

Immunohistochemical analysis of steroid receptors in ovaries of postmenopausal women - effects of aging and hormone status

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Summary. Current knowledge on immunolocalization and immunoexpression of steroid hormone receptors, especially estrogen receptor alpha (ER- α), progesterone receptor (PR) and androgen receptor (AR) in normal ovaries in postmenopausal women is not complete. The recognition of localization of these receptors in postmenopausal women is crucial, as many of these women receive estro-progestagene therapy, and its participation in the pathogenesis of ovarian cancer should be carefully studied. In our paper we present the results of immunohistochemical studies performed on samples from 100 post-menopausal women (aged: 48 to 60 years) who did not use hormonal therapy. The ovaries were removed during elective operation due to uterine leiomyoma, endometriosis and/or prolapsed uterine. PR, ER- α and AR were detected in the normal ovaries of postmenopausal women in stroma and in ovarian surface epithelium, as well as in its invagination and in epithelial inclusion cysts. The expression of PR and AR did not change, while the expression of ER- α decreased in time from menopause, and it was also detected in patients more than 10 years after menopause. Women older than 60 were not included in the study. The concentration of selected hormones was measured in the serum. The immunohistochemical expression of PR and AR were similar in all examined patients and did not correlate with FSH, LH, T, A, DHEAS concentrations in serum, while immunohistochemical expression of ER- α correlated with FSH, LH, T, A, DHEAS concentrations in serum. The significant correlation of decreasing expression of ER- α in normal ovarian tissue and

decreasing concentrations of T, A and DHEAS in serum were found, as well as increasing serum concentrations of FSH and LH.

Key words: Postmenopausal ovary, Immunohistochemistry, Steroid receptors, Progesterone receptor, Androgen receptor, Estrogen receptor α , Epithelial inclusion cyst, Pathology

Introduction

Steroid hormones interact with their receptors in nuclei that act as ligand - activated transcription factors. Receptors are located in the cytoplasm or in the nucleus as an inactive complex that is activated by a ligand and which subsequently binds DNA and activates transcriptional activity of selected genes.

The results of many immunohistochemical studies on animal models, as well as on human tissue samples, showed that immunoexpression and immunolocalization of steroid receptors is different in different tissues (Hiroi et al., 1999; Pelletier, 2000; Juengel et al., 2006; Brodowska et al., 2007; Starczewski et al., 2008). It mainly depends on sex, age, phases of menstruation cycle, and in the case of human patients – on the drug used (Yoshimura and Bahr, 1991; Słomczyńska, 2002; Narayana et al., 2005; Vaskivuo et al., 2005).

Detailed knowledge on localization of steroid hormone receptors in tissues is important, as steroid hormones modify numerous biological processes and participate in different pathologies, such as inflammation, hypertrophy, hyperplasia and neoplasia (Khan et al., 1997; Fendrick et al., 1998; Vanderhyden, 2005). Until now the dynamic of localization of estrogen

receptor ER- α and ER- β , progesterone receptor PR and androgen receptor AR in ovaries of reproductive women has been analyzed in relation to menstruation disfunctions, diagnostic and therapy of infertility and habitual miscarriages (Chakraborty and Gore, 2004; Deroo and Korach, 2006). The immunoexpression of ER, PR and AR in reproductive women clearly depends on menstruation cycle phases (Chakraborty and Gore, 2004; Narayanan et al., 2005). PR was detected in corpus luteum, in stroma cells and in surface epithelial cells, as well as in early stages of follicular development, in theca and granulosa cells (Akahira et al., 2002; Słomczyńska, 2002; Flototto et al., 2004). The presence of AR was shown in surface epithelial cells, stromal cells, theca and granulosa cells, and in early follicles where PR was present in almost all cells. In Graafian follicle the AR was detected only in antral part and in cumulus oophorus (Słomczyńska, 2002; Laszczyńska et al., 2008). Estrogen receptors in ovaries of reproductive women and of some mammal females (pig, rat, hamster and mouse) are located mainly in granulosa cells, theca cells, stromal cells, luteal and paraluteal cells of corpus luteum, as well as in surface epithelium cells (Pepe et al., 2002; Słomczyńska, 2002; Yang et al., 2002). In mammals in reproductive period the isoform ER β is the dominant estrogen receptor. The localization of ER β was shown on every stage of development of the follicle, from the primary follicle to the Graafian follicle, as well as on every stage of development of corpus luteum, while the presence of ER α was shown only in large, preovulatory follicle and early stage development of corpus luteum (Słomczyńska, 2002; Yang et al., 2002; Li et al., 2003).

