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Special aspects of steroid hormone receptors' expression in cases of cervical intraepithelial neoplasia depending on the papillomavirus infection progression in women with infertility

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The relevance of the study is contingent on insufficient study of the correlation between the reception of uterine cervix steroid hormones in cases of cervical intraepithelial neoplasia (CIN) and the level of human papilloma virus load in women with infertility. The goal of the research was to determine the peculiar features of cervix estrogen and progesterone receptors' expression at different severity levels of cervical dysplasia, depending on the progression of Papilloma Viral Infection (PVI) in women with infertility. The material for the study was 157 cervical biopsy specimens: 62 – with a mild CIN; 53 – with a moderate CIN; 42 – with a severe CIN. For the immunohistochemical (IHC) test we used primary monoclonal antibodies to estrogen (ER) (clone 6F11, Novocastra) and progesterone receptors (PR) (clone 1A6, Novocastra) as well as the latest generation imaging system UltraVision LP (Lab Vision), chromogen (DAB (Lab Vision)). With PVI-associated CIN with infertility a redistribution of steroid receptor expression was determined; thus, estrogen receptors are characterised by reduction of epithelial and appearance of stromal reaction. But the progesterone receptors' expression differs by appearance of positive epithelial expression with manifested reaction in the cervical stroma. Redistribution of steroid hormone reception under CIN is predominantly observed in women with tubal infertility. There was a significant decrease in the ER/PR index, with the lowest indicator in the group of patients with severe CIN. Negative expression of steroid hormone receptors is typical for high viral load, regardless of CIN severity level. Thus, in women with infertility, estrogen and progesterone receptors' expression depends on CIN severity level and PVI progression level. Determining steroid hormone receptors' expression under PVI-associated CIN in women with infertility may be used as an additional criterion for CIN severity level determination.

Keywords: estrogen receptors; progesterone receptors; cervical intraepithelial neoplasia; papilloma viral infection; infertility.

Introduction

A necessary condition for the full realisation of a woman's reproductive function is an adequate response of the endocrine system (Rojas et al., 2015). Cyclic changes that are observed in the female reproductive system depend on the level and predominance of the type of main hormones of the female body – estrogen and progesterone. These changes are expressed in differing degrees, which is determined by functional specialisation and receptor equipment of the epithelium, but differs in synchronicity and constancy (Berstein, 2000; Patel et al., 2015).

In the cervix in the normal menstrual cycle, hormone-induced structural changes are governed mainly by different amounts and biological properties of mucous (Najmabadi et al., 2020). In addition, under normal menstrual cycle conditions, estrogens stimulate the proliferation of squamous epithelium and increase cellular glycogen production (Blaskewicz et al., 2011). In the luteal phase, progesterone causes desquamation of glycogen-filled surface cells of the vaginal part of the cervix; on the one hand, it stimulates normal flora growth, and on the other hand, it is accompanied by epithelial layer thinning and intercellular junction extension, which contributes to migration of white blood cells (Elwood & Craig, 2003).

According to the Jenson's (1962) theory, sex hormones like all steroids, by contrast with other protein-peptide hormones, realise their effect on target organs by selective interaction with intracellular receptors (Elwood & Craig, 2003). Steroid hormone receptors are proteins that specifically and selectively bind the corresponding steroids after their penetration into the cell and thus provide their biological effects (affect cells' differentia-

tion, growth and adaptation upon changing metabolic conditions, and induction of protein biosynthesis) (Henderson & Feigelson, 2000).

Steroid hormones, in particular, estrogen and progesterone receptors, are of great importance for oncogenesis and tumour proliferation. There is a wide variety of experimental material on the molecular mechanisms of steroids' effect, but the question of how these hormones stimulate proliferation in cancer cells remains open to this date (Baik et al., 2016; Mehta et al., 2017, Son et al., 2018). Numerous studies indicate that malignant neoplasms of the female reproductive system in most cases are hormone-dependent. Modern molecular diagnostics of the estrogen receptor alpha (ER α) and progesterone receptor (PR) functions can be valuable for the choice of methods for cervical cancer treatment (Chung, 2015).

Obviously, increase in the amount of steroid hormones, in particular estrogens, is a necessary condition for initiation of the oncotransformation process in target cells (Grigorian et al., 2007, Hamid et al., 2009; Baik et al., 2016; Kumar et al., 2016). It was found that the level of their expression depends on the female patient's age – the older the age category, the higher is the level of representative cells (Henderson & Feigelson, 2000).

