ORIGINAL ARTICLE

UDC: 618.146-07
DOI: https://doi.org/10.2298/VSP200818087V

Application of P16, P63, cyclin D1 immunostaining and nuclear morphometric analysis for assessment of cervical dysplasia

Primena imunohistohemijskih markera P16, P63, ciklin D1 i morfometrijske analize u proceni težine displazije grlića materice

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Abstract

Background/Aim. Human papilloma virus (HPV) infection is the main etiological factor for the development of cervical precancerous dysplastic squamous intraepithelial lesions (SIL). The virus oncoproteins affect several proteins included in cell proliferation. The aim of this study was to evaluate application of immunohistochemical markers related to proteins of the cell cycle and, also, application of nuclear morphometric analysis for assessment of cervical dysplasia. **Methods**. Retrospective study included 78 women with detection of presence of high-risk HPV by polymerase chain reaction (PCR), with histopathology diagnosis low-grade SIL (LSIL) or high-grade SIL (HSIL). Immunohistochemical staining for p16, p63, cyclin D1 and morphometric analysis of the nuclear surface area were performed. The control group consisted of ten women without SIL and without HPV infection. This study was conducted in accordance with the Helsinki Declaration. **Results.** Comparing immunohistochemical expression of p16 and p63, highly statistically significant differences ($p < 0.001$) were established among the control, LSIL and HSIL groups, while cyclin D1 showed significant statistical difference ($p < 0.05$). Great variations were observed in nuclear morphology and nuclear surface area that had highly statistically significant differences ($p < 0.001$) among the control, LSIL and HSIL groups. **Conclusion.** This study demonstrated that immunohistochemical analysis of p16, p63 and cyclin D1 are important for diagnosis of dysplastic changes in cervical epithelium. Also, morphometric analysis of the nuclear surface area demonstrated a high significance for diagnosis of cervical dysplasia.

Key words:
diagnosis; immunohistochemistry; ovarian neoplasms; papilloma viridae; polymerase chain reaction; severity of illness index.

Apstrakt

Uvod/Cilj. Infekcija humanim papiloma virusima (HPV) je glavni etiološki faktor za razvoj displastičnih, skvamoznih intraepitelnih lezija (SIL) grlića materice. Virusni onkoproteini utiču na gene i proteine koji su uključeni u deobu ćelije. Cilj istraživanja je bio da se ispita efekat primene imunohistohemijskih markera koji se odnose na proteine uključene u ćelijski ciklus, kao i primene morfometrijskijske analize jedara u proceni težine displazije grlića materice. **Metode.** U retrospektivno istraživanje je bilo uključeno 78 žena kod kojih je potvrđeno prisustvo visoko rizičnih tipova humanih papiloma virusa (HPV) metodom *polymerase chain reaction* (PCR), a histološki je dijagnostikovana SIL niskog (LSIL) ili visokog (HSIL) stepena. Izvršene su imunohistohemijske analize p16, p63 i *cyclin* D1 i morfometrijska analiza površine jedara. Kontrolnu grupu činilo je 10 žena kod kojih nije potvrđeno prisustvo HPV virusa, niti SIL lezije. Studija je sprovedena uz poštovanje principa Helsinške deklaracije. **Rezultati.** Ustanovljena je statistički visoko značajna razlika u imunohistohemijskoj ekspresiji markera p16 i p63 ($p < 0,001$) između kontrolne grupe i LSIL i HSIL grupa i statistički značajna razlika u ekspresiji *cyclin* D1 ($p < 0,05$) između ovih grupa. Morfometrijska analiza površina jedara pokazala je visoko statistički značajnu razliku ($p < 0,001$) između ispitivanih grupa. **Zakljucak.** Studija je pokazala da imunohistohemijske analize p16, p63 i *cyclin* D1 imaju

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značaja u dijagnostici SIL grlića materice. Takođe, pokazano je da je morfometrijska analiza jedara cervikalnih ćelija veoma značajna u dijagnostici displazije.

Ključne reči:
dijagnoza; imunohistohemija; jajnik, neoplazme; papiloma virus, humani; polimeraza, reakcija stvaranja lanaca; bolest, indeks težine.

Introduction

One of the most common diseases of modern age is the cervical cancer and dysplasia of epithelium of cervical mucosa. Squamous cell carcinoma of the cervix in Serbia is among cancers with very high incidence and mortality [1, 2]. Preventive examinations of this disease decreased morbidity drastically. Health education of young people with information about risk factors also gives good results in the prevention of this serious disease.

