Colposcopic, Histologic and Immunohistochemical Assessment in Cervical Intraepithelial Lesions

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It is widely accepted that HPV infection precedes the occurrence of neoplastic disease in a varying time frame, and HPV testing can detect 30-100% more cervical precancers than conventional cytology and 20-50% more precancers than liquid-based cytology. Low-grade squamous intraepithelial lesions include the categories of mild dysplasia, respectively cervical intraepithelial neoplasia grade 1, and complementary, various descriptors indicating the presence of Human papilloma virus, such as koilocytotic atypia or condilomatous dysplasia. High-grade squamous intraepithelial lesions cytologically consist of moderate and severe dysplasia, respectively cervical intraepithelial neoplasia grade 2, 3 and carcinoma in situ. The purpose of our paper is to analyze the cytological and colposcopic characteristics in low-grade squamous intraepithelial lesions and high-grade squamous intraepithelial lesions cervical lesions and to accomplish histological and immunohistochemical correlations in these cervical intraepithelial lesions. Systematic three-step colposcopic evaluation using successively, normal saline with or without green filter, acetic acid and Lugol staining provides enhanced efficiency to the colposcopic examination and allows a more individualized and targeted surgical, medical or expectant management. Special microscopic techniques are very important in diagnosing and grading cervical intraepithelial neoplasia.

Keywords: cervical neoplasia, pathology, surgery, antibody, prognostic marker

Cervical cytology screening represents one of the major successes in cancer control and prevention [1]. The Papanicolaou (Pap) test is based on the principle that cells from the squamous epithelium repeatedly exfoliate, so that normal or abnormal epithelial cells are desquamated at the cervical surface and collected for cytologic examination [2].

The Bethesda System for reporting results of cervical cytology has been developed to provide a uniform terminology that would lead to a clear set of management guidelines for the patients [1].

The finding of dysplastic cells on a Pap test indicates the presence of these cells on the surface of the cervix or vagina, and for any degree of dysplasia, the hallmark cytologic feature is abnormal nuclear transformation [2].

Low-grade squamous intraepithelial lesions (LSIL) are characterized by nuclear enlargement, variable hyperchromasia and other nuclear changes, and koilocytoticchange consisting of perinuclear cavitation and peripheral dense rim of cytoplasm [1, 3, 4]. LSIL include the categories of mild dysplasia, respectively cervical intraepithelial neoplasia (CIN) grade 1, and complementary, various descriptors indicating the presence of Human papilloma virus (HPV), such as koilocytotic atypia or condilomatous dysplasia [2, 5, 6].

High-grade squamous intraepithelial lesions (HSIL) cytologically consist of moderate and severe dysplasia CIN2, CIN3 and carcinoma in situ [2]. HSIL cells are smaller and less mature, cells may occur singly or clustered, and the nuclear-cytoplasmatic ratio is higher than in LSIL, nuclear hypercromasia and irregular nuclear membranes being as well common [1, 7, 8].

HPV is an icosahedral, nonenveloped, double stranded deoxyribonucleic acid (DNA) virus, the genome of all HPVs consisting of an approximately 8-kilobase pair molecule of circular, double stranded DNA [9-11].

It is widely accepted that HPV infection precedes the occurrence of neoplastic disease in a varying time frame, and HPV testing can detect 30-100% more cervical precancers than conventional cytology and 20-50% more precancers than liquid-based cytology [12-14].

The purpose of our paper is to analyze the cytological and colposcopic characteristics in LSIL and HSIL cervical lesions and to accomplish histological and immunohistochemical correlations in these cervical intraepithelial lesions.

Experimental part

In this multicenter study (see affiliation of the authors) we included 114 women aged between 16-49 years, with positive LSIL or HSIL liquid-based cervical cytology (LBC). All of the patients included in the study underwent HPV testing and colposcopy.

DNA HPV genotyping was performed by amplification of the target DNA by polymerase chain reaction (PCR) technique and hybridization of nucleic acids for individual

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qualitative detection of 37 HPV anogenital types, in cervical cells collected in liquid media (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/MM9, 81, 82/MM4, 83/MM7, 84/MM8, IS39 and CP6108).

