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Expression of Estrogen Receptors (α, β), Cyclooxygenase-2 and Aromatase in normal endometrium and endometrioid cancer of uterus

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ABSTRACT

Purpose: Endometrial cancer (EC) is one of the most common malignancies of the female genital tract, but the etiology, especially its metabolism is still investigated. The aim of this study was to evaluate the presence and relative expression of Estrogen Receptors (α, β), Cyclooxygenase-2 and Aromatase in both endometrial cancer and normal mucosa.

Material/Methods: Two groups of women were selected for the study: 1) patients with endometrioid endometrial cancer (FIGO I; G1 - G3) (n=35) and 2) subjects with normal endometrial tissue (control group, n=29). The expression of Estrogen Receptors (ERα, β), Cyclooxygenase-2 (COX-2), Aromatase were estimated by Western blot analysis. Furthermore, the associations between FIGO classification (stage: Ia, Ib), tumor grade (G) and expression of ERα, β, COX-2, aromatase proteins were evaluated. Overall and disease-free survival curves were generated according to the Kaplan-Meier method. Median follow-up time of the patients examined in this study was 39 months.

Results: The relative expression of each examined protein was markedly higher in the endometrial cancer tissue as compared to the healthy endometrium. The trends towards greater expression along with a tumor progression was noticed (FIGO stage: Ia vs. Ib). Analysis of endometrial cancer risk factors and their influence on survival curves showed only an inverse significant correlations between obesity (BMI: 36.2; n=21) and disease-free survival in EC group (p=0.00872), but there was no significant association between obesity and overall survival (p=0.358).

Conclusions: Endometrioid endometrial cancer shows relatively higher expression of either ER, COX-2 and aromatase comparing to healthy mucosa, suggesting their involvement in tumor development and progression.

Key words: Endometrial cancer, Estrogen Receptors, Cyclooxygenase-2, Aromatase

INTRODUCTION

Endometrial cancer (EC) is the most common malignancy of the female genital tract, comprising more than 90% of all uterine neoplasms. Since the 90's, the mortality rate for endometrial adenocarcinoma has increased by 50%. Arising from the glands within the endometrium, it predominantly affects postmenopausal women, with a median age of incidence of 65-69 years in European Countries [1].

It is well recognized that estrogens (Estrone - E_1, Estradiol – E_2) play an important role in the development and progression of EC [2]. Results from many clinical and epidemiological studies demonstrated that excessive and/or prolonged exposure to estrogen (not opposed by progesterone) is an important risk factor for the development of EC, especially that of the endometrioid type (type 1 by Bokhman's classification) [3-5]. However, many other risk factors for EC development have been recently implied including: high

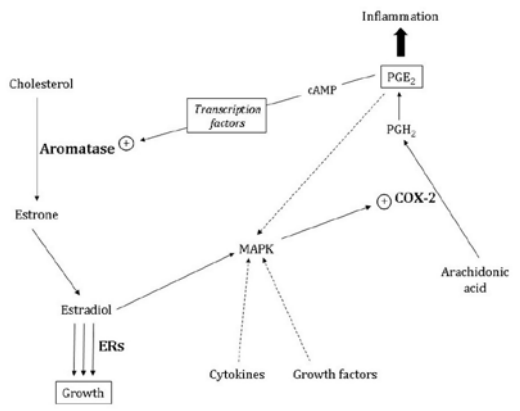
postmenopausal body mass (obesity), hypertension, hormone replacement therapy, anovulatory states, early menarche, late menopause and null parity [6-9].

It seems that molecular biology of EC has been extensively studied, but there are still some controversies [2, 10-12]. Recently, much attention has been paid to the specificity of in situ estrogen metabolism [2]. Results from different studies have demonstrated an increased tissue estrogen content in human endometrium compared to non-cancer tissues [13]. Others have also reported that the tissue concentration of E_1, E_2 and their sulfate metabolites were several times higher in tumor tissue than measured in serum [2]. These findings all indicated that intratumoral estrogen metabolism and synthesis are important in the etiology and progression of EC [14, 15].