There is very little data on immunolocalization and immunoexpression of the above-mentioned receptors in ovaries of postmenopausal women. Probably the changes of the structure and function of human ovary after menopause are related to changes of localization and expression of steroid hormone receptors. This is an important problem that requires further studies, since hormonal therapy is widely applied to patients in this period of life, and its effectors are steroid hormones receptors localized in the ovary. Moreover, as has been proved by many studies, in this group of women epithelial ovarian cancers are the most common neoplasm, and their development depends on concentration of steroid hormones in serum and on the presence of steroid hormone receptors (Saarikoski et al., 1982; Kanajet et al., 1985; Ahonen et al., 2000; Wright et al., 2001; Richer et al., 2002; Vang et al., 2006). The influence of time from menopause on presence and number of steroid hormone receptors is also interesting and has not been studied yet.

The detailed analysis of changes in the structure and function of the ovary is important for determining the localization and the expression of steroid hormone receptors in postmenopausal women compared to reproductive women. The early changes in the structure and function of the ovary are seen as early as during the

premenopausal period, about 5 years before menopause (Focchi et al., 1995, 1996; Brodowska et al., 2007). Generally, the differences in the structure of the ovary after menopause are seen in the cortex and in the medulla. The main changes are a blurred border between the cortex and the medulla, the diminished thickness of the cortex, the presence of epithelial inclusion cysts and epithelial invaginations, a lower number of follicles and fragmentation of corpora albicantia. The connective tissue in the medulla becomes fibrotic, the architecture of vessels is altered and their walls are hyalinized, which causes the narrowing of the lumen (Focchi et al., 1995, 1996; Brodowska et al., 2007). The change in the structure of postmenopausal ovary that seems to be important for carcinogenesis is the presence of inclusion epithelial cysts. Cysts that are lined by columnar or cuboid epithelium are localized mainly in the stroma below the surface epithelium and around corpora albicantia, and they are formed by metaplasia of cuboid epithelium or by invaginations of the surface epithelium (Auersperg et al., 2001). According to published data, 90% of epithelial ovarian cancers develop from these cysts and/or from surface epithelium (Aoki et al., 2000; Auersperg et al., 2001; Heller et al., 2003; Scott and McCluggage, 2006).

The aim of our study was to analyze the immunolocalization and the immunoexpression of steroid hormone receptors: estrogen receptor α (ER- α), progesterone receptor (PR) and androgen receptor (AR) in ovaries of postmenopausal women in relation to time from menopause and to the hormonal status measured by the concentration of selected hormones in serum.

Materials and methods

Patients

The study was performed on 100 post-menopausal patients (at least one year after menopause, aged: 48 to 60 years) operated in the Department of Reproduction and Gynecology of Pomeranian Medical University in Szczecin, due to uterine leiomyoma, endometriosis and/or prolapsed uterine between 2004 and 2008. The PAP smear and breast mammography were normal in all patients studied. Menopausal hormonal therapy was not applied since patients presented none or only mild symptoms of hormonal fluctuation. The approval from the local Bioethics Committee was obtained for the study.

Hormone serum concentration (E₂, T, A, FSH, LH and DHEAS) was analyzed in all women included in the study prior to the operation. During the laparotomy, the total hysterectomy or uterine corpus removal were performed. The morphologic and immunohistochemical analysis of the ovary was done: the morphology of the ovary was analyzed in standard HE slides, the immunoexpression and immunolocalization of ER- α , AR and PR were analyzed.

The women involved in the study were divided into

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3 groups (A, B, C) depending on the time from the last menstruation. Group A (50 patients) included women whose last menstruation was between 1 to 5 years earlier. In group B (30 patients) menopause appeared 5 to 10 years earlier and in group C over 10 years earlier.

