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The utility of p16, E-cadherin and Ki67 in cervical squamous intraepithelial lesions diagnosis

CRISTIANA SIMIONESCU[1)], CL. MĂRGĂRITESCU[1)], A. STEPAN[1)], CLAUDIA VALENTINA GEORGESCU[2)], MIHAELA NICULESCU[3)], MIHAELA MUNTEAN[4)]

[1)]*Department of Pathology, University of Medicine and Pharmacy of Craiova*

[2)]*Department of Histopathology, Emergency County Hospital, Craiova*

[3)]*Department of Anatomy, University of Medicine and Pharmacy of Craiova*

[4)]*Department of Histopathology, Emergency County Hospital, Slatina*

Abstract

In this study, we included 26 cases diagnosed as squamous intraepithelial lesions, which were examined histopathologically, and in terms of p16, E-cadherin and Ki67 immunoexpression. In low-grade lesions, p16 expression was limited to one third below the epithelium, E-cadherin has a membranous pattern and Ki67 proliferation index had low values. In high-grade lesions, the p16 diffuse stain was present in two thirds or all epithelium layers, E-cadherin expression became aberrant, with membranous and cytoplasmic pattern and Ki67 proliferation index was high. These biomarkers have proven useful to accurately assess the extent of lesions and to identify lesions with high risk of progression.

***Keywords*:** squamous intraepithelial lesions, p16, E-cadherin, Ki67.

Introduction

Despite the ease with which can be examined the uterine cervix (clinically, by colposcopy and cytology), cervical cancer is the second in frequency of malignant neoplasms in woman worldwide, constituting about 10% of all cancers [1]. Since the vast majority of cervical cancers are preceded by a long preclinical period, the detection of precursor lesions is crucial in preventing and reducing the incidence and mortality. Recent data from the *National Breast and Cervical Cancer Early Detection Program* indicated a rate of 2.9% for lesions of low grade squamous intraepithelial (LSIL) and 0.8% of high grade (HSIL), reported in all cervical lesions [2].

Squamous intraepithelial lesions comprises a spectrum of atypical changes begin with minimum marked abnormalities that progress to intraepithelial level and then to invasive carcinoma, thus being the precursors of cervical carcinoma [3, 4]. Initiation and progression of squamous intraepithelial lesions is the result of a multifactorial process in which the infection with Human Papillomavirus (HPV) plays a central role [3, 5]. High-risk HPV types (HPV–HR) are associated lesions that tend to progress, being present in 99% of squamous cell carcinomas of the cervix [6].

The correct diagnosis of precursor lesions (intraepithelial squamous lesions – SIL, cervical intraepithelial neoplasia – CIN) underlying programs of cervical cancer detection. "Gold standard" of diagnosed these lesions is the histopathological evaluation [7]. However, some studies have shown significant intra- and inter-observatory differences regarding histopathological diagnosis of lesions [8]. Moreover, the morphological criteria assessed do not provide information about the further development of these lesions, which may be the meaning of regression or progression to invasive disease [4].

The variability in histopathological assessment and difficulty in highlighting of oncogenic viral products, resulted in the need to identify biomarkers with high sensitivity and specificity for these lesions. There are studies that have attempted to highlight the individual or associated usefulness of p16, E-cadherin and Ki67 in SIL diagnosis and prognosis and were able to make arguments for these markers [9–11].

The purpose of this study is to assess the benefits of using a panel consisting of p16, E-cadherin and Ki67 for diagnosis and evaluation of SIL development.

Material and Methods

We performed a retrospective study that included 26 cases with squamous intraepithelial lesions of the uterine cervix. Biological material was represented by bioptic fragments or amputation pieces of the cervix,

performed in gynecological services, which were processed by common histopathological technique using 10% formalin-fixation, paraffin embedding, and Hematoxylin–Eosin staining. The diagnosis was made within the Pathology Laboratory of Emergency County Hospital, Craiova, in 2009. Histopathological grading assessment of SIL was aimed.

