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Original contribution

Immunohistochemistry expression of targeted therapies biomarkers in ovarian clear cell and endometrioid carcinomas (type I) and endometriosis☆,☆☆

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Summary Ovarian clear cell and endometrioid carcinomas (type I) are thought to develop from endometriosis. ARID1A loss of expression is known to be related to the promotion of the endometriosis carcinogenesis. Despite the diverse origins and prognosis of type I and type II carcinomas, surgery followed by platinum-based chemotherapy is the mainstay of treatment for both. Limited knowledge about the expression of targeted therapies' biomarkers prevents the use of such markers as potential guides for tailored treatment. This study aimed to evaluate the expression of *ARID1A* gene and target therapies biomarkers (VEGF, PD-L1, and PARP-1) in ovarian clear cell and endometrioid carcinomas and endometriosis, and its relationship with prognosis. Forty-six ovarian clear cell and endometrioid carcinomas, and 24 endometriosis foci samples retrieved from the same surgical specimens were studied. ARID1A, VEGF, PD-L1, and PARP-1 immunohistochemistry expression was compared in carcinomas and endometriosis with regard to the clinicopathological features and prognosis. We found that endometriosis was associated with increased rates of diagnosis of cancer in the initial stages ($P = .008$). Different levels of expression of all biomarkers were detected in clear cell and endometrioid carcinomas and endometriosis. However, only the VEGF expression level showed a significant increase in the carcinoma group when compared with endometriosis ($P = .0002$). PARP-1 overexpression correlated with worse progression-free survival ($P = .03$) and overall survival ($P = .01$). In conclusion, endometriosis and ovarian clear cell and endometrioid carcinomas exhibited

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ARID1A loss of expression, and VEGF, PD-L1, and PARP-1 expression. PARP-1 overexpression in clear cell and endometrioid carcinomas was associated with early recurrence and worse overall survival.

1. Introduction

In 2014, the World Health Organization accepted a new theory about ovarian carcinogenesis, which divides ovarian carcinomas into 2 main groups, namely, type I and II carcinomas. Type I carcinomas comprise endometrioid, clear cell, seromucinous, low-grade serous, mucinous carcinomas, and malignant Brenner tumors, whereas type II carcinomas mostly comprise high-grade serous carcinomas [1].

Clear cell and endometrioid ovarian carcinomas encompass most type I carcinomas and, in women with endometriosis, occur at twice the expected rate of the general female population [2]. There is sufficient evidence to consider endometriosis as the precursor lesion to those carcinomas (endometriosis-associated

