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Evaluation of Retinoblastoma Protein Expression in Endometrial Hyperplasia

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In our study we investigated immunohistochemical expression of retinoblastoma protein (pRb) in physiological endometrium (n=15), hyperplastic endometrium (n=61) and post-hormone replacement therapy (HRT) endometrium (n=24).

Methods: We performed an immunohistochemical analysis of pRb expression in 100 specimens of human endometrium.

Results: The average pRb immunoexpression index score in glandular endometrial cells and stromal endometrial cells were 57,699 and 21,191 respectively. The less intense pRb immunostaining for the glandular cells was displayed in women over 49 years old. The most intense staining effect of glandular cells was observed among patients with physiological endometrium, no older than 49 years of life. There was no statistically significant correlation between the obtained pRb immunoexpression and clinical data of the examined patients.

Conclusions: We conclude that immunohistochemistry is not sensitive and specific enough to reveal pRb alterations resulting in endometrium pathologies. Alternatively, pathological rearrangements of endometrium may be independent from the accuracy of the pRb control over restriction point in cell-cycle pathway.

Introduction

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

Menstruation is not always the effect of preceding ovulation. This kind of bleeding occurs without the control of corpus luteum or the hormone it releases, progesterone. Such a situation usually happens at the time of puberty or menopause, when endometrial proliferation results from a prolonged hormonal effect exerted only by estrogens. We can observe those abnormalities usually in hormonal disorders, during uncontrolled synthetic or natural estrogens treatment without progesterone balance.

Endometrial proliferation rearrangements caused by prolonged estrogens stimulation include a wide range of abnormalities with a relevant 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 [6]. The discussion over pathogenesis of adenocarcinoma of endometrium is still in progress. The most probable hypothesis of endometrial cancer etiology is based on the prolonged estrogens stimulation of endometrium in genetically-prone women. Endometrial hyperplasia is eventually considered as one of the stages of carcinogenesis [2, 3, 25]. It is worth pointing out that several cases of endometrial hyperplasia may persist unchanged for an unpredicted period of time, or undergo regression, either spontaneous or under the influence of exogenous progestagens. On the other hand, endometrial cancer may arise from atrophic endometrium without hyperplastic tendencies [24].

Because of the arguments mentioned above, in the eighties of the 20th century, some scientists questioned the concept of a continuous sequence of neoplastic changes. They believed there are two different, biologically independent pathologies of endometrium: hyperplasia and neoplasia [29]. Simultaneously with the evolution of opinions on the endometrial cancer pathogenesis, there were a few ideas how to classify clinical stages of endometrial hyperplasia. The aim of the developed classification would be identification of a group of patients most prone to endometrial cancer development.

The standard classification of endometrial hyperplasia dates from 1994, was established by International Society of Gynecological Pathologists and accepted by World Health Organization (WHO). It is based on standardization

of a diagram, according to which, while diagnosing, rearrangements of tissue structure and cytological disorders may be examined independently.

The classification included:

1. Simple hyperplasia without atypia (SH).
2. Simple hyperplasia with atypia (SAH).
3. Complex hyperplasia without atypia (CH).
4. Complex hyperplasia with atypia (CAH).

In order to observe subsequent stages of carcinogenesis, Kurman and colleagues analyzed material obtained from patients who were managed without a hysterectomy for at least one year after the primary diagnosis of hyperplasia. An increased risk of cancer development was seen in 1% of patients with simple hyperplasia, 3% with complex hyperplasia, 8% with atypical simple hyperplasia, and 29% with atypical complex hyperplasia [25]. According to Baak, the risk equaled respectively 0%, 17%, 7% and 45% [1]. Severe atypical hyperplasia is described as carcinoma in situ; however, this term is controversial.

A histopatological diagnosis of endometrial hyperplasia, especially atypical, is demanding. Overdiagnosing in those cases is pretty frequent [22].