The immunohistochemical processing was made on serial sections from each paraffin-embedded block using the EnVision technique for p16 and LSAB+ System–HRP for E-cadherin and Ki67. As antigen retrieval, we used citrate buffer solution pH 6 for p16 and Tris-EDTA solution pH 9 for E-cadherin and Ki67 and sections were boiled for 20 minutes at microwave. Sections were incubated for one hour with primary antibody (rabbit anti-human p16, 1/100 diluted, mouse anti-human E-cadherin, NCH 38 clone, 1/50 diluted, mouse anti human Ki67, MIB1 clone, 1/100 diluted, all antibodies have been from Dako). DAB (3,3'-diamino-benzidine tetrahydrochloride) was used to visualize the reaction, followed by counterstained with Hematoxylin. Negative external control was used by omitting the primary antibody.

Immunoreaction for p16 was considered positive when a diffuse nuclear and cytoplasmic staining was seen, being negative if the reaction was absent or focally distributed on small groups of cells [9]. Also was analyzed the immunostaining intensity, characterized as strong or weak. E-cadherin reactions were quantified by means of the marked cells' percentage: absence, <5% positive cells, 5–50% and respectively >50% [12]. The reaction was also analyzed in terms of homogeneity. A proliferation index was used to quantify Ki67 immunoexpression, represented by the number of marked dysplastic cells and the total number of cells ratio on 40-field microscope. Count was made on 10 fields, each including 1000 cells and we calculated the average value. Proliferation index was considered 1, 2 or 3 that were scored as 5%, 5–50% and >50% of cells [13]. The acquisition of the images was done with Nikon Eclipse E600 microscope and software program Lucia 5.

Results

Histopathological analysis of the 26 cases revealed the presence of LSIL (CIN I) and HSIL (CIN II and CIN III) in 14 cases, respectively 12 cases. The average age for diagnosis was 32 years for LSIL, respectively 37 years for HSIL. All lesions showed atypical cells and nuclear changes, and varying degrees of architectural change. In LSIL the changes have occupied the lower third of squamous epithelium which was observed at an increase parabasal immature cells, with obvious mitotic activity and in three cases we found atypical mitosis and multinucleated cells with 4–6 nuclei, representing LSIL with marked atypia (LSIL–MA). In 21 of the 26 SIL cases in the surface layer was present koilocytic atypia. In HSIL, atypical nuclear and cellular abnormalities and architectural maturation occupied by two thirds to full thickness epithelium (Figure 1).

