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### Indices of Immunohistochemical Markers in Women with Genital Viral Infection

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**Abstract.** The article presents the analysis of immunohistochemical markers indices in the uterine cervix tissue affected by genital viral infection. In morphogenesis and pathogenesis of cervical intraepithelial neoplasia associated with papillomavirus infection some inexplicable moments appear, namely prognosis of the development and course of disease relapses. Immunohistochemical techniques are often determinative in the diagnosis of cervical pathology. Analysis of immunohistochemical markers expression in dysplastic and neoplastic processes of cervical epithelium allow to determine the intensity of the mechanisms of proliferation, differentiation, apoptosis and malignant transformation when infected by human papilloma virus of high oncogenic risk. During an examination of reproductive age women with cervical disease of viral etiology study groups of patients were formed. Ki-67, p63 and p16INK4a markers were investigated in the women's cervical biopsy samples. Results of the study showed increased expression of specific antigens with the increase in cervical intraepithelial neoplasia severity. This may be the unfavorable prognostic indicator in terms of human papilloma virus infection.

**Keywords:** immunohistochemical markers; genital viral infection; cervical intraepithelial neoplasia.

#### Problem statement and analysis of the recent research

Genital papilloma viral infection (GPVI) plays a crucial role in the occurrence of background and precancerous diseases of the cervix uteri (CU) among women of reproductive age [4]. Today, the influence of highly endogenous types of human papillomavirus (HPV) on the development of cells neoplastic aberration and its further progression in cervical intraepithelial neoplasia and cervical cancer has been proven [6]. Slow but steady increase in incidence rate of cervical oncopathology forces to look constantly for new diagnostic approaches for early and effective verification of precancerous processes of cervix uteri. However, the diagnosis of neoplasia also depends on subjective factors, contributing to a false interpretation of cytological and histological conclusions. An important place in such cases is given to analysis of the expression of immunohistochemical markers which are considered to be indicators of proliferation and oncology transformation and allow objectifying of obtained pathomorphological data in order to avoid erroneous results [1, 2]. Antigens Ki-67, p63 and p16INK4a should be used to study changes in cervix uteri epithelium on the background of virus infection [3, 5].

**The objective of research** was to determine the expression of proteins p16INK4A, Ki67 and p63 in cervical biopsy samples of women who suffered from cervix uteri pathology with persistent papilloma virus infection of high carcinogenic risk at the stage of morphological diagnostics.

#### Material and methods of the research

We examined 130 women of reproductive age (at the age of 22 – 45) who were divided into 3 experimental groups: group I and group II (52 and 48) consisted of women who suffered from cervix uteri pathology affected by genital papilloma viral infection of cervix uteri and group III (control group) included 30 healthy women.

4-5  $\mu\text{m}$  thick sections were deposited on a glass slide previously modified with viscous liquid (poly-L-lysine) for immune histochemical study. After standard dewaxing it was heated on a water-bath with citrate buffer pH 6.0 and autoclaved. Monoclonal antibodies were used as primary to p63 (clone 4A4, DakoCytomation), Ki-67 (clone MIB-1) p16INK4A (kit for histological preparation, № K5334). Control reactions were performed to control the specificity of

immunohistochemical staining for each marker. Further processing was performed using imaging system LSAB2 and EnVision (DakoCytomation) during 10 minutes for each reagent with intermediate washing in Tris-buffered solution. The reaction with a chromogen was then performed, assessing the quality of interaction under the microscope for the period from 20 seconds to 3 minutes. For the differentiation of tissue structures sections were additionally stained with hemalum for 3 minutes. Dehydration and embedding in balsam was carried out according to conventional methods (S. V. Petrov, 2004; D. J. Dabbs, 2006). Cells that were considered to be positive with respect to markers expression were studied for 4-6 accidentally selected fields of microscope. After counting 300 histologically identified cells we determined expression indices based on the results of all the studied areas, including reactions in normal and neoplastic modified cells of epithelial layer.

