Insights into molecular pathways of endometriosis and endometriosis-related ovarian carcinoma

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Abstract

Background: Endometriosis is a benign estrogen-dependent gynecological disease involving components of the female genital tract (uterus, Fallopian tubes, ovaries, large, round, and utero-sacral ligaments) and intra- and extraperitoneal regions. Since the moment of its etiopathology has been identified, the intrinsic capacity of endometriosis malignant transformation has been hypothesized. Patients, Materials and Methods: Our study included a total number of 50 patients diagnosed with endometriosis (31 cases) and endometriosis-related ovarian carcinoma (EOC) (19 cases). A clinicopathological and immunohistochemical study directed towards the detection of atypical transitional lesions and the similitudes in epithelial–mesenchymal transition (EMT) phenomenon [E-cadherin/β-catenin/cytokeratin 18 (CK18)], apoptosis [B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X (Bax)], and hormonal dynamics mirrored by the immunomarkness of estrogen receptor (ER) and progesterone receptor (PR) in endometriosis and EOC glands and stroma has been performed. Results: Our study showed a higher immunomarkness of CK18 and E-cadherin in endometriosis than in neoplastic counterparts, while β-catenin had a stronger immunomarkness in tumors compared with endometriotic areas, with statistically significant differences between the studied groups. Bcl-2/Bax higher rate in endometriosis had a statistically significant association to a more aggressive tumor behavior (p=0.027). Stromal ER and PR immunomarkness has been significantly lower in endometriosis in comparison to tumor stroma (p=0.001) and PR stromal immunomarkness had been higher in more differentiated tumors compared to less differentiated types (p=0.005).

Conclusions: Our study supports that endometriosis is a precursor of EOC by the identification and the coexistence of both lesions in the investigated cases, the identification of intermediate lesions, as well as the expression of EMT immuno-markers, along with apoptosis and steroid receptors immunomarkness.

Keywords: endometriosis, endometriosis-related ovarian carcinoma, epithelial–mesenchymal transition, apoptosis, steroid hormones.

Introduction

Although endometriosis has been described in 19th century, it remains an enigmatic entity, because of its complex etiopathology, lack of specific diagnostic markers, and its potential association with specific malignancies. Both surgical and medical techniques are not addressed to etiology but to symptomatology. In this context, recurrences and symptoms persistence are frequently registered, making endometriosis a debilitating disease.

This disease is now considered to have a complex determinism, involving genetic, epigenetic, immunological, proteasic, angiogenetic, anti-apoptotic, and proliferative factors that confer multiple facets to the condition and that require a multimodal treatment approach.

In an effort to elucidate the pathogenesis of endometriosis, many theories as retrograde implantation, coelomic metaplasia, induction of endometrial proliferation, embryonic remnants, vascular and lymphatic metastasis, involvement of stem cells from either bone marrow or endothelial progenitors, have been proposed over the years, without reaching a consensus on the mechanism of producing this disease, but considering that it is actually a combination of these mechanisms and accordingly the pathogenesis of endometriosis being multi-factorial and multi-compartmental [1–3].

The malignant transformation of endometriosis is well known [4] and is attributed to two possible mechanisms: either endometriotic implants may directly undergo malignant transformation, or both processes share the same precursor mechanisms and/or predisposing factors with evolutionary molecular pathways divergence [5].

Moreover, based on genomics, transcriptomics, and proteomics, by correlation between genetic mechanisms related to endometriosis transition to malignancy, new algorithms of diagnosis and therapy can be achieved. Accordingly, a special focus has been given to search a possible similar molecular model in both diseases. Thus, the identification of some key molecules of endometriosis and endometriosis-related ovarian carcinoma (EOC) pathogenesis, one or more mechanisms could be validated.

In general, in the mechanism of implantation, three
phenomena are succeeding, namely, apposition, adhesion, and invasion [6]. During these phenomena dynamics, several adhesion molecules are involved, such as cadherins, selectins, integrins, galectins, heparan sulphate, and trophinin–tassin–bystin complex [6]. Considering the role of the process of epithelial–mesenchymal transition (EMT), added to its opposite mechanism, of mesenchymal–epithelial transition (MET) in endometriosis, E-cadherin, β-catenin, and cytokeratin 18 (CK18) are known to be useful markers in the study of endometriosis, demonstrating the initiation of early E-cadherin–β-catenin complex mutations in the EMT process. It is estimated that β-catenin mutations represent an early event in endometriotic-dependent ovarian carcinogenesis and CK18 expression is correlated with EOC stage progression along with EMT, as the malignant process is extending [7].

Apoptosis represents a component of tumor growth, considering its antagonism to cellular proliferation [8] and that it is frequently inhibited in variable types of tumors [9]. This process is prevented in malignant cells and is correlated to carcinogenesis [5, 10], being associated to malignant transformation in ovarian tumors [11], to high-grade tumors, and with a poor prognosis [12, 13]. Moreover, this process is associated to high-grade tumors, and with a poor prognosis in ovarian tumors [12, 13]. B-cell lymphoma 2 (Bcl-2) family maintains the balance between apoptosis and its inhibition, by pro- and anti-apoptotic molecules, in functional antagonism [8, 14, 15]. An apoptotic evasion mechanism has been proven, allowing the development of ectopic implants, in endometriosis. Furthermore, Bcl-2/Bcl-2-associated X (Bax) ratio is progressively increasing in EOC compared to endometriosis [16].