While using the WHO classification, there are difficulties in differentiating simple hyperplasia without atypia from complex hyperplasia without atypia. If the treatment of both disorders of endometrium is the same (because of a low risk of neoplastic transformation), it may be advantageous to stop separating atypical hyperplasia into simple and complex forms. It is logical to combine these two disorders into one nosological category. The calculated risk of transformation of atypical hyperplasia into malignant neoplasm equals 15-45% [12, 23]. That is why we can treat both groups as an early stage of carcinogenesis and classify them as a single pathology.

Taking into consideration the above arguments, a simplified classification suggested by European Group of Experts in Gynecological Pathology in 1999 seems very logical and convenient.

The classification includes two categories:

1. Endometrial hyperplasia (EH),
2. Endometrial neoplasia (EN).

Mutter, together with Endometrial Collaborative Group, also established a two-class system and divided pathological proliferation into:

1. Endometrial hyperplasia (EH),
2. Endometrial intraepithelial neoplasia (EIN).

The first group includes cases of endometrial hyperplasia without cellular atypia, which is benign and does not demand invasive treatment. The second group includes all the cases of hyperplasia with cellular atypia, where surgery is absolutely necessary.

Based on the findings of morphometric and clonal investigations, as well as examination of PTEN expression, Mutter and colleagues suggest that simple hyperplasia should not be treated as a premalignant lesion, but as a simple reaction to estrogens. They support the usage of the term 'intraepithelial neoplasia' for the precancerous lesion. Along with Mutter et al, the term 'hyperplasia' should be used only in case of reversible processes, vulnerable to hormonal modulation. It is obligatory to distinguish cancer from EIN because the treatment is different. While discussing pathologies of endometrial proliferation, the value of immunohistochemical markers is worth emphasizing. The discovery of these markers allowed for diagnosing precancerous lesions in preclinical stages. The tumor development suppressor gene called PTEN is inactive in 83% of cases of endometrial adenocarcinoma. The loss of PTEN protein appears at the beginning of carcinogenesis. That is why we can use negative PTEN expression to recognize neoplastic glandules. Immunohistochemical expression of PTEN is negative in 63% of EIN.

As the markers of morphologically latent neoplastic transformation we can also use cell-cycle proteins [7,13].

Proteins responsible for the stimulation of cell-cycle are the products of expression of proto-oncogenes group. Alternatively, the inhibition of cell-cycle depends on the protein products of suppressor genes.

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 form together various complexes that affect the metabolism of a cell.

The studies carried out to date indicate that there are three main ingredients of cell-cycle: cyclins, cyclin-dependent kinases (CDK) and inhibitors of CDK (CKI). Cyclin inhibitors are the most recently identified group and consist of the family of p21 proteins (p27 and p57) and the family of INK4 protein (p15, p16, p18, p19). There are also restriction points along the pathway of cell-cycle, in which cellular proliferation may be temporarily or permanently stopped. The most frequent reason for such inhibition is a defect of cell-cycle reactions or a defect of proteins responsible for the above reactions. The inhibition of cell-cycle allows the repairing systems to remove the defects or - if it is not possible - to provoke apoptosis of that particular cell.

The active cycle of cellular proliferation may be divided into four phases: mitosis (M), synthesis (S), a gap between mitosis and synthesis (Gap 1 or G1), and a post-synthesis gap (Gap 2 or G2). When a cell does not enter into any of the described phases of active proliferation, it is said to be in Gap 0 (G0).

Retinoblastoma protein (pRb) is a product of suppressor gene expression of the same name. The retinoblastoma gene (Rb) is located on the chromosome 13q14.2 and is the

first identified tumor suppressor gene in humans [9,15]. It includes 27 exons and its expression results in forming Rb protein consisting of 928 amino acids with the molecular weight of 105-110 kDa (depending on the degree of phosphorylation) [28]. The loss of both Rb gene alleles causes a phenotypic manifestation of a malignant tumor of the eyeball specific for children, which is called retinoblastoma. Rb protein consists of several functional domains. Domain A and B are strongly conservative in all the representatives of Eucaryota. They are responsible for creating the pocket domain, with which E2F transcription factors link [20].