Traditionally, cervical cancer is considered to be insensitive to steroids; cancer cell culture *in vitro* study has shown that addition of estrogen increases cell proliferation, which, in turn, is inhibited by progesterone. In printed publications, there are also a large number of contradictions in expression of these markers under normal and pathological cervix changes (Ramachandran, 2017). According to study results of various authors, the average level of estrogen receptors' expression in the multilayer flat non-keratinised unchanged cervix epithelium ranges 13–100% (Belokri-

nitskaia et al., 2005). As to progesterone receptors, these authors' discrepancies are even greater: expression indexes range from 0 to 100% (Coelho et al., 2004; Ikuo et al., 2006; Dorokhova et al., 2007). There was no statistically proven correlation between the steroid hormone receptor's level and menstrual cycle phase, menopause, etc. There is an assumption that the development of papilloma viral infection (PVI) depends on the sex hormones' level (Berstein, 2000; Belotserkovtseva & Leskova, 2010; Veropotvelian et al., 2012). Regarding the level and variant of estrogen receptors' expression upon pathological changes in the cervix, some studies have determined reduction in estrogen receptors' expression upon various degrees of cervical intraepithelial neoplasia (Dorokhova et al., 2007). At the same time, the peculiar features of estrogen and progesterone receptor's expression upon cervical dysplasia, depending on the PVI level progression in women with infertility, have not been determined.

The goal of research is to determine the peculiar features of cervix estrogen and progesterone receptors' expression at different severity levels of cervical dysplasia, depending on the PVI progression in female patients with infertility.

Materials and methods

The material for the morphological study was cervix specimens of 157 female patients with PVI-associated CIN obtained during diagnostic biopsies in infertility patients, including 62 female patients with mild CIN; 53 patients with moderate CIN and 42 patients with severe CIN. The female patients' average age was 27.5 years.

The Ethics Commission of Ivano-Frankivsk National Medical University, PHEI (Minutes No. 99/18 dated 07.02.2018) found that the study conducted was compatible with the basic bioethical standards of the Declaration of Helsinki adopted by the World Medical Association's General Assembly, the Council of Europe Convention On Human rights and Biomedicine (1977), the relevant provisions of WHO, the Council for International Organisations of Medical Sciences, the International Code of Medical Ethics (1983) and the laws of Ukraine.

To identify Human Papilloma Virus (HPV) we used specific quantitative diagnostics of polymerase chain reaction (PCR) with real-time hybridisation-fluorescence detection (Real-Time PCR) (Heid et al., 1996) applying the reagent kit (PCR kit "AmliSens HPV HCR screen-titre-FL", manufactured by the CSRI of Epidemiology of the Russian Federation) to identify 12 types of HPV-DNA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) in scrapings from the cervical canal, transformation zone and cervix abnormal areas. The final result was calculated automatically in logarithms of the virus genomic equivalents (Ig GE) normalised per 100 thousand (10^5) human genomes, distinguishing three types of viral load: < 3 Ig GE per 10^5 cells, $3-5$ Ig GE per 10^5 cells and > 5 Ig GE per 10^5 cells.

The immunohistochemical (IHC) test was performed on cervical tissue paraffin sections using standard method. We used primary monoclonal antibodies to estrogen (ER) (clone 6F11, Novocastra) and (PR) progesterone receptors (clone 1A6, Novocastra), using the latest generation UltraVision LP (Lab Vision) imaging system, chromogen (DAB (Lab Vision)). To differentiate tissue structures, the sections were additionally stained with Mayer's hematoxylin for 1-3 minutes. Cells with positive expression were studied in 4-6 microscope random fields of view. After counting of 300 histologically identified cells, we calculated expression indicators based on all the studied loci results, taking into account the reactions both in normal and dysplastic modified cells of the epithelial layer. Steroid hormone receptors expression was also evaluated in the stromal component.

Level of steroid receptors' expression was determined by the following scale: negative reaction - 0-10% of positively stained cells; positive reaction - more than 10% of positively stained cells (Dorokhova et al., 2007). The morphological study was performed on an AxioScop 40 (Zeiss) microscope at various magnifications (eye lens 10, field lens 10, 20, 40).