For ethical reasons a control group was not included in the study, as normal ovarian tissue from healthy, reproductive women could not be obtained

Hormone measurements

Total serum concentration of E_2 , T, LH, FSH was measured by electrochemiluminescence assay (ECLIA) with monoclonal antibodies by COBAS E analyzer (Roche Diagnostics GmbH, Poland). Sensitivity limit was 0.10 mIU/ml for LH and FSH, 0.069 nmol/L for T and 18.4 pmol/L for E_2 . 1.8% CV for LH and FSH, 4.6% CV for T and 5.7% CV for E_2 .

Total serum concentration of A and DHEAS was also measured by electrochemiluminescence assay (ECLIA) with monoclonal antibodies by COBAS E analyzer (Simens Immulite 1000, UK). Sensitivity limit was 0.3 ng/ml for A and 3 μ g/dL for DHEAS. 9.1% CV for A and 7.6% CV for DHEAS.

Immunohistochemistry

For immunohistochemical analysis of expression of steroid receptors (PR, ER α and AR) the paraffin-embedded specimens fixed in 4% buffered formalin were used. 5 μ m thick sections were cut and mounted on microscope slides. The slides were heated in citrate buffer, pH 9.0 for 30 min. in a water bath at 96°C. The sections were next incubated for 30 min. at room temperature in a humidified chamber with monoclonal

mouse anti-human antibody; for progesterone receptor (N 1630 Dako, Denmark), for estrogen receptor α (N 1575 Dako, Denmark) and for androgen receptor (M 3562 Dako, Denmark) respectively. Aminoethylcarbazole (AEC substrate chromogen) was used to visualize the immunohistochemical reaction (Dako LSAB 2 KIT/HRP). Finally, the sections were counterstained with Mayer's hematoxylin. After each step, the sections were rinsed with Tris-buffered saline (TBS). The negative control sections were incubated with TBS instead of the primary antibody.

Statistical analysis

All statistical analyses were done with Statistica for Windows PL. Quantitative variables were characterized by minimal and maximal values, arithmetic mean and standard deviation. The Shapiro-Wilk test was used to assess the distribution of tested parameters. The significance of difference between two samples was assessed with the t-Student test, the Mann-Whitney test and the Kruskal-Wallis test. Correlations were assessed using the Pearson correlation coefficient. P value less than $p < 0.05$ was considered significant.

Results

Hormone measurements in serum of the examined patients

The mean E_2 , FSH, LH, A, T, DHEAS serum concentrations of the examined patients are shown in Table 1.

The mean E_2 serum concentrations did not differ between groups, while the mean FSH, LH, A, T and

Table 1. The levels of E_2 , FSH, LH, A, T and DHEAS in serum of the postmenopausal women.

