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The analysis of hormonal status and vascular and cell proliferation in endometrioid endometrial adenocarcinomas

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Abstract

Endometrioid endometrial carcinomas (EECs) are the most common malignancies of the uterus. Hormonal dependence of EEC, in relation to biomolecular mechanisms involved in tumor progression, such as angiogenesis and cell proliferation, are aspects that can contribute to improving the prognosis of patients. We analyzed the immunoexpression of markers addressed to steroid hormone receptors [estrogen receptor (ER), progesterone receptor (PR)], angiogenesis [cluster of differentiation (CD)105/endoglin] and cell proliferation (Ki-67) in 50 EECs related to the histopathological prognostic criteria of the lesions. In this study, the ER and PR scores were higher in low grade and early stages EEC, the statistical aspects being variable. The CD105 microvessel density and the Ki-67 proliferation index were superior in high grade and advanced stages EEC, the statistical aspects being significant or at the limit of significance. The ER/PR and CD105/Ki-67 immunomarker groups indicated a positive linear intragroup relation and a negative linear intergroup relation, suggesting the presence of synergistic and antagonistic molecular mechanisms of tumor endometrial control that can be used to stratify patients for targeted therapy.

Keywords: endoglin, hormonal receptors, proliferation, endometrial carcinoma.

Introduction

Endometrial carcinoma makes up over 95% of uterine cancers, ranks 4th among malignant neoplasms in women worldwide and it is the most common malignant lesion in the female genital tract [1, 2].

According to the dualistic model of endometrial carcinogenesis, endometrioid endometrial carcinomas (EECs) are the prototype of type 1 endometrial carcinoma, representing 70–85%, the initiation being hormone-dependent related to the estrogen exposure unopposed by progesterone [1, 3–5]. EECs are tumors that have as a precursor lesion hyperplasia, generally with a good prognosis, with localized disease in 60–80% of cases, a 5-year survival rate of almost 90% and an overall survival rate of 95 [4, 6–10].

At the same time, some studies indicate that survival rates in some geographical areas have decreased in recent decades, with tumor subsets with a 5-year mortality rate of 15–25% [9, 11]. In this context, there have been numerous research directions to identify reproducible molecular prognostic factors that allow adequate treatment and which in recent years have materialized by establishing a classification based on the genomic analysis initiated by *The Cancer Genome Atlas* (TCGA) with identification of four groups of tumors in which EEC is included [5, 11]. However, EEC genetic and molecular markers are not included in routine clinical practice, and some studies indicate inconsistency in estrogen receptor (*ER*) and progesterone receptor (*PR*) gene expression and *TCGA* classification [11]. Moreover, there is no molecular classification of endometrial carcinomas based on immunohistochemical (IHC) markers like in breast cancers, although there are numerous proposals in this regard, especially based on the inclusion in such a panel of ER and PR, considered independent prognostic factors and less expensive than genetic determinations [8, 11–13]. The aspect may be due to inconsistencies in ER and PR expression in relation to EEC histopathological (HP) prognostic factors or in relation to other markers, which may be attributed to the number of cases used in studies, to the used clones, quantification methods or tumor heterogeneity, for which is not possible to generalize the hormone receptor status [8, 10, 11, 14, 15].

The hormonal dependence of EEG and the hypotheses issued in various experimental studies related to the molecular mechanisms of tumor development indicate that the link between tumor hormonal status, angiogenesis and cell proliferation should not be neglected, a less integrated investigated aspect in the literature [9, 16–18].

Aim

In this work, we investigated ER, PR, and Ki-67 immunoexpression in tumor cells, but also the expression of cluster

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of differentiation (CD)105 in endothelial cells in relation to EEC HP prognostic parameters to establish the link between hormonal status and vascular/cell proliferation rate, a tool that may be useful for patient stratification.

Materials and Methods

In this study were included 50 cases of EEC, diagnosed in the Laboratory of Pathology within Emergency County Hospital, Craiova, Romania, between 2017–2020. The biological material came from patients hospitalized and operated in the Departments of Gynecology or General Surgery, and it consisted in total hysterectomy samples, which after harvesting were treated with 10% formalin solution, paraffin embedded and Hematoxylin–Eosin (HE) stained.

HP evaluation of the tumors was made in agreement with the classification indicated by the pathologists specialized in female genital tract within *World Health Organization* (WHO) [19].

HP analysis followed the distribution of cases according to the degree of tumor differentiation and tumor stage, respectively in relation to the immunoexpression of ER and PR in the epithelial tumor compartment and markers specific for tumor vessels (CD105) and proliferative cells (Ki-67) (Table 1).