The results obtained during the study were statistically processed by variation statistical methods (arithmetic mean value, standard error, standard deviation, confidence interval). Probability of discrepancies between comparative groups was determined using the Student parametric criterion. Statistical check of hypotheses to determine differences between nonparametric features was performed using the χ^2 -criterion and the z-criterion. The correlation analysis was performed on the basis of parametric

correlation coefficient determination. The correlation accuracy was evaluated by the Student criterion. Results with $P < 0.05$ were considered statistically valid.

Results

The study of features of expression of steroid hormones in female patients with CIN and infertility has established a redistribution of steroid receptors' expression; thus ER is characterised by reduction in epithelial and stromal positive reaction. However, PR expression differs in positive epithelial expression appearance with expression of reaction in the cervical stroma. We established that upon mild PVI-associated CIN in 37.1% of cases there was positive ER expression ($>10\%$). Negative ER expression in this study group was observed in 62.9% of female patients. PR expression was present in 66 cases (42.0%). The highest proportion (64.3%, $P < 0.05$) was observed in cervical specimens with severe CIN. The vast majority (91 female patients) had negative PR expression, which constitutes 57.9%. Female patients with mild CIN predominated in this group (67.7%, $P < 0.05$, Table 1).

Table 1
Steroid hormones expression in the cervix in female patients with CIN and infertility (abs. numb., %)

CIN severity level	n	ER expression				PR expression			
		$<10\%$		$>10\%$		$<10\%$		$>10\%$	
		abs. numb.	%	abs. numb.	%	abs. numb.	%	abs. numb.	%
Mild	62	39	62.9	23	37.1	42	67.7	20	32.3
Moderate	53	38	71.7	15	28.3	34	64.2	19	35.9
Severe	42	33	78.6	9	21.4	15	35.7	27	64.3

According to the χ^2 -criterion method, there was no significant influence of the ER expression level on the CIN severity level ($\chi^2 = 5.99$, $P > 0.05$), but there was a significant influence of the PR expression level on the CIN severity level ($\chi^2 = 9.48$, $P < 0.05$). With CIN no significant effect of viral load on ER and PR expression level ($\chi^2 = 5.99$, $P > 0.05$) was detected by the χ^2 -criterion method.

Using χ^2 -criterion method, we found a significant influence of ER expression level on the type of infertility ($\chi^2 = 9.48$, $P < 0.05$) with mild CIN. Using χ^2 -criterion method, we found a significant influence of ER expression level on infertility type ($\chi^2 = 9.48$, $P < 0.05$) with moderate CIN, but no significant influence of infertility type on PR expression level ($\chi^2 = 9.48$, $P > 0.05$). In cases of severe CIN, the method of χ^2 -criterion did not reveal a significant influence of infertility type on ER expression level ($\chi^2 = 9.48$, $P > 0.05$), but it revealed a significant influence of infertility type on PR expression level ($\chi^2 = 9.48$, $P < 0.05$). Evaluation of the components of the exocervix hormone-receptor apparatus with CIN showed a significant reduction in ER volume content (twofold) ($P < 0.05$) as compared with PR (by 1.3 times) ($P < 0.05$). With PVI-associated CIN, there was a significant reduction in the ER/PR index, with the lowest indicator (0.3) in the group of female patients with severe CIN ($P < 0.05$, Table 2).

Table 2
Content of steroid hormone receptors in the cervix in women with CIN and infertility ($x \pm m$, in volume, %).

CIN severity level	n	Steroid hormone receptors		ER/PR index
		ER	PR	
Mild	62	1.9 ± 0.4	4.2 ± 0.5	0.4
Moderate	53	1.9 ± 0.4	4.3 ± 0.5	0.4
Severe	42	1.9 ± 0.1	6.3 ± 0.4	0.3

After evaluating the components of hormone-receptor apparatus of the cervical mucosa, we established the following types of expression: ER+PR+, ER+PR-, ER-PR+, ER-PR-. Under conditions of PVI containment, female patients with ER-PR-expression type predominated ($P < 0.05$), in particular, with mild CIN (41.9%) and with moderate CIN (54.7%, Table 3). Using χ^2 -criterion method, we found significant influence of hormone expression type on the CIN severity level ($\chi^2 = 12.59$, $P = 0.003$). Since estrogens and their metabolites are able to activate HPV-infected cervical epithelial cells apoptosis, there is a reduction in estrogen receptors' expression as and when precancerous changes in the cervical

epithelium progress. We noted the appearance of receptor-negative areas with CIN, especially in loci with morphological signs of PVI (koilocytosis, Fig. 1).

