Statistica 7 program.

**RESULTS**

**Immunohistochemical analysis.** The distribution of patients with respect to FIGO system staging and tumor differentiation was as follows: 14 (26.5%) patients were stage I/II and 39 (73.5%) patients — stage III/IV; 5 (9.4%) patients had well differentiated tumors (G1), 22 (41.5%) patients — moderately differentiated tumors (G2) and 26 (49.1%) — poorly differentiated (G3) tumors (Table 2). The median age was 51 years.

Table 2. Distribution of ovarian cancer patients according to the clinicopathological parameters

<table>
<thead>
<tr>
<th>Clinicopathologic parameters</th>
<th>Number of patients (%)</th>
</tr>
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<tbody>
<tr>
<td>Age ≤51</td>
<td>28 (52.8)</td>
</tr>
<tr>
<td>&gt;51</td>
<td>25 (47.2)</td>
</tr>
<tr>
<td>FIGO I</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>II</td>
<td>13 (24.5)</td>
</tr>
<tr>
<td>III</td>
<td>29 (54.7)</td>
</tr>
<tr>
<td>IV</td>
<td>10 (18.8)</td>
</tr>
<tr>
<td>Total</td>
<td>53 (100.0)</td>
</tr>
<tr>
<td>Tumor differentiation G1</td>
<td>5 (9.4)</td>
</tr>
<tr>
<td>G2</td>
<td>22 (41.5)</td>
</tr>
<tr>
<td>G3</td>
<td>26 (49.1)</td>
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</tbody>
</table>

Immunohistochemical expression of p53, p16$^{INK4a}$, pRb and cyclin D1 was examined in OC tissues with some exceptions (Table 3). The immunopositivity of p53 was revealed in 86.8%, p16$^{INK4a}$ in 92.5%, pRb in 51.0% and cyclin D1 in 83.3% of ovarian carcinomas (see Table 3). Figure 1 shows representative examples of strong p53 (Fig. 1, a), p16$^{INK4a}$ (Fig. 1b), Cyclin D1 (Fig. 1, c) and pRb (Fig. 1, d) staining in ovarian carcinomas.

Table 3. Immunohistochemical analysis of cell cycle regulatory proteins in serous ovarian cancer

<table>
<thead>
<tr>
<th>Protein</th>
<th>Number of positive samples (%)</th>
<th>Weak expression, n (%)</th>
<th>Mean values, LI, % (variation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (n = 53)</td>
<td>46 (86.8)</td>
<td>26 (45.1)</td>
<td>31.5±2.9 (4–73)</td>
</tr>
<tr>
<td>p16$^{INK4a}$ (n = 53)</td>
<td>49 (92.5)</td>
<td>24 (45.3)</td>
<td>32.5±2.9 (6–94.8)</td>
</tr>
<tr>
<td>pRb (n = 52)</td>
<td>27 (51.0)</td>
<td>24 (45.4)</td>
<td>13.5±2.7 (1–88)</td>
</tr>
<tr>
<td>Cyclin D1(n = 49)</td>
<td>40 (83.3)</td>
<td>22 (45.8)</td>
<td>46.8±4.7 (8–97)</td>
</tr>
</tbody>
</table>

Based on LI, the mean level of expression of the cell cycle proteins in OCs were as follow: p53 — 31.5±2.9%; p16$^{INK4a}$ — 32.5±2.9%; pRb- 13.5±2.7%; Cyclin D1 — 46.8±4.7%

An increasing trend between increasing median values of p53 and pRb and decreasing tumor differentiation was observed (Kruskall — Wallis test) (Table 4). Significantly higher median cyclin D1 value was observed in well and moderately differentiated carcinomas (Grade 1 and 2) compared with poorly differentiated tumors (Grade 3) ($p=0.05$). Median p16$^{INK4a}$ value was significantly higher (39.0 vs 21.0) in poorly differentiated tumors as compared to the moderately or highly differentiated tumors ($p=0.05$). A significant correlation between median values of the p53, pRb, p16$^{INK4a}$ and cyclin D1 expression, the median of patients’ age, and FIGO staging could not be confirmed to $p<0.05$.