Steroid hormones and hormone-like substances have a major role in endometrium normal function and their unbalance results in different endometrial diseases, including endometriosis. Numerous studies have demonstrated that estrogen receptor (ER) and progesterone receptor (PR) immunoeXpressions are associated with endometriosis and EOC [17, 18].

As a hormone-related pathology, endometriosis has an enhanced level of estrogens within the endometrial tissue, associated with an increased rate between ER-β and ER-α, thus resulting in a low PR immunoeXpression [19, 20]. Progesterone effect, modulated by the expression of both isoforms of the specific receptor, PR-A and PR-B, is involved in endometriosis and endometriosis-related carcinomas pathogenicity [21, 22]. It has been demonstrated that the cytokines production, in endometriosis, is the modified response to progesterone, with a characteristic decreased PR-B/PR-A ratio and reduced immunoreactivity of PR-B [19, 23]. In a close correlation to PR immunoeXpression, ER immunoeXpression has a major importance in clinicopathological manifestations of ovarian carcinoma (OC), including in that associated to endometriosis [21, 22]. Thus, it seems that alteration of steroid receptor immunoeXpression is correlated to ovarian endometriosis and endometriosis-related carcinogenesis.

**Aim**

Our study compared a group of cases with endometriosis and a group with ovarian malignancies associated with endometriosis in order to evaluate the immunoeXpression of some molecules of the EMT process (E-cadherin, β-catenin, and CK18) associated to apoptotic immunomarkers (Bcl-2 and Bax) and hormonal profile (ER and PR), providing correlations with molecular changes sequence during the pathogenic mechanism of these diseases.

**Patients, Materials and Methods**

**Patients**

We conducted a retrospective study on 50 patients diagnosed with endometriosis and EOC. Patients were divided in two groups. The first group comprised of 31 cases with endometriosis, which were diagnosed in the Department of Histopathology of the Elena Doamna Clinical Hospital in Iași, Romania, between January 2005 and April 2017. The second group consisted of 19 cases of OCs associated with endometriotic lesions, diagnosed between February 2013 and January 2016, in the Regional Institute of Oncology, Iași, which have been treated by total radical hysterectomy and pelvic lymphadenectomy.

The relevant clinicopathological data have been collected from the medical records in both groups (Table 1). Accordingly, age, parity, menopausal status, type (unifocal versus multifocal and cystic lesions), site (ovarian, cervical, tubal, and cutaneous), and associations with other gynecological pathologies have been recorded in endometriosis group. Supplementary to age, parity, menopausal status, data regarding tumor size, ovarian capsular invasion, histological types of EOC, International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d’Obstétrique – FIGO), tumor, node, metastasis (TNM) stages, and cancer antigen 125 (CA125) serum values have been recorded in EOC group.

| Table 1 – The main clinicopathological features in the study groups |
|---------------------------------------------------------------|
| **Endometriosis group (n=31 cases; median age: 36.61 years)** |
| **Endometriotic foci** | **Parity** | **Menstrual status** | **Associated lesions** |
| Parietal/mural | Ovarian uni/bilateral | Mixed-multifocal | Multiparous | Menopausal status | ULM | AD | OC | CC |
| 3 | 22 | 6 | 22 | 9 | 7 | 10 | 5 | 9 |
| **Epithelial ovarian tumors associated with endometriosis (n=19 cases; median age: 59.10 years)** |
| **Histological types** | **FIGO stages** | **Histological grading** | **TNM system** |
| Endometrioid carcinoma | Non-endometrioid carcinoma | I | II | III | GI | GII | GIII | T1 | T2 | T3 |
| 8 | 11 | 4 | 6 | 9 | 1 | 6 | 12 | 5 | 6 | 8 |

AD: Adenomyosis; CC: Chronic cervicitis; FIGO: International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d’Obstétrique); OC: Ovarian cysts; TNM: Tumor, node, metastasis; ULM: Uterine leiomyomas.
Methods

All cases were investigated by applying the immunohistochemical (IHC) method, using a panel of primary antibodies with the appropriate dilutions (Table 2).