Colposcopic assessment has been performed using a videocolposcopic equipment with the facility to capture and store images in order to set up the database of the study (table 1). Coloposcopic evaluation has been performed by first applying of the normal saline solution (0.9% Sodium Chloride Solution). The next step in the assessment of cervical lesions has been the application of 3-5% acetic acid, maintaining tissue contact for 2 minutes. The last stage in the colposcopic examination has been the application of diluted Lugol's iodine solution (iodine solution diluted half-strength or quarter-strength), checking that the patient is not allergic to iodine.

Pap test data, DNA HPV genotyping results and colposcopic assessment have been integrated, and case management was directed in accordance with current guidelines. Excision biopsy and ablative surgery have been performed. In our series we had cases diagnosed with LSIL and HSIL (CIN1, 2 and 3), and therefore, the techniques used for ablation surgery were loop electrosurgical excision procedure (LEEP) and needle excision, electrosurgical conization or *top hat* LEEP procedure (table 2).

All excised specimens were placed in a 10% neutral buffered formalin and sent to the pathology department. According to the current guidelines and protocol of this study, the patologist has focused on the attachement of squamous epithelium to the underlying stroma, the lateral thermal cut and the base of the excised specimen.

Following paraffin embedding, the blocks were sectioned by the HMB350 microtome equipped with a water-based section transfer system (STS microM). Sections of 5 microns have been made for histological and immunohistochemical techniques. Simple slides for hematoxylin eosin staining and poly-L-lysine slides for immunohistochemical stains were used. Hematoxylin-Eosin staining (Mayer's hemalum acid solution) was used for the progressive staining of cytoplasm and nuclei (crystallized hematoxilin 1.0 g, distilled water 1000 mL, sodium iodide ($NaIO_3$) 0.2g, aluminum sulphate 50.0g, citric acid 1g, chlorohydrate 50g). The staining technique included tissue deparaffinization in 3 successive baths of xylene, tissue hydration in 3 alcohol baths (100%, 90%, 70%), washing in distilled water, staining with Mayer's hemalum solution 10 min, flushing tap water in continuous jet for 15 min, staining with eozine - floxin working solution 20 seconds, short washing in distilled water, dehydration by successive passage through alcohol baths (70, 90, 100%), clarification in 3 xilen baths, (3x10 min) and Canada balsam mounting. For the immuno-histochemical staining the slides were dewaxed and hydrated as with the hematophilin-eosin technique. After hydration, the antigen was exposed by microwave heat, the *p*H of the solutions, their temperature and duration of treatment being the most important factors.

We used citrate solution [16] at *p*H 6 and EDTA *p*H 9. The technique used to prepare buffered citrate *p*H 6 [17] included sodium citrate 29.4g, HCl 1M 29.4g, distilled water 1L, adjusting the *p*H to 6 with 1M HCl. Furtheron, for the stock solution of citrate buffer (10x) *p*H 6 to expose the antigen, we used citric acid monohydrate 21.014g and distilled water 1000mL, following the *p*H adjustment with

Table 1
COLPOSCOPIC ASSESSMENT

Purposes of the colposcopic examination[1*]	Focus on	Solutions used	Magnification
- Cervix imaging - Describing the squamocolumnar junction - Identifying the transformation zone - Size, shape, contour, location and extent of the lesions - Pap test correlations - Therapeutic management	- Leukoplakia - Abnormal blood vessels	Normal saline (0.9% Sodium Chloride Solution)[2*]	Low (3.75x) Medium (7.5x) High (15x)
	- Differentiation between normal / abnormal epithelium - Tissue acetowhiteness	Acetic acid (3-5%)	Low (3.75x) Medium (7.5x) High (15x)
	- Iodine capture - Edges of the lesion - Features of the lesional contour - Lesion extension	Lugol's iodine (iodine solution diluted half-strength or quarter-strength)	Low (3.75x) Medium (7.5x) High (15x)
1* Colposcopy has been performed only by trained and certified physicians, according to the legislation; 2* Native and green filter assessment.			