Human papilloma virus (HPV) infection is the main etiological factor for the development of precancerous dysplastic squamous intraepithelial lesions (SIL) and cervical carcinoma. HPV is a large group of viruses specific for their tropism for epithelium. The different levels of oncogenic potential of HPV viruses are very important characteristic for research. In this moment, we know that a persistent infection in high-risk type of HPV, have a high tendency of dysplastic or neoplastic progress [3]. Typing of HPV may be performed as a big prognostic factor by several laboratory techniques, but the polymerase chain reaction (PCR) has been mostly used. HPV typing provides information relevant to the patient's clinical treatment [4].

One of the crucial pathogenic problem and characteristics of HPV is its ability to integrate itself in the human genome. This event is the main cause of cervical epithelial dysplasia. Cervical dysplasias are among the most common histopathological diagnoses of the females. In those cases, the virus affects cells structure and functions. Depending on the area affected, we have a diagnosis of low grade and/or high grade SIL (LSIL and HSIL, respectively). At the beginning of the pathogenesis of dysplasia, there are low-grade dysplasias. That level of pathophisiological and pathological changes occur at the level of the lower third of the full epithelial thickness. If dysplastic cells are present in the epithelial thickness greater than one third of the total epithelial thickness, then we are talking about severe dysplasia. The nuclei of dysplastic cells are irregular in many morphologic characteristics. Nuclei have different shape, larger area. Also nuclei are hyperchromatic and occupy a significant part of the cell volume like bigger nuclear than cytoplasmatic volume. Due to the specific pathophysiologic and genetic mechanisms in the cytoplasm, perinuclear clearance of the cytoplasm, called koilocytosis, can be seen morphologically. In cells with dysplasia, there is a disturbance of the cell cycle and maturation. Inadequate genetic regulation results morphologically like a stratification disorder. As well as, we can notice as very important morphological disorder, the presence of a pathological mitoses. The index of pathological mitoses determines the severity of dysplasia [5, 6]. It is interesting that on microscopic preparations we can notice zones of regular epithelium that directly extend to the epithelium with dysplasia. A p16 protein is very important “regulator” of cell-cycle. Normally, it prevents progression of cell cycle. That protein has the important role for inhibiting cyclin D-dependent protein kinases. After the integration of HPV into the nuclei of squamous cell genome, some of tumor suppressor genes are lost and cell cycle is deregulated. The progression from a transient to a transforming HPV infection is characterized by a strong increase of HPV E6/E7 mRNA and protein expression. Viral oncoprotein E7 disrupts pRb in transforming HPV infections. The disturbance of the Rb pathway leads to a compensatory overexpression of p16. It works by a negative feedback loop. p16-overexpression in cells is considered as a surrogate marker for deregulated E7 expression and hence for transforming HPV infections. Overexpression and cellular accumulation of p16 may be evaluated immunohistochemically and studies show that immunopositivity to p16 is an important marker [6, 7].

p63 is one of the members of the group of p53 genes. It participates in determining the proliferation of epithelial cells. p63 inhibits the cell cycle at a determined point and induces apoptosis. In that way, its influence is the inhibition of oncogenic mutations [8]. According to data from molecular cell biology, genetic models and clinic research, it appears that p63 may act as either an oncogene or a tumor suppressor gene in different scenarios. The role in promotion of tumorigenesis is by promoting cell proliferation and survival, and it depends of the whole p53 family [9].

Cyclin D1 is associated with the cyclin-dependent kinases (CDKs). It inhibits the retinoblastoma (RB) protein, and induces proliferation. Many additional factors have positive or negative effects on the control of cellular proliferation. Many researches have shown changes within the RB-p16-cyclin D1 pathways. Relatively few studies have investigated cyclin D1 expression within the normal cervix or SIL changes, and most of the latter have focused on the more common *in situ* and invasive squamous lesions [10].

The use of computer programs in the analysis of dysplastic nuclei is an imperative of the 21st century. This is a way of objectifying the process of assessing the degree of dysplasia. It is of special importance when the dysplasia reaches the junction/border of the lower with the middle third of the epithelium: the border of mild and severe dysplasia. For pathologist in routine practice, it plays a big role in the differential diagnosis in many different cases: dysplasia and immature squamous metaplasia with atypia, reactive atypia in atrophic epithelium is also unambiguous. Morphometric analysis is performed by analyzing morphometric software for cellular structures. For dysplasia of the cervical epithelium, the most important component is the analysis of cell nuclei. Comparison of numerical values of diameter,

volume and surface of nuclei and cytoplasm gives the possibility of easier diagnostics, especially since numerical values can be used. In addition, the advantage of morphometry is that the nuclei from the dysplasia zone can be compared by computer with the nuclei that are not dysplastic, which further verifies the diagnosis.