Table 2 – Antibodies types, clones, dilutions, and immunostaining pattern used in the immunohistochemical technique

| Antibody | Type | Clone / Manufacturer | Dilution | Immunostaining pattern |
|----------|------|----------------------|----------|------------------------|
| E-cadherin | MoAbs | 36B5 / Novocastra | 1/50 | Membrane |
| β-catenin | MoAbs | β-catenin-1 / Dako | 1/200 | Membrane and cytoplasm |
| CK18 | Mouse MoAbs | DC-10 / Novocastra | 1/100 | Membrane |
| Bax | Rabbit PoAbs | Polyclonal / Dako | 1/1500 | Cytoplasm |
| Bcl-2 | Mouse MoAbs | Bcl-2/100/D5 / Novocastra | 1/80 | Cytoplasm |
| ER | Mouse MoAbs | 6F11 / Novocastra | RTU | Nuclear |
| PR | Mouse MoAbs | PGR-312/16 / Novocastra | RTU | Nuclear |

Immunohistochemistry was performed on representative tissue samples of each type of lesion. Serial sections from each paraffin block, of 3–4 μm thickness, were cut and placed on slides SuperFrostPlus. The immunostaining was performed using the automated BenchMark XT system (Ventana Medical System, Inc., Tucson, AZ, USA), following standardization.

Negative controls, obtained by replacement of primary antibodies with distilled water, along with positive controls have been simultaneously run.

Semi-quantitative analysis

Semi-quantitative assessment of IHC reactions was performed using the score systems reported in the literature, for each immunomarker. The differences in quantification methods applied are justified by immunomarkers specific patterns of immunoreexpression, which require or not a double measurement (intensity and the percentage of positive cells).

The score systems took into consideration the immunostaining intensity multiplied with the percent of positive cells with a cut-off of 4 (<4 – negative and low score and ≥4 – high score). The immunostaining intensity for all investigated immunomarkers was considered as follows: 3 as a strong intensity, 2 as moderate, 1 as weak, and 0 as negative immunostaining.

The percentage of E-cadherin immunopositive cells was classified as 1 for ≤10%, 2 for 11–50%, and 3 for ≥50%. The percentage of cells which displayed a β-catenin immunopositivity was scored as following: 1 for <10%, 2 for 10–50%, 3 for 50–75%, and 4 for >75% positive cells. The percentage of cells which showed CK18 immunopositivity was evaluated as 4 in >76–100%, 3 in 51–75%, 2 in 26–50%, 1 in 6–25%, and 0 in 0–5% positive cells [7, 24, 25]. For Bcl-2 analysis, the percentage of immunopositivity was scored as 4 in >75%, 3 in 51–75%, 2 in 26–50%, 1 in 1–25%, and 0 in no positive cells. Score 0 has been considered as negative, and scores 1–4 have been considered as positive [26].

A score which was exclusively based on the immunostaining intensity has been used for Bax and accordingly, the cases have been divided into three categories, as follows: weak intensity (+), moderate intensity (++), and strong positivity (+++) [27].

Allred score [28, 29], based on index of positive cells, has been used for ER and PR immunostaining epithelial quantification, with the score considered as 0: none, 1: <1/100 (<1%), 2: 1/100–1/10 (1–10%), 3: >1/10–1/3 (10–33%), 4: >1/3–2/3 (33–66%), 5: >2/3 (>66%), added to immunostaining intensity. ER and PR were also qualitatively assessed in stromal cells.

Statistical analysis

Statistical data processing was performed by Statistical Package for the Social Sciences (SPSS) v. 19.0 (IBM SPSS Statistics), with mean, standard deviation, and the categorical variables as number (%) for continuous variables. The degree of correlation between the markers investigated and the clinicopathological findings was achieved by χ² (chi-squared) test or Fisher’s exact test. In order to evaluate continuous variables, Student’s t-test or the Wilcoxon rank-sum test were performed, with significant values in p<0.05.

Results

Results in endometriosis

E-cadherin expression had a homogeneous, membrane distribution throughout the entire endometriosis foci in glandular epithelium and had been also noticed in few stromal cells. The assessment of E-cadherin showed positive immunoreexpression in all cases, with moderate intensity in 18 (58.06%) cases and strong intensity in 13 (41.93%) cases (Figure 1A). The percentage of positive cells was higher than 50% in 23 (77.41%) cases (Table 3).

In endometriotic area, glandular epithelium showed homogeneous, membrane and cytoplasmatic β-catenin immunoreexpression (Figure 2). β-catenin immunostaining intensity was moderate in 14 (45.16%) cases and strong in 17 (54.83%) cases. The percentage of positive cells was over 50% in 14 (45.16%) cases. An increased β-catenin immunoreexpression could be observed as well in the periglandular stromal cells.

Most of epithelial cells in the endometriotic foci were strongly positive for CK18 and only a small proportion of cells, immunostained moderate or weak, CK18 intensity being moderate and weak in nine (29.03%) cases and strong in 22 (70.96%) cases. Immunoreexpression of CK18 was found to be homogeneous, with a higher intensity than E-cadherin and β-catenin immunoreexpression. The percentage of CK18 immunopositive cells was higher than 50% in almost all cases [29 (93.54%) cases] (Figure 3).

The correlation analysis between EMT immunomarkers expression (E-cadherin, β-catenin, and CK18) did not reveal any statistically significant associations.