Aromatase is an important, key enzyme which catalyses the conversion of androgens (androstenedione, testosterone) to E_1 and E_2 respectively (*Fig. 1*). Increased local tissue aromatase expression is also involved in the pathogenesis of several malignancies: breast cancer, liver, prostate, colon cancer [11]. Several studies, but not all have shown that the expression of aromatase in endometrial cancer tissues is significantly greater than in benign endometrial lesions [16, 17].

Among the many identified risk factors for type I EC, obesity seems to be the most important one (increases the risk of EC 3 – 10 times) [9]. One of the putative mechanisms involve the peripheral conversion of cholesterol to 4-androstenedione (17-ketoestosterone) in adipose tissue that leads to elevated level of estradiol. Another opinion explaining high level of estradiol has underlined a result of the direct carcinogenic effects of dietary N-6 polyunsaturated fatty acid, which are metabolized via cyclooxygenase (COX) and lipooxygenase enzymes. COX-2 gene has been characterized as highly associated with cellular growth and differentiation. Recent data have suggested that COX-2 has been involved in the process of malignant transformation and tumor progression, even in EC [18].

Figure 1. **A schematic diagram showing major interactions between estrogens, COX-2 and aromatase in normal tissue.**



Based on this findings and existing controversies we sought to investigate the relative expression of both: ER (α,β), COX-2 and aromatase in endometrial cancer at different stages of progression (FIGO stage: Ia, Ib). Additionally overall and disease-free survival curves were generated according to the Kaplan-Meier method.

MATERIAL AND METHODS

Patients and tissue

The present study conforms with the principles outlined in the Declaration of Helsinki and was approved by the Ethical Committee for Human Studies of the Medical University of Bialystok. All patients gave their informed consent prior to their inclusion in the study. Between 2006 to 2010 two groups of women were investigated in the Department of Gynecology and Gynecological Oncology, Medical University of Bialystok, Poland: 1) patients with endometrial cancer (n=35) and 2) subjects with normal endometrial tissue (control group, n=29). Women suspected to have the cancer of corpus uteri were examined in outpatient clinic and biopsies were then taken. Standard histopathological parameters of the tumors were determined by two independent pathologists. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO and staged according to the International Federation of Gynecology and Obstetrics system (2009) where FIGO Ia (n=21) was classified as tumor confined to the uterus, no or < ½ myometrial invasion, FIGO Ib (n=14) - tumor confined to the uterus, > ½ myometrial invasion. In the group of diagnosed endometrial cancer only type endometrioid, FIGO I, grade 1-3 were included in the study. Radical hysterectomy, bisalpingo-oophorectomy, pelvic lymphadenectomy and peritoneal washing as standard procedures were performed in those patients. Endometrial samples were collected just after removal of the uterus and immediately placed in liquid nitrogen. None of the patients from this group had also received preoperative chemotherapy, pelvic radiation and hormonal cancer therapy.

Control endometrial tissue was collected during non-oncological operations, mostly because of fibroids. Supracervical hysterectomy or total hysterectomy was performed and endometrial samples were stored as described above.

Median follow-up time of the patients examined in this study was 39 months (range, 5 – 61 months). Overall survival (OS) and disease-free survival (DFS) were calculated from the time of the first surgery to the recurrence and/or death or the date of last contact. Survival times of patients still alive or lost to follow-up were restricted to June 2011. In both groups examined, endometrial cancer risk factors such as age, the presence of hypertension, obesity (BMI), glycemia were retrieved and evaluated. Body Mass Index (BMI) was defined as the weight (kg) divided by the square of the height in meters (kg/m^2).