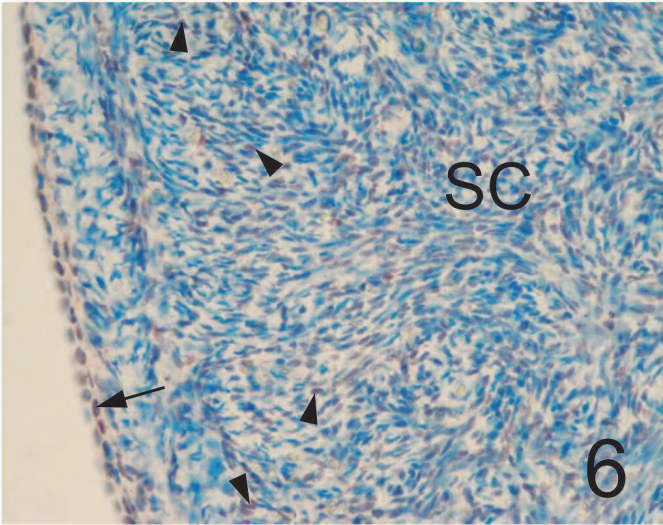
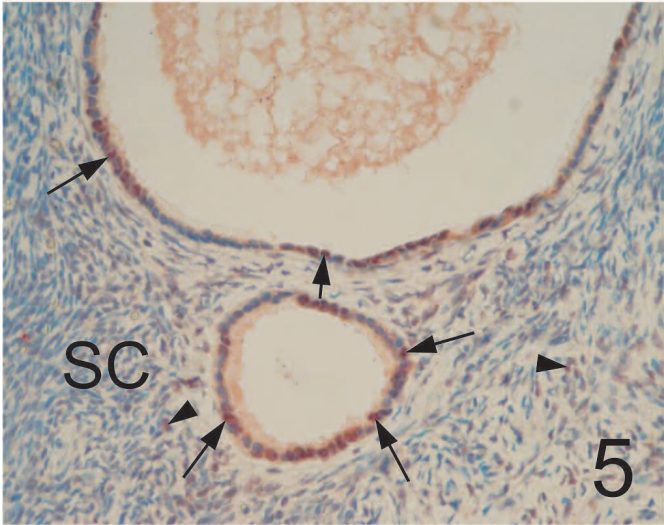
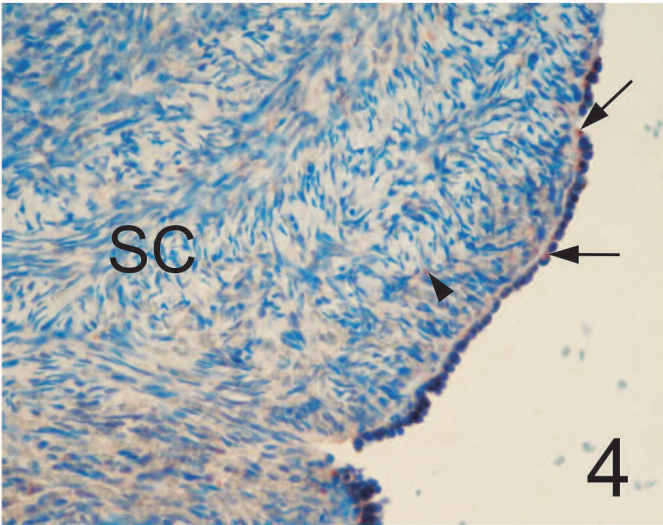
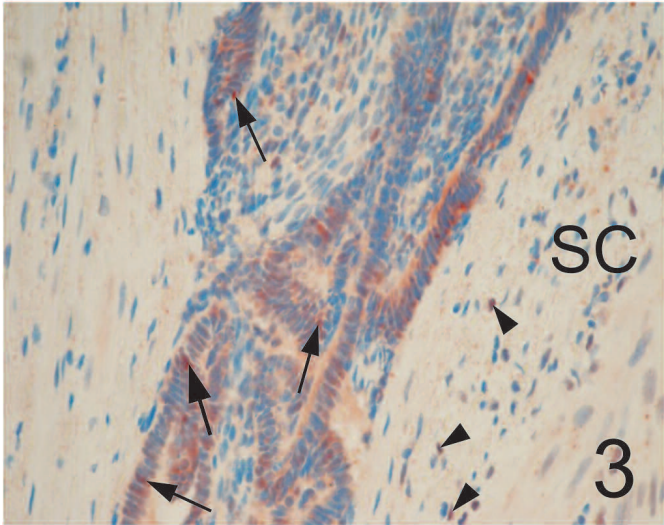
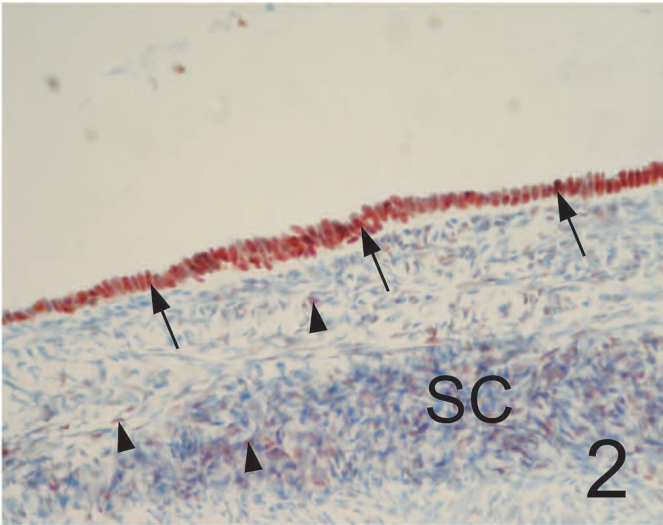
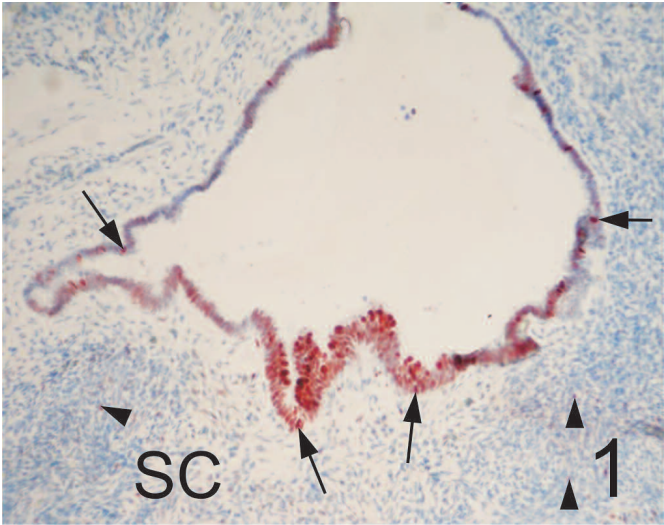
Group	No	E_2 (pg/ml)	FSH (mIU/ml)	LH (mIU/ml)	A (ng/ml)	T (ng/ml)	DHEAS (μ g/ml)
A	50	24.35 $\pm$ 10.29	30.80 $\pm$ 26.40**	18.05 $\pm$ 11.53**	2.55 $\pm$ 1.27**	0.45 $\pm$ 0.17**	99 $\pm$ 44.63*
B	30	25.05 $\pm$ 10.12	60.15 $\pm$ 14.38**	27.55 $\pm$ 7.00**	1.80 $\pm$ 1.17**	0.29 $\pm$ 0.07**	93 $\pm$ 32.2*
C	20	25.70 $\pm$ 8.85	70.35 $\pm$ 19.91**	46.75 $\pm$ 6.05**	1.20 $\pm$ 0.6**	0.23 $\pm$ 0.08**	52 $\pm$ 19.87*