Table 2
SURGICAL MANAGEMENT

Surgical technique	Colposcopic control	
LEEP	Single-pass	- Lugol's solution applied to delimit the margins; - Marking LEEP start and stop (3 o'clock and 9 o'clock); - Loop electrode; - 5-8 mm depth; - 4-6 mm outside the perimeter of the lesion - 30-50 Watts cutting mode; - Current applied before tissue contact; - Continous and smooth motion; - Bleeding control.
	Multiple passes	- Method of excision similar to that for single-pass; - Marking LEEP start and stop (6 o'clock and 12 o'clock); - 1 or 2 different loop sizes; - 2 or more specimens.
Needle excision ***Top hat* LEEP procedure** **Electrosurgical conization**	- Needle electrode and special sized and shaped electrodes; - Single or multiple excision pass; - Approximately15 mm depth; - Approximately 8 mm circle incision on the cervix; - 40-50 Watts cutting mode; - Continous motion; - Bleeding control; - Specimens placed in a 10% neutral buffered formalin.	

Primary antibody	Producer	Clone	Exposure solution	Primary antibody dilution	Secondary antibody
Anti KI67	Dako	MIB-1	EDTA pH 9	1:50	Monoclonal Mouse Anti-Human Ki67
Anti-HPV	Dako	K1H8	Citrate pH 6	1:50	Monoclonal Mouse Anti- HPV
Anti-BCL-2	Dako	124	EDTA pH 9	1:50	Monoclonal Mouse Anti-Human BCL-2 Oncoprotein
Anti CD20	Dako	L26	Citrate pH 6	1:50	Monoclonal Mouse Anti-Human CD20cy
Anti CK7	Dako	OV-TL 12/30	Citrate pH 6	1:50	Monoclonal Mouse Anti-Human Cytokeratin 7

Table 3
IMMUNOHISTOCHEMICAL PANEL OF ANTIBODIES

NaOH and 1:10 dilution with distilled water at the time of use. In order to prepare EDTA solution *p*H 9 (Tris-EDTA) we used ethylenediaminetetraacetic acid 0.372g, distilled water 1000ml and adjusting the *p*H to 9 with NaOH and HCl.

The slides were inserted into an end holder, and placed in the microwave oven (7 cycles x 3 min) at 650 W. After each cycle, the solutions of citrate / EDTA are completed. This is followed by the blocking of nonspecific background staining caused by the presence of tissue endogenous enzymes. Blocking is done for 30 min with 30% Perhydrol solution, diluted in distilled water (2 mL of 30% perhydrol, 100ml distilled water). The slides are washed in distilled water and then in Phosphate-buffered saline (PBS) (NaCl 0.12 M- 13.92g, $NaH_2PO_4 \cdot 2H_2O$ 0.01M - 3.12g, K_2HPO_4 0.04M -13.92g, distilled H_2O 200mL) [15].

Subsequent isomeric serum blocking with the secondary antibody (optimal dilution in PBS with 1% bovine albumin) is continued for 30 min at ambient temperature. Next, incubate with the primary antibody in optimal dilution (performed in 1xPBS + 1% albumin) overnight at 4 degrees C. The immunohistochemical study included a panel of antibodies presented in table 3.

The next day slides are washed in PBS (3x5 min), incubated with the biotinylated secondary anti-primary antibody in the optimal dilution (1: 200) for 30 min, washed in PBS (3x5 min), incubated with marked streptavidin reagent in optimal dilution (1: 200) for 30 min and washed again in PBS.

Subsequently, the background for the detection enzyme is added, the appearance and intensity of the signal is checked by microscope. The reaction stops in PBS. The diaminobenzidine solution is used as background.

After receiving the signal the slides are washed in distilled water, contrasted with hematoxyline, washed in tap water for blueing of the nuclei, dehydrated by passing through successive alcohol baths (70, 90, 100%), clarify in xylene (3x10 minutes), mount with Canada balsam.

The research meets the conditions of the ethical guidelines and legal requirements, and was approved by each Ethical Committee of the Universities of Medicine and Pharmacy (see authors' affiliations). Informed consent was obtained from every patient included in the study.

Results and discussions

Of the 114 patients included in the study, 41 (35.96%) had LSIL cytology and 73 (64.03%) HSIL (table 4). After DNA HPV genotyping we found 27 (23.68%) cases presenting HR-HPV (type 16, 18), 17 (14.91%) with other HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 66), and 21 (18.42%) presenting HR-HPV associated with other HPV types. Clinical characteristics correlated with cervical pathology are also presented in table 4.

