Introduction

Cervical cancer is still at the top of the list of oncologic diseases. Morbidity and mortality are significantly reduced by introducing a program using cytological methods for making early diagnosis of changes. The number of newly diagnosed cases in the world is still significant and there are approximately 400,000 cases annually [1].

IMMUNOHISTOCHEMICAL EXPRESSION OF P16INK4a IN INFLAMMATORY, PRENEOPLASTIC AND NEOPLASTIC CERVICAL LESIONS

IMUNOHISTOHEMIJSKA EKSPRESIJA P16INK4a U INFLAMACIJSKIM, PRENEOPLASTIČNIM I NEOPLASTIČNIM LEZIJAMA GRLIĆA MATERICE

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Summary

Introduction. High-risk human papilloma viruses play a main role in the development of cervical dysplasias and carcinomas. p16INK4a can be considered as a surrogate marker of active high-risk human papillomaviruses infection in dysplastic and neoplastic cells of the cervix. This study was aimed at determining the presence and level of p16INK4a expression in inflammatory, preneoplastic and neoplastic lesions of the cervix. Material and Methods. The study was performed on 109 samples of cervical biopsy. Cervical cancer was diagnosed in 36 patients, 34 patients had a preneoplastic change (dysplasia) in stratified squamous cervix epithelium and a nonspecific inflammatory process was found in 39 patients. In all samples, immunohistochemical analysis using antibodies to p16INK4a was performed. Results. The expression of p16INK4a was verified in all cases of cervical cancer (100%), in 67.65% of dysplastic cervical lesions and in 38.5% of inflammatory lesions. A statistically highly significant difference was found in the presence and level of expression among neoplastic, dysplastic and inflammatory lesions of the cervix (χ² = 76.02, p < 0.001). The expression was more frequent and had a higher level in neoplastic and high grade dysplastic lesions compared to expression in inflammatory lesions and low grade dysplasias. Conclusion. The analysis of the presence of p16INK4a can differentiate non-neoplastic, high grade preneoplastic and neoplastic changes of the cervix. The use of p16INK4a in interpreting borderline lesions of the cervix can enable a rational therapeutic treatment of patients. Key words: Squamous Intraepithelial Lesions of the Cervix; Uterine Cervical Neoplasms; Uterine Cervicitis; Immunohistochemistry; Cyclin-Dependent Kinase Inhibitor p16; Papillomavirus; Papillomavirus Infections; Biological Markers.

Sažetak

Uvod. Humani papiloma virusi visokog rizika imaju glavnu ulogu u nastanku displazije i karcinoma cerviksa. p16INK4a se može smatrati „surogat“ markerom prisustva aktivne humanih papiloma virusima visokog rizika u displastičnim i neoplastičnim celiijama grlića matrice. Cilj istraživanja bio je utvrđivanje prisustva i stepena ekspresije p16INK4a u inflamacijskim, preneoplastičnim i neoplastičnim lezijama grlića matrice. Materijal i metode. Istraživanje je izvršeno na 109 biopsijskim uzorcima grlića materice. Kod 36 pacijentkinja dijagnostikovan je karcinom grlića materice; kod 34 pacijentkinje je utvrđena preneoplastična promena (displazija) u pločastosojevitom epitelu grlića; a kod 39 utvrđen je nespecifični inflamacijski proces. U svim uzorcima je urađena imunohistokemijska analiza upotrebom antitela na p16INK4a. Rezultati. Ekspresija p16INK4a je verifikovana u svim slučajevima kod pacijentkinja sa karcinomom cerviksa (100%); u 67,65% slučajeva u displastičnim lezijama grlića; i u 38,5% slučajeva u inflamacijskim lezijama. Statistički visoko značajna razlika je prisutna u prisustvu i stepenu ekspresije između neoplastičnih, displastičnih i inflamacijskih lezija grlića matrice. Zakučak. Analizom prisustva p16INK4a može se diferencirati neneoplastična promena od preneoplastičnih promena visokog stepena i neoplastičnih promena na grliću materice. Uzrokom bi u interpretaciji graničnih lezija na cerviksu omogućava se racionalan terapijski tretman pacijentkinja. Ključne reči: Skvamozne intraepitelijalne lezije cerviksa; Karcinomi grlića matrice; Cervicitis; Immunohistokemijska; P16INK4a; Papilomavirida; Infekcije papiloma virusom; Biološki markeri