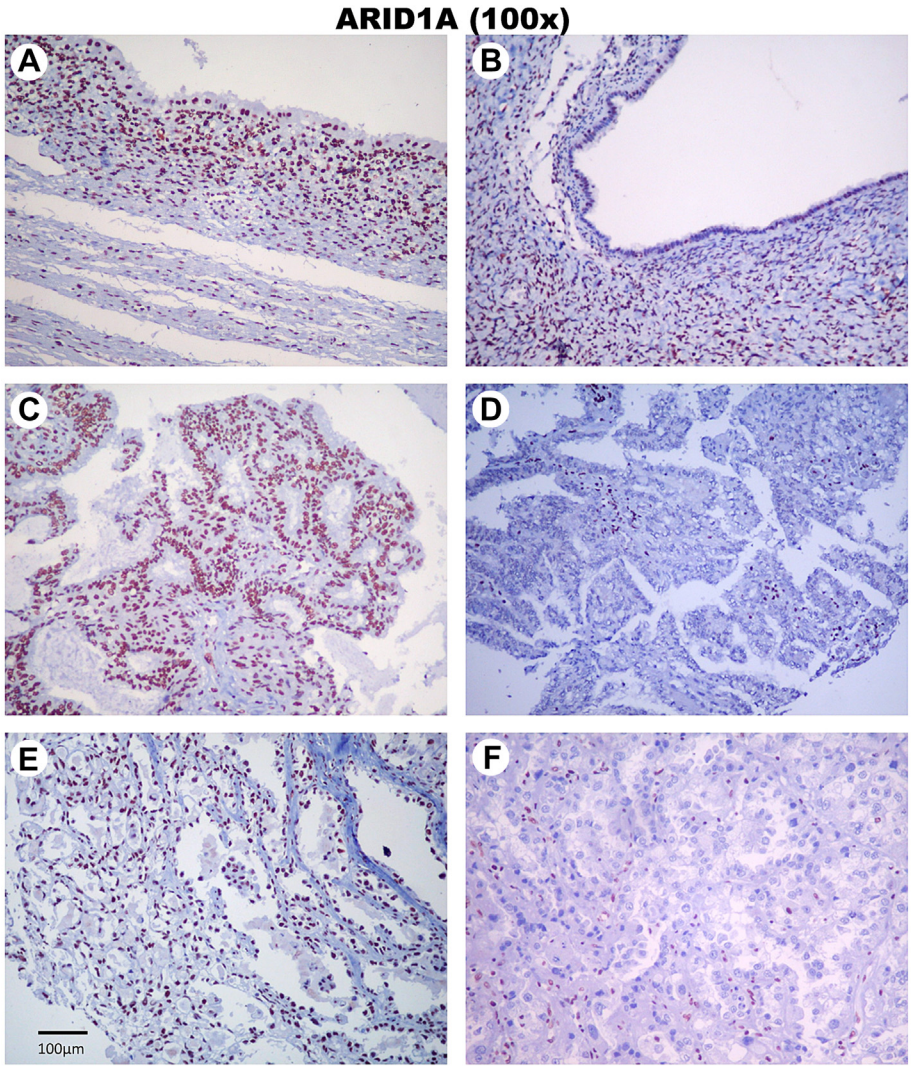

Fig. 1 ARID1A immunohistochemical expression. ARID1A: ovarian endometriosis with diffuse nuclear expression in the epithelium (A) and loss or focal nuclear expression (B). Note that the stroma, below the epithelium, show ARID1a-positive cells. Ovarian endometrioid adenocarcinoma with diffuse nuclear expression (C) and without expression (D). Ovarian clear cell adenocarcinoma with diffuse nuclear expression (E) and without expression (F). Rare positive cells are observed in tumor stroma in panels D and F. All figure magnifications ×100 (bar corresponds to 100 µm).

ovarian carcinomas [EAOCs]) [3-5]. A few molecular markers have been described bearing a relationship to endometriosis and the subsequent development of ovarian cancer [6,7]. Studies demonstrated an intrinsic connection between gene mutations in endometriosis and the acquisition of malignant features in ovarian tissues. Most notably, mutations of the ARID1A, KRAS, PTEN, and TP53 genes exhibit similar patterns between endometriosis and EAOC, with patterns that differ from those found in type II ovarian carcinomas [6,7]. Also, ARID1A mutations and the knockout of its expression seem to be directly related to the malignant transformation of endometriotic tissue [8,9].

Despite the knowledge of the diverse origins and clinical prognosis of type I and type II carcinomas, surgery followed by platinum-based chemotherapy is the mainstay of treatment for both entities [10]. Limited knowledge about the expression of targeted therapy biomarkers prevents the use of such markers as potential guides for tailored treatment regimens. Also, there is a lack of prognostic markers to guide follow-up schedules [11].

We therefore designed this study to evaluate the presence of ARID1A gene expression knockout by immunohistochemistry in endometriosis and EAOC specimens. We also aimed to assess the expression of biomarkers associated with potential targeted therapies (VEGF, PD-L1, and PARP-1) and their relationship with prognosis in these carcinomas. This knowledge may aid in the discovery of groups of patients at high risk for recurrence and may help with the development of further studies of targeted drug therapies for type I ovarian carcinomas.

