HPV genotyping and p16/Ki-67 dual staining in the detection of high-grade cervical lesion in patients with LSIL on Pap smear

LAVINIA-CRISTINA MOLERIU1), IOAN SAS2), ADRIAN CLAUDIU RAŢIU2), MĂDĂLIN-MARIUS MARGAN3), CRISTINA-ANAMARIA SECOSAN2), RĂZVAN-ŞTEFAN ILINA2), FLORIN-GEORGE HORHAT4), LOREDANA GABRIELA STANA5), DORIN GRIGORAŞ2), LAURENŢIU CORNEL PIRTEA3)

Abstract
p16/Ki-67 dual-stained cytology, either alone or combined with human papillomavirus (HPV) 16/18 genotyping, could be a useful tool for triage for colposcopy of HPV-positive patients. Based on this background, we aimed at comparing the diagnostic performance of the p16/Ki-67 dual staining test, and high-risk HPV test for the detection of high-risk cervical intraepithelial neoplasia (CIN2/3) in patients diagnosed with low-grade squamous intraepithelial lesion (LSIL) on Pap smear. We performed a retrospective study including 184 patients with LSIL cytology on Pap smear, of which 64 were referred for biopsy after colposcopy. Prior biopsy HPV genotyping and dual staining test were performed on all 64 patients. The mean age of the patients selected for conization was 36 years and seven months. The pathological exam showed that 28.13% (18/64) from the patients LSIL on cytology were actually having CIN2/3: 12 cases with CIN2, five cases with CIN3 and one case of in situ cervical carcinoma. HPV positive were 56.25% (36/64) of the patients with LSIL. The p16/Ki-67 dual staining test was positive in 29.69% (19/64) of the patients with LSIL. Among women with LSIL cytology, the sensitivity and specificity of the HPV genotyping test for predicting CIN2/CIN3 were 94.44% (17/18) and 58.7% (27/46), respectively. The sensitivity and specificity of the p16/Ki-67 dual staining test were 66.67% (12/18) and 84.78% (39/46), respectively. Our results agree with other data available in literature and suggest that the p16/Ki-67 dual staining test could be included in the management protocol of patients with modified cytology as a triage test before referring those patients for colposcopy.

Keywords: HPV genotype, p16/Ki-67 dual staining, cervical lesion, classification functions.

Introduction
Cervical cancer represents a major health issue, especially in developing countries. Due to the screening programs available in most countries, there is a great potential for the prevention or early recognition of cervical malignancy. The Pap smear is currently the first and worldwide-accepted screening method. The main risk for cervical cancer is represented by the persistent infection with high-risk human papillomavirus (HPV) types [1].

Therefore, many studies investigated and offered solid arguments for the use of HPV genotyping as a screening tool for cervical malignancy. Data available in literature suggests that cytology has a sensitivity that ranges between 47% and 62% and specificity between 60% and 95% for the detection of moderate and severe cervical dysplasia [2]. On the other hand, HPV genotyping has a higher sensitivity than cytology, but a significantly lower specificity, especially in young patients [3]. Patients classified as having low-grade squamous intraepithelial lesion (LSIL) on Pap smear need further investigations for the correct assessment of their status and, according to the data available in literature, 9% to 30% of them are actually having high-grade cervical intraepithelial neoplasia (CIN2/CIN3) [4].

To improve cervical cancer risk stratification, automated detection of dual p16/Ki-67 nuclear immunoreactivity in liquid-based Pap tests was investigated [5]. Several studies indicated that p16/Ki-67 dual staining cytology is useful to identify underlying high-grade CIN in Pap cytology cases classified as LSIL [6]. The simultaneous detection of p16 and Ki-67 expression within the same cervical epithelial cell is considered an indicator of high-risk cervical dysplasia that can be used in the management of women with LSIL Pap cytology results. A high level of concordance was found between dual staining and E6/E7 mRNA test [7]. Although initial studies suggested that p16/Ki-67 testing is not suitable for triage of women with atypical squamous cells of undetermined significance (ASCUS) or LSIL cytology [8], more recent data showed that p16/Ki-67 dual staining cytology, either alone or combined with HPV16/18 genotyping, could be a useful tool for triage for colposcopy of HPV-positive patients [9–11].

Based on this background, we aimed at comparing the diagnostic performance of the p16/Ki-67 dual staining
test, and high-risk HPV test for the detection of high-risk CIN2/3 in patients diagnosed with LSIL on Pap smear.

**Patients, Materials and Methods**

Patients’ selection: we performed a retrospective study. We included in our study 184 patients with LSIL cytology on Pap smear who were referred for colposcopy to the Department of Obstetrics and Gynecology, “Victor Babeș” University of Medicine and Pharmacy, Timișoara, Romania, between August 2015 and August 2016.