Western-blot analysis (ERs, COX, Aromatase)

Tissue samples were homogenized in ten volumes of ice-cold RIPA buffer (50 mM Tris – HCl, pH 7.4, 1% NP – 40, 0.25% DOC, 150mM NaCl), with the addition of protease inhibitors cocktail (1mM EDTA, 1mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin, 1μg/ml pepstatin). Next, samples were centrifuged at 10,000 g for 30 min at 4ºC. The supernatant was saved and the protein content was measured with BCA protein assay kit (Sigma). Bovine serum albumin (fatty acid free, Sigma) was used as a standard. Proteins (70 μg) were separated by SDS PAGE on 10% gel. Separated proteins were transferred on nitrocellulose membranes (BioRad) in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) at 30 V for 16 h at 4°C. The membranes were then incubated with 5% non-fat milk in TBS for 1 h. Then after membranes were incubated overnight at 4°C with primary antibodies against ERα (1:500), ERβ (1:1000) (Acris Antibodies) aromatase and COX (1:500) (Santa Cruz Biotech) or β-actin (loading control) (Sigma) and kept overnight at 4ºC. After three washings in TBS-T, membranes were incubated with appropriate alkaline phosphatase-conjugated secondary antibody (Sigma). Protein bands were scanned and quantified using a Gel Doc EQ system (Bio-Rad).

Statistical analysis

All data are presented as means ± SD. Statistical comparison was performed using Mann-Whitney test or Spearman Rank test to study the relationships between proteins. Association between stage: Ia, Ib, tumor grade (G) and expression of ERα, β, COX-2, aromatase proteins was estimated by non-parametric analysis of variance test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method and the statistical significance was calculated using the log-rank test. The results were considered significant when the $p<0.05$.

RESULTS

General characteristic of the patients

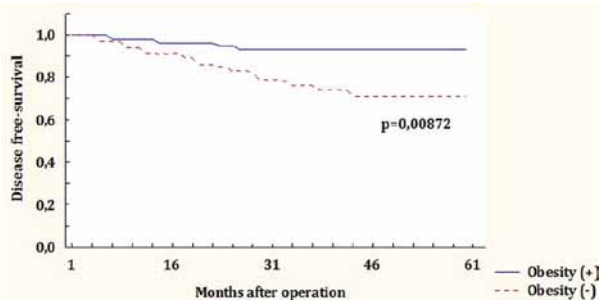
A total of 28 out of 35 (80%) patients with endometrioid endometrial cancer (EC) underwent complete surgery. The remaining 7 (20%) patients underwent total abdominal hysterectomy and bisalpingo-oophorectomy without lymphadenectomy because of obesity (BMI≥40). These patients received not complete surgery. In all cases first biopsy diagnosis (histology, stage, grade) were post-operatively confirmed and none of them changed their classification. Out of the 28 patients who underwent complete surgery, 22 were classified as FIGO I and G1, G2; final histology reports of 6 women confirmed FIGO I and G3.

Women who did not undergo complete surgery (n=7) were classified as FIGO I and G1, G2 and there were no cases of G3. None of the women from the group of patients who underwent complete surgery had lymph node metastases. Because of early stage (FIGO I) and no lymph node metastases all patients from endometrial cancer group did not receive any adjuvant therapy.

All selected patients in the control group (n=29) with normal endometrial mucosa had no hypertension or hyperglycemia nor obesity (BMI was 29.4), with the average age around 48.5 (range 37 – 59). The average age in the group of patients with endometrial cancer was around 58.9 (range 44 – 76) and there was no evidence of hypertension nor diabetes.

Among all analyzed endometrial cancer risk factors and their influence on survival an inverse significant correlation was detected only between obesity (BMI: 36.2; n=21) and disease-free survival in this group (p=0.00872), (*Fig. 2*). There were no other significant associations between obesity and overall survival of the patients (p=0.358; data not shown).

Figure 2. **Disease free-survival in EC patients with obesity (+) – BMI ≥ 30.**



ER (α,β), COX-2 and aromatase expression

The relative expression of all examined proteins was markedly higher in the endometrial cancer tissue (n=35) as compared to the healthy endometrium (n=29). The expression of both ERs isoforms (α, β) in endometrial cancer tissue was greater nearly 2-fold (ERα: 1.8-fold; ERβ: 1.7-fold; $p<0.0001$), (*Fig. 3* and *Fig. 4*) with a relative expression of ERα to ERβ in this tissue 1.4. In normal endometrium, ERα level was also at least 1.3-fold higher than ERβ. The expression of COX-2 in the endometrial cancer tissue was 1.6-fold higher than in the normal endometrium ($p<0.0001$), (*Fig. 5*). Western blot analysis revealed greater expression of Aromatase which was 1.5-fold higher in endometrial cancer than in normal mucosa ($p<0.0001$), (*Fig. 6*).