Table 1 – ***Monoclonal antibodies used and immunostaining protocol***

Antibody	Clone/ Manufacturer	Dilution	Pretreatment (microwaving)	External positive control
ER*α*	1D5/Dako (mouse anti-human)	1/50	Tris-EDTA solution, high-pH 9	Mammary gland
PR	PgR 636/Dako (mouse anti-human)	1/50	Tris-EDTA solution, high-pH 9	Mammary gland
CD105 (endoglin)	EP274/Abcam (rabbit anti-human)	1/100	Tris-EDTA solution, high-pH 9	Renal tissue
Ki-67	MIB-1/Dako (mouse anti-human)	1/100	Citrate solution, low-pH 6	Palatine tonsil

CD105: Cluster of differentiation 105; EDTA: Ethylenediaminetetraacetic acid; ER*α*: Estrogen receptor alpha; PR: Progesterone receptor.

The sequential steps of IHC technique were represented by dewaxing (xylene), rehydrating (alcohol solutions), blocking of the endogenous enzyme (hydrogen peroxide), blocking of the unspecific antigens [bovine serum albumin (BSA)] and incubating with primary antibodies in 4°C (12 hours) environment. The IHC system for reactions was represented by EnVision™ FLEX+ (K8002-code, Dako), and for visualizing the reactions it was used the 3,3'-Diaminobenzidine (DAB) tetrahydrochloride chromogen. In this study were used external positive and negative controls to validate the IHC reactions.

The assessment of the IHC reactions was done in accordance with the literature data. Allred score was used for the analysis of ER and PR, consisting in addition the score of the positive cells expressed as percentage from total cells (score 0: without staining; score 1: <1% cells; score 2: 1–10% cells; score 3: 11–33% cells; score 4: 34–66% cells; score 5: >66% cells) with the score of the reaction intensity (score 0: without staining; score 1: weak intensity; score 2: moderate intensity; score 3: strong intensity), the final score being considered negative for values of 0–2 and positive for values of 3–8 [20]. The microvessel density (MVD) was used to quantify the tumoral neoformation vessels, which result from the counting of stained vessels on 20× microscopic field (MF) and establishing a medium value of each case from the five most vascularized MFs [21]. The vessels' counting assessment was made blinded by the authors and supervised by a pathologist author, being included in the counting process only vessels with the entire circumferentially fair visible border, including reactions identified in the cellular level. Ki-67 was assessed as proliferation index (PI) in 20× MF, representing for each case the mean percentage value of positive cells from total cells on five MFs with maximum stainings.

We included in the study only primitive EECs, without any history of hormonal/oncological treatments and without a cancer history in another location.

For statistical analysis, we use the one-way analysis of variance (ANOVA), χ^2 (*chi*-squared) and Pearson's comparison/correlation tests, within Statistical Package for the Social Sciences (SPSS) 10 software, and the results were considered significant in the case of *p*-values less than 0.05.

The study was done with the patients informed consent and the local Ethics Committee for approval.

Results

In our study, the age of diagnosis for the investigated lesions was between 40 and 77 years, with a mean value of 62.3±6.9 years. Of the 50 EECs included, 26 (52%) cases were G1 tumors (well differentiated), 16 (32%) cases were G2 tumors (moderately differentiated), and eight (16%) cases were G3 tumors (poorly differentiated). In relation to the tumor stage, 24 cases were classified in stage IA, 19 cases in the stage IB, four cases in the stage II and three cases in the stage III, of which in two cases the invasion was present in the pelvic lymph nodes (Table 2).

Table 2 – ***Distribution of cases depending on tumor grade and tumor stage***

Tumor grade/Tumor stage	*IA*	*IB*	*II*	*III*
Well differentiated (G1)	15	11	0	0
Moderate differentiated (G2)	8	5	2	1
Poorly differentiated (G3)	1	3	2	2

ER nuclear immunoreaction was present in 43 (86%) cases, in parenchyma and tumor stroma, the negative cases being observed in moderately or poorly differentiated EEC. For the entire investigated group, the medium value of ER-positive tumoral cells was 53.4±16.9, with variable reaction intensity and an average Allred score of 6.1. Depending on the tumor grade, the highest values of the immunoreactions were observed in G1 EEC, with an average number of 62.1±11.5 positive tumor cells, predominantly high/moderate intensity and an average Allred score of 6.6. By comparison, for G2 and G3 EEC, the mean values were 40.8±15.7 and 39±16.7, with predominantly moderate/low intensity and average Allred scores of 5.4 and 5 (Table 3; Figure 1, A–C).

