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IMMUNOHYSTOCHEMICAL MARKERS OF ENDOMETRIAL PROLIFERATION ACTIVITY IN PREMENOPAUSAL WOMEN WITH NORMAL MENSTRUAL CYCLE

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Immunohistochemical markers of proliferative activity, such as p21 and Cyclin D1 genes, E-cadherin, β-catenin, Ki-67 proteins, estradiol and progesterone receptors in the normal endometrium at the proliferative and secretory phase of the menstrual cycle in women have been studied. The dynamics of estradiol and progesterone receptor expression was determined, and it was proved that progesterone receptor expression begins from the first week of the menstrual cycle and is the highest in the glands of the proliferative endometrium. The expression of the Ki-67 proliferation marker is also the highest in the endometrial glands at the proliferation phase and is significantly and reliably reduced in the secretory endometrium. As for the expression of the Cyclin D1 and p21 genes, their feedback was found –at the proliferative phase the expression of the Cyclin D1 gene increased, but p21 was mostly negative, at the secretory phase the activity of Cyclin D1 decreased significantly, and there was a tendency to increase in p21 expression. E-cadherin protein expression increased significantly at the secretory phase of the menstrual cycle, but no significant differences were found in the dynamics of β-catenin expression. The determined parameters of receptor and proliferative activity of the endometrium in healthy premenopausal women can be used as basic information for further study of the endometrial proliferation state in its hyperplastic and neoplastic transformation.

Key words: endometrium, estradiol receptors, progesterone receptors, p21 gene, Cyclin D1, E-cadherin, β-catenin, Ki-67.

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ІМУНОГІСТОХІМІЧНІ МАРКЕРИ ПРОЛІФЕРАТИВНОЇ АКТИВНОСТІ ЕНДОМЕТРІЯ У ЖІНОК ЗІ ЗБЕРЕЖЕНИМ МЕНСТРУАЛЬНИМ ЦИКЛОМ В ПРЕМЕНОПАУЗІ

Проведено вивчення імуногістохімічних маркерів проліферативної активності, таких як ген p21 та Cyclin D1, протеїнів Е-кадгеріну, β-катеніну, Кі-67, рецепторів до естрадіолу та прогестерону в нормальному ендометрію проліферативної та секреторної фази менструального циклу жінок пременопаузального віку. Визначено динаміку експресії рецепторів до естрадіолу та прогестерону, та доведено, що експресія рецепторів до прогестерону починається з першого тижня менструального циклу та є найвищою в залозах проліферативного ендометрію.

Експресія маркера проліферації Кі-67 також є найвищою в залозах ендометрію фази проліферації та суттєво та достовірно знижується у секреторному ендометрії. Що до експресії гену Cyclin D1 та p21, то було знайдено їх зворотній зв'язок – в проліферативній фазі підвищувалась експресія гену Cyclin D1, але p21 був переважно негативним, в секреторній фазі значно знижувалась активність Cyclin D1, та була наявна тенденція до збільшення експресії p21. Експресія протеїну Е-кадгеріну суттєво зростала в секреторній фазі менструального циклу, але в динаміці експресії β-катеніну не знайдено достовірних відмінностей. Визначені параметри рецепторної та проліферативної активності ендометрію здорових жінок пременопаузального віку можуть бути застосовані як базова інформація для подальшого вивчення стану проліферації ендометрію при його гіперпластичній та неопластичній трансформації.

Ключові слова*:* ендометрій, рецептори до естрадіолу, рецептори до прогестерону, ген p21, Cyclin D1, Е-кадгерін, β-катеніну, Кі-67.

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The endometrium in women of reproductive age is one of the key anatomical elements of the reproductive organs, which biology is aimed at the implantation of a fertilized ovum. From the standpoint of modern science, the endometrium is a multicellular system with cyclic changes in its structure and function in the menstrual cycle under the influence of steroid hormones, expression of various receptors and genes, numerous critical proteins and growth factors integrated into certain intracellular signaling pathways, which interaction regulates proliferation and differentiation of cells [2].

In women in the transition period of life from reproduction to menopause, hormonal, genetic and epigenetic disorders in the regulation of the cell cycle often cause hyperproliferative and neoplastic pathology of the endometrium, which is given considerable attention in the scientific literature of the last decade [4, 5, 9, 15]. Meanwhile, the interpretation by various researchers of individual components in the complex system of the cell cycle molecular regulation in pathologically transformed endometrium is not always unambiguous [8, 11, 14] and quite contradictory, due to, in our opinion, lack of standards for endometrial phenotype in women with preserved menstrual function. in premenopause. This was the basis for our study.