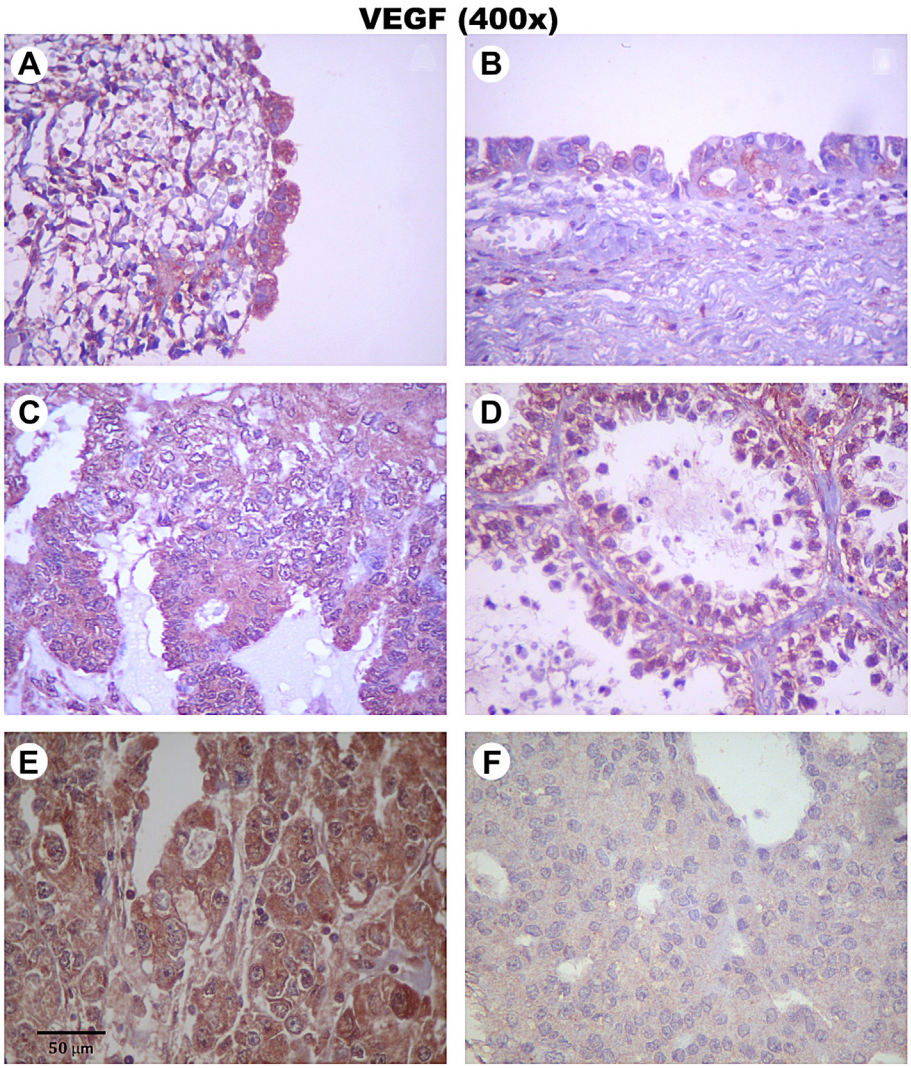

Fig. 2 VEGF immunohistochemical expression. VEGF immunohistochemical expression is cytoplasmic and varied in the intensity of the reaction and in the number of positive cells. The area of ovarian endometriosis shows strong and diffuse reaction in panel A and moderate and focal reaction in panel B. The endometrioid ovarian carcinoma has strong expression in panel C and moderate in panel D; the expression in clear cell carcinoma is strong in panel E and weak in panel F. All figure magnifications ×400 (bar corresponds to 50 µm).

2. Materials and methods

2.1. Sample collection

2.1.1. Tissue and patient data

After reviewing the historical medical files of the Women's Hospital of the University of Campinas, we found 273 cases of ovarian carcinomas that had undergone surgical resection between 1995 and 2016. Fifty (18.3%) of the 273 cases were clear cell or endometrioid carcinomas. These cases were selected for this reconstituted cohort study.

Slides were obtained from the surgical pathology files of the Laboratory of Pathology of the Clinical Hospital of the University of Campinas. The hematoxylin and eosin–stained sections were reviewed to confirm the histologic diagnosis (World Health Organization, 2014) [1], identify representative areas of the carcinomas, look for endometriosis foci associated with the tumors, and select more representative areas of the tumor for construction of the tissue microarray (TMA). We retrieved samples from formalin-fixed, paraffin-embedded tissue sections in 46 cases (22 clear cell carcinomas and 24 endometrioid carcinomas). Endometriosis foci were found in 36 of 46 of the cases. However, endometriosis samples could be retrieved from only 24 of these 36 cases. Therefore, we were able to compare biomarker expression in the carcinoma and endometriosis regions of the same surgical specimen for 24 cases. For this study, any foci of endometriosis found in surgical specimens were considered positive for associated endometriosis. We made the TMA blocks by taking 2 core tissue samples (2 mm in diameter) from the identified areas of donor paraffin blocks and arraying them into a new recipient paraffin block with a Sakura Tissue Microarray System (Sakura