Methods

The study was retrospective and included 88 patients treated during a period of 30 months. Of the selected specimens, 10 women were in the control group. The rest of the subjects (n = 78) who formed dysplasia group, entered the study after meeting the following criteria: 1) primary lesion that had not been treated before; 2) colposcopic finding suggestive of dysplasia; 3) Papanikolau (PAPA) test result (according to Bethesda criteria) showing some of the following – LSIL, HSIL, atypical squamous cells of undetermined significance (ASCUS); 4) performed PCR HPV typing, with proven presence of one of the following types: 16, 18, 31 or 33. The study did not include women with previously diagnosed and treated dysplasia, as well as women with squamous cell carcinoma.

The control group consisted of women who did not have HPV infection, nor dysplasia or any clinical data indicating the possibility of dysplastic process. These patients had symptoms of uterine myomas and due to that had underwent hysterectomy. The final histopathological findings of the control group cervix tissue denied any signs of dysplasia, and verified only mild nonspecific inflammation.

Biopsy samples of all patients who met the entry criteria underwent standard histological tissue processing, hematoxylin-eosin staining, immunohistochemical staining and morphometric tissue analysis. Histological diagnoses were established under the light microscope, according to the latest World Health Organization (WHO) classification criteria and were classified as LSIL or HSIL, and upon that, additional two groups were formed: LSIL group (n = 48) and HSIL (n = 30) [11].

Following antibodies were used for immunohistochemical staining: p16 (CINtec® E6H4 Histology Kit, Ventana), p63 (DAK-p63, DAKO), cyclin D1 (EP12, Cell marque, Milipore Sigma). Staining was performed according to manufacturers instructions (having internal and external control tissue), and interpreted as positive or negative staining according to reference guidelines: positive p16 staining was interpreted as nuclear and/or cytoplasmic [7]. p63 nuclear staining is considered as immunohistochemical positivity [9]. Cyclin D1–nuclear staining is considered as immunohistochemical positivity [10].

Morphometric analysis was performed using Image J software on digital microphotographs. Analysis was performed in the zone of the most intense dysplasia. The surface of the nucleus was analyzed, since its characteristics are the most important diagnostic criterion for diagnosing dysplasia and that the most important changes in dysplasia take place at the nuclear area.

The obtained results were compared among control group and dysplasia group. Statistical significance of the obtained results was analyzed by the methodology of analytical and descriptive statistics. A χ^2-test was used for statistical analysis of immunohistochemical results. The Kruskal Wallis test was used to analyze the morphometric results of the nuclei. The results are presented in images, numerically and graphically.

Results

Using the χ^2-test, it was verified that there is a highly statistically significant difference ($\chi^2 = 14.841$, $p = 0.001$) in p16 expression between patients without cervical epithelial dysplasia (control group), and those with LSIL and HSIL (Figures 1 and 2).

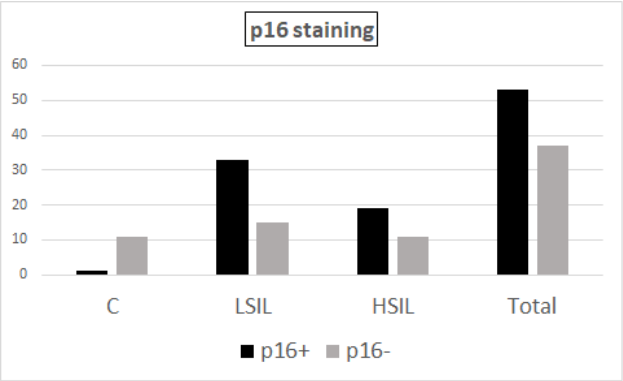

Fig. 1 – Results of p16 immunohistochemical staining (y-axis – number of women). LSIL – low-grade squamous intraepithelial lesions (SIL); HSIL – high-grade SIL.