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The prevalence of preneoplastic lesions of the cervix - cervical intraepithelial neoplasia (CIN) is different and depends on the socioeconomic status, geographical factors, and exposure to risk factors and ranges from 1.05% to 13.7%. CIN is most commonly diagnosed in 20-year old women, carcinoma in situ in women aged 25–35 years, and invasive cervical cancer is diagnosed after the age of 40 [2, 3].

Epidemiological risk factors for the development of CIN are similar to those for the development of cervical cancer: multiple sexual partners, early onset of sexual activity, high risk sexual partners, infection with human papilloma viruses (HPV), neoplasm of lower genital tract, previous sexual contacts with persons who had cancer of genital organs, exposure to sexually transmitted diseases, smoking, human immunodeficiency virus (HIV) infection, other forms of immunosuppression, multiparity and long-term use of oral contraceptives [4].

HPVs are the main etiological factor in the occurrence of CIN and cervical cancer. Most of the above mentioned social factors are insignificant in comparison with HPV infection. The analyses have confirmed the presence of HPV in more than 80% of cases in CIN lesions and 99.7% of all invasive cervical cancers. HPV infection is very common and varies depending on the age of the patient.

There are more than 120 types of HPV and half of them cause infections of the anogenital epithelium. Because of their malignant potential, HPVs are classified in low-risk and high-risk categories. In the category of low-risk HPVs, types 6,11,42,43,44 are present and are associated with genital warts and mild dysplasia (CIN 1). The category of high risk HPVs includes types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, which are associated with the development of severe grade of dysplasias (CIN 2 and CIN 3) and invasive cervical cancer [5].

Different markers and their expression in dysplastic epithelial cells were examined in cytological and histological materials (Ki 67, p53, Cdc6, Mcm5, MN) [6, 7].

Most studies emphasize the major role of HPV in the development of dysplasia and cervical cancer. High-risk HPV, mostly 16 and 18, have been identified in 99% of cervical cancers. Two viral oncogenes, E6 and E7, are expressed in the HPV infected cells. Products of viral oncogenes are involved in the mechanism of genesis of malignant cells. Oncogenic activity of the products of viral genes is based on the interaction with specific cellular proteins. E6 viral protein causes premature degradation of the p53 tumor suppressor protein. Similarly, E7 binds retinoblastoma (RB) protein and leads to the release of the E2F transcription factor. Functionally, E7 leads to the inactivation of the RB protein. Inactivation of the RB protein (due to E7 or mutations, deletion of the gene) leads to enhanced phosphorylation and expression of cyclin-dependent kinase (CDK) (CDK4 and CDK6). In a normal cell cycle, the activity of CDK4 and CDK6 is tightly regulated by a number of CDKI, including p16INK4a. If the RB protein is inactivated directly, the affected cells release large amounts of growth inhibitor including CDKI (p16INK4a). The cells proliferate despite high levels of p16INK4a.

The nature of expression of p16INK4a represents a negative feedback control of RB protein loss. Thus, the loss of function of RB protein results in increased p16INK4a. Inactivation of the RB protein with E7 viral protein leads to increased expression of p16INK4a, which can be a sensitive and significant biomarker of active expression of HPV oncogenes. Expression of p16INK4a may be considered as a marker of viral E7 protein activity in the cell. Therefore, p16INK4a may be considered as a “surrogate” marker for the presence of active high-risk human papilloma viruses (HR-HPV) infection in dysplastic and neoplastic cells of the cervix [1, 8–10].

