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Diagnostics of the severity of cervical intraepithelial neoplasia in women with infertility in the presence of papillomavirus infection

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Abstract

Objectives. To objectify the diagnosis of cervical intraepithelial neoplasia in the presence of papillomavirus infection in women with infertility by morphometric analysis of the cervical mucosa. Material and methods. The pieces of the cervix obtained during diagnostic biopsies of 157 infertile women with cervical intraepithelial neoplasia associated with papillomavirus infection were material for morphological examination. A specific quantitative diagnosis of real-time polymerase chain reaction with hybridization-fluorescence detection was used to identify papillomavirus infection. Immunohistochemical study was performed on paraffin sections of cervical tissue by conventional methods. Histological examination was performed on an AxioScop 40 (Zeiss) microscope. Metric parameters were calibrated on the tool for measuring "Mira". Results. Mild dysplastic ectocevix changes were generally characterized by preservation of anisomorphism and stratification of the surface and intermediate layers, focal basal cell hyperactivity with increasing nuclear-cytoplasmic ratio. The volumetric density of capillaries of a mucous membrane credibly increased in 2.8-times in moderate cervical intraepithelial neoplasia compared with the control group. The thickness of multilayered squamous non-keratinized epithelium with severe cervical intraepithelial neoplasia often did not differ from that with mild or moderate ones. Violation of histoarchitectonics due to loss of stratification and vertical anismorphism was noted in the ectocervix. There was a total basal hyperactivity, impaired maturation and differentiation of epithelial cells. Conclusions. The obtained morphometric data studies have revealed that disorders of the epithelial-stromal relationship especially in cases of severe cervical intraepithelial neoplasia may be one of the stages of carcinogenesis.

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Introduction.

The future of the state is determined by a set of political, economic, social factors that affect the demographic situation and health status of the population. Reproductive health is an integral part of the health of the nation as a whole and is of strategic importance for the sustainable development of society. According to the World Health Organization (WHO), reproductive health is a state of physical, mental and social well-being. Major reproductive health issues worldwide are: high maternal and infant mortality, high abortions, miscarriages, complications of pregnancy and childbirth, high prevalence of female and male infertility and sexually transmitted infections, including human immunodeficiency virus/acquired immunodeficiency syndrome, oncological morbidity of the reproductive sphere, etc [1, 2]. There is a tendency to increase the number of cases of female infertility in Ukraine. The cervical factor is among 22 causing factors of infertility, according to the WHO and occurs from 8 to 20% of infertility according to the literature [3, 4, 5, 6]. The greatest importance of cervical infertility belongs to hormonal disorders, cervicitis, cervical ectopia, polyps, cervical neoplasia and especially the consequences of aggressive treatment of cervical pathology [7, 8, 9, 10]. Cervical intraepithelial neoplasia (CIN), especially due to papillomavirus infection is considered a morphological substrate for the development of cervical cancer [11, 12, 13]. Analysis of recent studies shows the widespread use of molecular biological markers aimed at improving the diagnosis of CIN, as they can help to objectify the verification of the diagnosis [14, 15, 16, 17]. Despite the large number of studies on CIN, in particular in human papillomavirus infection (PVI), a number of issues remain under discussion, and the peculiarities of its morphology in women with infertility are absent.

Objectives.

To objectify the diagnosis of cervical intraepithelial neoplasia in the presence of papillomavirus infection in women with infertility by morphometric analysis of the cervical mucosa.

Material and methods (Case presentation).

