

IMMUNOHISTOCHEMICAL PATTERN OF PROTEIN P21, CYCLIN D1 AND CYCLIN E IN ENDOMETRIAL HYPERPLASIA

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Purpose: In our research we investigated immunohistochemical expression of cell cycle proteins protein p21, cyclin D1 and cyclin E in physiological endometrium (n = 15), hyperplastic endometrium (n = 61), and post hormone replacement therapy endometrium (n = 24).

Material and methods: We performed immunohistochemical analysis of selected cell cycle proteins in 100 specimens of human endometrium.

Results: The average immunoexpression index scores in glandular endometrial cells (GES) and stromal endometrial cells (SEC) were respectively: for p21- GES: $11.8 \pm 17.19\%$; SEC: $9.31 \pm 17.15\%$; for cyclin D1- GES: $9.25 \pm 18.41\%$; SEC: $3.22 \pm 11.46\%$; for cyclin E: GES: $26.42 \pm 27.47\%$; SEC: $4.61 \pm 7.90\%$. Statistical analysis disclosed more intense p21 glandular immunoreactivity among women with endometrial hyperplasia in comparison to other subpopulations. In the case of assessment of cyclin D1 immunoreactivity, there was no statistical correlation between analysed parameters. The average cyclin E immunoreactivity in endometrial glandular cells was significantly higher ($p = 0.003$) in women with endometrial hyperplasia and correlated with age.

Conclusions: Intensive immunoreactivity of cyclin E in glandular cells is typical for endometrial hyperplasia and can be treated as an objective indicator of this pathological process during histopathological diagnostic procedures. Immunoreactivity index of p21 and cyclin D1 is independent of the morphological pattern of human endometrium, patients' age and gynaecological history of patients.

Key words: carcinogenesis, cell cycle, endometrium, HRT, hyperplasia, neoplasia, regulatory proteins.

Introduction

Human endometrium is a special tissue of great proliferative and regenerative potential. This ability is the result of ovarian and pituitary hormone impact as well as central nervous system stimulation. These described factors cause changes typical for hormonal, two-phase endometrium with its breakdown during menstruation.

Endometrial proliferation rearrangements caused by prolonged oestrogen stimulation compose a wide range of abnormalities with a corresponding variety of morphological patterns. We can detect several types of pathological proliferation beginning with the ones slightly different from the late

proliferative phase endometrium to the complex types, difficult to differentiate from carcinoma [1]. The discussion over pathogenesis of adenocarcinoma of endometrium is still ongoing. The most probable hypothesis of endometrial cancer aetiology is based on the prolonged oestrogen stimulation of endometrium of genetically prone women. Endometrial hyperplasia is considered as possibly one of the stages of carcinogenesis [2-4]. It is worth pointing out that several cases of endometrial hyperplasia may persist unchanged for an unpredicted period of time or undergo regression either spontaneously or under the influence of exogenous progestagens. On

the other hand, endometrial cancer may develop on the base of atrophic endometrium without a hyperplastic tendency [5-7].

When discussing pathologies of endometrial proliferation the value of immunohistochemical markers is worth emphasizing. The discovery of these markers enabled us to diagnose precancerous alterations in the preclinical stage.

We can also use cell cycle proteins as markers of morphologically latent neoplastic transformation [7, 13].

The proteins of the cell cycle pathway play the role of several enzymes such as kinases, phosphatases and other regulatory proteins responsible for activation or deactivation of the latter [11]. The proteins may react chemically with each other or simply make together different complexes influencing the metabolism of a cell.

The studies carried out so far indicate that there are three main ingredients of the cell cycle: cyclins, cyclin-dependent kinases (CDK) and inhibitors of CDK (CKI). Cyclin inhibitors are the youngest identified group and consist of the family of p21 proteins (p27 and p57) and the family of INK4 proteins (p15, p16, p18, p19). The described proteins react with the cyclin-CDK complex and at the same time prevent the continuation of further cell cycle processes.