Rb protein stops a cell in phase G1 by inhibiting transcription of genes, whose protein products are essential to pass on from phase G1 to S. That is why Rb protein is thought to be the major inhibitor of restriction point (R) in phase G1 of the cell-cycle [9,15]. Point R divides the cell-cycle pathway into the part dependent on growth factors, and the part of late G1 phase, independent from those factors. Therefore, after crossing the restriction point, even if previous mutagens have been already deactivated, the cell continues the subsequent stages of neoplastic transformation. Active pRb inhibits E2F by attaching them to pocket domain. Only active E2F stimulates transcription of proto-oncogenes and eventually the synthesis of their protein products. As a result, the cell-cycle continues. In order to enable a cell to continue through the subsequent phases of the cell-cycle, the inactivation of pRb is necessary. This can be done by its complete phosphorylation. The enzymes responsible for the phosphorylation are linked together in complexes of cycline D1/CDK4 or 6 and cycline E/CDK2.

The rearrangements of the Rb gene structure resulting in alterations of point R control may provoke a cell to implement its own, coincidental pathway of proliferation, eventually leading to neoplasia. Adenocarcinoma of endometrium is nowadays the most common malignant tumor of the genital tract. It has been recently suggested that alterations of pRb activity may play a significant role in pathogenesis of endometrial adenocarcinoma [8]. However, investigations carried out to date with the use of immunohistochemical methods or molecular biology techniques have not yielded results entitling us to make a clear conclusion on the role of pRb in the pathogenesis of endometrial adenocarcinoma.

The aims of the present study were:

1. The investigation of immunohistochemical expression of retinoblastoma protein in samples of endometrium obtained from patients with physiological endometrium, with hyperplastic endometrium and from patients who underwent hormone replacement therapy (HRT),

2. The analysis of usefulness of retinoblastoma protein immunoexpression as a marker of pathological, endometrial proliferation.

Materials and Methods

We studied one hundred paraffin-embedded endometrial tissue samples retrospectively selected from the files of the Department of Pathology of Medical University of Łódź in Poland. The material was obtained by curettage because of metrorrhagia or abnormal images of endometrium seen during ultrasonographic examinations.

Formalin fixation, the process of embedding and hematoxilin-eosin staining were performed routinely. We examined hematoxilin-eosin stained samples with a light microscope Olympus BX/41. The standing classification we employed while making histopatological diagnoses was accepted by WHO in 1994.

In the current study, we investigated pRb immunohistochemical expression with the use of a DAKO EnVision detecting system. The staining was performed using the immunoperioxidase method with the following primary monoclonal antibody: Anti-Human Retinoblastoma Gene Product by DAKO. The slides were cut at 4 μm. After placement on adhesion glass and drying for 24 hours in the temperature of 56 Centigrade, the samples were deparaffinized in xylen and rehydrated through graded alcohols (96%, 80%, 70%, 60%). The activity of endogenous peroxidase was inhibited by 5 min. of incubation with 3% solution of perhydrol in methanol. Epitope retrieval of pRb in cellular nuclei was performed on all slides using the DAKO Target Retrieval Solution. First, the samples were heated for 30 min. in the temperature of 95 Centigrade. Second, after cooling, the sections were washed for 5 min. with 0.05 M TBS buffer (pH=7.6). Then, the sections were incubated with Anti-Human Retinoblastoma Gene Product (1:100 dilution) at room temperature for 30 min. The slides were washed twice with TBS buffer. In order to visualize the reaction between the antigen and antibody, a two-stage system of visualization (DAKO EnVision) was employed. The first phase of the process involved 30-minute incubation with a polymer marked with peroxidase and attachment of goat anti-anti-pRb immunoglobulins. Finally, the signal was detected by 3'3-diaminobenzidine as the chromogen for peroxidase activity and Meyer's hematoxilin as the counterstain. The sections were then dehydrated by graded alcohols (70%, 80%, 96%). After passing through solutions of acetones and xylens, the adequate sections were closed by mounting medium DPX.