Table 3 – ***Immunoexpression of analyzed markers depending on HP parameters in EECs***

HP parameters		ER (Allred score)	PR (Allred score)	CD105 (MVD)	Ki-67 (PI)
Tumor grade	G1	6.6	6.8	10.7±3	15.9±5.3
	G2	5.4	6.5	13.8±4	43.7±12.1
	G3	5	6	17.1±2.6	48.1±13.3
Tumor stage	IA	6.4	6.8	12±4.5	22±13.4
	IB	5.9	6.5	12.2±2.8	29.5±12.8
	II	5.6	6.6	16.5±4.4	52.5±6.4
	III	5	6.3	16.3±1.5	66.6±7.6

EECs: Endometrioid endometrial carcinomas; ER: Estrogen receptor; HP: Histopathological; MVD: Microvessel density; PI: Proliferation index; PR: Progesterone receptor.

In relation to the tumor stage, the highest values of ER expression were observed in EEC IA and IB stages, with a mean number of marked cells of 59±16.2 and 53.5±27.8, variable reaction intensity and Allred scores of 6.4 and 5.9. By comparison, for EEC in stages II and III, the values were 26.6±2.8 and 35, variable intensity of reactions and Allred scores of 5.6 and 5 (Table 3).

PR nuclear immunoreaction was present in 44 (88%) cases, in the parenchyma and tumor stroma, the negative cases being observed especially in moderately or poorly differentiated EEC. For the entire investigated group, the medium value of PR-positive cells in the tumor epithelial compartment was 60.5±13.6, and the average value of the Allred score was 6.6. In relation to the tumor grade, the percentage values of labeled cells were 63±13.5 for G1 EEC, 57.9±14.3 for G2 EEC and 56.4±11.4 for G3 EEC, while the intensity of the reactions was variable, and the average Allred scores for those three categories were 6.8, 6.5 and 6 (Table 3; Figure 1, D–F). Reported to the tumor stage, for stage I tumors the percentage of PR-positive tumor cells had a mean value of 60.7±14.8 for stage IA and 60.5±12.7 for stage IB, with variable reaction intensity and average Allred scores of 6.8 and 6.5. For stage II and III tumors, the mean values of the labeled cells were 60±21.7 and 60±5, while the mean Allred scores were 6.6 and 6.3, respectively.

Statistical analysis indicated significantly higher differences in ER scores in G1 EEC compared to G2/G3 tumors (p=0.003, χ^2 test) (Figure 2A). Although ER scores were higher in stages IA and IB compared to stages II/III, the differences were statistically non-significant (p=0.105, χ^2 test) (Figure 2B). In the case of PR, the differences were non-significant both in relation to the tumor grade (p=0.214, χ^2 test) and to the tumor stage (p=0.649, χ^2 test), although the mean values of Allred scores were higher in G1 ECC and in those in stage IA (Figure 2, C and D).

CD105 (endoglin) immunoreaction was present in the entire investigated group in the apical cytoplasm of the neoformation vessels' endothelium. The tumor new vessels presented variable dimensions and shapes, with aberrant morphology, often with the appearance of small, irregular, tortuous vessels, sometimes with a unicellular appearance or with the formation of a complex anastomosed network. For the analyzed group, the value of CD105 MVD was 12.7±4. Regarding the tumor grade, the highest mean values for CD105 MVD were present in the case of G3 EEC, respectively 17.1±2.6.

By comparison, in the case of G2 EEC, the average value was 13.8±4, and for G1 EEC, it was 10.7±3 (Table 3; Figure 3, A–C). Regarding the tumor stage, the highest values were identified in the case of stages II and III, respectively 16.5±4.4 and 16.3±1.5. By comparison, in the case of stages IA and IB, the mean values of CD105 MVD were 12±4.5 and 12.2±2.8.

Ki-67 immunoreactions were identified in all cases in the epithelial tumor compartment, but also in stromal elements. For the whole analyzed group, the mean value of Ki-67 PI was 30±17.4. Regarding the tumor grade, the mean values for Ki-67 PI were 15.9±5.3 for G1 EEC, 43.7±12.1 for G2 EEC and 48.1±13.3 for G3 EEC (Table 3; Figure 3, D–F). In relation to the tumor stage, the mean values were 22±13.4 for stage IA, 29.5±12.8 for stage IB, 52.5±6.4 for stage II and 66.6±7.6 for stage III.