We used Fisher’s exact test to analyze the relation between clinicopathologic parameters and patterns
of cell cycle protein p53, p16INK4a, pRb and cyclin D1 expression in OC (Table 5). We determined the pattern of p53, p16INK4a, and cyclin D1 expression to be significantly correlated with poor prognosis as assessed by Kaplan—Meier analysis.

We could detect a positive correlation between significant levels of p53 expression and poor prognosis in OC tissues (19.6±4.08%) as compared to HPV-negative (16.6%) (log-rank test $p=0.02$ and 0.05, respectively) (Fig. 2 and 3). In patients with highly and moderately differentiated tumors, a strong expression of cell cycle regulatory proteins was observed according to E6 HPV oncoprotein localization in ovarian cancer tissues

<table>
<thead>
<tr>
<th>Covariate</th>
<th>n</th>
<th>p53</th>
<th>p16INK4a</th>
<th>pRb</th>
<th>Cyclin D1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>14</td>
<td>44.8*</td>
<td>35.7*</td>
<td>57.2*</td>
<td>52.8*</td>
</tr>
<tr>
<td>III</td>
<td>29</td>
<td>44.8*</td>
<td>55.2*</td>
<td>55.6</td>
<td>44.8*</td>
</tr>
<tr>
<td>IV</td>
<td>10</td>
<td>50.0</td>
<td>50.0*</td>
<td>50.0*</td>
<td>30.0*</td>
</tr>
<tr>
<td>Fisher exact test</td>
<td>$p = 0.0006$</td>
<td>$p = 0.04$</td>
<td>$p = 0.02$</td>
<td>$p = 0.02$</td>
<td></td>
</tr>
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</table>

Survival analysis. Clinical follow-up data were available for 42 patients with a median follow-up of 24 months (range 12–84 months) after the date of diagnosis. Survival curves were evaluated according to the immunohistochemical expression levels of cell cycle proteins.

Ten OC patients died within 36 months after diagnosis (23.8%). The univariate analysis determined that a strong p53 and p16INK4a expression was associated with poor prognosis of ovarian cancer (log-rank test $p=0.02$ and 0.05, respectively) (Fig. 2 and 3). In patients with highly and moderately differentiated tumors, a strong expression of cyclin D1 was correlated with reduced overall survival (log-rank test $p = 0.04$) (data not shown).

**HPV status.** We detected the presence of HPV DNA in 9 serous ovarian carcinomas (17.0%) by subtype-specific PCR. Four patients had HPV type 16 (Fig. 4) and five patients had HPV type 18. The tissues from the same patients reacted positively to HPV 16/18 E6 antibody (Fig. 5). Diffuse staining was observed in 7 and focal staining in 2 ovarian carcinomas, respectively.

We could detect a positive correlation between significantly low levels of p53 expression in HPV positive OC tissues (19.6±4.08%) as compared to HPV-negative ovarian tumors (33.4±3.2%) ($p=0.04$) in our limited sample size. Interestingly, where HPV E6 oncoprotein
localization was focal, a high level of p53 expression (53.0 and 39.0%) was observed. In contrast, the tumors with diffuse E6 oncoprotein localization had low p53 expression (8–26.0%) (Fig. 5, d, e). In two of the HPV-positive samples with diffuse E6 staining we did not see any p53 immunopositivity (Table 6) (Fig. 5, b, c). There was no significant difference between the presence of HPV in the tumors and p16<sup>INK4a</sup>, pRb and cyclin D1 expression level (p<0.05). However a trend for negative (4 samples) or lower pRb expression (4.0–26.0%) where tumors had diffuse HPV E6 staining, compared to tumors with focal E6 staining (19.0 and 30.0%) (see Table 6) was observed. We could not confirm a significant association between the histological grade, FIGO stage, patients’ age and the presence of HPV in ovarian cancer tissue (p<0.05).