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

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

The characteristics of the employed antibody are shown in Table 1.

TABLE 1
Characteristics of anti-pRb immunoglobulin

Antigen	Retinoblastoma protein
Manufacturer	DAKO Corporation, USA
Type of serum	mice
Clone	Rb1F8
Isotype	IgG, kappa
Dilution	1:100
Epitope retrieval	microwaves
Type of expression	nuclear
Positive control (tissue)	nodular thyroid goiter

Retinoblastoma protein immunoexpression in collected endometrial specimens was estimated with the use of the MultiScan software. The computer system consisted of an IBM computer with a Pentium processor and image digitalization card by ADDA, a color TV camera by Panasonic and a light microscope (Jenval made in Germany). We used 250x magnification of light microscope to assess the sections.

pRb 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, the pRb immunoreactivity index score is expressed in percents.

Simultaneously, we carried out a semiquantative evaluation of immunoexpression. The criteria of that method were:

1. overexpression - pRb expression index score >50%,
2. expression - pRb expression index score between 10-50%,
3. lack of expression - pRb expression index score between <10%.

Results

One hundred of cases were retrospectively selected from the files of Department of Pathology at the Medical University of Łódź 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 - simple hyperplasia, 6 cases-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 secretory phase.

While dividing into the material several subgroups, we considered the following clinical data: age (the age of

TABLE 2
Rb protein immunoexpression index score in the group with non-hyperplastic endometrium

No	Histopathological diagnosis	Age	Number of			Index pRb [%]	
			pregnancies	deliveries	Miscarriages	G	S
1	FE	46	0	0	0	38.3	23.3
2	FE	44	3	3	0	60.4	41.5
3	FE	40	4	2	2	70.4	12.5
4	FE	51	4	2	2	60.1	22.8
5	FE	53	2	2	0	41.6	24.7
6	FE	51	0	0	0	-	-
7	FE	55	1	1	0	58	27.7
8	EF	45	0	0	0	58.4	22
9	SE	38	2	2	0	-	-
10	SE	51	2	1	1	70.1	8.2
11	SE	54	1	1	0	63.3	33.2
12	SE	54	1	1	0	51	31.8
13	SE	47	2	2	0	75.6	11.8
14	SE	49	3	2	1	59	6.1
15	FE	49	1	1	0	-	-

Abbreviations: FE - follicular endometrium, SE - secretory endometrium, G - glandular epithelium, S – endometrial stromal cells.

TABLE 3
Rb protein immunoexpression index score in the group who underwent HRT

No	Age	Number of			Index pRb [%]	
		pregnancies	deliveries	miscarriages	G	S
1	56	1	1	0	56.3	33.3
2	60	0	0	0	-	-
3	50	0	0	0	80	35.9
4	52	2	2	0	80.6	10
5	73	0	0	0	29.1	9.2
6	58	0	0	0	40	14.3
7	48	4	3	1	45.5	27
8	51	2	2	0	15.8	11
9	50	2	2	0	63	39.7
10	44	4	3	1	72.5	7.3
11	53	3	1	2	30.2	15.6
12	46	5	3	2	67.9	39.4
13	50	2	0	2	51.4	59.4
14	40	1	1	0	87.4	9.6
15	46	2	2	0	52.3	50.8
16	60	0	0	0	72.2	35.4
17	54	3	2	1	34.8	6.3
18	52	0	0	0	-	-
19	46	1	1	0	46.2	30.2
20	50	0	0	0	73.8	13.8
21	55	3	1	2	43.8	9.7
22	48	1	1	0	55.4	11.7
23	49	0	0	0	80.7	22.7
24	40	1	1	0	68	6.8

Abbreviations: G - glandular epithelium, S – endometrial stromal cells.