Bcl-2 and Bax had a variable cytoplasmic distribution, in the endometriosis foci, though some heterogeneous areas were focally identified. Bcl-2 immunoreexpression has been negative in 15 (48.38%) cases and positive in 16 (51.61%) cases of endometriosis (Figure 4), while Bax
showed negative or weak immunoeexpression in 14 (45.16%) cases of endometriosis and moderate or high immunoeexpression in 17 (54.83%) cases (Figure 5). The percentage of Bcl-2 immunopositive cells was over 50% in two (6.45%) cases (Table 3).

A negative Bcl-2 immunoeexpression associated a negative Bax immunoeexpression in two (13.33%) cases and Bax immunopositivity in 13 (86.66%) cases, while cases with positive Bcl-2 immunoeexpression associated a negative Bax immunoeexpression only in one case (6.25%) and Bax immunopositivity in 15 (93.75%) cases.

### Table 3 – Distribution of the investigated immunomarkers in endometriotic and EOC group

| Immuno-markers | Endometriotic group (n=31 cases) | CP features | EOC group (n=19 cases) | CP features |
|----------------|---------------------------------|-------------|------------------------|-------------|
|                | Distribution Intensity (%)      | p<0.05      | Distribution Intensity (%) | p<0.05      |
| E-cadherin     | Homogeneous, membrane glandular epithelium (all) and stromal cells (rare) | Positive (all cases) Moderate (18 cases) Strong (13 cases) Positive cells (23 cases / >50%) | Heterogeneous, membrane Negative (all cases) |
| β-catenin      | Homogeneous, membrane and cytoplasmic epithelium (all) and stromal cells (rare) | Positive (all cases) Moderate (14 cases) Strong (17 cases) Positive cells (14 cases / >50%) | Heterogeneous, membrane and cytoplasmic epithelium (all) and stromal cells Moderate to weak (three cases) Strong (16 cases) Positive cells (17 cases / >50%) |
| CK18           | Homogeneous, membrane glandular epithelium (all) and stromal cells (rare) | Positive (all cases) Moderate to weak (nine cases) Strong (22 cases) Positive cells (29 cases / >50%) | Homogeneous, membrane glandular epithelium (all) and stromal cells Moderate (five cases) Strong (14 cases) Positive cells (14 cases / >70%) |
| Bcl-2          | Heterogeneous, cytoplasmic glandular epithelium (all) and stromal cells (rare) | Positive (16 cases) Weak (11 cases) Moderate (three cases) Strong (two cases / >50%) Negative (15 cases) | Heterogeneous, cytoplasmic, finely granular pattern Weak to moderate (seven cases) Negative (12 cases) |
| Bax            | Weak/negative (14 cases) Moderate to strong (17 cases) | | Strong to moderate (12 cases) Negative/weak (seven cases) |
| ER             | Homogeneous diffusely nuclear (glandular epithelium) | Positive (22 cases) Negative (nine cases) | Homogeneous (ER)/heterogeneous (PR) Strong to moderate (15 cases) Negative/weak (four cases) |
| PR             | Positive (19 cases) Negative (12 cases) | | Strong (10 cases) Negative/weak (nine cases) |

Bax: Bcl-2-associated X; Bcl-2: B-cell lymphoma 2; CK18: Cytokeratin 18; CP: Clinicopathological features; EOC: Endometriosis-related ovarian carcinoma; ER: Estrogen receptor; NSSV: Non-statistically significant values; OE: Ovarian endometriosis; PR: Progesterone receptor; TG: Tumor grade. Student’s t-test or Wilcoxon rank-sum test, chi-squared test or Fisher’s exact test (p<0.05).

Figure 1 – (A) Positive homogeneous, apical membrane E-cadherin immunoeexpression in glandular epithelium of endometriosis; (B) Negative E-cadherin immunoeexpression in EOC, with weak positive luminal secretion. Anti-E-cadherin antibody immunostaining: (A) ×40; (B) ×200. EOC: Endometriosis-related ovarian carcinoma.
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Figure 2 – Strong, homogeneous membrane and cytoplasmic β-catenin immunoexpression in EOC (left) and surface epithelium in endometriosis (right). Anti-β-catenin antibody immunostaining, ×100. EOC: Endometriosis-related ovarian carcinoma.

Figure 3 – Limited CK18 immunoexpression in surface epithelium in endometriosis (right) compared to strong, homogenous, diffuse, membrane immunoexpression in EOC area. Anti-CK18 antibody immunostaining, ×100. CK18: Cytokeratin 18; EOC: Endometriosis-related ovarian carcinoma.

Figure 4 – Moderate intensity, heterogeneous cytoplasmic Bcl-2 immunoexpression in endometriosis (right) and weak to moderate immunoexpression in EOC (left). Anti-Bcl-2 antibody immunostaining, ×40. Bcl-2: B-cell lymphoma 2; EOC: Endometriosis-related ovarian carcinoma.

Figure 5 – Bax cytoplasmic weak immunoexpression in EOC (right) compared to moderate cytoplasmic immunoexpression in endometriosis (left). Anti-Bax antibody immunostaining, ×100. Bax: Bcl-2-associated X; Bcl-2: B-cell lymphoma 2; EOC: Endometriosis-related ovarian carcinoma.