The importance of p16INK4a expression in cervical tissue samples has been shown in many studies [11–13]. In cytology, the analysis of p16INK4a expression is done to assess the benefits in terms of triage of patients whose findings on routine cytology are interpreted as boundary cases. In most studies, the sensitivity is similar to HPV testing, but there is a significantly higher rate of specificity of p16INK4a expression in cytologic materials [13–15].

The aim of this study was to determine the presence of p16INK4a expression in inflammatory, preneoplastic (dysplasias) and neoplastic lesions of the cervix, and to determine whether the presence and level of expression of p16INK4a depend on the type of pathological processes in the cervix, the grade of dysplasia of the cervical epithelium and histological type and the grade of invasive cervical cancer.

Material and Methods

The study was performed on biopsy specimens of patients who were biopsied due to pathological Papanicolaou (PAPA) test or colposcopic findings at the Department of Gynecology and Obstetrics, Clinical Center of Banja Luka.

The patients’ age was taken from the outpatients’ protocols and referrals for histopathologic analysis. Analysis of biopsy tissue samples was performed at the Department of Pathology, Clinical Center of Banja Luka. The material obtained during biopsy was fixed in 10% formalin. After the routine processing, paraffin tissue blocks were made and cut on a microtome in serial sections and stained with hematoxylin and eosin (HE) staining method. The analysis was performed by a binocular micros-
cope, Leica (objectives 10x, 25x, 35x, 40x; 10x eyepieces) with the width of the visual field of 1.4 mm.

The nature of changes in the mucosa of vaginal portions was determined (mucosa without changes, inflammation, dysplasia in stratified squamous epithelium, invasive carcinoma of the cervix) for all patients. The classification was made according to the recommendations of the World Health Organization (WHO) [16].

Changes were classified into three groups:
- group I - 36 biotic samples - in which histological analysis revealed the diagnosis of a malignant epithelial tumor of the cervix. Cytological diagnosis and biopsy of changes localized in the cervix were used in the diagnostic procedure before surgical intervention. After the diagnosis had been confirmed and clinical stage of disease determined, the patients underwent surgery. After surgery, histopathological analysis of the removed material was performed, and definitive diagnosis and pathological stage of the disease were determined.
- group II - 34 biotic samples - in which the histological analysis confirmed the diagnosis of preneoplastic lesions (dysplasia) in the epithelium of the cervix.
- group III - 39 biotic samples - in which histological analysis revealed the diagnosis of inflammatory lesions in the cervical epithelium (non-specific inflammation).

In all patients in group I, the following morphological details were defined: type of material in which the definitive histological diagnosis was determined, histological type of the tumor, tumor grade according to the International Federation of Gynecology and Obstetrics (FIGO), depth of stromal invasion and the level of expression of p16INK4a.

The type and grade of dysplasia were determined in all subjects of group II.

The presence and type of benign lesions in the mucous membrane of the cervix were determined in all subjects of group III.

In all samples (from group I, II and III), immunohistochemical analysis was performed using antibody to p16INK4a (Dako, Denmark).

The anti-human p16INK4a monoclonal antibody (clone E6, MTM laboratories AG, Heidelberg, Germany) was used for immunohistochemical analysis. The tissue samples were cut in the microtome (Leica 2000) to the thickness of 2-4 microns and subsequently deparaffinized. Antigen unmasking was performed in a citrate buffer, 0.007M for 40 minutes at 97 degrees Centigrade, pH 6.0. Unblocking was done with 1.5% hydrogen peroxidase for 15 minutes. The slides were stained with primary monoclonal antibody (1: 1000 overnight at 4 degrees Centigrade), and visualization was performed with avidin-biotin kit (Ultra Vision Detection System).

The presence of p16INK4a expression was assessed in the following way:
The level of p16INK4a expression was evaluated semiquantitatively according to the percentage of epithelial cells showing the expression. Positive reaction involves intensive nuclear and cytoplasmic immunoreaction (coloration).