During neoplastic transformation, over-synthesis and hyperreactivity of cell cycle process promoting factors take place together with inactivation or lack of synthesis of cell cycle inhibitors [11, 12]. Oscillation of cell cycle protein levels throughout the phases of active proliferation and differentiation is characteristic for cells of human endometrium [13]. Endometrial adenocarcinoma is nowadays the most common malignant tumour of the genital tract among the populations of developed countries. Aberrations of cell cycle protein activity may initiate an independent, incidental process of proliferation that can constitute the key factor of pathogenesis of endometrial adenocarcinoma [11, 14, 15]. However, the studies conducted so far using molecular biology techniques and immunohistochemical methods have not revealed any clear results.

The aims of the current study were:

1. To investigate immunohistochemical expression of cyclin D1, cyclin E and protein p21 in samples of endometrium received from women with physiological endometrium, with hyperplastic endometrium and from patients who underwent hormone replacement therapy (HRT),
2. To analyse the utility of immunoexpression of studied proteins as objective markers of pathological, endometrial proliferation.

Material and methods

We studied one hundred paraffin-embedded endometrial tissue samples retrospectively selected from the files of the Department of Pathology of the Medical University of Lodz in Poland after acquiring the approval of an independent Ethics Committee and the formal consent of patients from whom the material came. Material was obtained by curettage carried out because of metrorrhagia or abnormal picture of endometrium during ultrasonographic examination.

Formalin fixation (4%), the process of embedding and haematoxylin-eosin staining were performed routinely. We examined haematoxylin-eosin stained samples with an Olympus BX/41 light microscope. The standing classification we used when making histopathological diagnoses was accepted by WHO in 1994.

In the current study, we investigated pRb, p21, cyclin D1 and cyclin E immunohistochemical expression using the DAKO EnVision detecting system. The staining was performed using the immunoperoxidase method with the primary monoclonal antibody Anti-Human Retinoblastoma Gene Product by DAKO, Anti-Human p21 (WAF/CIP1) by DAKO and anti-cyclin D1 and E by Novocastra. The slides were cut at 4 μ m. After applying to the adhesion glass and drying for 24 hours at a temperature of 56°C, samples were deparaffinized in xylene and rehydrated through graded alcohols (96%, 80%, 70%, 60%). The activity of endogenous peroxidase was inhibited by 5 min incubation with 3% solution of perhydrol in methanol. Epitope retrieval of pRb and p21 in cellular nuclei was performed on all slides using DAKO Target Retrieval Solution. The samples were heated for 30 min at a temperature of 95°C. The samples investigated for cyclin D1 expression were boiled with 0.001 M versenate buffer (EDTA) of pH 8.0, using power of 650 W for 20 min; for investigation of cyclin E expression, the samples were heated in a microwave oven 6 times with the 0.01 M citrate buffer of pH 6.0 using the following power levels: 150 W (5 min), 350 W (5 min), 450 W (5 min), 650 W (3 \times 2 min). Secondly, after cooling, all the sections were washed for 5 min with 0.05 M TBS buffer (pH = 7.6). Then the particular sections were incubated at room temperature for 30 min with: Anti-Human Retinoblastoma Gene Product (1 : 100 dilution), Anti-Human p21 (1 : 50 dilution), cyclin D1 (1 : 20), cyclin E (1 : 80). The slides were washed twice with TBS buffer. In order to visualize the reaction between antigen and antibody, a two-stage system of visualization (DAKO EnVision) was used. The first phase of the process

was based on 30 min incubation with a polymer marked with peroxidase and attaching goat anti-anti-pRb immunoglobulins. Eventually, the signal was detected by 3'3-diaminobenzidine as the chromogen for peroxidase activity and Meyer's haematoxylin as the counterstain. The sections were then dehydrated by graded alcohols (70%, 80%, 96%). After passing through solutions of acetones and xylenes, the respective sections were closed with mounting medium DPX.