Table 3
Steroid hormone receptors' expression
in women with PVI-associated CIN and infertility (n, %)

CIN severity level	n	Types of steroid hormones expression							
		ER+PR+		ER+PR-		ER-PR+		ER-PR-	
		abs. numb.	%	abs. numb.	%	abs. numb.	%	abs. numb.	%
Mild	62	6	9.7	16	25.8	14	22.6	26	41.9
Moderate	53	1	1.9	14	26.4	9	16.9	29	54.7
Severe	42	8	19.1	2	4.8	18	42.9	14	33.3

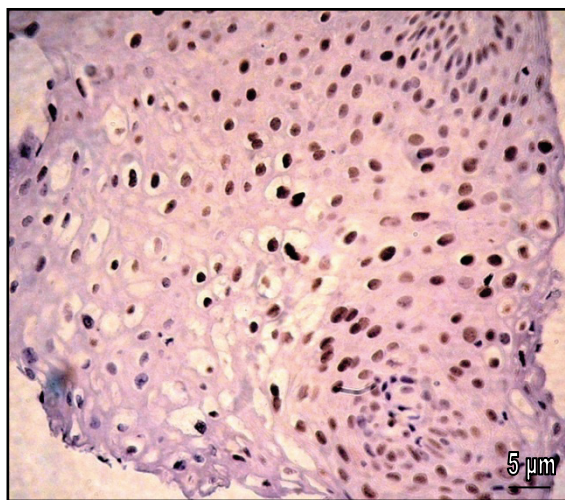


Fig. 1. ER-positive koilocytes and ER-negative areas in stratified squamous non-keratinised epithelium in woman with mild PVI-associated CIN: IHC method with primary ER antibodies; 25-year-old female patient with tubal-peritoneal infertility (4 years); viral load >5 Ig GE per 10⁵ cells, scale bar = 5 μm

With an increase in CIN severity level, there is a gradual reduction in the detection of ER-receptors with predominance of PR-receptors in stratified squamous non-keratinised epithelium (Fig. 2, 3).

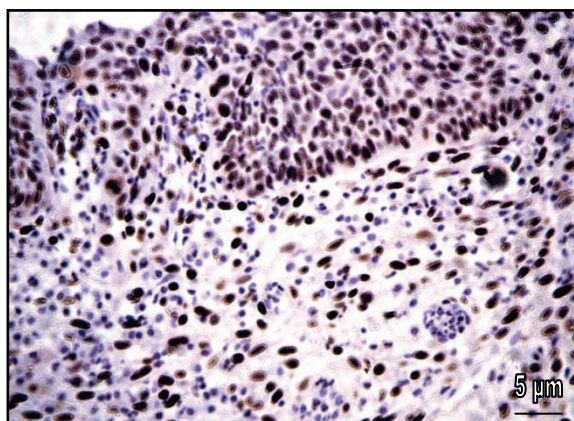


Fig. 2. Positive PR expression in squamous metaplasia locus and in inner layer of cervix mucous membrane in woman with moderate PVI-associated CIN: IHC method with primary PR antibodies; woman, 23 years, with primary tubal infertility (3.5 years); viral load 3–5 Ig GE per 10⁵ cells, scale bar = 5 μm

However, with severe CIN development, the level of estrogen receptors falls twofold upon slightly increased level of progesterone receptors.

With CIN in women with infertility and HPV infection, the correlation analysis results indicate a low feedback between dysplasia degree and ER expression level ($r = -0.18$, $P < 0.05$) and a moderate interrelation between dysplasia degree and PR expression level ($r = -0.40$, $P < 0.05$).

There is also a moderate feedback between viral load and estrogen receptors' expression level ($r = -0.31$, $P < 0.05$).