The cytological and coloposcopic findings (table 5, fig. 1-4) characteristic for LSIL and HSIL, respectively CIN1

Table 4
CLINICAL CHARACTERISTICS AND CYTOLOGY

Partner with lower genital tract lesion or condyloma - n (%)			8 (7.01)
Associated vulvar or vaginal HPV lesion - n (%)			11 (9.64)
Unexplained intercourse bleeding - n (%)			52 (45.61)
Erosion, ulceration or tumor at the gynecological speculum examination - n (%)			39 (34.21)
Type of cytology	LSIL n (%)	HSIL n (%)	
	41 (35.96)	73 (64.03)	
HPV testing	HR - HPV n (%)	Other HPV types*	HR - HPV + Other HPV types
	27 (23.68)	17 (14.91)	21 (18.42)
HR-HPV - High risk HPV, * Types - 31, 33, 35, 39, 45, 51, 52, 56, 58, 66.			

Table 5
COLPOSCOPIC FINDINGS

LSIL - n (%)		**HSIL - n (%)**	
Absent vessels	13 (31.7)	Coarse vascular changes	52 (71.23)
Fine mosaic	21 (51.21)	Coarse mosaic	23 (31.5)
Fine punctation	19 (46.34)	Coarse punctation	31 (42.46)
Flat contour	32 (78.04)	Smooth surface	38 (52.05)
High and irregular condymoma appearance	3 (7.31)	Iodine negativity	73 (100)
Pale and translucent acetowhite epithelium	38 (92.68)	Dense acetowhite epithelium	63 (86.3)
White matte epithelium	12 (29.26)	Yellow appearance (iodine) in a region previously dense acetowhite	48 (65.75)
Indistinct margins	11 (26.82)	Sharp outer border	69 (94.52)
Geographic margins	30 (73.17)	Lesion near the SCJ	17 (23.28)
SCJ - squamocolumnar junction			

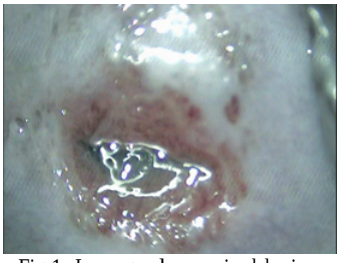

Fig.1. Low grade cervical lesion (CIN1) demonstrated at 9-3 o' clock after the application of normal saline and green filter. Blood vessels appear fine, thin, slightly dark , quite easy to distinguish from the green background. Magnification 15x.

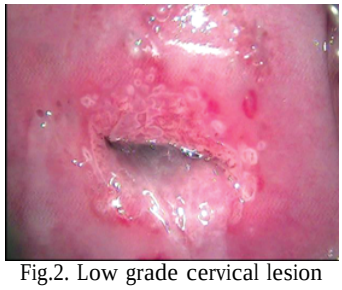

Fig.2. Low grade cervical lesion (CIN1). Colposcopy after the application of 3-5% acetic acid demonstrating map-like geographic borders and pale acetowhite color. Magnification 15x.

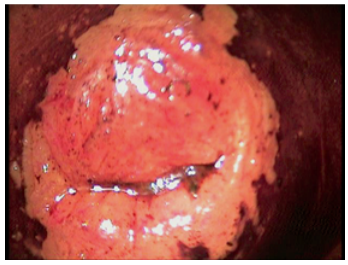

Fig.3. High grade cervical lesion (CIN2) following application of Lugol, mostly rejecting iodine solution. Areas of previously acetowhite epithelium appear yellowish. Magnification 15x.

Fig.4. High grade cervical lesion (CIN3) following application of Lugol's iodine solution, demonstrating smooth and irregular margins and sharp outer border. Magnification 7.5x.

and CIN2, 3 were consistent in 102 (89.47%) of the 114 cases in the study. Discordant cases were 7 (6.14%) HPV-HPV and other HPV types positive, respectively 5 (4.38%) HR-HPV positive.

Surgical management has been indicated in 79 (69.29%) cases, while conservative medical treatment was recommended in 35 (30.7%) cases. Surgical treatment has meant in 47 (59.59%) of the cases LEEP, 21 (26.58%) electrosurgical conization and in 11 (13.92%) cases *top hat* LEEP procedure.

Our study is limited by the rather low number of cases included, and this may impact the statistical power of our analysis.

An important advantage of this study is however, the complex analysis and correlations obtained in cervical intraepithelial neoplasia by LBC, colposcopy, histology and immunohistochemistry (IHC).