The negative control was established by sections in which anti-pRb immunoglobulins were replaced by TBS buffer. The further steps of the staining procedure were exactly the same as above.

The positive control was created by sections of nodular thyroid goitre that earlier expressed strong pRb immunoreactivity.

The characteristics of applied antibody are shown in Table I.

Cell cycle proteins' immunoexpression in collected endometrial specimens was estimated using the computer program MultiScan. The computer system consisted of an IBM computer with Pentium processor and card of picture digitalisation by ADDA, colour TV camera by Panasonic and light microscope (Jenaval made in Germany). We used 250 × magnification of light microscope to assess collected sections.

The cell cycle expression was evaluated by a quantitative method. Each time we described the percentage of immunopositive cells among 1000 glandular cells and 1000 stromal cells. Therefore, cell cycle immunoreactivity index score is expressed in percent.

Results

One hundred cases were retrospectively selected from the files of the Department of Pathology at the Medical University of Lodz in Poland. For immunohistochemical analysis we used paraffin-

embedded samples of endometrium, obtained by diagnostic curettage. There were 61 cases of histopathologically diagnosed endometrial hyperplasia without atypia (55 cases of simple hyperplasia, 6 cases of complex hyperplasia) and 24 cases of endometrial changes typical for HRT. The control group consisted of 15 cases of normal endometrium, including 6 samples of proliferative endometrium and 9 samples of endometrium in the secretory phase.

When dividing into several subgroups we took into consideration the following clinical data: age (the age of 49 was treated as the average age of menopause among Polish women), number of past pregnancies, deliveries and miscarriages.

The control group consisted of women at the average age of 46.5 (range 38-55). The number of pregnancies was in the range 0-4, deliveries 0-3 and miscarriages 0-2.

The investigated population who underwent hormone replacement therapy comprised 24 women (40-73 years old), with the history of 0-5 pregnancies, 0-3 deliveries and 0-2 miscarriages.

In the group of patients with endometrial hyperplasia we gathered women at the average age of 52.5 (wide range 24-81). The number of pregnancies was in the range 0-10, deliveries 0-8, miscarriages 0-2.

In the table II we depict the results of our immunohistochemical analysis.

During the statistical analysis it appeared that among women with endometrial hyperplasia no older than 49 the intensity of p21 immunostaining of glandular and stromal cells demonstrated strong statistical tendency ($p = 0.057$).

The average cyclin E immunoreactivity in endometrial glandular cells is significantly higher ($p = 0.003$) among women with endometrial hyperplasia. In the population of women with endometrial hyperplasia we also observed a correlation between cyclin E immunoreactivity and patients' age. The statistical analysis we carried out

Table I. Characteristics of used antibodies

ANTIGEN	PRB	P21	CYCLIN D1	CYCLIN E
Producer	DAKO Corporation USA	DAKO Corporation USA	Novocastra UK	Novocastra UK
Type of serum	mice	mice	mice	mice
Clone	Rb1F8	SX118	P2D11F11	13A3
Isotype	IgG1, κ	IgG1, κ	IgG2a	IgG2a, κ
Dilution	1 : 100	1 : 50	1 : 20	1 : 80
Epitope retrieval	microwaves	microwaves	microwaves	microwaves
Type of expression	nuclear	nuclear	nuclear	nuclear
Positive control (tissue)	nodular thyroid goitre	nodular thyroid goitre	breast cancer	placenta

Table II. The arithmetic averages of protein p21, cyclin D1 and cyclin E immunexpression index score in nuclei of glandular and stromal cells of endometrial epithelium together with their standard deviations