The statistical correlation analysis revealed statistically significant differences between Bax and Bcl-2 immunoexpression in the endometriosis group (p=0.020). The statistical correlation analysis between Bcl-2 and Bax and clinicopathological factors did not reveal any statistically significant associations.

The immunoexpression of ER and PR was diffusely nuclear and homogeneous through all endometriotic area. ER immunoexpression in endometriotic areas had negative score in nine (29.03%) cases and positive score in 22 (70.96%) cases (Figure 6), while PR revealed negative score in 12 (38.70%) cases and positive score in 19 (61.30%) cases (Figure 7). The statistical correlation analysis between ER immunopositive endometriotic cells and percentage of PR immunopositive cells and clinicopathological factors revealed statistically significant differences only in the ovarian location of endometriosis (p=0.004) (Table 3).

We identified also a particular immunoexpression pattern of ER and PR in the stromal cells. According to IHC evaluation of stroma component for both hormonal immunomarkers, the percentage revealed a moderate to strong, heterogeneous immunoexpression, with the following aspects: 10 (32.25%) cases were positive, 21 (67.74%) cases had negative ER immunoreaction, while 11 (35.48%) cases were PR positive and 20 (64.51%) cases were PR negative.

The statistical correlation analysis between stromal ER/PR immunoexpression and clinicopathological factors (age, parity, menopausal status, lesional type and site, association with other gynecological disease) did not reveal any statistically significant associations.

Results in EOC

E-cadherin distribution was heterogeneous throughout
the entire tumor cells, the immunoexpression being weak in 13 (68.42%) cases and moderate in four (21.05%) cases (Figure 1B). Although immunohistochemistry revealed a lower percentage of positive cells (less than 40%) in 13 (68%) cases and a higher one (more than 45%) in six (32%) cases, all cases have been considered as E-cadherin negative, as their scores have ranged between 0 and 4. In five (26.31%) cases, we noticed a very low positivity percentage (<10% of E-cadherin-positive tumor cells), and these cases associated high β-catenin immunostaining, in more than 50% of the tumor cells (Table 3).

β-catenin immunostaining was positive in all cases with moderate and weak intensity in three (15.78%) cases and strong in 16 (84.21%) of cases (Figure 2). The percentage of positive cells was over 50% in 17 (89.47%) cases with scores ranging from 4 to 12. In tumor cells, β-catenin showed homogeneous, diffuse expression with a more intense immunostaining compared with E-cadherin (Table 3).

CK18 immunostaining in all EOC cases had a moderate [five (26.31%) cases] or strong intensity [14 (73.68%) cases] (Figure 3). For most of the tumor area, the percentage of positive cells was higher than 70%, with homogeneous immunoexpression pattern and higher intensity than E-cadherin and β-catenin immunostaining.

The statistical analysis between the EMT immunomarkers investigated in EOC group revealed significant differences only when comparing E-cadherin with β-catenin ($p=0.0001$) and also when comparing E-cadherin with CK18 immunostaining ($p=0.0001$).

The correlation analysis between EMT immunomarkers (E-cadherin, β-catenin, and CK18) expression in EOC and clinicopathological characteristics (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values) did not reveal any statistically significant associations (Table 3).

The statistical analysis between the two studied groups showed significant differences not only for E-cadherin ($p=0.001$) but also for the other two EMT immunomarkers, namely β-catenin ($p=0.000112$) and CK18 ($p=0.032468$), in endometriosis versus EOC derived from endometriotic foci (Figure 8).
Bcl-2 immunoexpression has been negative in 12 (63.15%) cases and weak or moderate positive in seven (36.84%) cases of EOC (Figure 4), while Bax showed negative or weak immunoexpression in seven (36.84%) cases of EOC and moderate or high immunoexpression in 12 (63.75%) cases (Figure 5). Bcl-2 and Bax immunoexpression exhibited a heterogeneous, cytoplasmic, finely granular pattern, in tumor cells.

We observed an interesting immunoexpression pattern of Bcl-2 and Bax, namely groups of tumor cells that were positive for Bcl-2 (seven cases) were negative for Bax (seven cases), while groups of tumor cells that were negative for Bcl-2 (12 cases) were positive for Bax (12 cases). Furthermore, negative Bcl-2 cases associated moderately and increased Bax immunoexpression, whereas Bcl-2 immunopositive cases associated negative or low Bax immunoexpression in half of the cases (Table 3).

Cases with negative Bcl-2 immunopositivity associated Bax negativity in six (31.57%) cases and Bax positivity in six (31.57%) cases, while cases with positive Bcl-2 immunoexpression associated a negative Bax immunoexpression in one case (5.26%) and positive one in seven (36.84%) cases. No statistically significant differences have been registered between the immunoexpression of Bax and Bcl-2 in EOC. The statistical correlation analysis between Bcl-2 and Bax immunoexpression and clinicopathological factors did not reveal any statistically significant associations from a statistical point of view.