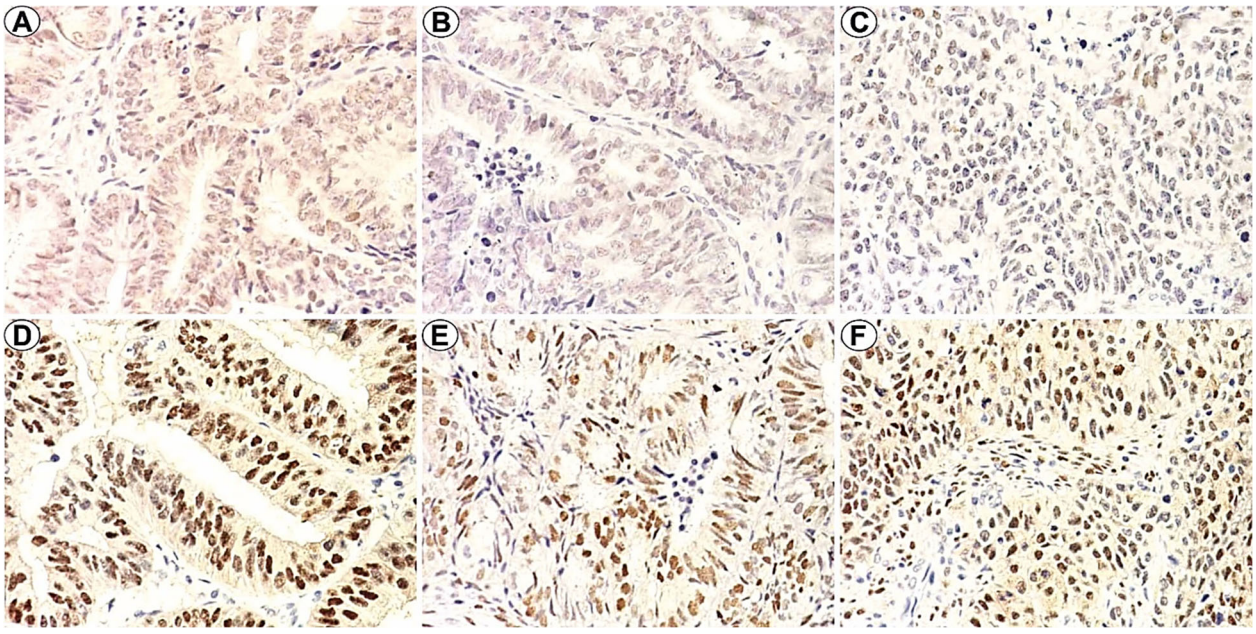

Figure 1 – ***ER immunostaining (×200): (A) G1 EEC; (B) G2 EEC; (C) G3 EEC. PR immunostaining (×200): (D) G1 EEC; (E) G2 EEC; (F) G3 EEC. EEC: Endometrioid endometrial carcinoma; ER: Estrogen receptor; PR: Progesterone receptor.***