The purpose of the study was to assess the expression dynamics of key biomarkers of endometrial proliferation and differentiation genetic and epigenetic regulation in the menstrual cycle in premenopausal women.

Materials and methods. The study was performed on the morphological endometrium material obtained by diagnostic biopsy in women with suspected hyperproliferative disease of the uterus in the gynecological department of the city clinical hospital No. 9, Dnipro, and gynecological department of Kyiv City Maternity Hospital No. 5 during 2014–2016. The design of the study and all the methods used in the study were approved by the Bioethics Committee of the above institutions and complied with the requirements of the Declaration of Helsinki.

Criteria for inclusion into the studied cohort of patients were: age of women 45–50 years, the presence of normal endometrial morphology according to the results of histological examination performed in the ISO 9001–2000 certified laboratory of pathomorphology and immunohistochemistry diagnostic center of Dnipropetrovsk Medical Academy, MOH of Ukraine, according to the standard method. Exclusion criteria were: the presence of pelvic inflammatory disease, tumor pathology of the uterus and ovaries, endometriosis of the uterus, severe somatic pathology, any form of endocrinopathy and metabolic syndrome.

For immunohistochemical (IHC) study, 40 samples of proliferative and 40 samples of secretory endometrium were selected (a total of 80 paraffin blocks). IHC studies of nuclear and cytosolic antigens were performed in paraffin sections of endometrial biological samples using monoclonal or polyclonal antibodies manufactured by "DakoCytomation" (Denmark), "TermoScientific" (USA) and using the "UltraVision LP" imaging system (LabVision) and LSAB2, EnVision (Dako) with the results interpretation according to standardized protocols [3].

The state of proliferation and differentiation in the studied tissues was assessed by the expression of their key molecular participants –receptors of estrogen (ERα) and progesterone (PGR), Cyclin D1 and p21 genes, Ki-67 nuclear antigen, E-cadherin and transmembrane glycoproteins E-cadherin and β-catenin. Because ERα and PGR are exclusively nuclear antigens, only the nuclear reaction was considered in the immunostaining assessment using its following ranges (at least in the 10 fields of view at × 400 magnification): negative reaction – 0-10 % of positively stained cells; positive reaction – more than 10 % of cells with immunohistochemical label. The averaged values of ERα and PGR (H-index) expression were calculated by a semi-quantitative method according to the formula: H=(% of unresponsive cells x 1)+(% of cells with moderate reaction x 2)+(% of cells with intense reaction x 3). Values from 0 to 50 points by the H-index were considered as the absence of of ERα and PGR expression, from 50 to 100 – the expression of antigens to receptors was considered weakly positive; from 100 to 300 points – positive [3]. The proliferation index (PI) based on the expression of Cyclin D1 and Ki-67 was calculated as the percentage of cells with an intranuclear reaction, regardless of the staining intensity, to the total number of cells based on the results of all studied areas in the endometrial samples [3]. Assessment of the p21, E-cadherin and β-catenin expression was performed by determining the percentage of IHC-positive cells to these antigens depending on of their staining intensity by the following criteria: no staining (no expression), weak staining (weak expression), moderate staining (moderate expression) and intense staining (significant expression).

Statistical processing of the results was performed using the licensed statistical software Statistica (version 6.1; Statsoft, USA) [1].

Results of the study and their discussion. The performed study proved that in the proliferative endometrium ER-positive were the majority of cells, both in the glands (83.8±5.1 %) and in the stroma (73±3.5 %). A significant number of ER-positive cells was found in the secretory endometrium – 74.8±3.9 % and 59.95±2.7 % in the glands and stroma, and the statistical difference between these indices at the first and second phase of the menstrual cycle was not detected ($p > 0.05$).

PGR expression was detected in 59.5±2.4 % of glandular cells and 58±2.2 % of stromal cells on the 5th–8th days of the menstrual cycle, and from the 9th to the 15th days the mean number of cells with a positive IGC response to this antigen increased by 89.2±4.8 % and 67.5±3.1 %, respectively. A significant number of PGR-positive cells remained throughout the secretory phase of the cycle, both in the glands – 83.3±4.1 %, and in the stroma – 63.7±1.9 %. But it should be noted that ER- and PGR-positive cells had a significant range of differences in the intensity of IHC staining from weakly positive to positive, therefore in our study a more indicative index became such integrated one as the H-index of sex hormone receptors' expression (fig. 1).