means $\pm$ SD; *: ($p < 0.05$); **: ($p < 0.001$)

Table 2. Immunohistochemical localization and expression of PR, ER- α and AR in the ovaries of postmenopausal women.

Postmenopausal women	Ovarian surface epithelium (OSE)			Epithelial inclusive cysts			Stroma		
	PR	ER α	AR	PR	ER α	AR	PR	ER α	AR
Group A	+++/>++/+	+++	++	+++	+++	++	+++/>++	+++/>++	++
Group B	+++/>++/+	++	++	+++	++	++	+++/>++	+++/>+	++
Group C	+++/>++/+	+/-	++	+++	+/-	++	+++/>++	+/-	++

Legend: very strong intensity (+++), strong intensity (++), weak intensity (+), lack of expression (-).



DHEAS serum concentrations significantly differed between the examined groups of women (between groups A and B, B and C, and A and C).

Immunohistochemical results

Immunohistochemical localization and immuno-expression of PR, ER α and AR in the ovaries of postmenopausal women were shown in Table 2.

In our study we showed the immunohistochemical nuclear expression of PR in ovarian surface epithelium (OSE), in epithelial inclusion cysts and in stromal cells. In OSE we observed different levels of nuclear expression of PR in each group that we graded as: very strong, strong and weak expression (Table 2). The expression of PR seems to be similar in the ovaries of women in group A, B and C. (Figs. 1, 2). The immunohistochemical nuclear expression of ER α was detected in OSE, in epithelial inclusion cysts and in stroma, (Figs. 3, 4). Expression of ER α seems to decrease in the ovaries of women 5 and 10 years after menopause. In some ovaries of postmenopausal women in group C nuclear expression of ER α was not detected. The immunohistochemical nuclear expression of AR was shown mainly in OSE, in epithelial inclusion cysts and in stromal cells. (Figs. 5, 6). Expression of AR was strong and seems to be similar in the ovaries of women in groups A, B and C.