Type of examined cells	PROTEIN p21			CYCLIN D1			CYCLIN E					
	glandular cells	stromal cells	stromal cells	glandular cells	stromal cells	stromal cells	glandular cells	stromal cells	stromal cells			
	≤ 49	> 49	≤ 49	≤ 49	> 49	≤ 49	> 49	≤ 49	> 49			
Age												
Control group (%)	3.33 ± 3.97	6.96 ± 4.73	0.36 ± 0.41	5.78 ± 13.67	1.33 ± 1.76	0.13 ± 0.05	0.3 ± 0.44	11.65 ± 11.91	11.38 ± 19.37	3.21 ± 5.82	6.78 ± 8.07	
Endometrial hyperplasia (%)	20.63 ± 24.93	9.72 ± 8.87	10.73 ± 21.15	11.59 ± 23.69	14.3 ± 26.22	0.39 ± 1.1	0.1 ± 0.03	51.5 ± 33.69	21.42 ± 17.55	5.16 ± 7.8	3.78 ± 8.01	
HRT (%)	11.42 ± 17.47	19.17 ± 26.15	11.42 ± 17.47	19.85 ± 26.05	7.71 ± 13.06	22.28 ± 32.07	12.4 ± 24.4	14.57 ± 26.12	11.14 ± 13.79	12.8 ± 11.74	0.9 ± 1.7	0.51 ± 0.71
Statistical significance - p			0.057									0.003

demonstrated that cyclin E immunoreactivity in endometrial glandular cells is far more intense in women under 49 than older ($p = 0.064$).

We did not find any statistically significant correlation between patients' parity and other features of examined subgroups. That is why we decided to omit such trait as parity in Table II to make it more clear.

Discussion

Anomalies of cell cycle control are nowadays stated to be the main cause of carcinogenesis [16, 17]. Cell cycle regulating proteins decide about setting off that is overpassing the restriction point (R). Physiologically, a cell passes through its restriction point when it is free of any defects, especially genetic ones. Passing through the restriction point is at the same time a potential risk of neoplastic proliferation. Point R divides the cell cycle into the stage dependent on growth factors and on the late G1 phase. It means that after passing through the restriction point, even if the previous mutagens do not influence the proliferation process any more, the considered cell will continue its proliferation [18-21]. In the case of most malignant tumours the function of the following genes is disturbed: RB, CCND1, p16 or CDK4. The mutations of genes responsible for encoding cyclin A and E and CDK-inhibitors such as p21, p27, and p57 are seldom, but they can incite carcinogenesis as a consequence of altered post-transcription preparation [22].

It has been proved that in most neoplastic tumours not only are the cell cycle stimulating factors of increased expression but inhibitors as well. It results in sooner passing of the restriction point and therefore enhanced cellular proliferation [23, 24].

Evaluation of cell cycle proteins using immunohistochemistry may be applied to research on cellular neoplastic proliferation tendency [25].

Protein p21

The gene encoding p21 protein (CDKN1) is located on chromosome 6 in the region of 6p21.2 [26]. The main function of this antiproliferative protein is blocking of cyclin/CDK enzymatic complex and consequently inhibition of the phosphorylation process of Rb protein in response to physical and chemical damage to the genome [27]. Protein p21 has an inhibiting impact on promoter regions of E2F factor dependent genes [28].

There are discrepancies in dependence between p21 cellular reactivity, cancer invasion and clinical

stage [30, 31]. The state of increased p21 immunoreactivity in hyperplastic tissues in comparison to physiological tissues is more frequently mentioned in the available literature. In our research less intense p21 immunoreactivity was elucidated in glandular endometrial cells obtained from post-HRT, over 49-year old women. On the other hand, the most intense p21 immunoreactivity was revealed in glandular endometrial cells from women not older than 49, with at least one labour in the past. It is worth pointing out that in the case of nulliparas under 49 with endometrial hyperplasia the amount of p21 in glandular cells was relatively high. Statistical analysis disclosed more intense p21 glandular immunoreactivity in the case of women with endometrial hyperplasia (not older than 49) in comparison to the control group and the post-HRT population. The lowest p21 immunoreactivity was detected in physiological stromal endometrial cells in the population of women not older than 49 with at least one labour in the past. Whereas the most intense immunoreaction for p21 in stromal cells appeared in the group of post-HRT women over 49 years old, who had delivered at least one child. Statistical analysis of our data did not reveal any considerable correlations between p21 immunoreactivity, endometrial pattern, patients' age and their obstetrical history.