49 was treated as the average age at menopause among Polish women), the number of past pregnancies, deliveries and miscarriages.

Tables 2-6 present the results of the investigated pRb immunoreactivity in endometrial tissue.

The analysis included the material obtained from 15 patients at the average age of 48.5 years (range 38-55). The number of pregnancies range between 0-4, labors 0-3, and miscarriages 0-2. In case of three patients, the result of immunohistochemical staining was not accepted as reliable.

The investigation included samples from 24 patients at the average age of 51.3 years (range 40-73). The parity of patients was between 0-5, the women had 0-3 labors and 0-2 miscarriages. In two cases, the findings of immunohistochemical staining could not be taken into consideration because of technical defects.

The analysis was carried in material originating from 61 patients at the average age of 49.4 years (range 24-81). The number of pregnancies range between 0-10, deliveries 0-8 and abortions 0-2. In up to 10 cases, the result of immunohistochemical staining was doubtful because of technical reasons and the slides were not taken into consideration in further discussion.

The mean pRb immunoexpression index score in glandular epithelial cells and standard deviation for all the investigated patients were 57.698 +/- 17.386, respectively.

Less intense pRb expression was demonstrated in endometrial glandular epithelium of the group of women over 49 years of age, with at least one labor in the past and who underwent HRT. The highest pRb expression was observed in the group of subjects no older than 49 years of life with physiological endometrium.

We did not observe any statically significant correlation between pRb immunoexpression in glandular epithelial cells and the microscopic picture of routinely stained endometrium, age or parity.

The mean pRb immunoexpression index score in stromal cells and standard deviation for all the studied cases were 21.191 +/-14.451, respectively.

Taking into account endometrial stromal cells, the lowest pRb expression was identified in the group of nulliparas over 49 years of age. The most intense pRb expression in-

TABLE 4
Rb protein immunoexpression index score in the group with endometrial hyperplasia (EH)

No	Histopathological diagnosis	Age	Number of			Index pRb [%]	
			pregnancies	deliveries	miscarriages	G	S
1	SH	44	0	0	0	38.5	16.5
2	SH	39	4	2	2	-	-
3	SH	64	0	0	0	38.2	7.2
4	SH	36	0	0	0	43	5.5
5	SH	45	0	0	0	19.1	3
6	SH	48	1	1	1	34.3	5.8
7	SH	45	1	1	0	66.8	11.1
8	SH	49	1	1	0	71.1	14.4
9	SH	50	0	0	0	-	-
10	SH	50	0	0	0	50	24.4
11	SH	46	0	0	0	20.7	4.5
12	SH	50	0	0	0	42.5	15.4
13	SH	42	3	3	0	18.7	6.2
14	SH	49	0	0	0	21.7	8.2
15	SH	50	1	1	0	52.2	26.2
16	SH	59	0	0	0	68.4	13.5
17	SH	64	0	0	0	75.6	10
18	SH	43	2	2	0	36.8	14
19	SH	56	2	2	0	-	-
20	SH	58	1	1	0	75.2	18.8
21	SH	50	0	0	0	63.9	9.8
22	SH	44	0	0	0	48.2	33.8
23	SH	45	0	0	0	96.6	50.1
24	SH	44	3	2	1	53.9	12.2
25	SH	81	0	0	0	65.6	10.5
26	SH	58	4	2	2	-	-
27	SH	48	5	4	1	76.6	34.5
28	SH	48	0	0	0	72	18.9
29	SH	54	0	0	0	-	-
30	SH	50	4	2	2	59.5	15
31	SH	50	0	0	0	42.1	22.2
32	SH	24	0	0	0	-	-
33	SH	47	0	0	0	58.4	8.7
34	SH	49	0	0	0	71.5	36.5
35	SH	65	4	2	2	78.3	10.1
36	SH	48	2	1	1	51.3	22.6
37	SH	53	0	0	0	-	-
38	SH	53	3	2	1	61.8	20.7
39	SH	49	1	1	0	63.5	19.5
40	SH	52	0	0	0	-	-
41	SH	39	1	1	0	66.6	22
42	SH	52	0	0	0	74.3	27.6
43	SH	39	1	1	0	70.4	22.7
44	SH	35	0	0	0	68.5	20.8
45	SH	50	3	3	0	61.5	29.6
46	SH	47	2	2	0	76.2	30.4
47	SH	49	3	3	0	95.2	98.5