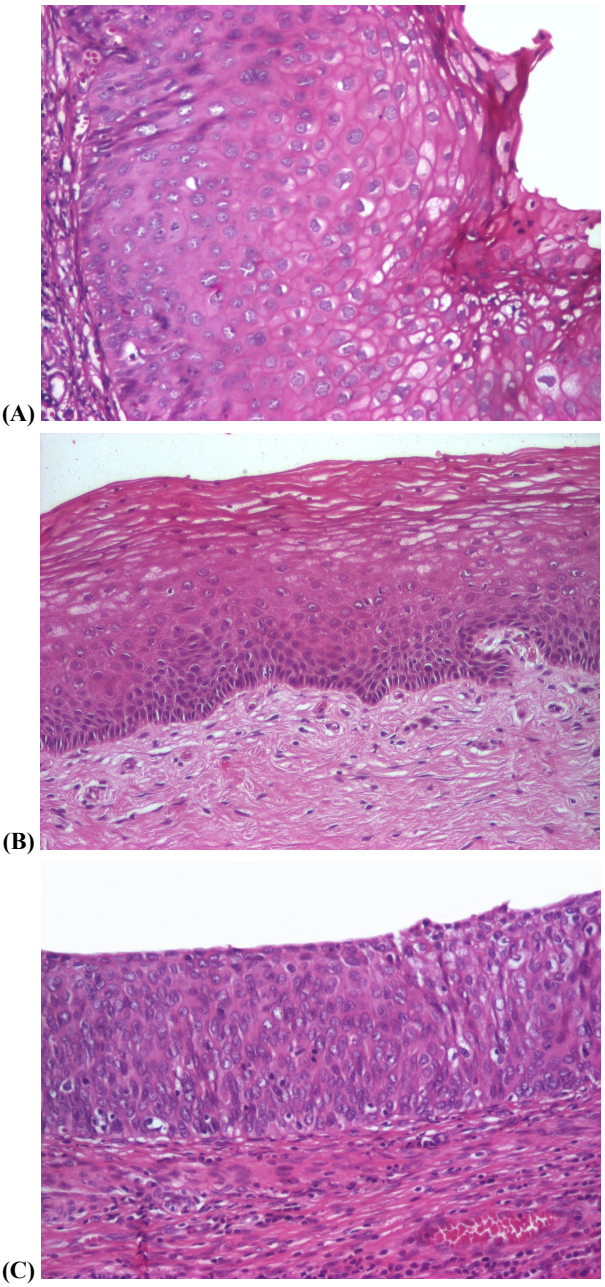

Figure 1 – ***Histopathological aspects in cervical squamous intraepithelial lesions: (A) LSIL-MA, ×100; (B) LSIL (CIN I), ×100; (C) HSIL (CIN III), ×100.***

Immunohistochemical analysis showed negative reaction for p16, E-cadherin and Ki67 in control cases. P16 immunoreaction was positive in 18 cases, representing 69.2%. Negative cases corresponded to some LSIL cases, in which the immunostain was only cytoplasmic or nuclear discontinuous, in small groups of cells in the lower third of the epithelium (Table 1).

Table 1 – ***P16 immunostain distribution based on the degree of lesions***

P16 immunostain	LSIL	HSIL
No. of positive cases	6	12
%	42.8	100

The p16-positive immunostain was diffuse and strong at nuclear and cytoplasmic level, without differences regarding the intensity of reaction in different SIL

degree. We also observed weak intensity stain in the cytoplasm and/or nucleus of fibroblasts, inflammatory cells and vascular endothelial cells. P16 immunostain was present in basal and parabasal cells from the lower third of the epithelium in LSIL cases and in HSIL cases, this was extended by two thirds to entire epithelium thickness. The p16 reaction was detected also in few epithelial cells from the upper layers but the stain intensity was weak (Figure 2).