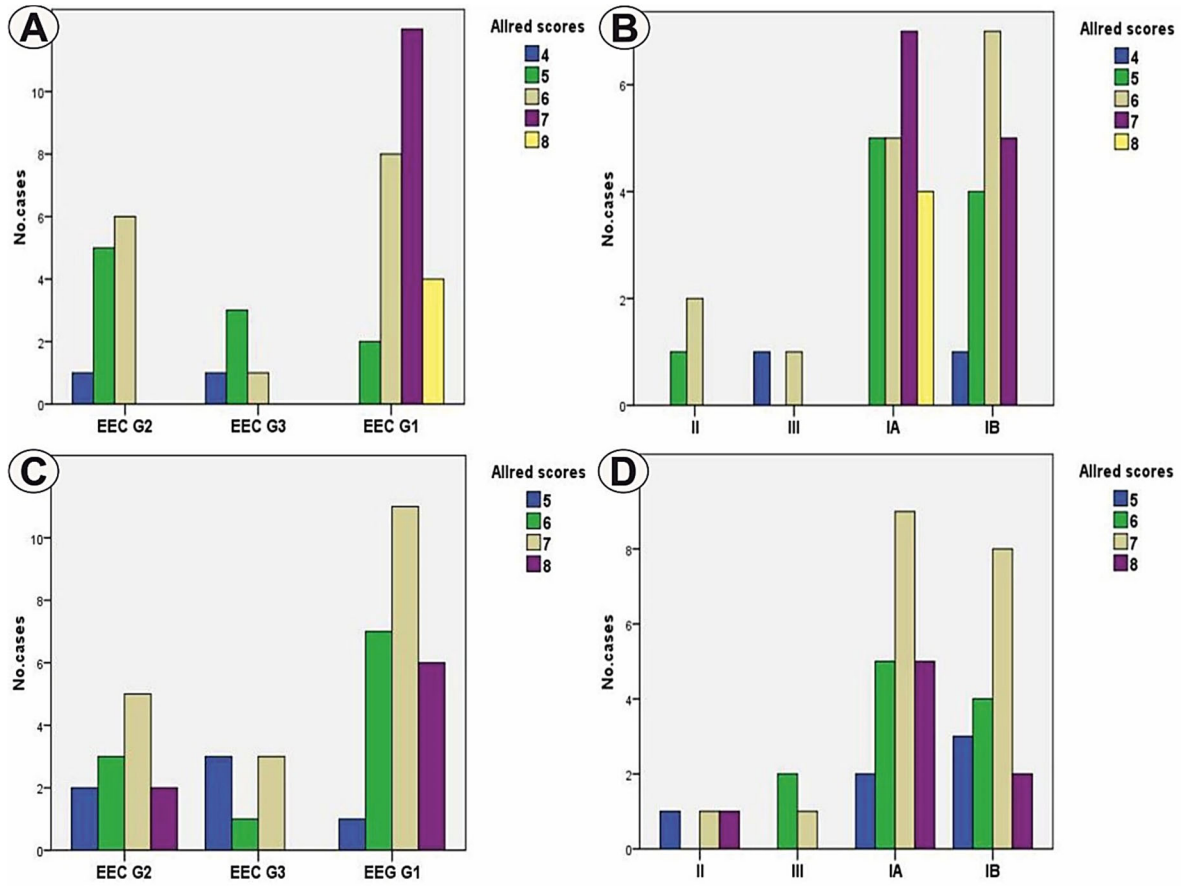

Figure 2 – ***(A) Cases distribution related on tumor grade and ER scores; (B) Cases distribution related on tumor stage and ER scores; (C) Cases distribution related on tumor grade and PR scores; (D) Cases distribution related on tumor stage and PR scores. EEC: Endometrioid endometrial carcinoma; ER: Estrogen receptor; PR: Progesterone receptor.***

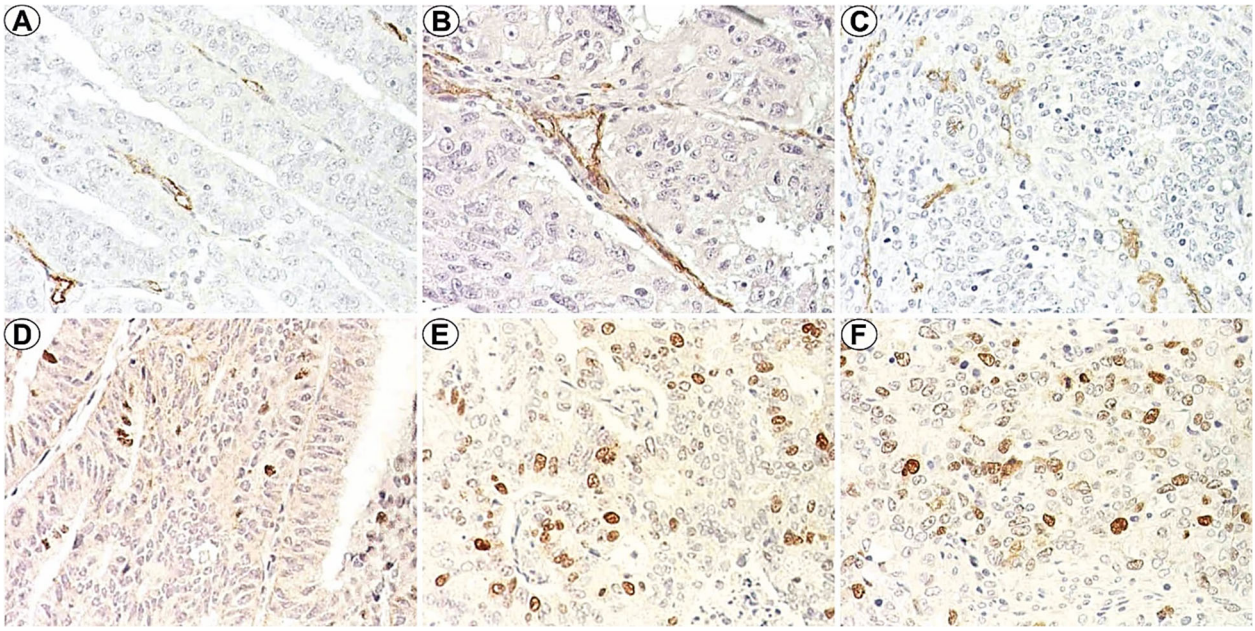

Figure 3 – ***CD105 immunostaining (×200): (A) G1 EEC; (B) G2 EEC; (C) G3 EEC. Ki-67 immunostaining (×200): (D) G1 EEC; (E) G2 EEC; (F) G3 EEC. CD105: Cluster of differentiation 105; EEC: Endometrioid endometrial carcinoma.***