The material for morphological examination were pieces of the cervix obtained during diagnostic biopsies of 157 women with infertility with PVI associated CIN, There were 62 patients with CIN of mild (CIN-I) severity, 53 patients with moderate CIN (CIN-II) and 42 patients with severe CIN (CIN-III) in this study. A specific quantitative diagnosis of real-time polymerase chain reaction with hybridization-fluorescence detection (Real-Nime PCR) was used to identify PVI with a set of reagents (PCR kit Russian Federation) to determine of 12 types of HPV DNA (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) in scraping from the cervical canal, transformation zone and pathological areas of the cervix. The final result was calculated automatically in the logarithms of the viral genom equivalents (GE) normalized per 100 thousand (105) human genomes, distinguishing three types of viral load: 5 Ig GE per 105. Pieces of the cervix were fixed in 10% neutral formalin, dehydrated through a series of alcohols of growth increasing concentration poured into paraffin and prepared serial sections 5-6 µm thick, stained with hematoxylin and eosin, picrofuxin by Van Gieson for morphological examination. Immunohistochemical study was performed on paraffin sections of cervical tissue by conventional methods. Monoclonal antibodies to Ki-67 (clone MIB-1, Dako) and p63 (clone 4A4, Dako) were used. Control studies were performed for each marker to exclude false-positive or false-negative results. The proliferative activity of Ki-67 was evaluated depending on the number of cells with labeled nuclei to the total number of epithelial cells and the prevalence of cells with nuclear expression in the layers of the ectocervix. The expression level of Ki-67 was assessed by the prevalence in the epithelial layer: low - positively stained cells occupy <1/3 of the epithelial layer; medium - 1/3 - 2/3 epithelial layer and high >2/3 epithelial layer. Proliferative activity was considered high when staining more than 50% of cells, low - when staining less than 10%, the value of 10-50% was considered as an intermediate zone. Proliferation indices (PIs), p63 were calculated as the percentage of cells with a positive nuclear reaction, regardless of the intensity of staining, to the total number of cells on average according to the results of all studied areas. To assess the expression level of p63, the following range was used: low level - less than 30% of cells with a positive nuclear reaction; moderate - 30-75% of positively stained cells; high level - more than 75% of cells with positive expression.

Histological examination was performed on an AxioScop 40 (Zeiss) microscope at original magnification x 20. Metric parameters were calibrated on the tool for measuring "Mira" (test control) with image analysis based on the software UTHSC SA Image Tool for Windows. The height of the multilayered squamous non-keratinized epithelium (MSNE), the height of the basement membrane were measured, the density of the mucous membrane capillaries and the ratio of the height of the MSNE to the volumetric density of capillaries of a mucous membrane (VDCMM) were determined. The obtained results were subjected to statistical processing by methods of variance

statistics (arithmetic mean, standard error, standard deviation, confidence interval). Testing statistical hypotheses to determine the differences between nonparametric features was performed using the χ2-criterion and the z-criterion. Correlation analysis was performed based on the determination of the parametric correlation coefficient. Probability was assessed by Student's t test. Results with p <0.05 were considered statistically significant. Construction of a mathematical model, regression analysis, the values of multiple regression coefficients were determined using the program SPSS. The obtained data were processed using Microsoft Access software, Microsoft Excel 2010 (license № 01631-551-3027986-27852).