ER and PR immunopositivity has been noticed in both tumor cells and stroma. ER and PR immunopositivity has been positive, exhibiting a nuclear immunostaining in the tumor cells. The distribution of ER was predominantly homogenous, while PR showed a predominantly heterogeneous immunoexpression (Table 3). ER immunoexpression in epithelial tumor areas had negative score in four (21.05%) cases and positive score in 15 (78.94%) cases (Figure 6), while PR immunoexpression revealed negative score in nine (47.36%) cases and a positive score in 10 (52.63%) cases (Figure 7).

For both immunomarkers, ER and PR, we identified a moderate to strong, heterogeneous expression in tumor stroma. ER/PR ratio in stromal cells have been positive in more than 50% of cases (n=11; 57.89%). Negative immunoexpression has been registered in eight (42.10%) cases.

There have been no statistically significant differences registered between the immunoexpression of ER in EOC and clinicopathological factors (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values).

Regarding PR immunoexpression, significant differences have been registered between more differentiated tumors compared to less differentiated tumors (p=0.027) and accordingly, the statistical analysis revealed significant associations between PR tumor cells immunoexpression and tumor grading.

The stromal expression for both steroid immunomarkers (ER and PR) has been significantly higher in EOC when compared to endometriosis stroma, p=0.001 and p=0.000, respectively. Moreover, the statistical analysis between stromal PR immunoexpression and tumor grading revealed significant associations (p=0.005).

**Discussions**

Endometriosis is a benign disease with an increasing incidence, probable due to the progresses registered in tools of diagnosis and population enhanced accessibility to these means.

The epidemiological data identified common risk factors, such as short menstrual periods, early menarche, nulliparity, late menopause, which are common for both endometriosis and OCs and there are also common potential protective factors for both diseases, such as oral contraceptives, high parity, tubal ligation, and hysterectomy [30].

The current state of knowledge shows that there are two pathways that appear to be involved in the endometriosis potential to progress towards neoplasia: either malignant transformation, perhaps through an atypical transitional stage, or a common precursor mechanism or predisposing factors is common in both processes, with a consecutive molecular divergence [31].

EOC occurs in 60–80% of cases in association with atypical endometriosis. Numerous studies have tried to identify a common model for endometriosis and EOC. The association of these two diseases has been identified most frequently in endometroid OC, clear cell carcinoma (CCC), seromucinous carcinoma, endometrioid stromal sarcoma, and Müllerian adenosarcoma [32].

In most cases, these tumors are developed during the first decade from endometriosis diagnosis (70%), and moreover they frequently display an intermediary stage of atypical endometriosis associated in most cases (60%) [32]. Supplementary, because of endometriosis intrinsic invasive capacity and high metastatic ability, its biological behavior shows a strong homology with cancers [33].

Since a multitude of etiopathogenic mechanisms was proposed for endometriosis, a variable involvement of multiple processes may be related to location and lesional type, with the possibility that some of the phenomena may represent, in fact, consequences of an initial lesion.

One mechanism that has aroused the interest of researchers in the last decade is that of EMT, and its reverse process, MET, these being studied mainly in the carcinogenesis and metastasis process. EMT is a strictly controlled, reversible process characteristic for the embryogenesis period and can be also found during adulthood in epithelia with fast cell regeneration, such as epidermis and intestinal villi, in wound healing, and in pathological processes, such as fibrosis and inflammation [25]. EMT also occurs during carcinogenesis, being characterized by changes that give malignant cells a high potential for invasion and metastasis, respectively [25].

As E-cadherin is expressed in all epithelial cells, being involved in the maintenance of polarization and of cellular integrity [34], its loss represents a marker of EMT process [34]. An ablation of E-cadherin expression is noticed in EMT and, therefore, cells gain an increased mobility, allowing them invasion and metastasis abilities [34]. Thus, E-cadherin decreased expression is involved in a decisive way in the pathogenic mechanism of endometriosis, cells that lose expression having the same invasive and metastatic phenotype as carcinomatous cells [34].

Because ovarian endometriosis represents a clonal proliferation of cells with genetic alterations; this disease...
is considered a true neoplasm, a precursor of OC [7]. It is estimated that β-catenin mutations represent an early event in the endometriotic-dependent ovarian carcinogenesis sequence [7].

Endometriosis has been documented as a precursor lesion in 20–40% cases of endometrioid type and 40–55% of clear cell carcinoma (CCC) type of OC [7]. It has been also demonstrated that these malignancies are associated with endometriosis in 5–10% of cases, adding an intermediary phase of atypical endometriosis only in 0.7–1.6% of cases, mainly in cases suffering from longstanding endometriosis [7].

Oncogenic mutations of phosphorylation site of β-catenin [catenin beta 1 (CTNNB1) gene] result in a stable protein formation, detected in 40–60% of EOC, in 52.4% of associated endometriosis, and in 73.3% of associated atypical endometriosis [7].