In the group of endometrial cancer, we observed statistically significant positive correlations between high expression of all examined proteins and obesity (BMI≥30; $p<0.05$; data not shown).

There was no statistical correlation of the altered expression of ERα, β, COX-2, aromatase and stage of endometrial cancer (FIGO Ia, FIGO Ib). However, analyzing ERα, β, COX-2, aromatase expression and grading (G1, G2,

Figure 3. **Expression of ERα in normal endometrial mucosa and endometrial cancer.**

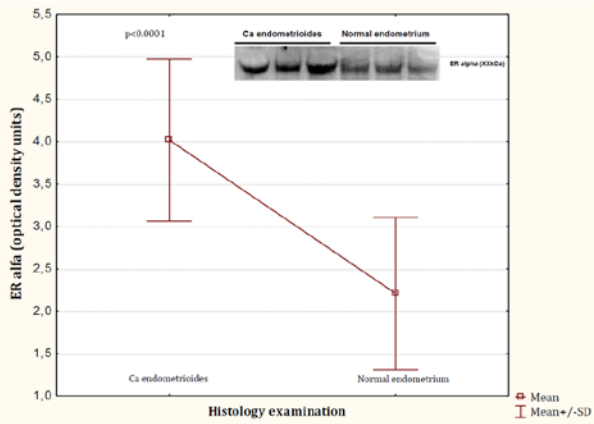


Figure 4. **Expression of ERβ in normal endometrial mucosa and endometrial cancer.**

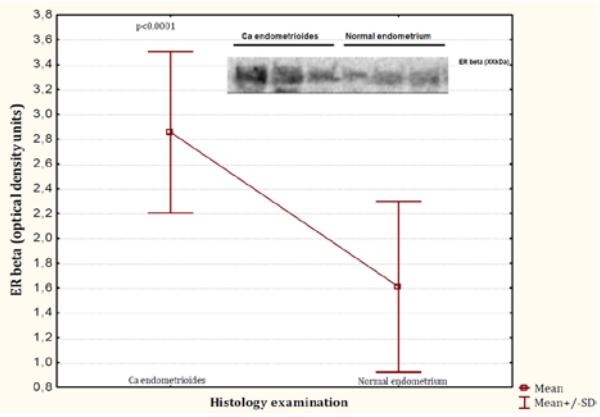


Figure 5. **Expression of Aromatase in normal endometrial mucosa and endometrial cancer.**

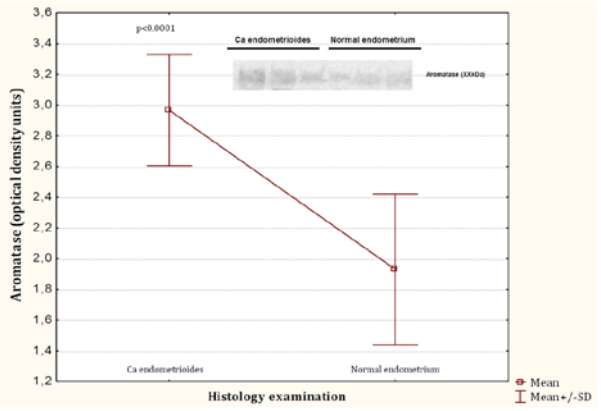
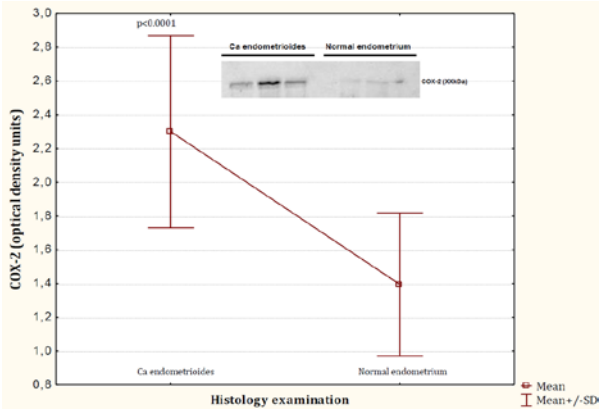