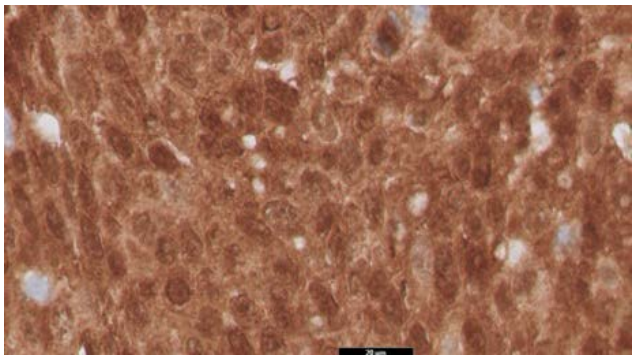

Fig. 2 – Positivity of p16 in cervical squamous cells (hematoxylin-eosin, ×60).

Expression of p63 showed highly statistically significant difference ($\chi^2 = 17.639$, $p = 0.000$) among the control, LSIL and HSIL groups, when compared by χ^2-test (Figures 3 and 4).

In patients without cervical epithelial dysplasia (control group), compared with those in the LSIL and HSIL groups, χ^2-test revealed statistically significant difference in cyclin D1 expression ($\chi^2 = 7.483$, $p = 0.024$) (Figures 5 and 6).

Kruskal Wallis test revealed highly statistically significant difference ($p < 0.001$) in the nuclear surface area

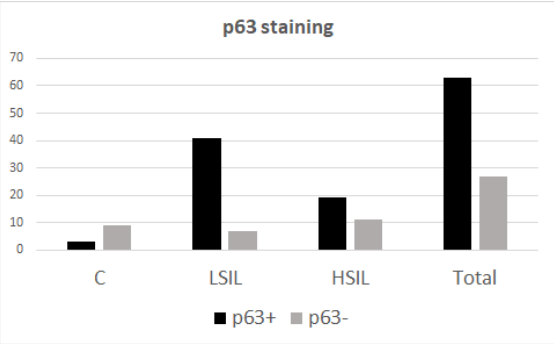

Fig. 3 – Results of p63 immunohistochemical staining (y-axis – number of women). LSIL – low-grade squamous intraepithelial lesions (SIL); HSIL – high-grade SIL.

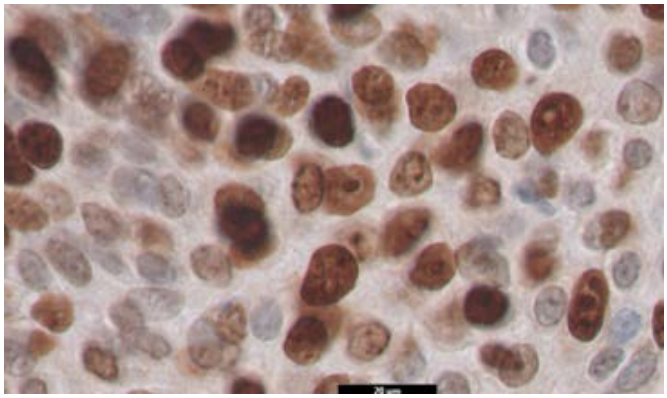

Fig. 4 – Positivity of p63 in cervical squamous cells (hematoxylin-eosin, ×60).

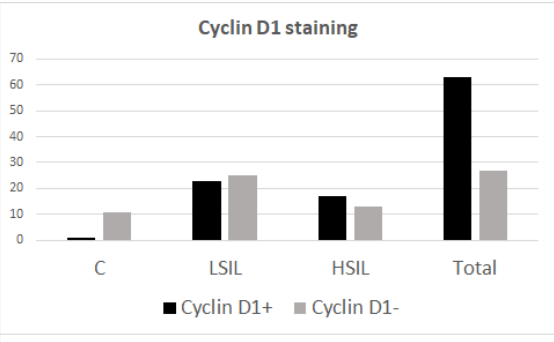

Fig. 5 – Results of cyclin D1 immunohistochemical staining (y-axis – number of women). LSIL – low-grade squamous intraepithelial lesions (SIL); HSIL – high-grade SIL.

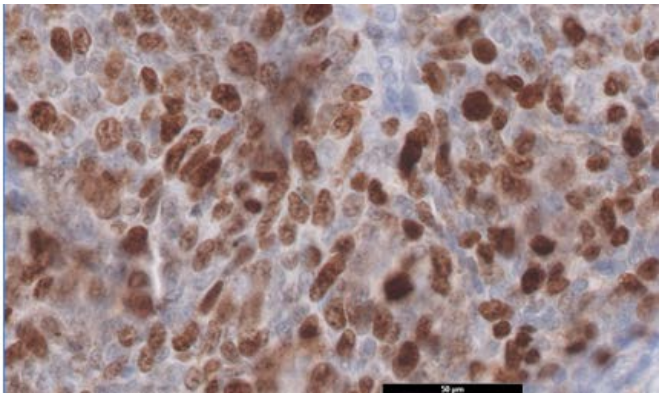

Fig. 6 – Positivity of cyclin D1 in cervical squamous cells (hematoxylin-eosin, ×40).