The level of expression was evaluated to be:
- Score 0- <1% of positive epithelial cells;
- Score 1- 1-5% of positive epithelial cells;
- Score 2- 5-25% of positive epithelial cells;
- Score 3- >25% of positive epithelial cells.

Statistical analysis was performed using SPSS, version 17.0. The descriptive statistical analysis (average value) was used to describe the overall sample as well as individual groups. The differences between the groups were analyzed by using the following tests: Chi square, Wilcoxon signed-rank test and Mann Whitney test.

**Results**

Our study included biotic material of 109 patients, who had been operated at the Clinical Center of Banja Luka, Department of Obstetrics and Gynecology. The analysis was done at the Department of Pathology and the definitive histopathological diagnosis was established. The study was performed during the period from January 2006 to December 2013.

The first group consisted of materials of 36 patients, who were operated due to invasive squamous cell cervical carcinoma.

The second group consisted of materials of 34 patients who were diagnosed with preneoplastic lesion in cervical samples.

The third group consisted of materials of 39 patients in whom morphologically nonspecific inflammation in the samples of the cervix was diagnosed.

The youngest patient was 23 years old and the oldest 86 years old.

The average age of all patients was 50 years. The youngest patients were in group II and group I, whose average age was 37 and 50 years, respectively. The average age in group III was 61 years (Table 1).

The patients in group III were the oldest and they had the diagnosis of nonspecific inflammation. The patients in group III were operated mainly due to the prolapse of uterus, which is most commonly diagnosed in postmenopausal women, thus explaining the age structure of this group of patients. The average age of patients in group II was 37 years and they were diagnosed to have dysplasia of the squamous epithelium of the vaginal portion. The average age of patients in group I, with the diagnosis of squamous cell carcinoma, was 50.78 years.

Mann-Whitney U test was used to determine whether there was a difference in the age of patients having the confirmed diagnosis of invasive squamous cell cervical cancer and preneoplastic lesions. The test showed a statistically significant difference, being U = 214.00, p <0.005.

The average age of patients with invasive squamous cell carcinoma of the cervix was higher (46.56 years) compared to the patients with preneoplastic lesion (23.79 years).
In most patients in group I, the uterus, as surgical material, was used for the analysis in 33 (91.67%) cases, whereas a conical section of vaginal portion was used in only 3 (8.33%) patients. In all patients of group II (n = 34), surgical material on which the analysis was done was a conical section of the vaginal portion of the cervix. In all 39 patients in group III, surgical material for the analysis was the uterus (Table 1).

Squamous cell carcinoma was diagnosed in 35 (97.22%) patients from group I, and only one case (2.78%) of adenosquamous carcinoma was found. As for group II, the diagnosis of severe grade of dysplasia of squamous epithelium (CIN 3) was made in 17 (50%) patients, a moderate dysplasia of squamous epithelium (CIN 2) and mild dysplasia of squamous epithelium (CIN 1) was found in 10 (29.41%) and 7 (20.59%) cases, respectively. Histological diagnosis of chronic nonspecific inflammation of the cervix was made in all 39 (100%) patients from group III (Table 1).

The tumors in samples of group I were most frequently moderately differentiated. A medium grade of differentiation was present in 27 (75%) patients. Grade III was verified in 7 (19.44%) patients, while grade I was seen in only 2 (5.56%) cases (Table 1).

The depth of invasion in the analyzed material ranged from 2 mm to 66 mm. The average depth of invasion in all patients in group I was 14.43 mm. Most of the patients, i.e. 32 (88.9%), had invasion of cervical stroma with cancer tissue larger than 5 mm (Table 1).