### Results of the research and their discussion

In order to objectificate pathomorphological data, immune histochemical study with the analysis of the proliferative activity indicator of the cervix uteri mucous membrane epithelium – Ki-67 and of the markers responsible for oncology transformation – p63, p16 INK4a was performed for all patients. As a result of this study semiquantitative analysis of the markers showed the increase in the intensity of their staining with condylomata for p16 INK4a with CIN for Ki-67 and p63. Antigen Ki-67 is an epitope of nuclear and nucleolar protein and its expression is a universal marker of cell proliferation. According to the obtained data, immuno-positive reactions with antibodies to Ki-67 were detected in all cases of hyper- and neoplastic processes, and, moreover, with the increase of neoplasia severity the number of cells positively reacting to the protein was significantly increasing when compared to the norm ( $p < 0.05$ ). Since antigen Ki-67 is able to express itself in fractions of proliferating cells, Ki-67 positive cells were found in women of the control group that could be explained by the possible processes of proliferative division in the setting of normal epithelial tissue. However, with the increase in neoplasia severity, the number of cells positively reacting to Ki-67 also increased significantly ( $p < 0.001$ ) in comparison with the control group (Table 1).

Table 1

The expression of Ki-67 cervix uteri epithelium in the examined women  
(absolute number, %)

Cervical Pathology	Number of observations (n = 130)	The level of Ki-67 expression					
		low <10		middle 10–50		high >50	
		abs.	%	abs.	%	abs.	%
Endocervicosis	25	16	64.0	8	32.0	1	4.0
Condylomata	26	8	30.8	12	46.1	6	23.1
CIN-I	28	24	85.7	4	14.2	0	0
CIN-II	21	7	33.3	11	52.4	3	14.3
Without pathology	30	30	100.0	0	0	0	0

Ki-67 hyperexpression was considered to be prognostically unfavorable sign of the disease. In case of CIN-I low level of expression (in 85.7% of cases) was usually diagnosed, when the proportion of Ki-67 positive nuclei was below 10% and all of them were mainly concentrated in isolated parabasal layers of exocervix and in places of epithelizing endocervicosis or squamous metaplasia. Index of exocervix proliferative activity thus constituted  $9.30 \pm 0.55\%$  and  $9.10 \pm 1.07\%$  for the first and second groups. In case of CIN-II and condylomata, expression level was moderate, namely 52.4% and 46.1% of cases with the index of proliferative activity in the first and second group constituting  $19.23 \pm 1.17\%$  and  $18.80 \pm 1.13$  for CIN-II and  $18.53 \pm 1.28\%$  and  $18.30 \pm 1.37\%$  for condylomata respectively (Table 2).

Table 2

Ki-67 proliferation index of cervix uteri epithelium in the examined women ( $M \pm m, \%$ )

Cervical Pathology	Examined Group		
	Control (n=30)	I (n=52)	II (n=48)
Endocervicosis	$3.22 \pm 0.21$	$12.21 \pm 1.3^*$ (n=13)	$11.80 \pm 1.33^{*o}$ (n=12)

Condylomata	3.22±0.21	18.53±1.2* (n=14)	18.30±1.37*° (n=12)
CIN-I	3.22±0.21	9.30±0.55* (n=14)	9.10±1.07*° (n=14)
CIN -II	3.22±0.21	19.23±1.2* (n=11)	18.80±1.13*° (n=10)

Note. 1. \* – difference reliability when comparing indicators in group I and II with control group ( $p < 0.001$ ); 2. ° – difference reliability when comparing indicators in group I and group II ( $p > 0.05$ ).

According to the table 1 and table 2 in case of CIN-II the level of Ki-67 expression ( $p < 0.001$ ) increased in comparison with the control group and CIN-I, while cells with positive expression were also identified in the exocervix intermediate layer. The number of Ki-67 positive nuclei was increasing, the expression level ranged from 10% to 50% in 52.4% of cases, and was high and amounted to over 50% in 14.3% of cases. Index of proliferative activity amounted to  $19.23 \pm 1.17\%$  and  $18.80 \pm 1.13\%$  in the first and second group.