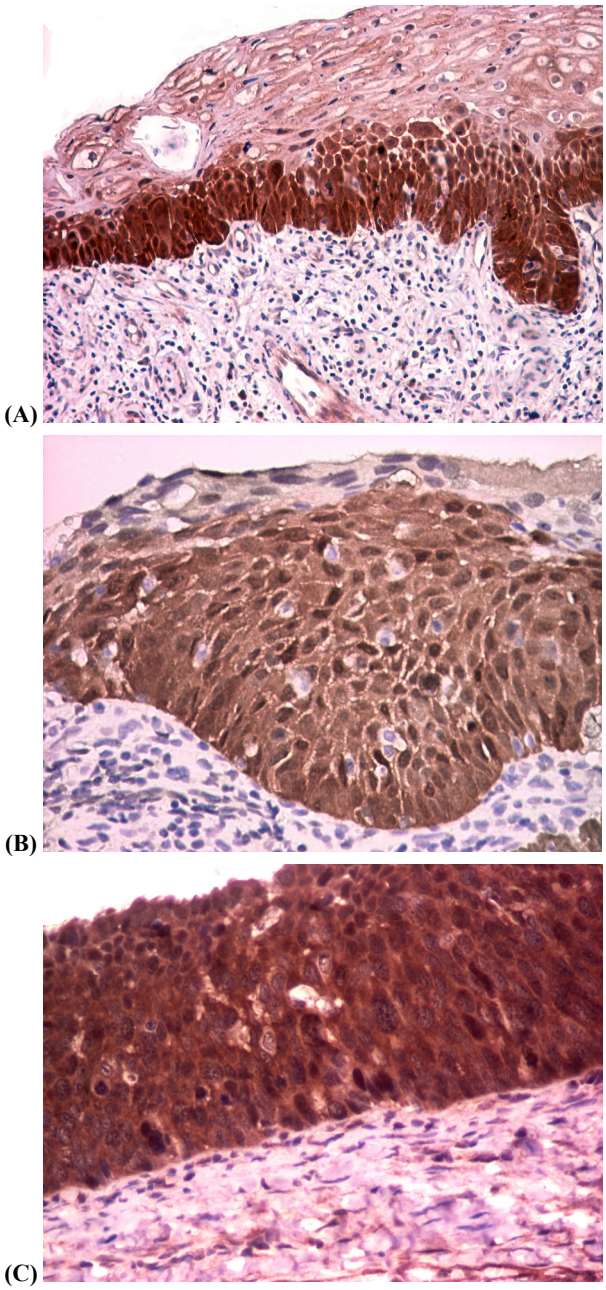

Figure 2 – ***P16 immunoexpression in cervical squamous intraepithelial lesions: (A) LSIL (CIN I), ×40; (B) HSIL (CIN II), ×100; (C) HSIL (CIN III), ×100.***

The E-cadherin immunostaining has been observed in 22 cases, representing 84.6% of evaluated cases. Negative cases corresponded to high-grade lesions. In LSIL-positive cases, the reaction was present in the basal, parabasal and intermediate cells and the number of marked cells was >50%. In the three LSIL cases with atypical mitosis, the stain was located at membrane and cytoplasmic level and it was heterogenous within the meaning of presence of some areas without immunoreaction and the number of positive cells was variable, between 5–50%. The same stain pattern of reaction was present in HSIL positive cases (two cases), the rest having less then 5% marked cells. As SIL grade increased the number of cells that have lost E-cadherin expression was higher or its expression was an abnormal one at cytoplasmic level (Table 2, Figure 3).