Expression of p16INK4a in materials of group I was present in 36 (100%) cases. In all samples, diffuse cytoplasmic and nuclear positivity (high level of expression) was determined, more than 25% of tumor cells were positive (Figure 1). A diffuse, high expression was found, which was semiquantitatively graded as score 3 in all samples. The level of expression in this group did not depend on the histological type and grade of the tumor. Expression of p16INK4a in the materials of group II was present in 23 (67.65%) cases. The expression ranged from focal, low expression which was semiquantitatively graded as 1 in 5 (14.7%) cases to high expression which was graded as 3 in 16 (47.06%) cases. Expression was not present in 11 (32.35%) cases (Table 1, Figure 1).

Expression of p16INK4a in materials of group I was present in 36 (100%) cases. In all samples, diffuse cytoplasmic and nuclear positivity (high level of expression) was determined, more than 25% of tumor cells were positive (Figure 1). A diffuse, high expression was found, which was semiquantitatively graded as score 3 in all samples. The level of expression in this group did not depend on the histological type and grade of the tumor. Expression of p16INK4a was rarely present in materials of group II

<table>
<thead>
<tr>
<th>Table 1. Characteristics of patients</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
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</thead>
<tbody>
<tr>
<td>Number (n) / Broj (n)</td>
<td>36</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>The average age / Prosečna starost</td>
<td>50</td>
<td>37</td>
<td>61</td>
</tr>
<tr>
<td>Type of the material / Vrsta materijala</td>
<td>Uterus / Uterus</td>
<td>/</td>
<td>39 (100%)</td>
</tr>
<tr>
<td>/ Conical section / Koničan isečak</td>
<td>3 (8.33%)</td>
<td>34 (100%)</td>
<td>/</td>
</tr>
<tr>
<td>Histological diagnosis / Histološka dijagnoza</td>
<td>Squamous cell carcinoma / Skvamozen karcinom</td>
<td>Dysplasia in squamous epithelium / Displazija u skvamozenom epitelu</td>
<td>Chronic non specific inflammation / Nesplicitno zapaljenje</td>
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<td>/ 35 (97.22%)</td>
<td>34 (100%)</td>
<td>39 (100%)</td>
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<tr>
<td>Grade / Stepen</td>
<td>I</td>
<td>II</td>
<td>III</td>
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<td>/ 2 (5.56%)</td>
<td>7 (20.59%)</td>
<td>/</td>
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<tr>
<td>/ 27 (75%)</td>
<td>10 (29.41%)</td>
<td>/</td>
<td></td>
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<tr>
<td>/ 7 (19.44%)</td>
<td>17 (50%)</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Average depth of invasion / Prosečna dubina invazija</td>
<td>14.43 mm (range 2 – 66 mm / raspon 2 – 66 mm)</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>Expression of p16INK4a / Ekspressija p16INK4a</td>
<td>36 (100%)</td>
<td>23 (67.65%)</td>
<td>15 (38.46%)</td>
</tr>
</tbody>
</table>
with a low grade of dysplasia (CIN 1), and if it was present, it had a lower level in the patients who were diagnosed with moderate and severe grade of dysplasia (CIN 2 and CIN 3), \( \chi^2 (3, N = 34) = 11.09, p < 0.05 \) (Table 2).

Table 2. Level of expression of p16INK4a in samples in groups I, II and III

<table>
<thead>
<tr>
<th>Level of expression of p16INK4a</th>
<th>Total/Ukupno</th>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
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<tr>
<td>---------------------</td>
<td>---------------</td>
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<tr>
<td><strong>Group I %</strong></td>
<td></td>
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<tr>
<td>0%</td>
<td>0</td>
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<tr>
<td>100.0%</td>
<td>100.0%</td>
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<tr>
<td><strong>Total %</strong></td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100.0%</td>
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<tr>
<td><strong>Group II</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>54.5%</td>
<td>42.9%</td>
</tr>
<tr>
<td><strong>Total %</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>100.0%</td>
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<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>93.8%</td>
<td>100.0%</td>
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<tr>
<td><strong>Group III</strong></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>61.5%</td>
<td>33.3%</td>
</tr>
</tbody>
</table>