It is well known that p53 protein is responsible for discontinuation of cellular proliferation partly by affecting genes of restriction point control. Considering the fact that p21 is a target protein of p53, cells lacking p21 may demonstrate deficiency of properly functioning p53 [32].

Palazzo *et al.* observed p21 immunoreactivity in completely differentiated cells of physiological human endometrium and endocervix [29]. Passive glandular and stromal cells of endometrium displayed varied p21 immunoreaction. Regular glandules with groups of cells positively stained for p21 bordered cells with p21 negative reaction. Immunoreactivity of p21 decreased in cells of proliferative endometrium. This effect could be monitored only in the case of several cells. Taking into account p21 ability of inhibition of cyclin-CDK complex we can elucidate that p21 plays an important role in restriction of endometrial proliferation. However, lack of p21 in completely differentiated cells suggests contribution of other factors in regulation of the cell cycle in human endometrium and endocervix. It is possible that p21 reactivity in examined cells is not high enough to be detected by immunohistochemical staining methods. In the case of endometrial and endocervical cancer, p21 immunoreaction is local and limited to small populations of cells. The presence of p21 immuno-

reactive cells in samples of cancers may be the proof for existence of non-proliferating subpopulations of tumour cells. The cells with negative immunoreaction for p21 demonstrate immunoreactivity for Ki-67, which can prove their increased mitotic activity. The positive immunoreaction for p21 in completely differentiated endometrial cells is frequent. Its immunoreaction negatively correlates with mitotic activity in endometrial hyperplasia and cancers, which is confirmed by the Ki-67 antigen reaction. In view of endometrial hyperplasia, endometrial and endocervical cancer, p21 immunoreaction is reduced and depicted only in small groups of cells. The loss of p21 expression is an argument for a more aggressive type of tumour. It can be approved that p21 inhibits the process of carcinogenesis.

Cyclin D1

Proto-oncogene encoding cyclin D1 (CCND1/PRAD1) is located on chromosome 11 in the region of 11q13 [26]. During observation of progression of physiological endometrium up to complex hyperplasia, the amount of cyclin D1 increases gradually regarding intensity of staining reaction and number of stained cells. There was no difference observed in immunoreactivity between complex hyperplasia and endometrial cancer. That is why the authors assume that dysregulation of cellular proliferation reaches a peak at the stage of hyperplasia. Biological differences between complex hyperplasia and endometrial cancer probably result from the influence of other factors and processes. Such a pattern of immunoreactivity may indicate that intense cyclin D1 staining reaction reflects an early stage of carcinogenesis. Data gathered by the authors support the thesis concerning complex hyperplasia as a precancerous phase.

In our research we decided to combine endometrial simplex hyperplasia and endometrial complex hyperplasia in one group for the reason that the repeatability of distinguishing these two histopathological patterns is very low. There is a need for further research on the role of cyclin D1 as a biomarker simplifying the diagnosis of EIN, a pathology requiring operative treatment. At the same time we should answer the question whether cyclin D1 immunoreaction would facilitate the diagnosis of disorders which react to hormonal treatment and regress after progestagen therapy. It was discovered that in some cases the results of the immunohistochemical reaction can enable identification of local areas with increased amount of cyclin D1, which are the areas

with already malignantly changed endometrium. These pathologically changed areas of endometrium cannot be classified using only standard morphological criteria. Therefore, cyclin D1 can become a practical biomarker in gynaecological oncology [33].

In our study we did not reveal any statistically significant correlation between cyclin D1 activity in glandular endometrial nuclei, morphological pattern of endometrium, patients' age and their parity. Taking into consideration stromal endometrial nuclei, statistical analysis did not disclose any significant association between cyclin D1 immunohistochemical reactivity, endometrial morphology, women's age and their parity either.