48	SH	69	2	2	0	-	-
49	SH	59	4	2	2	74.2	33.7
50	SH	48	10	8	2	52.5	28.5
51	SH	45	0	0	0	53	19.1
52	SH	49	1	1	0	59	10.4
53	SH	47	1	1	0	63.9	24.2
54	SH	51	0	0	0	70.2	16.7
55	SH	54	0	0	0	48.4	24
56	CH	49	0	0	0	52.1	9.5
57	CH	77	2	2	0	-	-
58	CH	49	3	2	1	72.6	16.1
59	CH	51	0	0	0	52.5	27.7
60	CH	48	2	2	0	66.6	29.6
61	CH	54	0	0	0	37.5	15.6

Abbreviations: SH - simple hyperplasia; CH - complex hyperplasia; G- glandular epithelium; S- endometrial stromal cells.

TABLE 5

Arithmetical averages of pRb immunoexpression index score in the nuclei of glandular cells of endometrial epithelium and the standard deviations

Parity	Age	Control group [%]	HRT [%]	Hyperplasia[%]
at least 1 labor	≤49	66.35 +/- 7.987	61.900 +/- 14.567	60.889 +/-17.803
	>49	57.35 +/-9.943	46.357 +/- 21.922	66.100 +/- 9.778
Nulliparas	≤49	48.350 +/-14.213	80.700 (1 case)	51.023 +/-22.966
	>49	no cases	57.750 +/- 20.677	56.092 +/- 14.052

TABLE 6

Arithmetical averages of pRb immunoexpression index score in the nuclei of stromal endometrial cells and standard deviations

Parity	Age	Control group [%]	HRT [%]	Hyperplasia [%]
at least 1 labor	≤49	17.975 +/- 15.945	22.850 +/- 16.586	23.483 +/- 20. 536
	>49	24.733 +/- 9.028	17.943 +/- 13.099	22.014 +/-8.315
Nulliparas	≤49	22.650 +/- 0.919	22.700 (1 case)	18.085 +/- 14.296
	>49	no cases	28.000 +/- 19.214	17.277 +/-7.145

dex score was depicted in the group of patients over 49 who underwent HRT. Statistically, there were no significant correlations between pRb immunoreactivity in the stroma of endometrium and microscopic picture of the material, age of examined women and their parity.

Discussion

The physiological human endometrium is characterized by phases of active proliferation and differentiation, controlled by cell-cycle proteins receiving information within oscillating levels of ovarian, pituitary and central nervous system hormones [9, 15]. The knowledge of regulatory mechanisms underlying the subsequent stages of the cell-cycle has been widely developed in recent years. It has been mainly the result of introduction of immunohistochemical staining methods and molecular biology techniques, employed in the examination of cell genome and subsequent stages of gene expression.

Immunohistochemical staining is commonly used in microscopic diagnostics of neoplasms. The investigation

of cell-cycle proteins activity using immunohistochemical staining methods may be employed during research of neoplastic predisposition of cells and tissues.