Table 2 – ***The E-cadherin immunoexpression score***

No. of marked cells / Lesion	<5%	5–50%	>50%
LSIL	-	+	+
HSIL	+	+	-

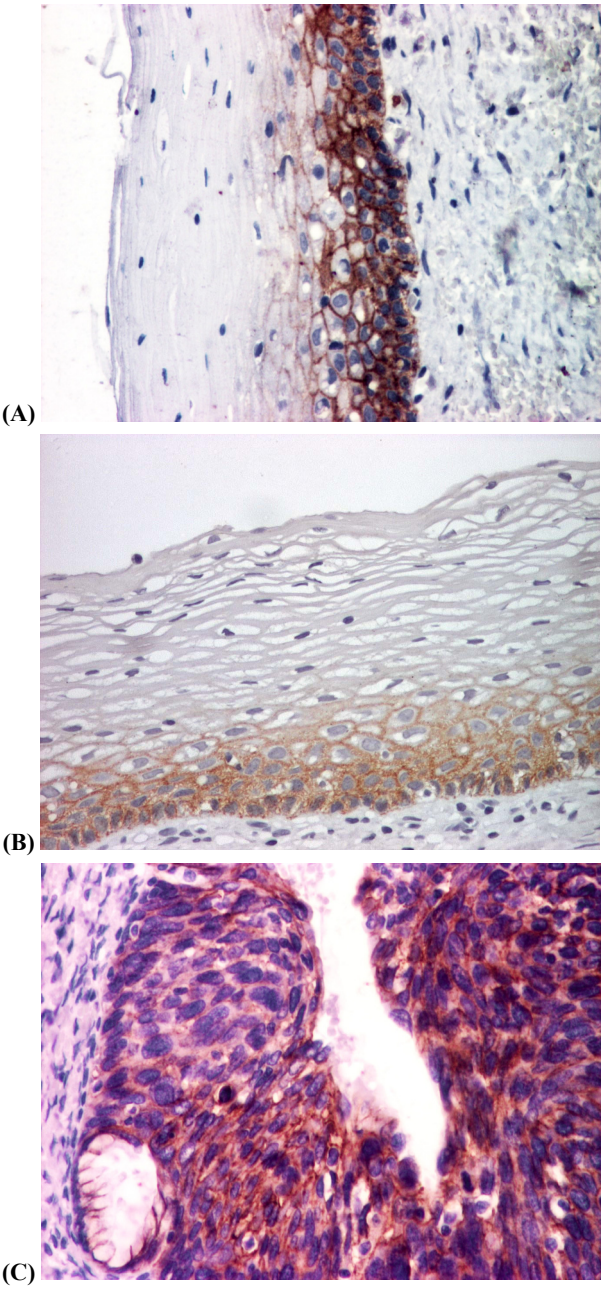

Figure 3 – ***E-cadherin immunoexpression in cervical squamous intraepithelial lesions: (A) LSIL (CIN I), ×100; (B) LSIL-MA, ×100; (C) HSIL (CIN III), ×100.***

Ki67 immunoreaction was positive in all analyzed

cases with homogenous or granular nuclear location. Its presence in the nucleus of stromal inflammatory elements was positive internal control for reaction. The staining was positive in basal and parabasal cells in LSIL cases, encompassing all or interlayer thickness epithelium in HSIL cases. Ki67 proliferation index was significantly higher in high-grade lesions (2 or 3), compared to low grade (1 or 2) (Table 3, Figure 4).

Table 3 – *Ki67 proliferation index in SIL lesions*

Lesion	LSIL	HSIL
% Marked cells	3–42	>42
Ki67 proliferation index	1 or 2	2 or 3

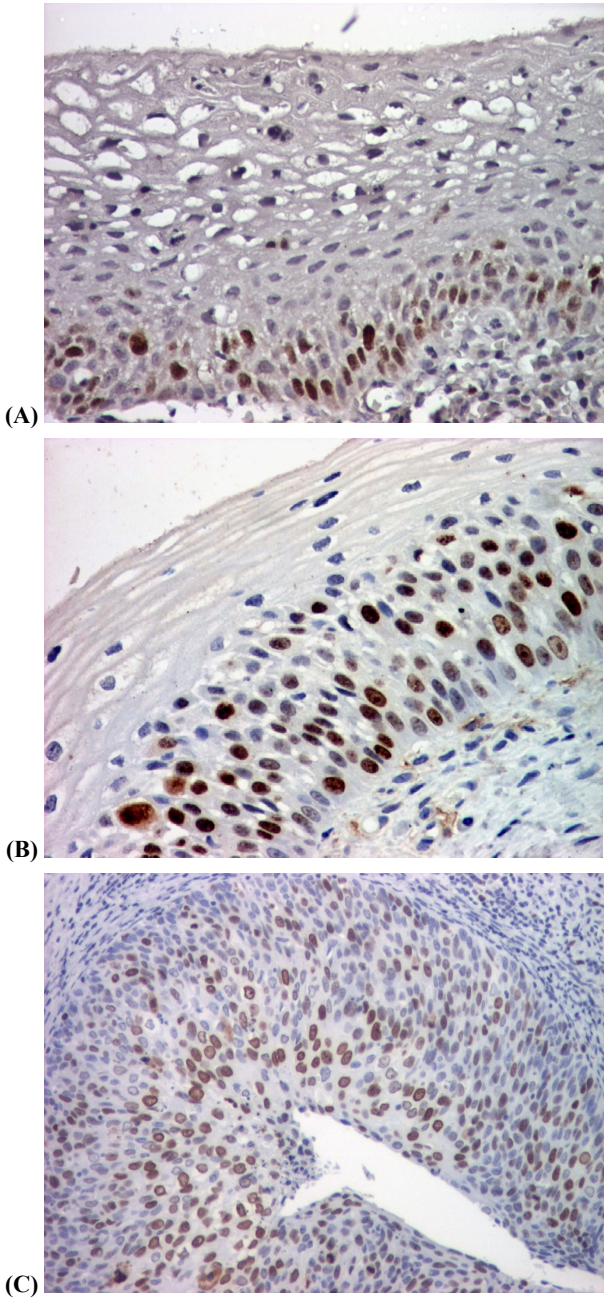

Figure 4 – *Ki67 immunoexpression in cervical squamous intraepithelial lesions: (A) LSIL (CIN I), ×100; (B) HSIL (CINII), ×100; (C) HSIL (CIN III), ×100.*