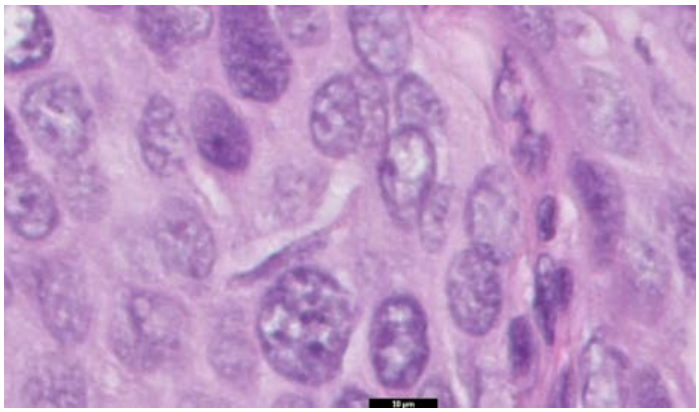

Fig. 7 – Nuclear characteristics and enlargement in squamous intraepithelial lesions (SIL) (hematoxylin-eosin, ×80).

of the cervical epithelial cells among examined groups. Morphological changes on epithelial cells with dysplasia were clearly visible (Figure 7).

Discussion

Cervical cancer is one of the most common deadly diseases in female population. Also, in 21th century, it is a disease with a poor prognosis. High-stadium of the disease is a bad prognostic factor. HPV is the main etiological factor for the disease. But cervical cancer is final pathological event. LSIL and HSIL are without symptoms. They occur from the moment of HPV infection and can be present for many years before the cancer develops. Developing a methodology for histopathological diagnosis of these lesions and recognizing prognostic factors for the development of HPV-induced lesions are very important. They provide information of the necessary treatment of precancerous lesion. This prevents cancer. Intraepithelial lesions are treated with both classical and modern technological methods [4].

The epithelium of cervix is stratified squamous, or columnar. The region where cylindrical epithelium continues with squamous epithelium is the most fragile location for pathologic process, especially for dysplasias. This is the place where the most frequent premalignant lesions occur. But as with all viral infections, the effect of the virus depends on both general immunity and local immunity. Protection against non-HPV sexually transmitted disease and the presence of normal microflora in the female reproductive organs also make prevention of cervical epithelial dysplasia and cervical cancer development. It is also easier to diagnose dysplasia if there is no associated inflammation and infection with non-HPV microbiological etiological factors. Reparative atypia and various forms of metaplasia can be a factor of complication in the diagnosis of cervical epithelial dysplasia.

The use of immunohistochemical markers in modern pathology is imperative. This includes the diagnosis of pathological conditions of the cervix. Immunohistochemicaly, analyzed markers in nondysplastic epithelial cervical cells – p16, p63 and cyclin D1, if are positive, depending on the marker used (except p63), are present in a small percentage of cells located in basal layers. This results is in accordance with literature data [10–14].

The p16ink4a/RB pathway is of a great significance. As a result, its malfunctions can be a good diagnostic marker for making prognosis of premalignant lesions [15]. Some authors who studied immunoexpression of p16 showed a linear increase in this expression from LSIL to HSIL [12, 13].

In our study, it was verified that there is a highly statistically significant difference in p16 expression in patients without cervical epithelial dysplasia, with LSIL and with HSIL. Our results are similar to above mentioned, and many other stating that p16 expression increases with the progression of dysplastic changes in cervical squamous epithelium. From other experimental and literature data and review articles, following should be kept in mind, regarding p16 expression – its level can both increase or decrease depending on tissue. There are great variations in interpretation of what p16 positivity is in some studies. Therefore, it is important to find panel of biomarkers necessary for the diagnosis of cervical epithelial dysplasia especially for the differential diagnosis of the degree of dysplasia in cases borderline from LSIL to HSIL 4, [14–17]. Our result show that p16 expression is one of the most significant biomarkers and diagnostic methods for HPV. This was confirmed both in accordance with the fact that p16 can rarely be positive in the cervical epithelium without dysplasia, but in a different percentage and a different microscopic image of the visualization of it. That is why we have to be very careful with interpretations of this immunohistochemical marker. Also, we have to find the best supplemental markers [13, 17].