Inclusion criteria: age between 18 and 65 years, LSIL on Papanicolau smear, patients with indication for biopsy after colposcopy. Exclusion criteria: pregnancy, previous surgical treatment for cervical dysplasia, patients with no suspect lesions on colposcopy.

Conventional Papanicolaou cytology was performed and evaluated according to the criteria of Bethesda 2001. All patients were evaluated by colposcopy and International Federation for Cervical Pathology and Colposcopy (IFCPC) criteria were used. All colposcopic examinations were performed by the same team, with expertise in colposcopy. Patients with colposcopic suspect lesions were referred for biopsy. All biopsies were performed by the same team of surgeons. Specimens were send to the Department of Pathology and were all interpreted by the same pathologist. The HPV genotyping and immunocytochemistry were performed on all patients with indication for biopsy before the procedure. All samples were examined using LINEAR ARRAY® HPV Genotyping Test (CE-IVD), based on reverse hybridization of amplicons. The DNA of 37 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108) was detected in cervical samples by multiplex polymerase chain reaction (PCR) targeted to the conserved L1 region of the viral genome. The Gene Amp PCR System 9700 was used for genotyping test according to the manufacturer’s instructions. Automated hybridization and detection of HPV DNA was done on the ProfiBlot 48 (Tecan Trading AG, Zurich, Switzerland). Immunocytchemistry analysis was performed using the CINtec® PLUS Cytology kit (Roche MTM Laboratories, Heidelberg, Germany) according to the manufacturer’s instructions. The kit contains a ready-to-use primary antibody combination: a mouse monoclonal antibody (clone E6H4) directed to p16 protein and a rabbit monoclonal antibody (clone 274-11 AC3) directed against Ki-67 protein. As a visualization reagent, the kit provides a polymer reagent conjugated with horseradish peroxidase (HRP) and affinity-purified goat anti-mouse fragment antigen-binding Fab’ antibody fragments for the detection of p16 antibodies and a polymer reagent conjugated with alkaline phosphatase and affinity purified goat anti-rabbit Fab’ antibody fragments for the detection of Ki-67. Using the two working solutions provided in the kit, HRP-mediated conversion of 3,3’-diaminobenzidine (DAB) chromogen and alkaline phosphatase-mediated conversion of Fast Red chromogen result in the water-insoluble colored end-products: the brown staining of p16 and the red staining of Ki-67. Slides for p16/Ki-67 dual staining were performed using SurePath specimens stabilized with CytoRich Fluid (BD) within one month of sample collection. One section for each selected case was stained with p16/Ki-67 dual test. Ki-67 expression within the nucleus was marked with red chromogen and p16 cytoplasmic expression was marked with brown chromogen. Each sample was considered positive when both markers were observed within the same cells (Figure 1a). Cases without any double-immunoreactive cell were considered negative (Figure 1, b and c).

Informed consent was obtained from every patient prior to his or her inclusion in the study. All procedures have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments and were approved by the Institutional Review Board and Ethical Committee of “Victor Babeș” University of Medicine and Pharmacy, Timișoara.

Statistical analysis was conducted using SPSS ver. 17 and Microsoft Excel.

**Results**

A total of 184 patients with LSIL were evaluated by colposcopy. A total of 64 patients were referred for biopsy after colposcopy. The rest of the patients were excluded from the study. Prior biopsy HPV genotyping and dual staining test were performed on all 64 selected patients. The mean age of the patients selected for conization was 36 years and seven months. The pathological exam showed that 28.13% (18/64) from the patients LSIL on cytology were actually having CIN2/3: 12 cases with CIN2, five cases with CIN3 and one case of in situ cervical carcinoma.

56.25% (36/64) of the patients with LSIL were HPV positive. The p16/Ki-67 dual staining test was positive in 29.69% (19/64) of the patients with LSIL.

Positive dual staining test was associated with histology as follows: 12 cases with CIN2/3 were positive at the dual staining test at six cases with CIN2/3 on were negative. On the other hand, 39 cases with CIN1 were negative and seven cases with CIN1 were positive at the dual staining test (Table 1).

<table>
<thead>
<tr>
<th>HPV+</th>
<th>HPV-</th>
<th>Total</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN2/3</td>
<td>17</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>CIN1</td>
<td>19</td>
<td>27</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>28</td>
<td>64</td>
</tr>
</tbody>
</table>

The HPV genotyping was correlated with histology, as follows: 17 cases with CIN2/3 were HPV positive and only one case with CIN2/3 was HPV negative. From the 46 cases with CIN1, 19 were HPV positive and 27 were HPV negative (Table 2).

<table>
<thead>
<tr>
<th>HPV+</th>
<th>HPV-</th>
<th>Total</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN2/3</td>
<td>17</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>CIN1</td>
<td>19</td>
<td>27</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>28</td>
<td>64</td>
</tr>
</tbody>
</table>
In our group, the sensitivity and specificity of the HPV genotyping test for predicting CIN2/CIN3 were 94.44% (17/18) and 58.7% (27/46), respectively. The sensitivity and specificity of the p16/Ki-67 dual staining test were 66.67% (12/18) and 84.78% (39/46), respectively.