The retinoblastoma gene consists of 27 exons and is a relatively big gene of a complex structure. Immunohistochemical staining of pRb is a less demanding and more comfortable method used to investigate the Rb gene expression. Unfortunately, this procedure is not sensitive and specific enough. The majority of described Rb mutations results in complete lack of its expression and afterwards deficiency of Rb protein. It is represented immunohistochemically as a negative staining effect in the analyzed sample. However, some of Rb gene disorders cause only rearrangements of Rb protein structure, which might not affect its immunohistochemical reactivity. In such situations, immunohistochemical methods do not guarantee differentiation between mutated protein and that of unchanged configuration. In order to properly control the staining procedure, it is absolutely necessary to pay attention to the immunoreactivity of the entire examined material, especially when it is of lower intensity. This kind of procedure will guarantee avoidance of errors in discriminating between technical errors of staining and absolute lack of immunoreactivity.

Rb protein, as it has been already mentioned, inhibits transcription of genes, whose protein products are essential in enabling cell to continue the cell-cycle process and pass on from phase G1 to phase S. It has been proven that insufficiency of this function may play a significant role in the process of carcinogenesis of many human neoplasms, such as retinoblastoma, liposarcoma, osteosarcoma, carcinoma of the bladder, kidney, esophagus, gallbladder and hepatocellular carcinoma [4, 14, 18, 26, 27]. Any rearrangements in the structure of the Rb gene or pRb may be of a prognostic importance. Shimizu and colleagues demonstrated lack of pRb immunoreactivity in 13.5% of the examined cases of gallbladder carcinoma and hyperreactivity in 48.6% of cases, which correlated with increased aggressiveness, shortened survival time and metastases in local lymph nodes [21].

The existence of casual nexus between disorders in pRb control of restriction point and development of endometrium pathology, such as hyperplasia or adenocarcinoma was suggested a few years ago. So far, only a few studies employing the previously described methods have been carried out. All of them concentrated rather on the pathogenesis of endometrial adenocarcinoma. However, all the presented findings vary as to the appearance of the positive staining effect and particular pRb index score and do not allow us to formulate significant conclusions.

In 1990, Sassano and colleagues were among the first investigators to examine three cases of endometrial carcinoma and one case of endometrial hyperplasia for abnormalities of the Rb gene using Southern blot hybridization [16]. They reported no big rearrangements in any of cases.

Three years later, Enomoto and colleagues reported an extensive analysis of the Rb status in nine cases of endometrial carcinoma and three cellular lines of this tumor [8]. They found no big alterations of the Rb gene by Southern blot hybridization, but two primary endometrial carcinomas demonstrated loss of Rb mRNA by dot blot analysis. Further study of Rb mRNA by RT-PCR and direct sequencing demonstrated deletion of exon 21 in one additional case of primary endometrial carcinoma and deletion of exon 8 in one of the endometrial carcinoma cell lines. The authors concluded that the alterations of the Rb gene structure, as well as Ki-ras activation play a significant role in etiology of endometrial adenocarcinoma.

Karin Milde-Langosch and colleagues investigated eight cases of proliferative endometrium, none of which revealed pRb immunoexpression, nine cases of secretory endometrium, one sample of which was pRb-negative, 11 cases of hyperplasia with one negative pRb slide, and 36 samples of adenocarcinoma with only one negative staining for pRb. Contrary to Enomoto's findings, Milde-Langosh demonstrated a high level of Rb mRNA by RT-PCR in the analyzed lines of adenocarcinoma cells [8].

Semczuk et al. carried out a research on an extensive population by immunohistochemical methods and molecular biology techniques [19]. In 45 cases of endometrial cancer out of 46 examined, they noted positive pRb immunostaining of endometrial glandular cells. In the remaining case, only stromal cells were positively stained. In some samples, neoplastic proliferation was associated with hyperplastic lesions. Immunoreactivity of those parts was extensively heterogeneous. Similarly as in the rest of the group, there was no statistically significant correlation between pRb expression and other clinical data of the patients. Semczuk took also into consideration the allelic status of the Rb gene. He demonstrated a statistically important association between loss of Rb heterozygosity (LOH-Rb) and lack or reduced pRb immunoreactivity in endometrial carcinoma samples. In another investigation, Semczuk and colleagues observed a coexistence of point mutations of the Ki-ras gene and extreme pRb immunoreactivity of neoplastic cells [20].