There was a difference in the presence and level of expression of p16INK4a among three examined groups, \( \chi^2 (6, N = 109) = 76.02, p < .001 \). The patients with neoplastic and preneoplastic changes had expression of p16INK4a more frequently and more intensely than the patients with nonspecific inflammation. The difference in the presence and level of expression of p16INK4a was found between groups I and II, \( \chi^2 (3, N = 70) = 25.65, p < .001 \). The patients with preneoplastic lesions rarely had p16INK4a expression, and the level of expression was lower in the patients with invasive cervical squamous cell carcinoma (Table 2).

By examining the differences in the level of expression of p16INK4a between group I and group II, with the grade of dysplasia CIN 1, a statistically significant difference, \( \chi^2 (2, N = 43) = 43.00, p < .001 \) was found. The patients with preneoplastic lesion and grade of dysplasia CIN 1 had a lower level of expression of p16INK4a than those patients with invasive squamous cell carcinoma of the cervix (Table 2).

There was a statistically significant difference in the level of expression of p16INK4a, \( \chi^2 (1, N = 63) = 14.00, p < .001 \) between group I and II, with grades of dysplasia CIN 2 and CIN 3. The patients with

**Figure 1.** A. Squamous cell carcinoma, HE x 200; B. Squamous cell carcinoma, high level of expression of p16INK4a, anti-p16 x 200; C. Dysplasia in the squamous epithelium of the cervix of high grade (CIN 3), HE x 200; D. Dysplasia in the squamous epithelium of the cervix of high grade (CIN 3), high level of expression of p16INK4a anti-p16 x 200.

**Slika 1.** A. Carcinoma squamosum, HE x 200; B. Carcinoma squamosum cervicis - visok stepen ekspresije p16INK4a, anti p16 x 200; C. Displazija u skvamoznom epitelu cerviksa visokog stepena (CIN 3), HE x 200. D. Displazija u skvamoznom epitelu visokog stepena (CIN 3) - visok stepen ekspresije p16INK4a u displastičnom epitelu grlića materice, anti-p16 x 200.

**Figure 2.** A. Nonspecific chronic cervicitis, HE x 200; B. Nonspecific chronic cervicitis, the image of expression of p16INK4a (score 2), anti p16 x 200.

**Slika 2.** A. Cervicitis chronica non specifica, HE x 200; B. Cervicitis chronica non specifica - prikaz ekspresije p16INK4a (skor 2), anti p16 x 200.
preneoplastic lesion and grade of dysplasia CIN 2 and CIN 3 had a lower level of expression of p16INK4a than patients with invasive squamous cell carcinoma of the cervix (Table 2).

No significant difference in the presence and level of expression of p16INK4a, $\chi^2 (1, N=53)= 1.16$, $p <.142$ was found between group I and II with the grade of dysplasia CIN 3 (Table 2).

There was a statistically significant difference in the level of expression of p16INK4a, $\chi^2 (3, N=75) = 75.00$, $p < .001$ between group I and III. The patients with invasive squamous cell carcinoma of the cervix had a higher level of expression of p16INK4a compared to the patients with nonspecific inflammation (Table 2).

A statistically significant difference in the presence and level of expression of p16INK4a, $\chi^2 (1, N=73)=24.16$, $p < .001$ was found between groups II and III. The patients with nonspecific inflammation rarely had p16INK4a expression, and the expression level was lower than in patients with preneoplastic lesions (Table 2).

No statistically significant difference, $\chi^2 (1, N=46)= 0.37$, $p = .54$ in the level of expression of p16INK4a was found between group III and group II, who were diagnosed with a low grade of dysplasia (CIN 1) (Table 2).