As it is shown in Fig. 1, ER-positive gland and stroma cells in the proliferative endometrium had higher H-index (193.1±12.2 and 166±9.7, respectively) compared to the secretory endometrium (125.4±5.7 and 122.4±4.8, respectively), and both in the glands and in the stroma, the differences between the

proliferative and secretory phases were significant ($p<0.05$). Unexpectedly, there was a relatively high expression of PGR by H-index in the glandular epithelium (193.2±8.5) and stroma (178.7±6.3) detected in the proliferative endometrium, which tended to decrease in the secretory endometrium (140, 2±4.4 and 116.6±3.1, respectively), but due to a significant difference in this index in the studied endometrial samples, this difference was not reliable.

The Cyclin D1 gene is considered to be a direct initiator of cell proliferation [13, 9]. In the studied endometrial samples, a positive IHC response to the Cyclin D1 antigen was observed in both glandular epithelial cells and stroma cells at the both phases of the menstrual cycle. However, if in the proliferative endometrium moderate expression (in the amount of 10–25 % of all the studied cells) of the Cyclin D1 gene in the glands is determined in 27 (67.5 %) out of 40 morphological samples, and in other 13 (32.5 %) cases only weak (single positive cells), in the secretory endometrium, on the contrary, moderate expression of Cyclin D1 was only detected in 9 (22.5 %) cases, and a much larger number of endometrial samples were with weak expression of this marker – 31 cases (77.5 %) (fig. 2a). Decreased expression of Cyclin D1 at the second phase of the menstrual cycle may be associated with the activation of the p21 gene, which belongs to the group of suppressor genes that control the state of cell proliferation [2].

According to our results, the expression of p21 antigen in the glands of the proliferative endometrium was absent in 27 (67.5 %) out of the 40 drugs studied, in 13 (32.5 %) cases it was weak (fig. 2b). It is noteworthy that in the secretory endometrium in 10 (25 %) of the studied drugs, i.e. in every fourth case, there was a moderate expression of p21, which was absent in the proliferative endometrium. Accordingly, the number of secretory endometrium samples with low expression of this antigen decreased slightly – in 8 (20 %) cases and its absence in 22 (55 %) cases. Dynamic change in the expression of the p21 gene occurs exclusively in the cells of the endometrial glands, in the structure of the stroma, the expression of Cyclin D1 and p21, was only found in single cells at the both phases of the cycle.

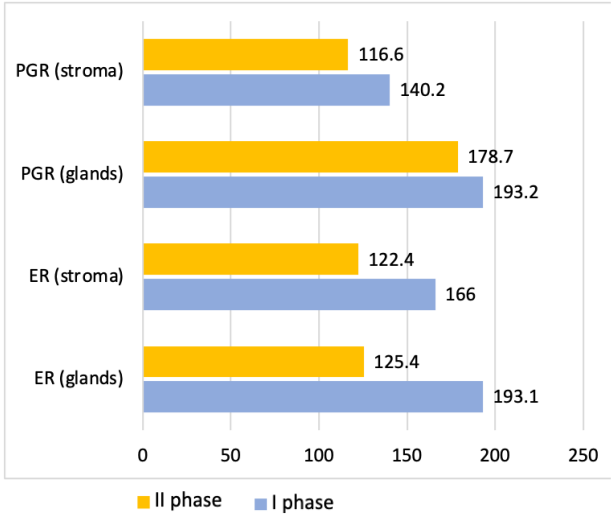

Fig.1. Expression of ER and PGR in cells of unaltered endometrium (phases I and II of the menstrual cycle), H-index.

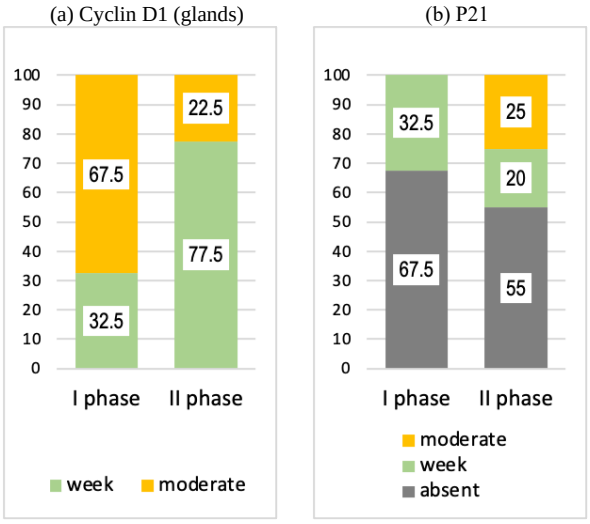

Fig.2. Indices of the IHC reaction intensity with Cyclin D1 (a) and) P21 (b) in cells of unaltered endometrium (phases I and II of the menstrual cycle), % of cells.