The proliferation index varied widely (3–22%) in LSIL lesion, the percentage of positive cells being maximum in cases with clearly koilocytosis, acanthosis and papillomatosis. Also in HSIL lesions with atypical mitosis, the proliferation index was high. In HSIL cases, Ki67 index was also variable in HSIL cases but had values over 42%. In LSIL, we observed rare cells with nuclear stain in the superficial layers of epithelium.

Discussion

There are numerous data in literature that they refer to the expression of proteins promising as regards the assessment of SIL progression potential. Among them, p16 seems to perform most of the criteria that define a good biomarker for this purpose. It is therefore important that such an antigen to be part of a broader investigation panel, which also must include other proteins with significant expression to histopathological lesions, as E-cadherin or Ki67.

P16 is part of cascade cell cycle regulators, which control the transition cell from G1 to S phase [14]. Cyclin D and cyclin-dependent kinases make complexes that phosphorylate pRB (retinoblastoma protein) and ensure cell proliferation to which p16 has a negative regulation role, being so implicated in carcinogenesis process [15].

In this study, we analyzed p16 immunoexpression for 26 cases diagnosed with SIL. P16-positive immunoreaction was present in 69.2% of lesions, LSIL was positive in 42.8% cases, and 100% of the HSIL cases, with specific disposal and proportional with the severity degree of lesions.

Many studies, which investigated p16 immunoexpression, indicated the potential of this biomarker for SIL diagnosis and prognosis. Gurrola-Díaz CM *et al.* [16] reported CIN II overstatement on a group of 78 of patients and improve interobservatory agreement regarding diagnosis made by p16 marking. Tringler B *et al.* [17] realized a study that included 19 cases with CIN I changes and p16-positive rate of 74.3% and 46 cases of CIN II/III, which were 100% positive, while in the normal squamous epithelium the antigen has not been expressed. Nam EJ *et al.* [18] analyzing 31 cases diagnosed with CIN remarked the higher degree of lesions the stronger p16-immunostain was present.

Other studies investigated the role of p16 in dysplastic lesion progress, in context of the HPV–HR presence [4, 9, 18, 19]. Dürst M *et al.* [20], with the conclusion that the majority of CIN I lesions were associated with HPV, suggested the possibility that genomic integration to be achieved from this stage and the expression of viral oncogenes to be restricted to cells of the superficial layers. When the oncogenes are expressed in the basal and parabasal layers of CIN lesions, cell cycle disturbances occur and this are translated by characteristic morphological feature [3]. It is shown that not all CIN I lesions associated with HPV–HR and p16-positive reaction are progressing, but in most cases with the diffuse p16-immunostain this process occurs [4]. Klaes R *et al*. realized a study in 2001 and observed that in CIN I lesions are two subtypes: some that reflect HPV-infection, but with restriction of viral oncogenes expression and p16-negative immunostain and others

with disturbed expression of HPV-oncogenes, resulting in loss of interference putative cellular factors and p16-positive immunostain. Thus, the rate of progression to high-grade lesions occurs especially when the HPV–HR sequence is activated to basal and parabasal cells level and p16-overexpression is present [9]. The same author concludes that p16 is useful not only for the delineation of low and high-grade intraepithelial squamous lesions, but also for detect the low-grade lesions with increased risk of progression to high-grade lesions.