LBC has many advantages compared to the conventional Pap smear, including more complete collection of exfoliated cells to slides, random and presumably more representative transfer of the cells to slides, and improved microscopic visualization [18, 19]. In our series all cases were examined by LBC, giving greater accuracy to the cytological examination.

Moreover, the published data show that the monolayer slides obtained from a liquid-based collection environment are more effective than the results of the conventional smear screening method [20].

HPV testing can detect 20-50% more cervical precancers than LBC alone, and normally a suitable sample for cytology is also an adequate pattern for HPV testing [12].

In PCR technique, the target DNA is selectively amplified by repeated cycles of temperature changes that facilitate denaturation, primer hybridization and primer extension by DNA polymerase, in order to produce amplicons, that will rise almost exponentially [12, 21]. Furthermore, Lorincz showed the fact that after 30-40 cycles, more than one billion amplicon copies of the original target DNA may be produced [12].

In this study, together with LBC, we used DNA HPV genotyping carried out by amplification of the target DNA by PCR technique and hybridization of nucleic acids for individual qualitative detection of 37 HPV types, these data significantly contributing to the case management and the accuracy of our study.

The utility of colposcopy as regards its ability to early detect cervical dyplasias increased over time due to the implementation of the risk score, which allows to integrate the colposcopic diagnosis with the corresponding histopathological diagnosis [20].

Bornstein et al. in 2012, concluded that colposcopic findings are able to be assessed into a colposcopic diagnosis degree, ranging from minor findings to invasive cancer or miscellaneous conditions such as erosion, condyloma, polyp, cyst, endometriosis, inflammation, vaginal stenosis or congenital transformation zone [20, 22].

It is widely accepted the fact that Lugol's iodine solution test is a minimally invasive, simple, quite quick, easy and harmless assessment [23, 24], providing rather encouraging diagnostic and therapeutic results in CIN.

The colposcopic examination protocol of our study included visualization of the cervix using normal saline solution and green filter, acetic acid test followed by Lugol test, this approach allowing the systematic recognition of suggestive changes for a particular types of CIN.

Almost all women diagnosed with HSIL are managed by either ablative or excisional surgery, since regression of CIN2 and especially CIN3 is improbable and the risk of progression is notably higher [25].

In our series we performed active management for all HSIL cases but also for 6 (14.63%) cases with LSIL, considering HPV persistence, associated conditions, age, patient's express option and exclusion of the perspective for a future pregnancy.

In the classic Hematoxilin-Eozin staining technique, it is observed that epithelial cells have changed their normal structure, and the nuclear-cytoplasmic ratio is in favor of the nucleus. These abnormalities are especially present in the epithelial basal layers, but there are also submucosa outbreaks (fig. 5).

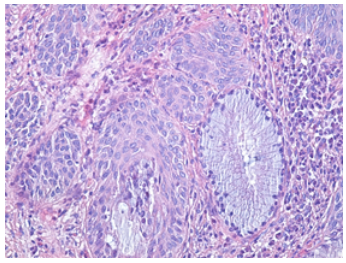

Fig 5. Low grade cervical lesion (CIN1). Changes in the nuclear-cytoplasmic ratio of squamous cells are observed. HE staining, ×200.

With the anti-Ki67 antibody we demonstrated that cells with nuclear-cytoplasmic alterations are present at different stages of the division, increasing directly proportional to CIN, and being present in a relatively low number in CIN I and in an increased number in CIN 2/ 3, the response being correlated with the BCL-2 oncoprotein positivity [26, 27] (fig. 6, 7).

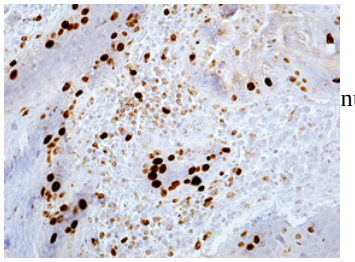

Fig 6. Low grade cervical lesion (CIN1). Cells with nuclear-cytoplasmic changes, diffuse disseminated in submucosa, are observed. Immunostaining with anti-Ki67 antibody, × 100

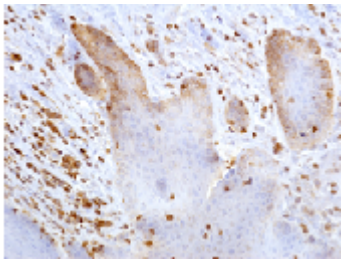

Fig 7. Low grade cervical lesion (CIN1). BCL-2 positive in the basal epithelial layers and in tumor cells, but also in other connective tissue cells. Immunostaining with anti-BCL-2 antibody, × 100

Anti-BCL-2 antibody reacts strongly in CIN 2, but mostly in CIN 3 [28, 29].