The statistical analysis indicated significant differences of the mean values of CD105 MVD in relation to the tumor grade, the highest values being observed in the case of G2/G3 EEC (p<0.001, one-way ANOVA test) (Figure 4A). Also, the values for CD105 MVD were higher in stage III and II EEC, compared to stage IA/IB tumors, the aspect being at the border of significance (p=0.072, one-way ANOVA test) (Figure 4B). For Ki-67, the mean PI values were significantly higher in the case of high-grade G2/G3 EEC (p<0.001, one-way ANOVA test) and in advanced stages II/III (p<0.001, one-way ANOVA test) (Figure 4, C and D).

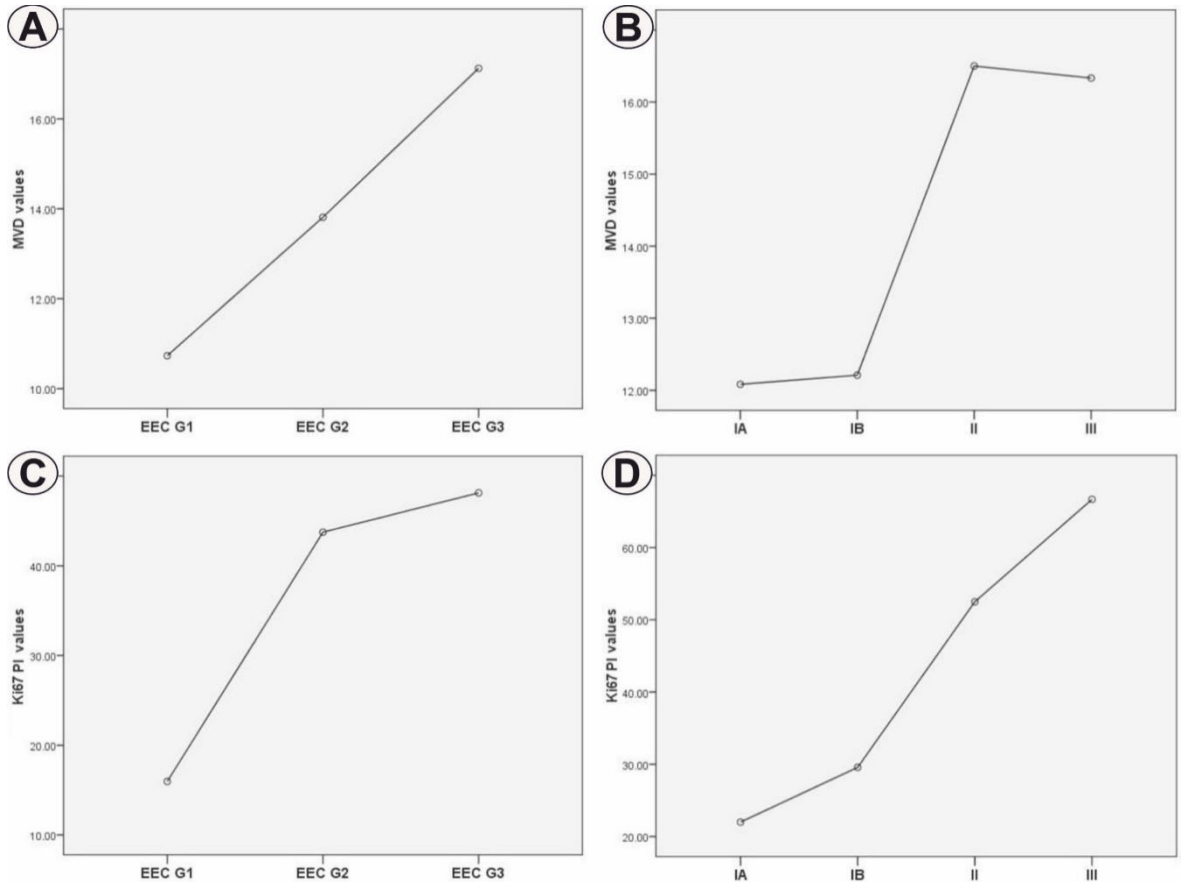

Figure 4 – ***(A) Cases distribution related on tumor grade and CD105 MVD values; (B) Cases distribution related on tumor stage and CD105 MVD values; (C) Cases distribution related on tumor grade and Ki-67 PI values; (D) Cases distribution related on tumor stage and Ki-67 PI values. CD105: Cluster of differentiation 105; EEC: Endometrioid endometrial carcinoma; MVD: Microvessel density; PI: Proliferation index.***

The percentage analysis of Allred scores with the values of CD105 MVD and Ki-67 PI, indicated significant negative linear correlations or at the border of significance in the case of ER with Ki-67 (p<0.001, Pearson's test) and CD105 (p=0.069, Pearson's test) and of PR with Ki-67 (p=0.05, Pearson's test). We also found significant positive linear correlations between ER and PR values (p<0.001, Pearson's test) but also between CD105 and Ki-67 values (p<0.001, Pearson's test).