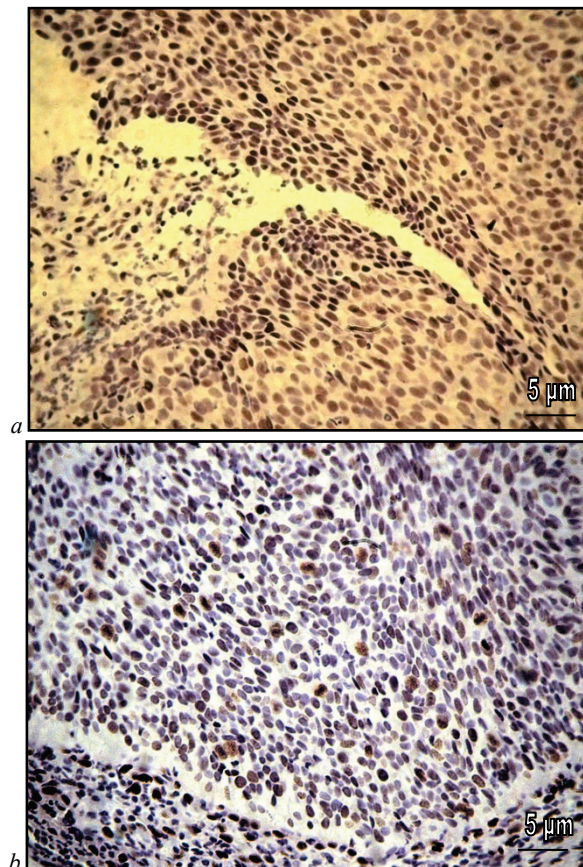


Fig. 3. Negative ER expression (a) and positive PR expression (b) in stratified squamous non-keratinised epithelium in woman with severe PVI-associated CIN: IHC method with primary ER antibody; woman 28 years, with primary hormonal infertility (7.5 years); viral load 3–5 Ig GE per 10⁵ cells, scale bar = 5 μm

Discussion

For five decades, researchers have been investigating the role of steroid hormones in cancer development, but the problem remains unsolved (Berstein, 2000; Henderson et al., 2000; Churuksaieva et al., 2010). A large amount of data has been collected regarding the indirect role of steroids in emergence of many human tumours. In oncoepidemiological studies, the “enhanced hormonal stimulation” concept was proposed to be used in the analysis of breast cancer, ovarian cancer, endometrial cancer, and other causes and mechanisms of malignant neoplasms (Samoilova & Kostrova, 2009; Coelho et al., 2014; Patel et al., 2015).

Papilloma viral infection of the genital tract is distinguished among the exogenous etiological factors in dysplasia development with subsequent cervix oncotransformation (Alp, 2012; Boda et al., 2018). Human Papillomavirus is an oncovirus that contains protein and DNA and is a part of the Papoviridae family. The genome is represented by a circular double-stranded DNA that has three functional areas: LCR (long control region), Early (E), and Late (L). The LCR region is involved in the regulation of virus gene transcription. The E-region includes E1, E2, E4, E5, and E7 genes. The L1 and L2 genes encode the structural proteins of viral capsid.

The leading role in retransformation belongs to the E-region proteins. And the trigger factor is E1 gene mutation which promotes the HPV DNA integration into the host cell's chromosomes. This process can also be accompanied by the E2 protein inactivation, of which functional activity loss leads to excessive E6 and E7 expression, which directly trigger neoplastic aberration processes (Fontecha et al., 2016). E6 and E7 oncogenic properties are due to their ability to form complexes with negative

cell growth regulators – p53 protein (for E6) and Rb protein (for E7). P53 and Rb – are normally responsible for differentiation and growth of cells, which due to mutation or damage turn into oncogenes, causing uncontrolled proliferation. Interacting with p53 and Rb proteins, E6 and E7 proteins cause their deactivation. This leads to apoptosis prevention, suppression of interferon production, prolongation of the cell's life through telomerase activation, enhancement of cell proliferation, disruption of the protective regulatory mechanisms that ensure DNA repair, which results in genome destabilisation, inducing chromosomal mutations and aberrations of host genes, which is an endogenous factor in tumour progression (Habbous et al., 2012).