The role of cyclin D1 in endometrial carcinogenesis has not been elucidated so far.

Cyclin E

The proto-oncogene encoding cyclin E (CCNE) is located on chromosome 19, in the region of 19q12-19q13 [26]. The product of its expression is a 48 kDa protein comprising 250 amino acids [34]. The complex of cyclin E/CDK2 is essential for the elongation phase of the DNA replication process [35] and for the phosphorylation of Rb protein [36].

The key results of our research are the assessments of cyclin E immunoreactivity in human endometrium. In our investigation we assessed considerably more intense cyclin E immunoreactivity ($p = 0.003$) in hyperplastic endometrium. Furthermore, in the group of women with hyperplastic endometrium we observed a correlation between cyclin E reactivity and patients' age. It arises from the statistical analysis that cyclin E immunoreactivity in hyperplastic endometrium is clearly more intense in women below 49 ($p = 0.064$). Whereas we did not find a significant correlation between the amount of cyclin E in stromal endometrial cells, endometrial morphology, patients' age and data from their obstetrical history. In accordance with data available in PubMed, our observation was based on examination of the most numerous population.

Taking into consideration endometrial cancer, Kato *et al.* observed a correlation between high intensity of cyclin E immunoreactivity and neoplastic invasion of endometrial muscle and lymphatic vessels [37]. There was no such association with appearance of endometrial hyperplasia, existence of progesterone or oestrogen receptors, or postmenopausal character of endometrial morphology. In view of data collected by Milde-Langosh, intensive cyclin

E reactivity of the tissue accompanied low level of oestrogen and progesterone receptor expression [38]. Kim *et al.* retrospectively evaluated cyclin E immunoreactivity of breast cancer cells with the frequency of cancer recurrence. They demonstrated that high expression of this cell cycle protein in breast cancer may help to predict future recurrence [39]. Keyomarsi demonstrated that cellular concentration of cyclin E in breast cancer assessed by Western-blot analysis strictly correlated with the survival rate [40]. Patients with low levels of cyclin E showed remarkably longer survival rate independently from appearance of metastases in lymphatic nodes. All patients with low level of cyclin E survived 5 years after initial diagnosis. That is why cyclin E can be treated as an important prognostic factor. Sutherland claims that cyclin E is a more specific and sensitive factor than any other discovered and used so far. Not the presence of metastases but the high intensity of cyclin E immunoreactivity determines the prognosis and helps in making the decision about the method of treatment to apply [41].

Many studies have confirmed the prognostic value of cyclin E, especially important in histopathological diagnostics. Evaluation of increased cyclin E cellular immunoreactivity frequently correlates with clinical stage of the cancer, its aggressiveness, presence of lymphatic metastases, poor differentiation and higher mortality rate. The usefulness of cyclin E as a diagnostic marker is unquestionable. There is a possibility that cyclin E immunoexpression can play an important role as a diagnostic and prognostic factor during diagnosis of precancerous changes in human endometrium.

Conclusions

Immunoreactivity of cell cycle regulatory proteins p21, cyclin D1 and cyclin E evaluated in samples of physiological and hyperplastic endometrium and endometrium treated with hormone replacement therapy is different considering not only glandular cells of endometrium but stromal endometrial cells as well. Intensive cellular immunoreactivity of cyclin E is typical for endometrial hyperplasia and can be treated as an objective indicator of this pathological process during the microscopic diagnostic procedure of endometrial disturbances. The values of immunoreactivity index of p21 and cyclin D1 do not depend on the morphological pattern of human endometrium, patient's age and data from gynaecological and obstetrical history of patients. Considering the results of the immunohistochemical staining process for p21 and

cyclin D1 activity this method is not sensitive or specific enough to be treated as significant during diagnostic procedures of endometrial pathologies.

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