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Peculiarities of the expression P16^{ink4a} under cervical intraepithelial neoplasia in women with reproductive disorders

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Abstract
We conducted comprehensive clinic-morphological study of 250 women with cervical intraepithelial neoplasia (CIN) with reproductive function disorders, of which in 157 (62, 8%) female patients was identified DNA of human papilloma virus (HPV) of high carcinogenic risk (HCR). We established the presence of HPV DNA in 62 female patients (56, 4 %) – under mild CIN, in 53 (61, 2%) – under moderate CIN, and 42 (77, 7%) – under sever CIN.
The obtained results of immunohistochemical study indicate that the p16^{ink4a} marker can be used as an additional criterion for the diagnosis of cervical intraepithelial neoplasia associated with papilloma viral infection and for the determination of the dysplastic process degree. Use of p16^{ink4a} allows assessing the potential of dysplasia in relation to the development of cervical cancer and, consequently, to choose an adequate therapeutic tactics of this disease in women with infertility.

Keywords: RPC, cervical intraepithelial neoplasia, infertility.

1. Introduction
Among exogenous etiological factors in the development of dysplasia with subsequent oncologic transformation of cervix we differentiated papilloma viral infection of the genital tract. The human papilloma virus is oncovirus that contains protein and DNA, and is a part of the Papovaviridae family [1].
The genome is represented by a circular double-stranded DNA, which has three functional areas: LCR (long control region), Early (E), and Late (L); LCR area is involved in the regulation of viral genes transcription. Region E includes genes E1, E2, E4, E5, E7. Genes L1 and L2 encode structural proteins of the viral capsid. The leading role in neo transformation belongs to the E region proteins. The E1 gene mutation is the trigger factor that facilitates the integration of HPV DNA into the chromosome of the host cell. This process may be accompanied by the protein E2 inactivation, loss of functional activity of which leads to over-expression of E6 and E7, which directly trigger the processes of neoplastic aberration [2-4]. Oncogenic properties of E6 and E7 are stipulated by their ability to form complexes with negative regulators of cell growth – p53 protein (for E6) and Rb protein (for E7). P53 and Rb usually encode for differentiation and growth of cells, which in the result of mutations or damage turn into oncogenes, causing uncontrolled proliferation. Interacting with proteins p53 and Rb, E6 and E7 proteins cause their dehydration [5, 6]. This leads to the prevention of apoptosis, suppression of interferon production, prolongation of cell life through the activation of telomerase, enhancing of cell proliferation, violation of protective regulatory mechanisms that ensure DNA repair, that leads to destabilization of the genome, induces chromosomal mutations and host genes aberrations, which is an endogenous factor in neoplastic progression. Detailed molecular analysis of this activity allowed us to identify biomarkers, in particular cyclin-dependent kinase p16^{ink4a} [7-10].
The aim of our study is to identify the peculiarities of cyclin-dependent kinase p16^{ink4a} expression inhibitor with the possibility of using it as an additional criterion in the diagnosis of cervical intraepithelial neoplasia associated with human papilloma viral infection in women with infertility.

2. Material and Methods
We conducted comprehensive clinico-morphological study of 250 women with CIN under disorders of the reproductive function. We diagnose CIN using the World Health Organization tumours classification: Pathology and genetics of breast tumours and female genital organs tumours, International classification of diseases of 10th revision (1995) and the International histological classification of tumours No. 13.

Depending on the CIN severity all the cases were divided into three study groups: the 1st group – 110 cases with mild CIN (CIN-I); the 2nd group – 86 cases with moderate CIN (CIN-II); the 3rd group – 54 cases with severe CIN (CIN-III). The women's average age was 29, 4±1, 3 years.

58, 4% of women suffered from primary infertility, 41, 6% – from secondary infertility. Among the causes of primary infertility prevailed tubal peritoneal factor (46, 6%), which was detected in 68 women. Among the examined women tubal peritoneal factor as a primary, was found in 27, 2%, hormonal infertility was diagnosed in 37 female patients (14, 8%), combined factors occurred in 24 cases (9, 6 %). In the structure of secondary infertility has dominated the pipe factor (60, 6%), peritoneal infertility observed in 24.0% of patients. In secondary infertility we did not observe women with reproductive disorders associated with endometriosis, immune genesis and unspecified forms. In the total number of the women examined secondary tubal infertility constitutes 25, 2%, peritoneal – 10, 0%, combined occurred in 3, 6% of female patients.

The control group consisted of 30 women with reproductive disorders, in which under histological examination of cervical biopsies awe did not detect any cervical pathology. The average age of female patients in the control group totalled 23, 9±0,82 years.

All female patients were examined to identify human papilloma viral infection. To identify the PVI, as the most sensitive method, was used polymerase chain reaction with hybridization-fluorescent detection in real-time. We identified 12 types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 types) of DNA of human papilloma virus (HPV) with high carcinogenic risk. Selected types are of high oncogenic property with respect to neoplastic transformation and development of cervical carcinoma.