The results of positive immunohistochemical detection of reactions with antibodies to Ki-67 were presented as nuclei clearly stained in brown color (nuclear pattern). This reaction differed in intensity and prevalence in case of various histological diagnosis. In some cervix uteri biopsy samples we observed alternation of intra nuclear reaction of Ki-67 with Ki-67-negative epithelial cells. At all stages of neoplastic processes the number of cells with positive reaction to Ki-67 was progressively increasing in comparison with the norm. Immunohistochemical technique also allowed to verify the specific (coilocyte atypia, polynuclear cells) and nonspecific (consolidation of pavement cells of different layers, uneven cytoplasm staining) signs of cervix uteri viral infection in examined patients. Thus, using immune histochemical reaction to Ki-67 it was possible to estimate the number of proliferating cells by means of diagnosing the degree of proliferation affected by the neoplasia. Ki-67 expression increased due to the increase in the level of oncology transformation of cervix uteri epithelium.

The study of p63 protein expression and defining the relation between the protein and the development of neo-, dis- and hyperplastic processes were also important for our work (Table 3).

Table 3

Cervix uteri epithelium p63 expression in the examined women  
(absolute number, %)

Cervical Pathology	Number of observations (n = 130)	The level of expression p63					
		low <30		middle 30–75		high >75	
		abs.	%	abs.	%	abs.	%
Endocervicosis	25	21	84.0	4	16.0	0	0
Condylomata	26	15	57.7	7	26.9	4	15.4
CIN-I	28	25	89.3	2	7.1	1	3.6
CIN -II	21	2	9.5	15	71.4	4	19.0
Without pathology	30	30	100.0	0	0	0	0

Examining the patients a significant increase in the p63 expression with progression of pathological process ( $p < 0.001$ ) was observed: if low expression level (<30%) was recorded in 21 (84.0%) patients, moderate (30-75%) was observed in 4 (16.0%) patients, high (> 75%) was absent in case of endocervicosis, in case of CIN-II 15 (71.4%) women had moderate and 4 women (19.0%) – high p63 expression and low level was observed in 2 (9.5%) patients. Women of the control group had low level of positive p63 protein expression in all cases.

Analyzing the results of protein p63 quantitative examination, women of groups I and II showed significantly higher levels of expression in case of all forms of dysplastic and neoplastic processes ( $p < 0.001$ ) in comparison with the results of the control group. High expression of this protein was detected in the I and II group of patients with condylomata ( $39.40 \pm 1.75\%$  and  $39.80 \pm 1.60\%$ ) and endocervicosis ( $27.80 \pm 1.76\%$  and  $26.30 \pm 1.73\%$ ). However, the highest rates of expression were observed in patients with moderate neoplasia, which amounted to  $54.70 \pm 3.20\%$  in group I and  $53.80 \pm 3.07\%$  in group II. Groups were almost identical, as evidenced by their unreliable difference ( $p_1 > 0.05$ ) (Table 4).

Table 4

Level of of cervix uteri epithelium p63 expression in the examined women  
(M±m, %)

Cervical Pathology	Examined Group		
	Control (n=30)	I (n=52)	II (n=48)
Endocervicosis	0.50±0.03	27.80±1.76* (n=13)	26.30±1.73*° (n=12)
Condylomata	0.50±0.03	39.40±1.75* (n=14)	39.80±1.60*° (n=12)
CIN-I	0.50±0.03	19.30±0.83* (n=14)	18.70±0.96*° (n=14)
CIN-II	0.50±0.03	54.70±3.20* (n=11)	53.80±3.07*° (n=10)

Note. 1. \* – difference reliability when comparing indicators in group I and II with control group ( $p < 0.001$ ); 2. ° – difference reliability when comparing indicators in group I and group II ( $p > 0.05$ ).

Evaluating immune histochemical staining p63 positive cells with brown nuclei of varied intensity in different layers of the cervix uteri epithelium were microscopically detected; their number was increasing in the neoplasia focuses. High level of protein p63 expression was observed in 3.6% of cases of CIN-I, in 15.4% of cases of condyloma, in 19.0% of cases of CIN II. Coilocytes with perinuclear zone of enlightenment and macronucleus were clearly differentiated.