A statistically significant difference, $\chi^2 (3, N=66) = 32.27$, $p < .001$, was found in the level of expression of p16INK4a between group III and group II (CIN 2 and CIN 3). The patients with nonspecific inflammation had a lower level of expression of p16INK4a compared to patients with preneoplastic lesion and grade of dysplasia CIN 2 and CIN 3 (Table 2).

**Discussion**

Cervical cancer incidence has remained unchanged for years in many poor countries without organized screening programs, while in developed countries, including the United States, the number of cases and deaths is constantly decreasing, now being below 10 per 100 000 women. This decrease in mortality and morbidity rate of this cancer in the second half of the last century can be explained by the organized screening program in most developed countries [1, 16]. The incidence of this cancer in the Republic of Srpska is 19.1 according to the data of the Institute of Public Health [17]. The incidence of cervical cancer ranges from 4 to 14 per 100 000 women in developed countries, where the screening program is developed and funded by the state, while the incidence of this disease is up to 10 times higher in developing countries.

Nevertheless, there are difficulties due to the costs associated with screening programs, limited accuracy of cytology and complications associated with unnecessary treatments, which prompted the research and development of new, more effective preventive tests for the diagnosis of changes that precede cervical cancer. During the last two decades there has been a considerable progress in understanding HPV infection and advances in molecular technology (diagnostics). New methods (techniques) have been developed and used in screening, alone or together with cytological techniques. Some of these tests are HPV deoxyribonucleic acid (DNA) screening tests that have been used successfully for triage and are more reliable than cytological techniques [18].

Morphological interpretation of lesions in the cervix is not completely reliable. The lack of morphological interpretations is obvious at borderline lesions (in cytology and histology), where the differences are significant. It is often impossible to predict the further evolution with morphological interpretation [14, 15].

Klaes et al. have developed an analysis of interpreting biotic samples in HE treated samples, followed by interpretation of samples treated with HE and p16INK4a. The following diagnoses were determined on cervical samples: normal findings (without dysplasia - non-dysplastic lesions/NDL/), cervical intraepithelial neoplasia (CIN 1, CIN 2, CIN 3) and cervical cancer. One of the pathologists who analyzed the material frequently used the NDL category (15%). Two pathologists rarely used the diagnosis of CIN 3 (<20%), and they used diagnosis of CIN 2 more frequently than other pathologists. The concordance in diagnosing invasive carcinoma was high (94%), while the concordance in interpreting CIN lesions ranged from 35% (CIN 2) to 72% (CIN3) on materials processed only with HE method [19]. The application of immunohistochemical techniques and p16INK4a decreased the difference in interpretation between pathologists. The concordance in all categories was above 90%. They concluded that the use of p16INK4a reduced the difference in interpreting lesions of the cervix between pathologists [19]. Similar results were reached by other authors [20–22].

New biomarker, p16INK4a, is an inhibitor of cyclin-dependent kinases, whose increased expression is present in cancer and precancerous lesions of the cervix. The increased presence of p16INK4a can be demonstrated in different ways: by immunocytochemical and immunohistochemical methods and the enzyme-linked immunosorbent assay (ELISA) [1, 5, 23]. The use of liquid cytology together with immunocytochemical examination of the presence of p16INK4a expression is very effective in detecting precancerous and cancerous lesions of the cervix [11, 24]. The sensitivity ranged from 0.59 to 0.96 and specificity between 0.41 and 0.96 in detecting dysplastic lesions [25, 26].

The expression of p16INK4a in the materials of the group I was present in all 36 (100%) samples. In all samples, a high level of expression (more than 25% of tumor cells positive) was found. The expression was high, cytoplasmic and nuclear.

Sano et al. demonstrated the expression of p16INK4a in all carcinomas of the cervix, and it
was high in 97% of cases. Expression of p16INK4a in CIN lesions ranged from 53% in CIN 1 lesions up to 100% in CIN 3 lesions [27].