For immunohistochemical studies we used monoclonal antibodies against $p16^{ink4a}$ (kit for histological preparations, No. K5334). We conducted the incubation of the sections with the primary antibody in humid chambers at a temperature of 23 – 25 ^{0}C within 30 minutes. Antibody titers were selected individually for each marker using, as solvent, a special solution antibody diluents (Lab Vision). We conducted the next stage of immunohistochemical studies using the visualization system of the latest generation Ultra Vision LP (Lab Vision). For differentiation of tissues structures sections were additionally stained with Mayer's haematoxylin (hemalum) within 1-3 minutes.

Subsequent dehydration and dipening in balm was carried out according to common methods. We studied cells with positive expression in 4-6 microscope's random fields of view.

To assess the level of expression of $p16^{ink4a}$ we used the following scale: negative reaction – up to 5% of positively stained cells, focal – up to 80% of cells with positive staining; diffuse – more than 80% of cells with positive immunohistological marker.

3. Results and Discussion

Analysis microbiological studies data showed that in157 (62, 8%) of women was identified HPV DNA. We established the presence of HPV DNA in 62 female patients (56, 4%) under CIN-I, in 53 (61, 2%) – under CIN-II, and in 42 (77, 7%) – under CIN-III. The HPV detection rate increases with the severity of the neoplastic process under CIN-II in 1, 1 times as compared with the CIN-I, under CIN-III in 1, 4 and 1,3 times, respectively, to the CIN-I and CIN-II indices.

Analyzing the quantitation data of HPV we determined three variants of viral load (copies of Ig HPV/10^5 cells). In 52 women (33,1%) we detected HPV with viral load < 3 copies of Ig HPV/10^5 cells, 3-5 copies of Ig HPV/10^5 cells were determined in 65 female patients (41,4%) and in the 40 of the examined female patients (25, 5%) was detected viral load > 5 copies of Ig HPV/10^5 cells.

Table 1: The viral load of HPV in the examined female patients (abs. nmb, %)

Copies of Ig HPV/10^5 cells	Groups under study						Total (n=250)	
	CIN-I (n=110)		CIN-II (n=86)		CIN-III (n=54)			
	abs.	%	abs.	%	abs.	%	abs.	%
>3	22	35,5	17	32,1	13	30,95	52	33,1
3-5	29	46,8	21	39,6	15	35,7	65	41,4
>5	11	17,7	15	28,3	14	33,3	40	25,5
Total	62	56,4	53	61,2	42	77,7	157	62,8

Analysis of the viral load distribution to the CIN severity showed that the three groups dominated quantitative value of 3-5 copies of Ig HPV/10^5 cells (41,4%): under CIN-I this indicator was observed in 46,8%, under CIN-II – in 39,6% and under CIN-III – in 33,3%.

Expression of $p16^{ink4a}$ was observed focally, in separate cells groups of the basal and parabasal layers. Mainly a mixed response was manifested, which was characterized by simultaneous coating of both cell nuclei and cytoplasm with a predominance of the latter. Sometimes we detect singular cells of type koilocyte, with positive $p16^{ink4a}$ expression in the intermediate layer and around subepithelial vascular pedicles.

When studying the $p16^{ink4a}$ expression, we observed positive status in 145 CIN cases that constitutes 92, 4%. The focal level was observed in 88 female patients (60, 7%), diffuse – in 57 cases (39, 3%).

Under CIN-I we detected negative $p16^{ink4a}$ expression in 9 cases (14, 5%). The positive marker expression was determined in 53 women (85,5%): positive focal reaction was observed in the majority of cases (36), which constitutes 58,1%, diffuse expression of the marker under study was identified in 17 female patients (27,4%), at that we noticed mixed type of $p16^{ink4a}$ excision (Table 2).

Table 2: Expression of p16^{ink4a} of the cervical surface epithelium under CIN associated with the PVI in women with infertility (abs.nmb, %)

Groups under study	n	p16 ^{ink4a} expression level					
		Negative		Focal		Diffuse	
		abs.	%	abs.	%	abs.	%
CIN – I	62	9	14,5	36	58,1	17	27,4
CIN – II	53	3	5,7	34	64,2	16	30,2
CIN – III	42	0	0,0	18	42,9	24	57,1
Control	15	15	100,0	0	0	0	0

Under CIN-II changes the intensity and the distribution of the P16 ^{ink4a} marker, which is more common. In 64, 2% cases we observed focal reaction with simultaneous nucleus and cytoplasm staining. The frequency of detection of the marker with the diffuse type of expression increases (30, 2%). Identification of negative expression under CIN-II decreases in 2, 5 times as compared to CIN-I.