Progression of cells with nuclear-cytoplasmic changes is strongly influenced by the presence of HPV. Immunohistochemical reactions which detects the HPV response, is of real importance in the diagnosis and treatment of cervical lesions, and with the anti-HPV antibody, the positivity of the reaction in lesional and perilesional modified glandular cells has been demonstrated (fig. 8).

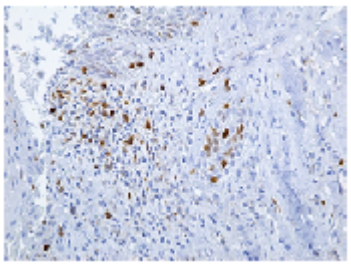

Fig 8. CIN1. HPV positive in tumor and peritumoral glandular cells. Immunostaining with anti-HPV antibody, × 100

T lymphocytes, B lymphocytes and macrophages are are very numerous in perilesional regions, especially in CIN 2/ 3 (fig. 9) [30].

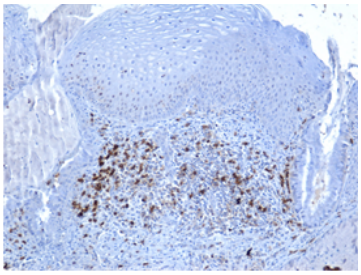

Fig 9. CIN1. Diffuse B lymphocytes spread in the peritumoral infiltrate. Immunostaining with anti-CD20 antibody, × 100

Also, the expression of scuamo-columnar junction with the Cytokeratin 7 (CK7) marker is associated with an increased rate of subsequent high-grade squamous intraepithelial lesion, suggesting that CK7 may inform risk stratification for CIN1 (fig. 10) [31].

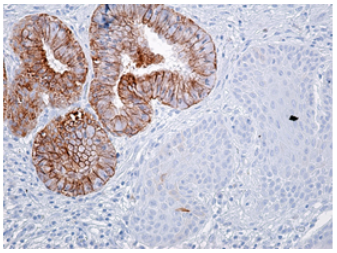

Fig 10. CIN1. Normal glandular structures highly positive with Citokeratin 7 and tumor glandular structures negative at Citokeratin 7. Immunostaining with anti-CK7 antibody, × 200

IHC uses a step-by-step approach from a set of generic markers having the advantage of the high sensitivity and specificity, and the classical correlation with morphological parameters, having no diagnostic value on its own, but definitely compleing the histopathological examination [32].

The pathological specimen assessment certainly offers important elements for dignosis, but IHC techniques provide additional information on prognosis, or might be adapted and considered retrospectively to examine tissues, thus allowing a more accurate diagnosis [32, 33].

Conclusions

LBC together with DNA HPV genotyping provides increased accuracy in the detection of cervical intraepithelial lesions. Systematic three-step colposcopic evaluation using successively, normal saline with or without green filter, acetic acid and Lugol staining provides enhanced efficiency to the colposcopic examination and allows a more individualized and targeted surgical, medical or expectant management. Surgical management of HSIL under colposcopic control is important for achieving disease free margins in cervical intraepithelial lesions. Significant correlation between cytology, colposcopy, histology and IHC in our study support that association between LBC with correct sampling to ensure good quality specimens, together with a rigorous colposcopic examination are reliable solutions for diagnosis or selective and adequate surgical management.

Special microscopic techniques are very important in diagnosing and grading CIN. Hematoxylin - Eozine staining revealed changes in nuclear - cytoplasmic ratios of cells in the lesion areas. IHC has shown that the proliferation of altered cells is increased and correlated with the intensity of BCL-2 and HPV oncogene.

Inflammatory response is strongly positive in the perilesional area, and CK7 is expressed in normal glandular cells and negative in cells with dysplastic changes, this being a prognostic marker in CIN I.

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