In our study, expression of p16INK4a was present in 67.65% of patients with CIN lesions. It should also be noted that expression was not present in most cases of CIN 1 lesions (57.14%), and, in cases where it was verified, the expression had low level, i.e. expression in <5% of positive epithelial cells (42.86%). A similar expression of p16INK4a was found in CIN 2 lesions; it was absent in 60% of cases, low expression was found in 20% of cases, and a moderate level of expression (5 to 25% of the cells showed expression) was seen in 20% of cases. Expression of p16INK4a in CIN 3 lesions was verified in 94.12% of cases. Expression was not observed in one case only. Our results differ from the results reached by Sano et al., who have verified the expression of p16INK4a in a high percentage of CIN 1 (54% of samples) and CIN 2 (94% of samples) [28]. Our results are consistent with the results of Izadi-Mood et al. [1].

Statistical testing found that there was a statistically significant difference in the expression of p16INK4a in relation to the grade of dysplasia. There was a significant difference in expression of p16INK4a in the patients who were diagnosed with cancer in relation to all dysplastic lesions (considered together). The difference in expression was not present only when groups with cancer and severe grade dysplasias were compared. In our study, a statistically significant difference was found in expression and the level of expression of p16INK4a between the patients with the diagnosis of inflammation (cervicitis) and the patients who were diagnosed with invasive cancer or dysplasia. In addition, a statistically significant difference was not found in expression of p16INK4a among the patients with established inflammation and those who had a low grade of dysplasia or CIN 1. The results are similar to those found in the work of Agoff et al. They proved that the level of expression of p16INK4a and Ki-67 highly correlated with the grade of dysplasia in the cervical epithelium and HR-HPV infection [28].

Thus, for lesions which were in cytological materials interpreted as atypical squamous cells of undetermined significance (ASC-US), those lesions interpreted as atypical squamous cells when it is not possible to exclude high-grade squamous intraepithelial lesion (ASC-H) and squamous intraepithelial lesions of low grade (LSIL) should also be processed with immunocytochemical techniques, and the level of expression of p16INK4a should be assessed. Lesions morphologically interpreted as a low (CIN 1) and moderate (CIN 2) grade of dysplasia should be processed by immunohistochemical techniques in histological materials as well, and the level of expression of p16INK4a should be assessed before planning any treatment of the patient [10].

Our study can contribute to a more reliable differentiation of “borderline cases” of lesions in the cervical epithelium, both on cytological and histological materials. The application of immunocytochemical and/or immunohistochemical analysis with an antibody to p16INK4a can help to differentiate lesions which are cytologically interpreted as atypical squamous cells of undetermined significance or atypical squamous cells and histologically interpreted as preneoplastic lesions (CIN 1, CIN 2). In addition, false diagnosis of reactive changes and their classification as preneoplastic lesions in histological materials can be prevented. Unnecessary treatment (usually surgical) can also be prevented. And vice versa, it will help to prevent classification of dysplastic lesions as non dysplastic lesions, and therefore enable timely and appropriate treatment [10].

Our study, as well as many others, has proved that the determination of expression of p16INK4a is a sensitive and specific method for identification of dysplastic and tumor cells in histological samples.

Conclusion

Expression of p16INK4a in inflammatory lesions of the cervix is rarely present; it is focal and has a low intensity.

Expression of p16INK4a in preneoplastic lesions of the cervix is often present. The presence and level of expression of p16INK4a depend on the grade of dysplasia. Expression of p16INK4a in severe grade of dysplasia is present, diffuse and has a high intensity, while in low grade dysplasia it is rarely present, focal and has a low intensity.

Expression of p16INK4a in neoplastic lesions of the cervix is present, diffuse and has a high intensity.

Expression of p16INK4a in neoplastic cervical lesions does not depend on the histological type and grade of the tumor.

Inflammatory changes may be differentiated from preneoplastic changes of severe grade and neoplastic changes in the cervix by analyzing the presence of p16INK4a.

The use of p16INK4a in interpreting borderline lesions on the cervix allows adequate therapeutic treatment of patients.

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