Under CIN-III we also observed redistribution of p16^{ink4a} expression. In this group under study the number of epithelial cells with mixed type expression increases in 2,1 times as compared to CIN-I and in 1,9 – to CIN-II.

We noted certain peculiarities of the expression of P16^{ink4a} depending on viral load. In 100.0% of cases we noted negative expression under CN-I in women with identified HPV of HCR with viral load <3 copies Ig of HPV/10^5 cells (Table 3). The highest percentage (75, 9%) of marker focal expression is observed in women with viral load 3-5 copies Ig of HPV/10^5 cells. Diffuse level was equally noted (41 2%) in women with medium and high level of viral load that in 3 times exceeds the rate, in which the indentified quantitative indicator <3 copies Ig of HPV/10^5 cells.

Table 3: The level of p16^{ink4a} expression of the cervical surface epithelium under CIN-I depending on viral load (abs.numb%)

Copies of Ig HPV/10^5 cells	p16 ^{ink4a} expression level					
	Negative n=9		Focal n=36		Diffuse n=17	
	abs.	%	abs.	%	abs.	%
<3 (n=22)	9	100,0	10	2,8	3	13,6
3-5 (n=29)	0	0,0	22	75,9	7	41,2
>5 (n=11)	0	0,0	4	11,1	7	41,2

Under CIN-II the analysis of the distribution of p16^{ink4a} expression depending on viral load showed the following: 100, 0% negative level of expression occurs in three cases with the lowest viral load.

A proportion of female patients (43, 8%) with diffuse type expression and viral load >5 copies Ig of HPV/10^5 cells increases as compared to CIN-I (Table 4).

Table 4: The level of p16^{ink4a} expression of cervical surface epithelium under CIN-II, depending on the viral load (abs.nmb.%)

Copies of Ig HPV/10^5 cells	p16 ^{ink4a} expression level					
	Negative n=3		Focal n=34		Diffuse n=16	
	abs.	%	abs.	%	abs.	%
<3 (n=17)	3	100,0	11	32,4	3	18,8
3-5 (n=21)	0	0,0	15	44,1	6	37,5
>5 (n=15)	0	0,0	8	23,5	7	43,8

Under CIN-III in 100, 0% cases we observed positive status of dysplastic epithelium in relation to p16^{ink4a} expression. Detailed distribution of p16^{ink4a} expression of cervical surface epithelium under CIN-III depending on viral load is presented in Table 5.

Table 5: The level of p16^{ink4a} expression of cervical surface epithelium under CIN-III, depending on the Viral load (abs. nmb, %)

Copies of Ig HPV/10^5 cells	p16 ^{ink4a} expression level					
	Negative n=0		Focal n=18		Diffuse n=24	
	abs.	%	abs.	%	abs.	%
<3 (n=13)	0	0,0	8	44,4	5	20,8
3-5 (n=15)	0	0,0	9	50,0	6	25,0
>5 (n=14)	0	0,0	1	5,6	13	54,2

Female patients with viral load <3 copies Ig of HPV/10^5 cells as in the previous groups under study the focal type of p16^{ink4a} expression is predominant and was observed in 8 cases (61,5%). But under high viral load, which was observed in 14 female patients, we noted the highest rate of diffuse p16^{ink4a} expression – 92, 9%.

The absence or weak pl6^{ink4a} expression under mild CIN, which was observed in 9 women, was obviously due to the viral load <3Ig of HPV/10^5 cells, in the result of which may not have occurred P16^{ink4a} super expression. According to the Ordi J. and co-authors also noted that in 72% of the biopsies of the cervix with CIN-I morphological signs in women infected by low-risk oncogenic types of HPV expression of pl6^{ink4a} was absent, diffuse reaction was determined only in 4%. In 22% of women infected by high-risk HPVs with CIN-I morphological features pl6^{ink4a} expression was not observed [1]. Available data that intraepithelial lesion low-grade is caused by the high risk types of HPV, are negative in relation to p16^{ink4a} are subject to spontaneous regression in 60% of women, while under CIN-I with diffuse staining p16^{ink4a} have a higher tendency to CIN progression. Thus, p16^{ink4a} expression increases with cervical intraepithelial neoplasia severity growth. CIN-I and CIN-II are characterized mainly by the presence of focal type and different intensity of p16^{ink4a} expression. Heavy CIN is

characterized by intense diffuse MBE staining. The presence of $p16^{ink4a}$ in cells of exocervix basal layer points to the HPV of HCR integration into the genome of proliferating cells and the possibility of disease progression.

4. Conclusion

1. $p16^{ink4a}$ marker can be used as an additional criterion for the diagnosis of cervical intraepithelial neoplasia associated with the HPV and establishing the degree of dysplastic process.
2. The obtained results allow to assess dysplasia potential in relation to the development of cervical cancer and, therefore, to choose an adequate therapeutic tactics of this disease in women with infertility.

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