Results.

Mild CIN were generally characterized by preservation of anisomorphism and stratification of the surface and intermediate layers, focal basal cell hyperactivity with increasing nuclear-cytoplasmic ratio. The nuclei of cells of the basal and parabasal layer were with a clear chromatin structure of the nucleoli. Basal cell cytoplasm had basophilic color. There was an increase in mitotic activity in hyperplasia cells. The dominant morphological feature was an increase in the thickness of the MSNE with CIN-I. On average the thickness of MSNE increased 2.9 times compared with control samples of the cervix (p <0,05). The VDCMM increased in 2.9 times in comparison with the control group (6.93 ± 2.3 vs. 2.35 ± 0.4; p <0.05). The ratio of the thickness of the stratified squamous epithelium to the relative VDCMM increased from 94.03 ± 11.5 in the control group to 107.7 ± 14.3 (p <0.05). The thickness of MSNE with CIN-II increased in comparison with MSNE of control group and CIN-I (p <0,05). CIN-II was characterized by a violation of vertical anisomorphism and stratification of the lower layers of the stratified squamous epithelium due to basal cell hyperactivity. The basal layer was represented by 9-12 rows. Hyperplasia cells of the lower layers were oriented perpendicular to the basement membrane. The nuclei of basal cells were hyperchromic, surrounded by a narrow rim of the cytoplasm, but there were also normo- and hypochromic nuclei. A subnuclear vacuolation of the cytoplasm was observed in some cases. The number of cells with figures of mitosis increased in the direction to the basement membrane. The number of atypical mitoses increased slightly compared with CIN-I. Superficial and intermediate layers of the epithelial layer were with preserved stratification. The cells were placed horizontally in relation to the basement membrane. There were small cells with pyknotic nuclei of different shapes and sizes with intense eosinophilic cytoplasm (dyskeratocytes) in the cells of the upper and intermediate layers. Diskeratocytes were located mainly in complexes. The basement membrane was thickened in the form of a homogeneous oxyphilic layer, but it was also sharply tortuous in cases of condyloma vegetation or presence of acanthotic cords. It was represented by bundles of collagen fibers, which were sharply compacted or swollen. Collagen fibers were fibrous and fragmented in areas of edema. Sharply swollen fibroblasts with focal plasmolysis and karyolysis were found in such areas. The VDCMM credibly increased 2.8-times with moderate CIN compared with the control group (6.93 ± 2.6 vs. 2.73 ± 0.4). The ratio of the thickness of the stratified squamous epithelium to the VDCMM decreased to 83.7 ± 14.3 (p <0.05). The thickness of MSNE with severe CIN often did not differ from that with mild or moderate ones. But it was at the same level with the indicators of the control group in 35.2% of cases. Violation of histoarchitectonics due to loss of stratification and vertical anismorphism was noted in the ectocervix. There was a total basal hyperactivity, impaired maturation and differentiation of epithelial cells. Dysplastic cells occupied a larger thickness of the epithelial layer, with the exception of a few (2-3) surface layers, represented by mature cells that maintained a normal structure. The arrangement of cells was chaotic. Dysplastic cells were polymorphic and had different sizes. The majority of cells with large hyperchromic nuclei were surrounded by a narrow rim of the cytoplasm. The nuclear-cytoplasmic ratio changed in favor of the nucleus. The nuclei were deformed with multiple invaginations and protrusion of the nucleolema in some cells. Such cells were found in the upper layers of the epithelial layer. Severe CIN was characterized by an increase in the VDCMM in 4.9 times (13.63 ± 2.4 vs. 2.73 ± 0.4 in the control group). The ratio of MSNE height to VDCMM significantly decreased to 31.2 ± 13.6 (p <0.05) (Figure 1).