**DISCUSSION**

One of the most important issues still unresolved in the treatment and management of OC is our inability to determine earliest changes that lead to the disease and target it for treatment. Researchers and physicians have not been able to develop targeted, optimized, risk-adjusted strategies for its treatment and then prevention of recurrent OC. Thus, the identification of reliable biomarkers which can be used to generate targets for OC treatment represents an urgent necessity not only for translational researcher but also for clinical oncologists. We tried to address this issue by studying an expression of cell cycle regulatory proteins and E6 HPV16/18 in a panel of 53 OC samples collected in our Institute and linked to clinical outcome data.

The cell cycle is controlled by a series of checkpoints that guide the cell’s transition through its cycle [20]. Each of those checkpoints represents an orderly interaction between cyclins, cyclin-dependent kinases, and their inhibitors. Disruption of any of these components of cell cycle progression can cause a deregulation of normal cell cycle progression. This dys-

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**Fig. 5.** Immunohistochemical detection of HPV 16/18 E6 oncoprotein in ovarian cancer tissues. a — positive control from cervical neoplasia (CIN III). In addition to the epithelium, endothelial cells of the blood vessels show E6 immunopositivity; b — HPV E6 oncoprotein diffuse immunopositivity in glandular structures of well differentiated ovarian cancer and absence of oncoprotein in stroma. c — the same tissue used for E6 staining in panel b showing p53 negative expression; d — E6 oncoprotein diffuse immunopositivity in moderately differentiated ovarian carcinoma; e — p53 weak expression in the same ovarian carcinoma tissue used for E6 staining in panel d. Magnification x400
disruptions of the p16-CDK4/CyclinD1 pathway may be sufficient for malignancy to develop and with additional mutations or other environmental triggers, progress. Several studies point to the cell cycle regulatory proteins including Cyclin D as possible useful markers in various cancers [21–23]. In this retrospective study, the expression of cell cycle proteins p53, p16\(^{INK4a}\), pRb and cyclin D1 in a series of 53 ovarian serous carcinomas and the status of HPV was examined. The immunohistochemical results were analyzed and correlated with the clinicopathological data. In our series of experiments, we observe strong p53, p16\(^{INK4a}\), pRb and cyclin D1 expression in about 50% of OC tissues examined with a significant correlation between p53, p16\(^{INK4a}\) and cyclin D1 status, histological grade and clinical stage of OC.

Although limited by samples size, our results indicate that high expression of Cyclin D1, p16\(^{INK4a}\) and p53 correlate with poor prognosis. In particular, our analysis demonstrated that localization and increased expression of these key cell cycle G1/S phase transition regulators is linked to more aggressive disease and a lower expression can be correlated with longer survivals. We were able to show that high Cyclin D1 expression was a significant indicator for poor prognosis in patients with early stages of disease or well differentiated tumors (see Table 5). These results are consistent with several studies indicating that cyclin D1 overexpression is an early event in ovarian carcinogenesis [7].

The literature evaluating and implicating p16\(^{INK4a}\) as both a diagnostic and prognostic marker in OC is accumulating. p16\(^{INK4a}\) is a cyclin-dependent kinase IV inhibitor and is expressed in a limited range of normal tissues and tumors [24]. Therefore its dysregulation in various tissues is thought to be associated with malignant changes. The normal function of p16\(^{INK4a}\) is negative regulation of cell cycle. It has also been reported to be expressed in tissues that are developmentally regulated to senesce [24]. Interestingly, we observe an overexpression of p16\(^{INK4a}\) in the late stages of OC. Our immunohistochemical screen revealed that p16\(^{INK4a}\) is either weakly or strongly upregulated in 92.5% OC tissues studied. The prognosis for patients with strong p16\(^{INK4a}\) expression was poorer than those with weak expression of p16\(^{INK4a}\) [25, 26]. The normal localization of p16\(^{INK4a}\) has been reported to be nuclear [24]. We observed mostly nuclear-cytoplasmic p16\(^{INK4a}\) staining in ovarian cancer cells. One of the reasons for this observation might be either due to very high nuclear localization of this protein leading to a leakage during tissue preservation process, or an aberrant overexpression of p16\(^{INK4a}\).