E-cadherin is a 120 kD transmembrane adhesion molecule being encoded by a chromosome 16q22.1 located gene. Is mainly located in Ca^{2+}-dependent adherent junctions and concentrates at intercellular contact sites the epidermal growth factor receptor [21]. Through its extracellular domain is involved in intercellular adhesion by osmophilic Ca^{2+}-dependent interactions, while the intracellular domain binds to actin cytoskeleton via catenins [21]. E-cadherin plays important role in intercellular adhesion of epithelial cells, in epithelial polarization achievement, differentiation and stratification [22, 23].

In SIL lesions, we found E-cadherin positive reaction in 84.6% of analyzed cases, the number of labeled cells and stain pattern being different, depending on SIL grade.

E-cadherin immunoexpression studies in cervical squamous lesions are controversial. Thus, Daraï E *et al.* [24] performed a study that included 138 HPV+/- SIL lesions and notes the increase of E-cadherin immunoexpression with SIL grade and found no significant differences between the two groups of patients. In 1995, Vessey CJ *et al.* [25] making a study which included cases of CIN, invasive carcinomas and metastases, found an increased cytoplasmic considered aberrant expression of E-cadherin with grade intraepithelial lesion and the loss of its expression at the membrane in most invasive and metastatic lesions. At the same results reached Faleiro-Rodrigues C, in 2004, studying E-cadherin, CD44 and CD44v6 immunoexpression in squamous preinvasive and invasive lesions of the cervix [26]. In a study performed in 1996, Jeffers MD *et al.* [10] demonstrate the abnormal expression of E-cadherin and all catenins subunit in SIL and invasive lesions of the cervix. It establishes the E-cadherin expression absence in the superficial layers of cervical dysplasia only in a small number of cases, most of them having a membranous and cytoplasmic diffuse immunoexpression. Immunoexpression and E-cadherin normal function are not always congruent. Although E-cadherin immunoexpression can be maintained, its function may be impaired due to disruption of other components of the intercellular adhesion system, like catenins, could explain such abnormal stain patterns present in this study.

Ki67 antigen is a non-histone protein short-lived, expressed during the cell cycle phases G1, S, G2/M, but not in G0 phase [27].

In the study group of 24 patients diagnosed with SIL, Ki67 immunoexpression was present in all cases proportional to the severity of lesions and cell proliferation index was greater the degree of lesions was increased. In LSIL, proliferation index was below 20% and in cases where atypical mitoses were present, it reached similar HSIL cases values.

Bulten J *et al.* [28] found the percentage of Ki67 labeled cells in CIN III was 2.5 times higher when compared with CIN I and comparable and even higher stain in cases of CIN II/III to invasive lesions. Sahebali S *et al.* [11] found that Ki67 reaction had a higher density in cases of CIN associated with HPV–HR compared with those associated with HPV–LR. In 1999, Walboomers JM *et al.* remarked some of the conditions under which isolated using Ki67 index for grading CIN lesions is limited, such immature squamous metaplasia with reactive epithelium, Ki67-positive cells to the surface layers in case of tangential section, menstrual cycle [6].

All this studies support Ki67 as a specific marker for the morphology of CIN lesions, but used alone is insufficient sometimes for lesions grading and subsequently to adopt a particular therapeutic protocol.

Conclusions

The role and significance of p16 in epithelial development dysplastic lesions and correlations with morphological features of SIL lesions its recommended as a marker with high specificity and sensitivity. P16 and Ki67 immunostain was positive in the dysplastic epithelial layers and increased with lesion grade, with a direct proportional relationship between the two biomarkers. Although was not observed a direct correlation between E-cadherin expression and SIL grade, there was an abnormal membranous and cytoplasmic stain in aggressive lesions. Practical usefulness of a panel consisting of p16, E-cadherin and Ki67 in cervical preinvasive lesions is supported by accuracy of the results in terms of grading and assessment of future development of lesions.

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Corresponding author

Cristiana Simionescu, Professor, MD, PhD, Department of Pathology, University of Medicine and Pharmacy of Craiova, 66 1 May Avenue, 200628 Craiova, Romania; Phone/Fax +40251–599 228, e-mail: csimionescu2004@yahoo.com

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