The expression of PR and AR were similar in all examined groups and they did not depend on FSH, LH, T, A, DHEAS concentrations in serum, while the expression of ER- α was different in all examined groups and depended on FSH, LH, T, A, DHEAS concentrations in serum.

Discussion

Current knowledge on localization and expression of steroid hormones in normal ovaries of postmenopausal

women is still incomplete. Published data is limited to comparison of distribution and expression of AR, PR and ER in epithelial tumors and/or stromal tumors, as well as normal ovaries of postmenopausal women. In our study we analyzed correlations of localization of the above mentioned receptors in normal ovaries of postmenopausal women with concentration of selected hormones in serum and length of period of time from menopause. Localization and expression of AR, PR and ER- α were mainly shown in surface epithelium cells, in stromal cells and in inclusion cysts lined by metaplastic epithelium. The expression of PR i AR did not change, while the expression of ER- α diminished with time from menopause (Brodowska et al., 2007; Starczewski et al., 2008). Thus, the patients 5 and 20 years after menopause showed similar localization and expression of PR and AR in ovaries.

In women 5 years after menopause the expression of ER- α was shown in OSE, in stroma and in epithelial inclusion cysts, and the expression was stronger than in women between 5 and 10 years after menopause. It should be stressed that the localization of ER- α was confirmed in cuboid and columnar epithelium and in the above-described inclusion cysts. In women over 10 years after menopause (but not older than 60 years old) only scattered ER- α positive cells were found in OSE and in the stroma. The relation of expression of ER- α and estradiol serum concentration was not confirmed and this concentration was low in studied groups and did not differ in time from menopause (Brodowska et al., 2007), although patients older than 60 were not included in our study. The correlation between diminishing concentrations of T, A and DHEAS and rising concentrations of FSH and LH in serum in time from menopause and expression of PR and AR in normal ovary was not found. However, this type of correlation was found for expression of ER- α . The lowest concentrations of T, A and DHEAS and the higher concentrations of FSH and LH was in serum, the

Fig. 1. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). Immunohistochemical nuclear localization of PR in the epithelial inclusion cyst, very strong expression of PR (arrows). Expression of PR in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 2. Ovary of group C postmenopausal women (examined more than 10 years after menopause). Immunohistochemical localization of PR in nuclei of ovarian surface epithelium, very strong expression of PR (arrows). Expression of PR in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 3. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). Immunohistochemical localization of ER α . Very strong nuclear staining is present in some columnar epithelial cells (invaginations of ovarian surface epithelium, metaplastic changes) (arrows). Nuclear expression of ER α in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 4. Ovary of group C postmenopausal women (examined more than 10 years after menopause). Immunohistochemical localization of ER α in nuclei of ovarian surface epithelium (arrows) and in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 5. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). Immunohistochemical localization of AR. Strong nuclear staining is present in some columnar epithelial cells (invaginations of ovarian surface epithelium metaplastic changes, epithelial inclusion cysts) (arrows). Nuclear expression of AR in some stromal cells (arrowheads). Stromal cells (SC). x 400

Fig. 6. Ovary of group C postmenopausal women (examined more than 10 years after menopause). Immunohistochemical localization of AR in some nuclei of ovarian surface epithelium (arrows) and in some stromal cells (arrowheads). Stromal cells (SC). x 400

weakest was the expression of ER- α . A similar correlation was also found by Motta et al. who proved the relationship between the concentration of DHEAS in serum and the expression of PR in ovaries of women after menopause (Motta et al., 2002). In their study the lowest was the concentration of DHEAS in serum and the strongest was the expression of PR in ovarian cells. In published data it can be found that the localization and the expression of PR in postmenopausal women was analyzed only in fat tissue, and they were significantly lower if the time from menopause was longer (Meza-Mundo et al., 2002). There are no publications on localization of ER and AR in normal tissues and organs of postmenopausal women. Steroid hormones and their receptors play a pivotal role in carcinogenesis in ovary and breast (Saarikoski et al., 1982; Kanajet et al., 1985; Ahonen et al., 2000; Wright et al., 2001; Richer et al., 2002; Vang et al., 2006). In studies by Ho, 2003 and Zheng et al. 2007 it was shown that estrogens, gonadotropins and androgens are main factors of neoplastic transformation in surface epithelium of the ovary, while progesterone and GnRH play a protective role (Ho, 2003; Zheng et al., 2007).