The alterations of E-cadherin, β-catenin, and CK18 are correlated to the progression along EMT process and to stages progression of EOC. An increased level of E-cadherin and β-catenin has been registered in the endometriosis group of our study and gradually decreased with the stages evolution of cases diagnosed with malignancy. This data suggests that its loss occurs late during the EMT process, being evident in the carcinogenesis process associated with endometriosis. Furthermore, the negative E-cadherin immunostaining score in our EOC group demonstrates the existence of the cadherinic switch, characteristic of the EMT process. Statistical analysis revealed in the investigated cases a significant difference between the E-cadherin immunostaining score in endometriosis versus EOC (p < 0.001). We found that β-catenin showed a marked immunointensity in the endometriosis group, showing a slight decrease in the EOC group. β-catenin percentage of immunopositive tumor cells registered a slight decrease in EOC group. Statistical analysis revealed significant differences between the group of cases diagnosed with endometriosis and EOC (p < 0.000112). This suggests the gradual loss of epithelial features as the EMT process extends. Moreover, β-catenin registered 94.73% immunopositivity in endometrioid versus 100% in CCC, suggesting its value as a poor prognosis factor in EOC.

CK18, a type I cytokeratin belonging to the cytoskeleton, expressed both in glandular endometriotic cells [35] and in carcinomatous cells, is considered an immunomarker of morphological heterogeneity and of neoplastic changes [36], including those of OCs [37]. CK18 maintains its expression in endometrioid carcinomas or in those with an endometrioid component.

CK18 showed a high immunostaining index and percentage of positive cells as a specific immunomarker of endometriotic epithelial cells, demonstrating its involvement in apoptosis in the investigated cases [38], with statistically significant differences between the endometriotic and EOC groups (p = 0.032468). In our study, in both components, intensity and immunopositive tumor cells, have registered evident changes in malignant group. Moreover, the malignant group showed a progressive reduction of the immunostaining index with the stage. This demonstrates the loss of epithelial phenotypic characteristics and the acquisition of a stromal phenotype, gradually according to the malignant process extension.

Although alterations of E-cadherin, β-catenin, and CK18 have been recorded, the correlation analysis between EMT immunomarkers expression in endometriosis did not revealed significant differences but revealed statistically significant differences in EOC group, between E-cadherin and β-catenin, on one hand, and between E-cadherin and CK18 immunoexpression, on the other hand, in EOC group, in our study.

The correlation analysis between EMT immunomarkers (E-cadherin, β-catenin, and CK18) expression in EOC and clinicopathological characteristics (age, parity, menopausal status, tumor size, ovarian capsular invasion, histological type of EOC, FIGO, TNM stages, and CA125 serum values) did not reveal any statistically significant associations, suggestive of other factors interventions in this process.

Apoptosis, as a pivotal mechanism of regulation of variable cellular populations, normal and pathological, is mainly based on the antagonism between Bcl-2 and Bax [39]. Bel-2 is preventing apoptosis without any effect on proliferation and protects against deoxyribonucleic acid (DNA)-induced apoptosis [40]. It is considered that Bcl-2 has a relevant anti-apoptotic activity, depending on Bax involvement [41, 42]. Due to apoptosis involvement in carcinogenesis, tumor promoter or suppressor roles are attributed to Bcl-2. Due to its pro-apoptotic role, Bax has the characteristics of tumor suppressor role [43]. The endometriotic cells show a weak Bax immunoexpression, associated to a high Bcl-2 immunoexpression [44]. An enhanced rate Bel-2/Bax has been demonstrated in EOC, representing a possible prognosis immunomarker and, at the same time, creates the premises for Bcl-2 antagonists and/or Bax agonists therapeutic use [8]. Furthermore, the Bel-2/Bax ratio immunoexpression in EOC compared to endometriosis reflects the decreased ovarian cell sensitivity to apoptotic stimuli. In agreement with this data, it has been demonstrated that spontaneous endometriosis may be induced by Bax and may be prevented by Bcl-2 [16].

The expression of pro- and anti-apoptotic immunomarkers revealed low levels of Bax, associated with a slight increase of Bcl-2 in the epithelial component of the investigated cases. The data obtained are consistent with literature reports showing that the relationship between anti-apoptotic and pro-apoptotic factors appears to be involved in the etiopathogenesis of endometriosis [44, 45]. It has been also demonstrated that Bcl-2/Bax high rate in endometriosis is correlated to a high malignant potential [8]. The statistical correlation analysis revealed statistically significant differences between Bax and Bel-2 immunoexpression in the endometriosis group (p = 0.020). Amplification of Bcl-2/Bax ratio demonstrates the progressive decrease in ovarian cell sensitivity to apoptotic signaling and contributes, along with the alteration of steroid receptor, to the transition mechanism to malignancy, opening new perspectives of prognosis and therapy improvement.

Although a significant difference has been identified between Bax and Bel-2 immunoexpression in endometriosis, no statistically significant differences have been registered between the immunoexpression of Bax and Bel-2 in EOC. Furthermore, no statistically significant differences have been registered between Bax and Bel-2 immunoexpression and clinicopathological characteristics in both groups, suggesting the intervention of other factors.