HPV by producting E6 and E7 oncoproteins affects tumor suppressor genes in the cell. Several of those genes are genes of p63, cyclin-dependent kinase inhibitors (p21, p27, p16) and retinoblastoma (pRb). That promotes malfunction of normal cell cycle and inhibits the repair of DNA damage. Some of the consequences of this happening may induce proliferation or cell apoptosis [18].

According to the literature, we may conclude that p63 expression is evidently increased in cervical intraepithelial neoplasia [8]. Our study did not deviate from those literature data. Expression of p63 showed highly significant statistical difference among the control group, and LSIL and HSIL groups. p63 positivity in the control group was smallest, compared to the LSIL and HSIL groups, which is also in accordance with theoretical and experimental scientific data. When comparing number of positive and negative cases among all analyzed cases, and just in the LSIL and HSIL groups, we observed predominance of p63 positive cases in dysplastic cells, which showed to be statistically highly significant. Functional and biochemical alterations of cell cycle components are one of the crucial pathogenesis mechanisms of this virus [19]. The expression of p63 is a significant marker for diagnostics of cervical carcinomas [8].

HPV induced deviations of E2F referred before, in concept of p53 family deregulation, stimulates the activation of cyclin-dependent kinase inhibitor [18]. The malfunction of RB-p16-cyclin D1 pathways results in abnormalities in cell cycle. The mechanism of occurrence is by increasing activity of cyclin-D1 [10]. Some authors have shown correlation between degree of dysplasia of cervical epithelial cells and cyclin E [13]. Some authors have shown immunoexpression of cyclin D1 in dysplastic cells and found that in contrast to p16, immunoreactivity of cyclin D1 is decreased in relation to the degree of dysplasia [12]. Our study showed statistically significant difference in cyclin D1 expression between the group of patients without cervical epithelial dysplasia and the LSIL and HSIL group. Our results show predominance of cyclin D1 positive cases when observing total sample, as well as when comparing number of positive and negative cases among the control and LSIL or HSIL groups, which was proven statistically highly significant. The number of positive cases in our HSIL group showed slight decline, compared to the LSIL group, but still it was significantly higher compared to controls. It appears that in some other cervical lesions, expression of cyclin D1 decreases as the dysplastic process progression occurs [10]. The explanation may be in different immune statuses – general and local. An explanation should also be in gene mutations. Laboratory standardization also plays a huge role in research results.

Morphological changes on epithelial cells with dysplasia are clearly visible. They exist in the nucleus and in the cytoplasm. The nuclear cytoplasmic ratio has also changed. The architecture of epithelial cell stratification in dysplasia is disturbed. Increased mitotic count and koilocytosis were presented also. The differential diagnosis of dysplasia in relation to reparative processes and inflammation is sometimes difficult. Also, metaplasia together with inflammation makes it difficult to diagnose dysplasia and gradation of dysplasias. Exclusion of the subjective component of the pathologist in the diagnosis is the basic goal of the pathology. The 21st century provides an opportunity to use software that can help a pathologist in the process of excluding the subjective component in making a diagnosis.

Morphometric analyzes of different types are applied and improved. Their improvement is a promising factor for the accuracy of pathological diagnosis.

Histologically, dysplasias were classified as LSIL or HSIL, according to its nuclear and cytoplasmatic features and the thickness of epithelium that was affected. In the LSIL group, changes were present in lower third of epithelium, while in the HSIL group, it involved whole epithelial thickness. Our results showed highly statistically significant difference between the nuclear surface area of the cervical epithelial cells in the LSIL and HSIL groups and epithelium without dysplasia. Out of total number of analyzed cases, 10 were in the control group, 48 in the LSIL group and 30 in the HSIL group. In specimens of the LSIL and HSIL groups, standard histopathological examination detected nuclear changes that were proven by morphometric analysis. Nuclear surface area increased significantly compared to the control samples. The research results, the economic factor and the availability of morphometry makes this methodology most promising.

Conclusion

This study demonstrated that immunohistochemical analysis of p16, p63 and cyclin D1 is important for diagnosis of dysplastic changes in cervical epithelium. Also, morphometric analysis of the nuclear surface area demonstrated its high importance for diagnosis of cervical dysplasia.

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Received on August 18, 2020
Accepted on August 27, 2020
Online First August, 2020