In a study of pRb expression in physiological human endometrium, Cordon-Cardo and Richon reported a homogenous pattern of staining [5]. Unfortunately, they neither specified in which phase of menstrual cycle the material was sampled, nor give any further details regarding their findings or techniques.

Nieman et al. observed intense pRb immunoexpression in 10/10 cases of proliferative endometrium, whereas eight cases of secretory endometrium appeared to dem-

onstrate less intensity or be completely negative [17]. The authors explain that their results are relatively synchronous with the cell-cycle pathway, in which pRb level oscillates and achieves the maximum value during S and G2/M phase. In the proliferative phase of menstrual cycle, endometrial cells are continuously ready to divide and actively proliferate, what provides another explanation of Nieman's findings. In 66 out of 70 cases of endometrial cancer and in all ten samples of hyperplasia, immunohistochemical staining for pRb was positive. However, this time the reactivity pattern was heterogeneous, what may be the effect of asynchronous metabolic processes of the particular endometrial cells with the defects of cell-cycle regulation. Some cases of endometrial cancer showed an altered pattern of reactivity characterized by areas of typical heterogeneous reactivity adjacent to areas of absent pRb expression. The zones of absent staining presumably represent clones of a cell with the Rb gene mutation, hindering its proper expression. The authors suggest that during progression of carcinogenesis, the Rb gene is deactivated. The fact that the pattern of altered reactivity is only observed in high-grade neoplasms may provide additional support for the latter theory.

The most probable explanation for the wide range of the observed results is a low number of particular investigated populations and varied analytical methodology. Another reason for such a situation is the possibility of rearrangements in the Rb gene structure, which results in alterations of pRb activity, but is not strong enough to change the structure of Rb protein so that it could be detected immunohistochemically [10, 21].

In studies conducted to date, investigators have concentrated mainly on pRb expression in cells of endometrial adenocarcinoma. Rb protein immunoexpression in unchanged endometrium or in endometrium with benign disorders has been generally treated as a control. Only Semczuk in one of his researches did observe a statistically significant association between the pRb immunoexpression index score and clinical data of the analyzed patients. However, all the groups studied to date have not been numerous enough so that the results of statistical test could be treated as reliable [20].

Aiming at achieving more reliable results and formulating firm conclusions, in our investigation we analyzed the most numerous to date population of women with endometrial hyperplasia, a group of previously not studied patients who underwent hormone replacement therapy (HRT) and the control group of patients with physiological endometrium. Moreover, we independently investigated pRb immunoexpression in the nuclei of glandular epithelium cells and in the nuclei of stromal cells in endometrium. In the present study, for all the considered patients the average pRb immunoexpression index scores in glandular cells of endometrium and stromal cells were 57.698 +/- 17.386 and 21.191 +/- 14.451 respectively. Less intense pRb immunostaining for the glandular cells was observed in the group of women over 49 years of age, who gave birth to at least one infant and underwent HRT. On the other hand, less intense pRb expression for stromal cells was revealed in nulliparas over 49 years of life. The most intense staining effect of glandular epithelium cells was observed among patients with physiological endometrium no older than 49 of age. The highest pRb expression index score for stromal cells was shown in the population of women over 49 who underwent HRT.

Despite the great number of collected material and adherence to appropriate rules during its analysis, statistical tests did not reveal any significant correlations between the obtained pRb expression index scores and histopathological diagnoses or other clinical patient data in any group.

Summing up, the immunohistochemical method seems to be not sensitive enough and specific enough to reveal pRb activity alterations resulting in endometrial pathologies. Alternatively, pathological rearrangements of endometrium may be independent from the accuracy of pRb control over restriction point in the cell-cycle pathway.

We suggest that any further studies of this problem should refer to the genome of endometrial cells and ought to be conducted employing molecular biology techniques.

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