Estrogens action, modulated by specific receptors, ERs, has effects on the production of cytokines and of...
apoptotic phenomena, in endometriosis [46], counter-balanced by progesterone action, modulated by their counterpart specific receptors, PRs [20, 47–49]. Different from eutopic endometrium, where steroid receptors level may be correlated with endometrial cycle phases, ER being strongly expressed in proliferative stage and decreasing its expression in both epithelial and stromal components in secretory phase, no cyclical changes have been noticed in endometriosis [50]. Moreover, a generally more reduced ER immunoexpression has been observed in endometriosis [50, 51]. It is well-recognized the estrogen value as a stimulator factor of cell proliferation, in ovarian milieu, of the mobility of the malignant population of cells, and of the inhibition of the intercellular adhesions [22, 52]. Although in the secretory phase of eutopic endometrium, PR is strongly immunoexpressed, mainly in the stromal component, no cyclical changes of these receptors have been noticed in endometriosis [50], PRs showing a weak immunoexpression [50, 51]. Relatively new data have demonstrated the correlation between ER or PR immunoexpression and their clinical impact in OC [21, 22].

In the current study, the hormonal receptors immunoexpression has been noticed in both components of endometriosis, epithelial and stromal. In comparative terms with their immunoexpression in eutopic and normal endometrium, a degree of difficulty in their interpretation occurs, considering that the hormones are responsible for the cyclical stimulation of proliferation and regeneration and of secretory function, in the secretory phase, respectively. Moreover, another recent theory, based on comparative studies between the different endometriotic locations and eutopic endometrium, is focused on paracrine inhibition of steroid receptors immunoexpression in ovarian endometriosis [51].

Consequently, although the steroid receptors levels are high in ovarian endometriosis, a moderately increased immunoexpression has been observed in our group of study, without any statistically significant correlations with clinicopathological characteristics. Supplementary, PR immunoexpression has shown statistically significant differences according to ovarian location of endometriosis (p=0.04). This finding is validating PR role in endometriosis mechanism, on one hand, and on the other hand, supports the influence of paracrine factors in ovarian microenvironment, responsible for differences observed from other locations, demonstrating a high susceptibility of ovarian endometriosis to malignant transformation.

ER immunostaining score in tumor cells was high in most cases of our study, displaying a strong nuclear immunostaining, demonstrating the hormonal influence in ovarian malignancies etiopathogenesis. A high epithelial immunoexpression of ER has been also detected in endometriosis, but displaying a heterogeneous immunoexpression. Although we would expect a concordance with literature data, no significant difference has been noticed between ER immunoexpression in tumor cells of endometrioid OC compared to non-endometrioid type in the investigated cases. These discrepancies are attributed probably to the limited number of cases available for our study or to the cut-off value.

Another interesting finding has been that of a weaker stromal ER immunoexpression in endometriosis in comparison to the tumor stroma (p=0.001) and its significance can be related to a less responsive histological area to the hormone stimuli or maybe to a much lower immunoexpression of an ER isoform.

Regarding FIGO staging, no significant difference has been observed between PR immunoexpression in EOC epithelial and stromal cells in early stages compared to late stages in the studied group, although literature data show a higher PR immunoexpression in endometrioid histological pattern, in the absence of peritoneal metastases, showing a high correlation with tumor cells proliferation inhibition and with metastases development [22, 53, 54]. However, significant differences have been noticed between the epithelial (p=0.027) and stromal PR immunoexpression (p=0.005) in EOC with more differentiated histological types, suggesting a partial protective role of progesterone in carcinogenesis.

PR stromal immunoexpression has been significantly lower in endometriosis in comparison with tumor counterpart (p=0.000), possible in correlation with an unbalanced estrogenic stimulus in endometriosis pathogenesis and a decreasing role of this stimulus in the development of EOC. The weaker PR immunoexpression both in epithelial cells and stromal elements of endometriosis suggests that loss of PR immunoexpression may be attributed to early genetic changes prior to morphological atypia, as an important IHC marker in endometriosis malignant transformation risk [55]. The IHC expression of steroid hormone receptors, associated to clinicopathological findings supports the transition mechanism of ovarian endometriosis towards EOC, with a potential use for a better evaluation of prognosis and opens new perspectives for the development of new therapies.

Conclusions

Endometriosis can be considered a precursor lesion of EOC, as demonstrated in this study, and IHC expression of E-cadherin, β-catenin, Bcl-2, Bax, ER, and PR in corroboration with clinicopathological features supports the mechanism of transition of ovarian endometriosis into EOC, provides new prognosis tools, and opens new therapeutic perspectives. Considering the value of ovarian endometriosis as a precursor lesion for a large spectrum of OCs, the understanding of mechanisms which are involved in these diseases has also a prevention value for ovarian malignancies, with a beneficial effect for populations at high-risk.

Conflict of interests

The authors declare that they have no conflict of interests.

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