We compared the two different diagnostic tests, the HPV test and the p16/Ki-67 dual staining test, using the area under a receiver operating characteristic (ROC) curve (AUC). We calculated the estimated sensitivity and specificity for each test and we plotted the ROC curve (Table 3 and Figure 2). In the first test, AUC is 0.766 and for the second test, AUC is 0.757, where the perfect diagnostic ability has the value 1.

### Table 3 – Sensitivity and specificity values for HPV genotyping and p16/Ki-67 dual staining test

<table>
<thead>
<tr>
<th>Coordinates of the curve</th>
<th>Test result variable(s)</th>
<th>Positive if greater than or equal to</th>
<th>Sensitivity 1 – Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPV positive</td>
<td>-1.00</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>0.944</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>p16/Ki-67</td>
<td>-1.00</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.50</td>
<td>0.667</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.00</td>
<td>0.000</td>
</tr>
</tbody>
</table>

#### Discussion

Cervical cancer rarely occurs in the absence of HPV infection. HPV infection persistence is the most important ethnological factor and is dependent on type, age of the patient and viral load [12]. Several studies highlighted the possibility of infection persistence after the excision of the cervical lesion with safe margins that is also related to high-risk HPV types [13]. Those aspects are very important for oncogenesis and somehow explain the high sensitivity of HPV genotyping in the detection of high-grade cervical lesions. On the other hand, most HPV infections are cleared spontaneously by a competent immune system within the first two years from the time of infection. A very significant proportion of sexually active women get infected with HPV at some point in their lives but most infection are cleared especially in younger females, and this way the development of...
cervical high-grade lesions is stopped. This explains the lower specificity rate of HPV genotyping for the detection of high-grade cervical lesions.

A sensitive problem that represents a major concern is the fact that an important proportion of the patients categorized by Pap cytology as having LSIL are actually having CIN2/3. Any method that would detect this group of patients has a real potential to be included in the protocol of screening for cervical cancer.

Immunohistochemistry has a consolidated role in modern pathology and oncology. It is useful for accurate diagnosis and for the identification of potential treatment targets in ovarian [14–16], skin [17], lung [18], prostate [19] and breast [20] malignancies. Immunohistochemistry is emerging as a new and promising method for screening in cervical cancer. A significant advantage is the fact that the specimens for liquid based cytology can be used for immunohistochemistry and no further visit to the specialist is necessary.

Immunocytochemical p16/Ki-67 dual staining is useful for the early recognition of the oncogenesis onset in cervical cells. The overexpression of p16 is associated with the increased activity of the E7 oncoprotein, caused by persistent infection with high-risk HPV types, and Ki-67 is a marker cell proliferation. The co-expression of p16/Ki-67 in the same cell is considered a marker for cell cycle deregulation. The use of Pap cytology for screening identifies a category of patients categorized with LSIL, that must be further investigated by HPV typing and colposcopy. Those with colposcopic findings suggestive for disease will be referred for biopsy, and the rest will be followed. The HPV genotyping has high sensitivity for CIN2/3 but low specificity, especially in young patients with transitory high-risk HPV infections. This means that many patients with no disease will be referred for unnecessary colposcopy. There is a real need for a method that identifies with higher specificity the group of patients that need to be referred for further investigations. The co-expression of p16 and Ki-67 within the same cervical epithelial cell is a morphology-independent marker of cell-cycle deregulation. The use of the dual staining immunocytochemistry can distinguish between p16 positive cells generated by dysplasia and squamous metaplastic cells or endocervical cells that overexpress physiologically p16 [21].

The p16/Ki-67 immunocytochemical reaction seems to have higher specificity but with a slightly lower rate of sensitivity compared with the high-risk HPV DNA test. An important practical aspect is the fact that the dual staining test can be performed from the same liquid-based sample used for cytology or HPV typing. Therefore, the patient does not have to return to the consultation room for another visit.

Conclusions

Our results agree with other data available in literature, and suggest that the p16/Ki-67 dual staining test could be included in the management protocol of patients with modified cytology as a triage test before referring those patients for colposcopy.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Lavinia-Cristina Moleriu and Ioan Sas equally contributed to the manuscript.

Funding

This article was supported by the grant P IV-CI-PDCC-02 2015/2016, “Victor Babeş” University of Medicine and Pharmacy, Timișoara, Romania.

References


**Corresponding author**
Răzvan-Ștefan Ilina, MD, PhD, Department of Obstetrics and Gynecology, “Victor Babeș” University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timişoara, Timiş County, Romania; Phone +40744–868 913, e-mail: razvanilina@yahoo.co.uk

Received: October 20, 2016

Accepted: July 3, 2017