Since our analysis determined a significantly positive correlation between high p16\(^{INK4a}\) expression, late stage disease and poor outcome for patients, we posit that this might represent either a mutant form of the protein or an as yet uncharacterized function of the native protein.

Disruptions of the p16-CDK4/CyclinD1 pathway (pRb pathway) and the p14ARF-DM2-p53 pathway (p53 pathway) are important mechanisms in the development of malignant tumors including ovarian malignancies [27]. Each member of p53- and pRb-pathways has regulatory roles in initiation and progression of tumor growth [28]. We confirm this finding with our own data where p53 was present but at a lower intensity in high grade (1/2) tumors and was highly expressed in poorly differentiated tumors. These results indicated that p53 plays a critical role in later stages where the disease is progressing.

The high risk HPVs are high etiologic risk factors for development of malignancies in the lower female genital tract [9]. The significance of high risk HPVs in upper genital tract, including ovarian cancer is controversial. A literature review shows highly variable reported frequency of HPV infection in ovarian carcinomas from 4.2 to 37.5%, while many other reports failed to confirm HPV DNA in ovarian neoplasms [13–17, 29]. In this study PCR analysis demonstrated HPV DNA in 17.0% (9 from 53) ovarian serous carcinomas. All the HPV 16 and 18 DNA positive OC tissues showed immunopositivity for E6 oncoprotein. In ovarian cancer samples, HPV E6 staining was adjacent to peritumoral area and was detected in glandular structures of ovarian carcinomas and was absent in stroma. The protein products of high risk HPV E6 and E7 oncoproteins disturb the function of key cell cycle regulators p53 and pRb as has been elegantly demonstrated in cervical cancer model [30]. The HPV E6 gene product interacts with wild type cellular p53 protein and subverts its function [30]. Oncoprotein E7 forms a complex with pRb leading to its functional inactivation through proteolytic degradation in cervical cancer model [30]. Interestingly, in our small subset of nine samples that tested positive for diffused HPV E6 staining, we observed an acute downmodulation of p53 expression. Since our p53 antibody (clone DO7) recognized both the wild type and the mutant form of p53 [18, 19] we reason that detection of p53 in HPV positive tissues might represent the presence of mutant p53. The p16\(^{INK4a}\), pRb and cyclin D1 expression levels were not significantly different in HPV-positive and negative ovarian cancer tissues. A strong expression of p16\(^{INK4a}\) has been reported in HPV positive cervical cancer [31, 32]. The proposed mechanism postulates that the upregulation of p16\(^{INK4a}\) in cervical cancer is due to the modulation of pRb by the viral E7 gene product [30]. The high expression of p16\(^{INK4a}\) in HPV- positive tumors in our study might still result from HPV E7 modulation, however, a larger sample set needs to be analyzed to determine the correlation with a statistical significance.

In conclusion, we demonstrate that a majority of moderately and poorly differentiated ovarian carcinomas are characterized by strong expression of p53 and p16\(^{INK4a}\) proteins, while strong staining with cyclin D1 antibody was observed in well differentiated tumors. This indicates that an aberrant expression of cyclin D1 might be an early event in the OC development. We show that, patients with high positivity for p53, p16\(^{INK4a}\) and Cyclin D1 had a poor prognosis and reduced overall survival. The presence of high risk...
HPV 16 and 18, although controversial, was detected in a small subset of nine samples with significantly low p53 expression profile. These findings, although in need of confirmation on a larger pool of cases, indicate that p53, p16INK4a and Cyclin D1 expression and HPV may represent a promising tool toward the identification of patients with poorer prognosis who may benefit from more aggressive therapy and HPV screening.

ACKNOWLEDGEMENTS

We would like to thank Dr. Tanja Pejovic from Oregon Health & Science University, USA for helping with HPV16 E6+HPV18 E6 antibody, primers for HPV 16/18 E6 amplification and valuable commentaries on the paper.

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