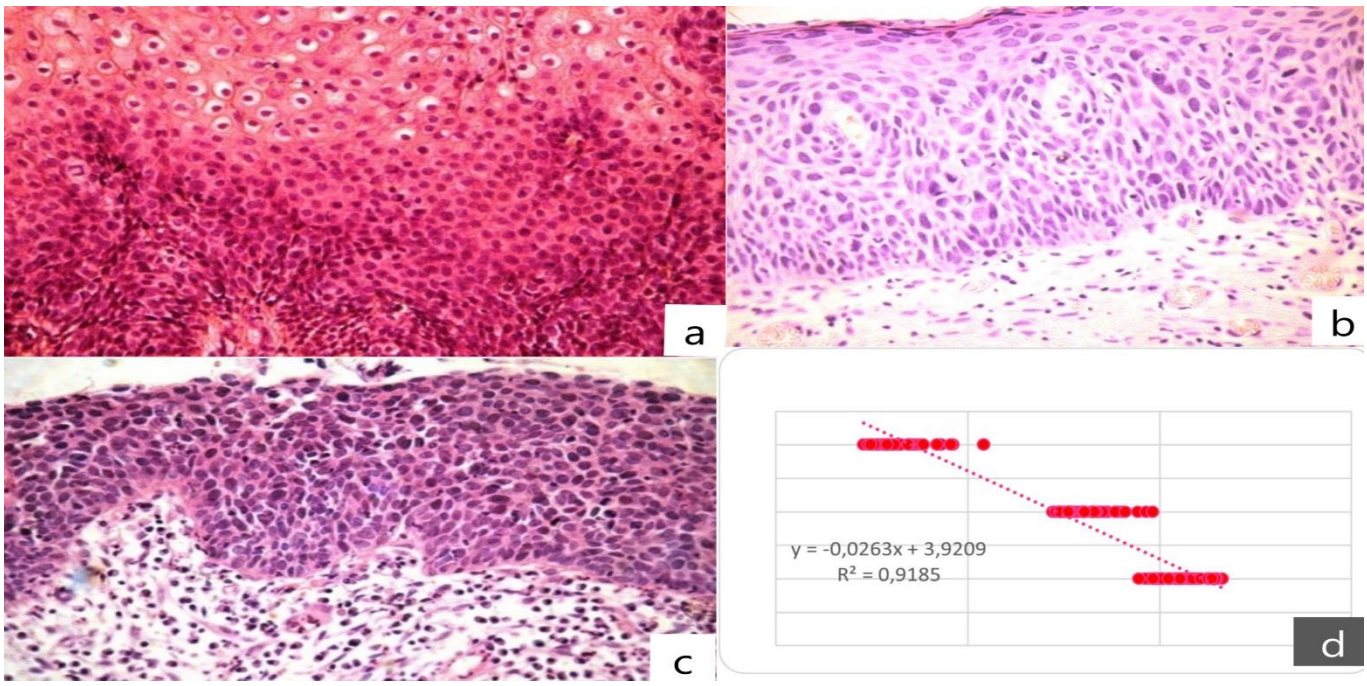

Figure 1. Cervical intraepithelial neoplasia (CIN), associated with papillomavirus infection in the infertile women: a – CIN of mild severity, b – CIN of moderate severity, c – severe CIN , d) connection between CIN severity and ratio of the multilayered squamous non-keratinized epithelium height to the volumetric density of capillaries of a mucous membrane. a-c – hematoxylin and eosin-stained section; original magnification x 20.

Immunohistochemical study of cervical tissue using the marker Ki-67 revealed a significant ($r = 0.96$, $p < 0.05$) increasing in expression according to an increase in severity of CIN. Analysis of the expression level showed that this marker contributed to a more complete determination of the severity, because the placement of Ki-67 positive cells corresponded to the general histological principle of CIN division. At the same time, at CIN variability of Ki-67 expression was noted. Thus, in the cervical biopsies of patients with CIN-I there was an increase in the intensity of binding of the proliferation marker of Ki-67 by epithelial cells of the basal and parabasal layers. This was characterized by a low level of expression and occupied no more than 1/3 of the of the integumentary layer. Also, a marker of cell proliferation was detected in the intermediate layer of the ectocervix, and it was also observed in the nuclei of superficial epitheliocytes in 4.8% of cases. Ki-67 was characterized by medium and high levels of expression with CIN-II (Figure 2).

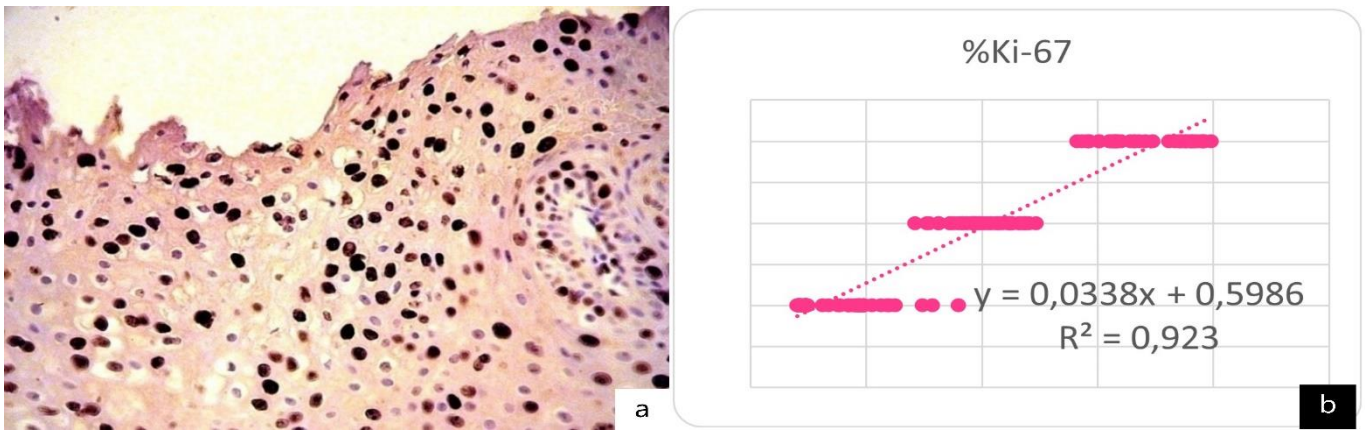

Figure 2. High level of Ki-67 expression with cervical intraepithelial neoplasia associated with papillomavirus infection of moderate severity. a - immunohistochemical study, original magnification x 20, b - connection between CIN severity and Ki-67 expression values.