Figure 6. **Expression of COX-2 in normal endometrial mucosa and endometrial cancer.**



G3) showed that all of the proteins were elevated with tumor progression compared within normal tissues. There was also observed a trend for G1 tumors (n=18) to express higher ERα, β, COX-2 proteins level than G2 and G3 of endometrial cancer (n=17) (data not shown).

Spearman Rank correlation analysis showed significantly positive relationship between all analyzed elements with strong correlation of ERα with aromatase and ERα with COX-2 in the group of EC ($p<0.05$), (*Tab. 1*).

Table 1. **Rang Spearman correlations |r| between Aromatase, COX-2, ERα and ERβ proteins in endometrial cancer group; all values are statically significant ($p<0.05$) within endometrial cancer group.**

Correlation (r)	Rang Spearman correlation – endometrial cancer			
	Aromatase	COX-2	ERα	ERβ
Aromatase	1	0.683	0.764	0.634
COX-2	0.683	1	0.822	0.555
ERα	0.764	0.822	1	0.649
ERβ	0.634	0.555	0.649	1

DISCUSSION

Endometrial cancer, especially FIGO type I is commonly classified as "endocrine-dependent malignancies" [19-21]. Current theory regarding the risk of developing endometrial cancer emphasizes the importance of high estrogen exposure unopposed by progesterone throughout a woman's life [22, 23]. This impaired ER vs. progesterone balance results in higher mitotic activity in endometrial tissue and therefore increases the risk of neoplastic transformation [24]. Several studies have demonstrated that E_2 and E_1 levels in tumor tissues were higher than in normal endometrial mucosa [13, 25, 26]. They have demonstrated that human endometrial carcinoma tissue contains functioning enzyme system required for local biosynthesis of estrogen. Among these enzymes, aromatase, 17β-hydroxysteroid dehydrogenases and steroid sulfatase are the major enzymes playing an important role in the formation of biologically active estrogen – estradiol [2, 11, 18, 23]. Our study confirmed greater expression of ERα proteins (1.8-fold) and of ERβ (1.7-fold) in endometrial cancer tissues. Other authors have also shown positive correlations of ERs mRNA, higher expression of ERs proteins and increased level of E_1 and E_2 in endometrial tumor [13, 27, 28]. In this type of cancer

Ito et al. [2] noticed also statistically significant association between expression of ERs proteins, increased level of E_1 and E_2 and obesity. In our study, in the group of endometrial cancer we observed statistically significant correlations between high expression of all examined proteins and obesity (BMI≥30; $p<0.05$). Additionally, an inverse correlation was also detected between obesity (BMI: 36.2) and disease-free survival (DSF) in this group ($p=0.00872$), with no other significant associations between obesity and overall survival of these patients ($p=0.358$). Presented results were similar to recently published data, in which examined Japanese women with endometrial carcinoma (obesity was defined by Japan Society for the Study of Obesity as BMI≥25) patients with endometrial malignancies had high BMI, but paradoxically obese women had longer DSF compared to non-obese patients with cancer [29]. Anderson et al. [30], demonstrated also that DSF increased significantly and recurrence rate decrease with increased BMI. Similar results were published by Everett et al. [31], in whose study, women with BMI>40 had a lower recurrence rate compared with those with BMI<30 (4.7% vs. 13%), although there was no statistical significance of those estimated values.