The results of the current study in relation to published data suggest that the expression of ER- α and AR in ovarian surface epithelium could be unfavorable, while the expression of PR could be protective in aspects of ovarian carcinogenesis. The results of our studies showed strong or very strong expression of PR in ovaries of postmenopausal women independently of time from menopause.

Has also been proved that more than 90% of ovarian cancers develop from ovarian surface epithelial cells and in epithelial cysts (Scully, 1995; Aoki et al., 2000; Heller et al., 2003; Scott and McCluggage, 2006). The frequency of ovulation, epithelial trauma and reepithelization and exposure of surface epithelium to the estrogen rich follicular fluid may contribute to carcinogenesis (Fathalla, 1971). Some authors report that loss of ER- α , PR and AR mRNA expression in human ovarian surface epithelial cells may be responsible for neoplastic transformation in these cells (Lau et al., 1999). Yang et al. showed that in Chinese women in reproductive age with ovarian cancer, the phenotype ER(+) / PR(+) is the most important prognostic factor (Yang et al., 2009). Trapani (1992) in studies focused on comparison of localization of steroid hormones in normal ovarian tissue and in ovarian tumors showed that the percentage of PR-positive cells in normal ovary is about 84%, and the percentage of ER-positive cells is about 35%. Similar values were found in benign ovarian tumors, while in malignant tumors the percentage of PR-positive cells was as low as 42%, and the percentage of ER-positive cells was slightly higher. He pointed out that the lower expression of steroid hormone receptors the higher malignancy of analyzed tumors was (Trapani, 1992). Similar results were found by Hogdall et al. (2007), who reported a higher number of ER-positive cells in higher stage ovarian tumor and a higher number

of PR-positive cells in the lower grade tumors (Hogdall et al., 2007). Based on 15 years of studies on long-term survival, proliferative activity, grade and apoptosis in ovarian tumors, Munstedt et al., (2000) reported that the most favorable phenotype is ER(-) / PR(+) (Munstedt et al., 2000). It is also known that the percentage of progesterone and androgen receptor-positive tumors was significantly higher in reproductive patients than in menopausal ones (Munstedt et al., 2000; Hogdall et al., 2007).

Some publications show that a low concentration of ER (less than or equal to 10fmol/mg cytosol protein) is a favorable prognostic factor in serous ovarian cancer, especially in advanced stages, while the presence of PR is not related to survival (Geisler et al., 1996). The analysis of endometrial type of ovarian cancers showed the AR-expression in 70-95% of cancers, and that AR-associated protein 70 (ARA70) expression might be involved in the etiology/progression of ovarian cancer (Shaw et al., 2001). It was proved that in metastatic ovarian cancers the presence of ER and AR significantly lowered the effectiveness of chemotherapy (Hamilton et al., 1983).

Having compared our results (the normal ovarian cells in women aged 48-60 show the phenotype PR(+) / AR(+) / ER- α (+) in all studied groups) to the results of other authors (Makhova et al., 1984; Trapani, 1992; Geisler et al., 1996; Lau et al., 1999; Munstedt et al., 2000; Hogdall et al., 2007) we state that neoplastic transformation can occur in ovarian cells especially when estro-progestagen therapy is used. Whereas our study was done on a group of post-menopausal women that had never used a hormonal therapy. Thus it would be interesting to analyze the immunoexpression and immunolocalization of steroid hormone receptors in normal ovaries of postmenopausal women with and without hormonal therapy, especially when the published data shows that estrogens in hormonal therapy can increase the risk and mortality in ovarian cancer (Cunat et al., 2004). The results of our study also contribute to knowledge of the aging processes in human gonads, in structural as well as functional aspects.

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