In women, endocrine profile has a direct impact on the PVI development: on the one hand, estradiol is rightly linked with estrogen receptors and, interacting with them, affects cells' metabolic and proliferative activity. The P-450 cytochrome enzyme system converts estradiol into two main metabolites: 16 α -hydroxysterone (16 α -OH) and 2-hydroxysterone (2-OH). It has been found that where there is active expression of HPV proteins, there is a high risk of 16 α -OH synthesis, which is similar to that in breast cancer cells. That is, infection of the cell leads to change in estradiol metabolism towards the predominant synthesis of 16 α -hydroxysterone. Here-with, HPV E7 gene has an estradiol-dependent expression character, and a stable complex of estradiol receptor-16 α -hydroxysterone (ER16 α) interacts with E7 gene regulatory region, initiating an increase in its expression, thus providing favourable conditions for growth of malignant tumours (Fontecha et al., 2016). On the other hand, the effect of steroid hormones on the immune system is based on maintaining the balance of Th1/Th2-links immune response under PVI. A large number of estrogen receptors have been identified on CD8+ T cells, i.e. on cells of a cytolytic/ suppressive nature. Estradiol stimulates an antigen-specific immune response by activating CD4+ T cells and parallel inhibition of CD8+ T cells (Ambühl et al., 2017).

Progesterone and pregnane-type acetylated progestogens activate glucocorticoid receptors and exert glucocorticoid-like immunosuppressive effect, including inhibition of T-cell activity, increase in tumour induction and lymphocytopenia. As for the humoral component of immune response, progesterone indirectly, through activation of second-type T-helper cells and secretion by them of IL-4 and IL-5, contributes to the differentiation of B cells in their antibodies synthesis, affects the inflammatory response by activating the production of IL-1 monocytes, α -TNF (Belokrinitskaia et al., 2005; Bhat et al., 2014).

With pathological changes in the cervical epithelium, the number and functional activity of sex hormone receptors may change, which in turn leads to changes in proliferative processes intensity (Ikeda et al., 2004; Ikuo et al., 2006; Son et al., 2014).

HPV-induced cervical lesions are closely linked to hyperestrogenemia, which is accompanied by the conversion of E2 to the aggressive metabolite 16 α -OHE1, which determines E2 prolonged effect and activates HPV E7 gene expression, which in turn initiates pathological proliferation mechanisms of cells, blocks immune protection factors and determines cells' tumour transformation (Henderson et al., 2000; Churuksaeva et al., 2010). This explains the dominant lesions of the estrogen-sensitive zone of cervical epithelium transformation, where the highest level of steroid hormone's expression is noted. Estrogens and their metabolites can activate apoptosis of HPV-infected cervical epithelial cells, in which case there is reduction in expression of estrogen receptors as and when precancerous changes of the cervical epithelium progress (Ramachandran, 2017). Appearance of receptor-negative areas can initiate progress of pathological changes in the cervix (Dorokhova et al., 2007). In our study, we noted the appearance under CIN of receptor-negative loci, especially in areas with morphological features of PVI (koilocytosis). Upon increase in dysplastic process, we noted a gradual reduction in the detection of ER-receptors with a predominance of PR-receptors in stratified squamous non-keratinised epithelium. However, with the development of severe dysplasia, the estrogen receptors' level falls twofold with a slightly increased level of progesterone receptors. Reduction of steroid reception level upon neoplastic transformation of the cervix may indicate the formation of autonomous "self-sustaining" growth due to locally producing growth factors and cytokines.

Conclusion

The study of the characteristic features of steroid hormones' expression in women with CIN and infertility established the redistribution of steroid receptors' expression; thus, ER is characterised by a decrease in epithelial and the appearance of stromal positive response; instead, PR expression differs in the appearance of positive epithelial expression, with a pronounced reaction in the cervical stroma. Estrogen receptor redistribution in cases of CIN is most common in women with tubal infertility: with mild CIN, negative ER expression was found in 59.0%, with moderate CIN – in 68.4%, and with severe CIN – in 51.5% of female patients. Positive epithelial PR expression was also predominantly found in women with tubal infertility, especially with moderate CIN – in 58.8% and severe CIN – in 51.9%. Evaluation of the cervix hormone-receptor apparatus components showed a significant reduction in ER volume content (two-fold) as compared with PR (1.3 times). With PVI-associated CIN, there was a significant reduction in the ER/PR index, with the lowest indicator in the group of female patients with severe CIN. Negative ER and RR expression is predominantly observed in female patients with a high value of viral progression regardless of CIN severity level. Determining steroid hormone receptors' expression in cases of PVI-associated CIN in women with infertility can be used as an additional criterion for determining CIN severity level.

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