Although the expression of Ki-67 was detected in the lower third of the epithelial layer in 13.2% of cases. Analysis by the method of χ2 - criterion revealed a significant effect of the severity of dysplasia on the proliferation index (χ2 = 9.48, p <0.05). The identified levels of oncoprotein p63 expression clearly correlated (r = 0.94, p <0.05) with the degree of CIN. The highest percentage (91.9%) with an expression level <30% was found in the group of patients with CIN-I. The expression level was 30-75% in 96.2% of cases with a moderate degree of CIN. The highest level (> 75%) of nuclear reaction was observed in 78.6% of patients with CIN-III (Figure 3). The results of the correlation analysis between the studied indicators showed the presence of a pronounced credible relationship between CIN and the ratio of MSNE / VDCMM (r = 0.95, p <0.05), the prevalence of Ki-67 expression in MSNE (r = 0.70, p <0.05), expression levels of Ki-67 (r = 0.96, p <0.05), p63 (r = 0.94, p <0.05). After a regression analysis, the regression equations of the dependence of the CIN severity on MSNE / VDCMM, Ki-67 and p63 were determined:

y = [-0,0263* X1+3,9209], coefficient of determination R^2 = 0,9185,

y = [0,0338* X2 + 0,5986], coefficient of determination R^2 = 0,9,

y = [0,03* X3 + 0,3088], coefficient of determination R^2 = 0,88,

when y – degree of CIN severity, X1 – value of MSNE / VDCMM,

X2 – value of Ki-67, %, X3 – value of p63, %.

A generalized regression model was created to determine the severity of CIN:

Y= [1,6094-0,00877*X1+0,011267*X2+0,01*X3]

The adequacy of the constructed mathematical model was proved by Fisher's criterion (F= 5123,064, F*=2,66, p=0,00000001). Coefficient of determination was R^2= 0,99.

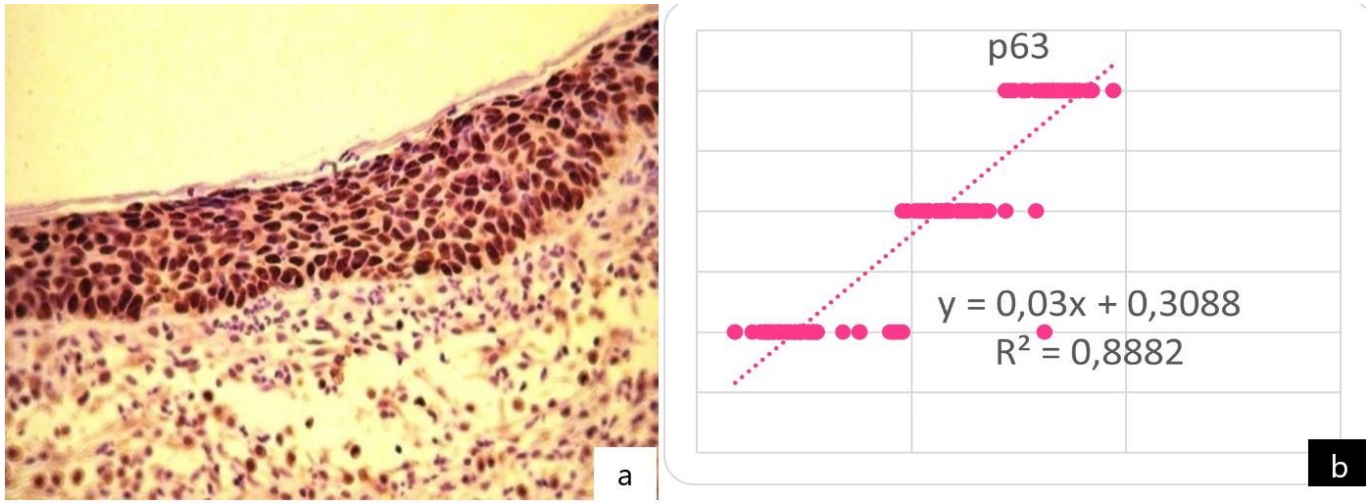

Figure 3. p63 hyperexpression in the cervical mucosa with positive reaction of cells groups in the cervical stroma with severe cervical intraepithelial neoplasia associated with papillomavirus infection. a - immunohistochemical study, original magnification x 20, b - connection between CIN severity and p63 expression values.

Conclusions.

The obtained morphometric data of the cervix with cervical intraepithelial neoplasia in women with infertility indicate changes in the epithelial-stromal relationship in the cervical mucosa. Disorders of the epithelial-stromal relationship especially in cases of severe cervical intraepithelial neoplasia may be one of the stages of carcinogenesis as evidenced by the ratio of multilayered squamous non-keratinized epithelium height to the volumetric density of capillaries of a mucous membrane and the expression of Ki-67 and p63.

The developed regression model which is based on the studied morphometric parameters reliably allows to determine the degree of CIN associated with papillomavirus infection in infertile women.

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