A more well-known marker of proliferation is the expression of the protein Ki-67, which is present at all active stages of the cell cycle (G1, S, G2, M). The study results of the Ki-67 protein expression in the proliferative endometrium of premenopausal women (fig. 3) showed a predominant activity of the cell cycle in the glands (57.8±3.6 %) than in the stroma (9.3±1.4 %; $p<0.05$).

In the secretory endometrium, the number of Ki-67 positive cells in the glands was not significant, but slightly less (41.6±2.5 %; $p>0.05$), while in stromal cells these fluctuations were not recorded (proliferative and secretory endometrium – 9.8±2.9 % and 9.3±1.4 % respectively; $p>0.05$). According to the data obtained, the Ki-67 antigen expression in the glands of the proliferative and secretory endometrium was, by 6.2 and 4.2 times, respectively, greater in number of cells detected than in the stroma, indicating the predominant proliferation of the endometrium glandular component.

Transmembrane glycoproteins E-cadherin and β-catenin play a key role in cells' signaling of their normal differentiation, maintaining normal adhesion in epithelial cells and inhibiting their proliferation by contact inhibition [14, 15]. A positive IHC reaction with E-cadherin in the glands was detected by us in all the studied endometrial samples.

In the proliferative endometrium in 6 (15 %) samples IHC-reaction with E-cadherin was weak, in the other 34 cases (85 %) – moderate (fig. 4a). In the secretory endometrium, the expression of E-cadherin

increased significantly, as evidenced by the detection of significant IHC staining in 15 (37.5 %) endometrial samples and preservation of a moderate IHC response in 25 (62.5 %). At the end of the menstrual cycle, in most of the samples studied, the expression of E-cadherin decreased to weak, which probably indicated a partial loss of adhesive cells by glandular cells and was one of the mechanisms for the endometrium functional layer rejection during menstruation.

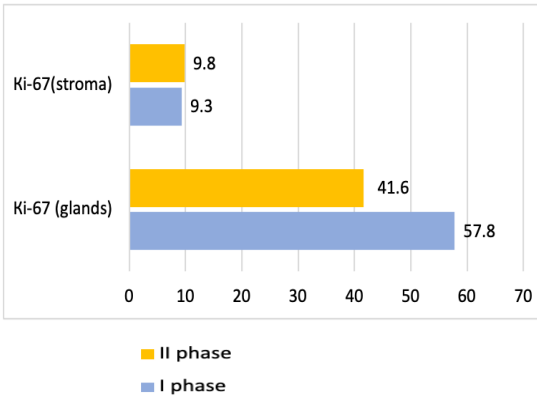

Fig.3. Indices of Ki-67 protein expression in cells of unaltered endometrium (phases I and II of the menstrual cycle), % of positive cells.

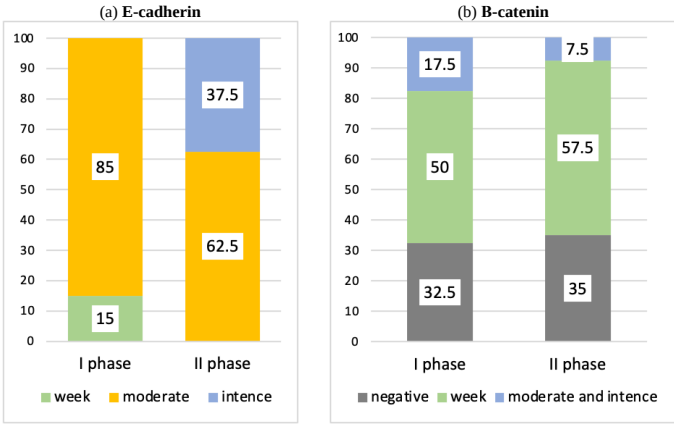

Fig.4. Intensity indices on the IHC reaction with E-cadherin (a) and β-catenin (b) glycoproteins in cells of unaltered endometrium (phases I and II of the menstrual cycle), % of cells.

No significant difference in the number of cells with the expression of β-catenin in the glandular epithelium in different phases of the menstrual cycle was detected. The number of tested samples with a negative IHC reaction to this antigen was almost the same in the proliferative (13 cases – 32.5 %) and secretory endometrium (14 cases – 35 %). According to the cycle phase, the IHC-reaction of β-catenin expression was weak in 20 (50 %) and 23 (57.5 %) samples, but there was moderate and intense expression of β-catenin in the proliferative endometrium – by 2.3 times more than in the studied samples (7 cases – 17.5 %) than in the secretory endometrium (3 cases – 7.5 %) (fig. 4b).