In order to analyze the pathogenesis of cervical pathology of viral etiology, we studied biological aspects of cyclin-dependent kinase inhibitor (CKI) p16 INK4a, which was considered to be an indirect marker of active oncogenic expression of human papilloma virus of high cancerigenic risk. Determination of p16 INK4a expression in the cervix uteri tissue allowed us to specificate aspects of cervical neoplastic processes. It is generally accepted that p16 staining in healthy stratified squamous epithelium is completely absent or, in some cases, is focal positive. In particular, at the microscopic level, in case of squamous metaplasia in the control group, we observed physiological p16 expression due to processes of transdifferentiation in 1 (3.3%) woman. Instead, in case of endocervicosis, condylomata and neoplasia of different degrees, expression intensity was growing from weak to diffuse in all cases. The level of p16 INK4a of cervix uteri epithelium in women with dis- and neoplastic processes was significantly different from the level of p16 INK4a of cervix uteri epithelium in the control group where expression was positive and low only in 3.3% of cases. In case of endocervicosis low rates of p16 in 100.0% of cases were also recorded. High expression level was observed in 61.5% of cases with condylomata, 60.7% of cases with CIN-I. Hyperexpression of p16 protein was detected in 61.9% in case of CIN-II (Table 5). Analyzing the indicators of p16 INK4a of cervix uteri epithelium we found significant increase in number of cells which were positive to p16 INK4a in case of dis- and neoplastic processes of cervix uteri, that significantly differed from the control group ( $p < 0.001$ ) (Table 6).

Table 5

Expression level of cervix uteri epithelium p16 INK4a (absolute number, %)

Cervical Pathology	Number of observations (n = 130)	The level of p16 INK4a expression					
		low <10		middle 10–20		hyperexpression >20	
		abs.	%	abs.	%	abs.	%
Endocervicosis	25	25	100.0	0	0	0	0
Condylomata	26	3	11.5	16	61.5	7	26.9
CIN- I	28	3	10.7	17	60.7	8	28.6
CIN- II	21	0	0	8	38.1	13	61.9
Without pathology	30	1	3.3	0	0	0	0

Table 6

Index of cervix uteri epithelium p16 INK4a in the examined women (M±m, %)

Cervical Pathology	Examined Group		
	control (n=30)	I (n=52)	II (n=48)
Endocervicosis	0.40±0.04	9.75±1.09* (n=13)	9.30±1.01*° (n=12)
Condylomata	0.40±0.04	20.30±1.0* (n=14)	20.80±1.04*° (n=12)
CIN-I	0.40±0.04	19.60±1.27* (n=14)	19.20±1.38*° (n=14)
CIN-II	0.40±0.04	30.40±1.53* (n=11)	29.80±1.60*° (n=10)

Note. 1. \* – difference reliability when comparing indicators in group I and II with control group ( $p < 0.001$ ); 2. ° – difference reliability when comparing indicators in group I and group II ( $p > 0.05$ ).

In tissue specimens the difference between neo- and dysplastic processes of epithelium consisted in p16 INK4a staining, namely diffuse and focal in case of condylomata and CIN and sporadically in case of endocervicosis. Immune histochemical study of p16 INK4a allowed to define neoplastic process foci, limits of its distribution and also allowed to establish a connection between its expression and severity of pathological changes of the epithelium. High levels of positive protein expression allowed differentiating in an authentic and accurate way of neoplastically changed epithelium from dystrophic one, primitive or interjacent squamosal metaplasia and atrophic process phenomenon.

Analysis of immune histochemical markers expression makes it possible to diagnose the involvement of certain individual proteins in different pathological processes and to clarify the role of molecular structures in the course and support of different body functions.

### Conclusions

Thus, the results of the study showed high sensitivity of immune histochemical study in the verification of dysplastic and neoplastic processes of cervix uteri. Analysis of immune histochemical markers expression makes it possible to diagnose the involvement of certain individual proteins in different pathological processes and to clarify the role of molecular structures in the course and support of different body functions. Thus, using immune histochemical reaction to Ki-67 it is possible to estimate the number of proliferating cells by means of diagnosing the degree of proliferation affected by the neoplasia. Immune histochemical study of p16INK4a allowed to define neoplastic process foci, limits of its distribution and also allowed to establish a connection between its expression and severity of pathological changes of the epithelium.

Ki-67, p63 and p16INK4a expression is significantly increasing due to the increase in the level of oncology transformation of cervix uteri epithelium.

**Prospects for further research** involve studies of immune histochemical markers indicators in the diagnostics of cervical pathology of viral origin.

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