Discussions

The age of diagnosis of EEC is variable, with an average of 55–60 years, and the HP prognostic factors of the lesions are represented by tumor grade, tumor stage, HP type, local tumor extension and lymph node invasion [2, 4]. In this study, the mean age of diagnosis was 62.3±6.9, most of the lesions being well differentiated and invading the myometrium.

The hormonal influence on the normal and tumoral endometrium has been reflected over time in numerous studies that have analyzed the immunoexpression of ER and PR and their isomorphs, some authors suggesting the usefulness of introducing the two markers in clinical practice to assess prognosis and evaluate therapeutic efficacy [4, 8–11, 13, 22–24]. The markers used in this study addressed to isomorphs ERα for estrogen and both PR-A/PR-B for progesterone.

In our study, the expression of ER was observed in 86% of cases and of PR in 88% of cases, the expression of the two markers being concordant and in a positive linear relation. In both cases, the IHC scores were higher in the case of G1 EEC and in stage I but were statistically significant or at the limit of significance only in the case of ER.

Positivity rates for ER and PR in type I endometrial carcinoma are described as over 90%, although some studies support a rate of 60–70% [9, 24]. EECs have the highest positivity rate for steroid receptors [22], their expression being completely lost in a small number of cases, even if they are advanced or deeply invasive [11].

ERα is necessary for the basic development of the endometrium and ERβ for morphological differentiation and functional maturation [4]. The estrogenic excess uncontrolled by progesterone seems to be involved in the appearance of EEC, in aggressive carcinomas being reported the decrease or absence of progesterone with the role of growth inhibition, promotion of cell differentiation and regulation of tumor invasion [4, 15]. However, there are studies that indicate that hormonal levels of estrogen in the two types of endometrial carcinomas are similar, and that only 20% are due to excess estrogen, which may suggest alternative mechanisms of tumor transformation [24].

At the same time, the expression of ERα, PR-A and PR-B receptors are associated with the degree of differentiation, the response to therapy or vascular invasion and the metastatic potential of endometrial carcinomas [22, 25]. Thus, some authors indicate decreased PR-A and ERα expression in carcinomas compared to non-tumoral and

hyperplastic endometrium, and in high-grade carcinomas compared to low-grade carcinomas, especially due to decreased stromal expression of receptors, an aspect that may be related of the epithelial–mesenchymal transition phenomenon at the level of the tumor advancing edge [4, 8, 23, 24, 26–28]. ER and PR expression are generally consistent because *PR* gene transcription is induced by estrogen and inhibited by progestin [23].

Studies to date on endometrial carcinogenesis indicate that the proliferative effect of ERα is inhibited by the dominant activity of PR-A, while PR-B may have a protective antagonistic effect or a synergistic effect with estrogen, depending on the competition between the two isomorphs [4]. However, the information is controversial regarding the PR expression, some studies indicating the loss of expression in the EEC, while others show the presence of the PR-A or PR-B expression in advanced EEC [29]. Moreover, in the study led by Kreizman-Shefer *et al*. different expression of PR-A and ERα is found in different tumor compartments [4].

Some studies indicate the association of ER and PR with the tumor stage, with the cervical invasion but not with the depth of the myometrial invasion [9, 22, 24]. In our study, significant differences were present when comparing stage I (A/B) with stages II/III. In the same context, there are studies that indicate the absence of PR-A in advanced carcinomas, as opposed to PR-B that can be expressed even in metastases, while others indicate decreased PR-B in poorly differentiated and invasive lesions [30, 31].

Angiogenesis is a sequential process in which new vessels are formed from pre-existing vessels that serve the tumor and are connected to the extratumoral vessels. The angiogenic switch is important for tumor survival, progression and metastasis [32]. Tumor angiogenesis can be quantified by evaluating the expression of proangiogenic growth factors and their receptors, as well as by MVD, which indicates the number of neoforming tumor vessels. Thus, it is indicated the higher the number of vessels, the deeper myometrial invasion, the higher grade, the higher the risk of metastases and a reserved prognosis [16].