This difference in the qualitative nature of β-catenin expression in the proliferative and secretory endometrium may indicate its involvement in cell cycle regulation, as one of the main targets of ß-catenin is the Cyclin D1 transcription gene [8, 14].

It is known that cell proliferation in the reproductive organs is associated mainly with the influence of sex hormones on the genome mediated by interaction with nuclear ER and PGR, which state of expression depends on the signal response formation in the form of cell cycle activation [2, 4, 10]. The dominant receptor to estrogen in the endometrium is ERα. ERα-binding estrogen is a driver of proliferation, while progesterone, which acts through progesterone PGR receptors, counteracts proliferation by inducing differentiation involving, E-cadherin and Wnt / β-catenin signaling [6, 14].

We were the first to show that the promoter effect of the estrogen-ER complex on cell proliferation in the endometrium coincides in time with the expression level of the Cyclin D1 gene, which is directly associated with cell cycle activation, and the level of progesterone receptor expression – with p21 gene activation in the secretory endometrium.

These genes can be considered part of a central cell cycle control system that regulates DNA replication and segregation, as well as cell distribution. It should be emphasized that despite the existing opinion that the expression of PGR in endometrial cells rises mainly under the influence of endogenous estradiol, which level grows significantly closer to ovulation [4, 7], in our study it was shown that significant expression of PGR was observed on the first week of the menstrual cycle, which gives reason to believe that the expression of receptors for steroid hormones is a deterministic function of the cell genome.

Because ERα is essentially a transcription factor, while PGR function is associated with the activation of cell differentiation genes [13], it can be assumed that cell proliferation and differentiation occurs in both phases of the menstrual cycle, as evidenced by sufficient expression levels of Ki-67 proliferation markers and E-cadherin and β-catenin differentiation markers in both proliferative and secretory endometrium.

According to the study performed, one of the important characteristics of the endometrial phenotype in premenopausal women is the expression of E-cadherin and β-catenin glycoproteins, which are involved in signaling cells for their normal differentiation. The transmembrane glycoproteins E-cadherin and β-catenin play a key role in maintaining normal adhesion in epithelial cells and inhibiting cell proliferation by contact inhibition, as it has been proved that proliferation mechanisms are excluded by

close cell contact [5, 13]. Thus, detection of the E-cadherin and β-catenin expression in the endometrium may be a signal to determine the normal differentiation state of its cells.

E-cadherin (epithelial cadherin) encodes E-cadherin-glycoprotein, which consists of an extracellular domain, a cytoplasmic domain and a single transmembrane domain [12, 13]. The extracellular domain reaches the cell surface to bind to the cadherins of neighboring cells by lateral dimerization. This leads to the formation of a cadherin-cadherin bond and, as a result, – to intercellular adhesion. The cytoplasmic domain consists of a perimembrane domain and a catenin-binding domain that interacts with β-catenin, which promotes protein clustering and stabilizes cell adhesion.

Cytoplasmic β-catenin level controls cell adhesion and E- cadherin content. In normal cells, much of the β-catenin is found in the cell membrane in cadherin-related form, but it is believed that E-cadherin can control cell growth indirectly by isolating β-catenin in the adhesive complex and thus limiting the activity of growth initiating factors.

It has also been proved that one of the main targets of ß-catenin is the Cyclin D1 gene, and as a consequence, this leads to the activation of cyclin-dependent kinases (Cdk), responsible for the transition of the cell cycle from phase G1 to phase S, i.e. to cell proliferation [5, 13]. It is possible that the so-called Wnt/β-catenin signaling pathway maintains the balance between estrogen-induced proliferation and progesterone-induced differentiation in the endometrium.

Conclusion

The study results permitted to formulate the main characteristics of the endometrium phenotype in women with a preserved menstrual cycle in premenopause. The main one is the preservation of key molecular mechanisms, the so-called "estrogen-dependent signaling pathway of cell proliferation", as evidenced by the high level of ER and PGR expression in the glandular epithelium and stroma throughout the menstrual cycle, and the manifestation of the Cyclin D1 gene and the p21 gene expression level, which control the cellular transition from the G0-phase of the cell cycle to the S-phase, Ki-67 nuclear antigen, which belongs to the regulatory proteins of the G1-M phase of the cell cycle, E-cadherin and β-catenin, glycoproteins which are involved in signaling cells for their normal differentiation.

The information obtained in the performed study on the epigenetic proliferative profile of the endometrium in women with the preserved menstrual function in premenopause can be used as a basis for determining the phenotype of the transformed endometrium in premenopausal women.

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