There are some studies presenting interrelation between ERs, COX-2 and aromatase in cancer tissue [11, 16, 18, 32]. However, there is still controversy in terms of its prognostic significance and whether it is an early or late event in the development of EC [10, 16, 18, 32]. Interestingly, hormonal control of COX-2 has been described in the literature but not fully explored [18]. Niwa et al. [33], showed that 17-β estradiol inhibits COX-2 mRNA expression in cell culture and that it is potent as steroids (dexamethasone) in inhibiting both COX-2 mRNA expression and prostaglandins production. However, Zhuo et al. [34] identified an alternative mechanism of hormonal control that is related to the chronically elevated gonadotropin and luteinizing hormone levels that promoted morphologic as well as functional differentiation of endometrial stromal cells into decidua. Our study showed, that human endometrial cancer was characterized by increased expression of COX-2 as compared with normal endometrium. Increased expression of COX-2 in EC is not the case in all studies as some show no change in COX-2 expression in the endometrial cancer or the endometrial hyperplasia [35]. Others detected COX-2 mRNA and protein expression by immunohistochemical analysis in 8 out of 11 endometrial cancers, but found no expression in 3 samples of normal tissues [36]. Fujiwaki et al. [37, 38] reported increased COX-2 expression in 63 patients with EC (51%). Ferrandina et al. [39] found that COX-2 positivity occurred more often in endometrial cancer with extrauterine involvement and in those with deep myometrial invasion. These findings suggest that COX-2 expression may correlate with tumor aggressiveness [39, 40]. In our study, we analyzed FIGO I (stage) endometrial cancer, grade: G1, G2, G3 and ERα, β, COX-2, aromatase expression and there was no statistically association between Ia and Ib stage. However, a trend was noticed for G1 tumors to express higher ERα, β, COX-2 proteins level than grade 2 and 3 of EC. In accordance, some studies showed elevated expression of ERα in G1 tumor compared within normal endometrium and higher grade tumors, with a significant negative association between ERα expression and tumor grade 2 and 3. However, it is not the case in all patients since Pathirage et al. [41] reported also no statistically significant differences in ERβ expression between any of the samples and there was a similar trend for G1 tumors to express only higher ERβ levels than G2, G3 tumors. Orejuela et al. [35] in a similar analysis have ascertained no differences detected in COX-2 expression on the basis of histological grade or stage of the cancer.

In our study, endometrial carcinoma was also characterized by increase expression of aromatase proteins (1.5-fold higher in patient with cancer) and this observation is in line with other studies [42-46]. As already mentioned in the introduction, it is a key enzyme in estrogen synthesis with significantly higher level of expression in cancer of uterine corpus than in healthy endometrium. Ito et al. [2], demonstrated that aromatase expression was significantly increased, both at the protein and mRNA levels at the site of invasion in endometrial cancer. This may suggest an induction of aromatase expression by the tumor cells and/or stromal interaction between neoplastic and stromal cells. Contrary to the study by Ito et al. [2], Tarkowski et al. [47] reported that expression of aromatase was up-regulated in normal compared to neoplastic tissue. These discrepancies probably arose based on the use of non-specific antibodies [2]. Segawa et al. [48] found a significant correlation between aromatase expression in stromal cells and poor prognosis in patients with EC. It was also observed that this positive correlation had indicated that local aromatase expression has played an important role in tumor progression through the formation of in situ estrogens [48]. In our study, we observed a significantly higher expression of aromatase in EC compared with healthy mucosa. Pathirage et al. [41] also reported that aromatase is up-regulated but only in G1 of endometrial carcinoma.

Recent publications have shown some advantages in using COX-2 inhibitors for the treatment of breast cancer, but in combination with aromatase inhibitors [17, 49]. COX-2 inhibitors have effected intratumoral aromatase, angiogenesis and apoptosis. It was also found that increased prostaglandin synthesis from COX-2 expression prompts formation of aromatase. This seems to be important in the treatment of endometrial cancer due to the importance of local steroid production in EC [18].

CONCLUSIONS

Our study shows that there is relatively higher expression of both ER, COX-2 and aromatase in endometrial cancer tissue comparing to healthy mucosa. This finding suggests their local involvement in tumor development and progression. It may also imply the possibility of protective effects of COX-2 and aromatase inhibitors as potential targets in the treatment of EC.

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