Endoglin (CD105) is a receptor of tumor growth factor beta (TGFβ) being one of the most dedicated antigens for the assessment of neoformation tumor vessels, more than other pan-endothelial markers, such as CD31 or CD34 [33, 34]. Endoglin participate in the normal vessels initiation and expansion being present in proliferative endothelium during angiogenesis [33, 34].

In this study, CD105 MVD was superior in the case of G2/G3 EEC in advanced stages II/III. The results are consistent with previous studies in which CD105 MVD was superior in aggressive EEC, especially at the advancing edge [35].

MVD in endometrial carcinogenesis comes with controversial results. Thus, in some studies, CD105 marked more vessels than CD31 and was positively associated with tumor grade, depth of invasion, lymphovascular invasion, tumor stage, and the presence of lymph node metastases [36], while in others there was no association with the clinicopathological factors of endometrial carcinomas [37].

The relation of hormone receptors with tumor endometrial angiogenesis is a rare topic in the literature. Our study indicated a negative linear relation of CD105 expression quantified by MVD with hormone receptor expression.

The role of estrogen and progesterone in endometrial angiogenesis is also controversial. ER is involved in the activation of numerous growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), involved in numerous biomolecular processes, including proliferation and tumor angiogenesis [23]. In an experimental study in mice, progesterone induced the most intense endothelial proliferation, estrogen inhibited the progesterone-induced angiogenesis, and progesterone-induced angiogenesis was only partially mediated by VEGF [17]. Estrogen seems to be linked to the stimulation of angiogenesis in the proliferative phase, and progesterone in the secretory phase [38]. Some studies indicate that progesterone has effects especially on vascular density, while estrogen on vascular permeability [17]. It appears that estrogen initially stimulates VEGF and VEGF receptor 2 (VEGFR2), only to later have an antagonistic effect [17].

Because endothelial cells express ER, estrogen stimulates endothelial proliferation and can initiate the production of new vessels directly, and it has been proposed that estrogen regulates or promotes endometrial angiogenesis by regulating glandular and stromal expression of VEGF [39, 40]. At the same time, estrogen may have different effects on angiogenesis depending on the receptor to which it binds, respectively ERα inhibits and ERβ stimulates angiogenesis [39], and this aspect is also argued by the fact that intensely positive ERα tumors respond better to treatment if VEGF, EGF or FGF-induced signaling pathway inhibitors are also administered [23].

In our study, Ki-67 PI was significantly superior in G2/G3 EEC in advanced stages II/III, with a positive linear relation with CD105 MVD and a negative linear relation with the expression of the investigated hormone receptors. This protective PR effect is also suggested in other studies in which one or both PR isomorphs are lost in endometrial carcinomas [13].

In various studies, Ki-67 expression has been correlated with HP grade, depth of myometrial invasion and risk of recurrence of endometrial carcinomas [4, 41]. The increased expression of Ki-67 in the tumor tissue compared to the non-tumor tissue must be carefully analyzed in relation to the phases of the menstrual cycle, the values being high in the proliferative phase and low in the secretory phase [4]. The Ki-67 index above 20% is usually associated with aggressive behavior of endometrial carcinomas [10]. Ki-67 expression is also higher in G3 compared to G1/G2 endometrial carcinomas [8] and correlates with myometrial, cervical and lymph node invasion [10] or with clinical stage [42].

Over time, there have been studies that have proposed antibody panels that can be used to assess the prognosis of endometrial carcinomas. Thus, Saito *et al*. and Guan *et al*. considered ER and PR as independent prognostic factors for endometrial carcinomas, directly related to patient survival [11, 13]. Canlorbe *et al*. indicated the concordance of ER, PR and Ki-67 expression with tumor grade, showing the association of ER/PR with low-grade tumors limited to the uterine body and CD105 MVD and Ki-67 with high-grade tumors in advanced stages, results consistent with

those from our study [8]. Moreover, in our study, the group was a homogeneous one that included only EEC.

The results of this study integrated in the literature support the need to introduce a group of antibodies with clinical applicability and general prognostic potential for endometrial carcinomas.

Conclusions

The study indicated decreased expression of the investigated hormone receptors and increased vascular and cell proliferation of high-grade, advanced EEGs. There is a negative linear relation between the protective ER/PR hormonal status and the tumor aggressiveness CD105/Ki-67 immunomarkers. We found concordance of hormone receptor expression and between the proliferative markers. The panel used in this study represents the expression of synergistic and antagonistic control mechanisms on the tumoral transformed endometrium, being usable for stratifying the patients for specific therapy.

Conflict of interests

The authors declare that they have no conflict of interests.

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