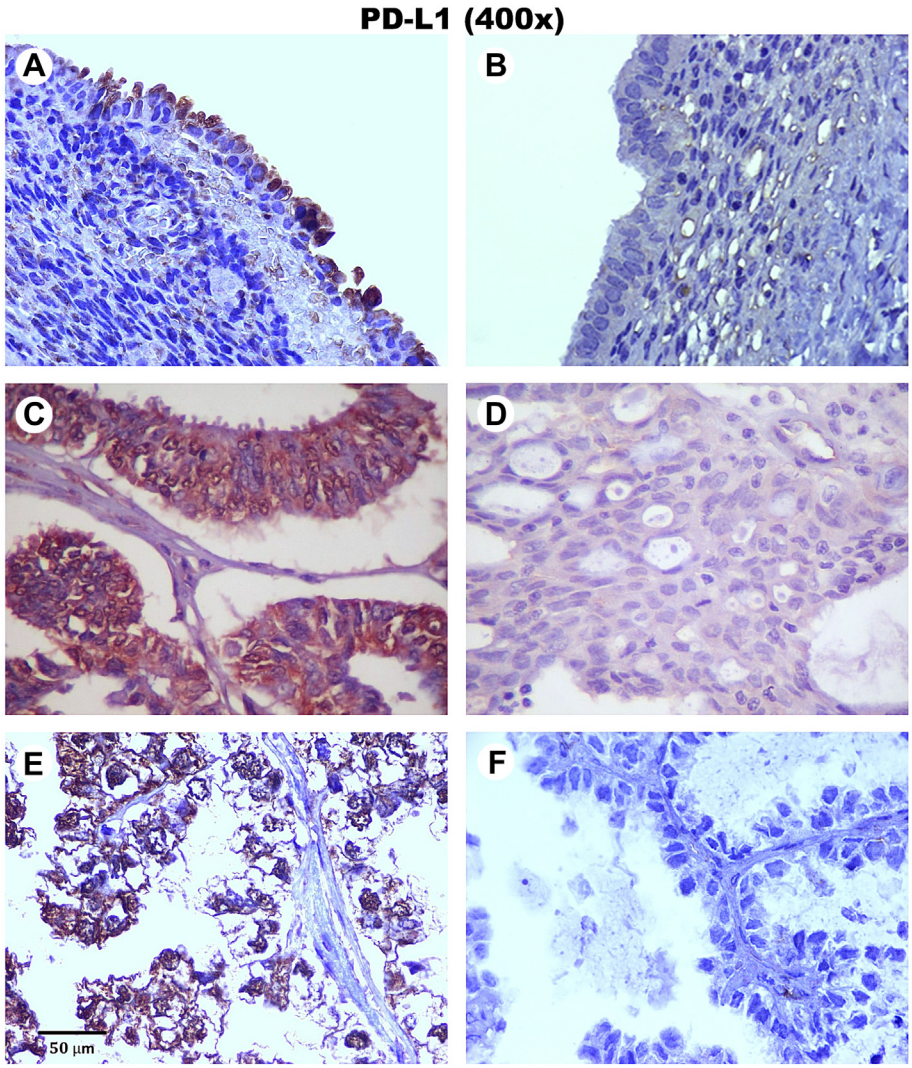

Fig. 3 PD-L1 immunohistochemical expression. PD-L1 immunohistochemical expression is observed in the cytoplasm or cellular membrane: in ovarian endometriosis, the expression is strong (A) or lacking (B); in the endometrioid carcinoma of the ovary, the expression varied from strong and diffuse (C) to weak or absent (D); in ovarian clear cell carcinoma, the expression is strong (E) or lacking (F). All figure magnifications ×400 (bar corresponds to 50 μm).

Finetek USA, Inc). Sections (4 μm) were obtained from TMA blocks and stained with hematoxylin and eosin to confirm tumor presence and perform the immunohistochemical reactions.

The clinical data were obtained from the patients' medical records comprising age, body mass index, parity, menopause, serum cancer antigen 125 levels, tumor histologic type and grade, and International Federation of Gynecology and Obstetrics (FIGO) stage. Dates of the last follow-up, recurrence, and death were also collected. Progression-free survival (PFS) was calculated as the number of months from cancer diagnosis to recurrence, and overall survival (OS) was calculated as the number of months from cancer diagnosis to death or last follow-up. Cases were followed up until April 2017.

The School of Medical Sciences of the University of Campinas review board approved this study under No. 68150617.7.0000.5404.

2.1.2. Immunohistochemical reactions

We used the following primary antibodies to perform the immunohistochemical reactions: anti-ARID1A rabbit monoclonal antibody (EPR13501; product no. ab182560; Abcam, Cambridge, MA) diluted 1:1000 in citrate buffer, VEGF (SP28) rabbit monoclonal antibody (product no. MA5-14573; Thermo Fisher, Fremont, CA; Ready-to-Use for Immunohistochemical Staining), anti–PD-L1 rabbit monoclonal antibody (28-8; product no. ab205921; Abcam) diluted 1:100 in EDTA buffer, and anti-PARP rabbit monoclonal antibody (E102; product no. ab32138; Abcam) diluted 1:50 in citrate buffer. The prepared tissue sections were fixed on poly-L-lysine coated slides overnight at 37°C. The paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a sequence of alcohols. Antigen retrieval in citrate buffer (pH 6.0) was used after the sections were treated in a microwave at 95°C for 30 minutes to unmask the epitopes;

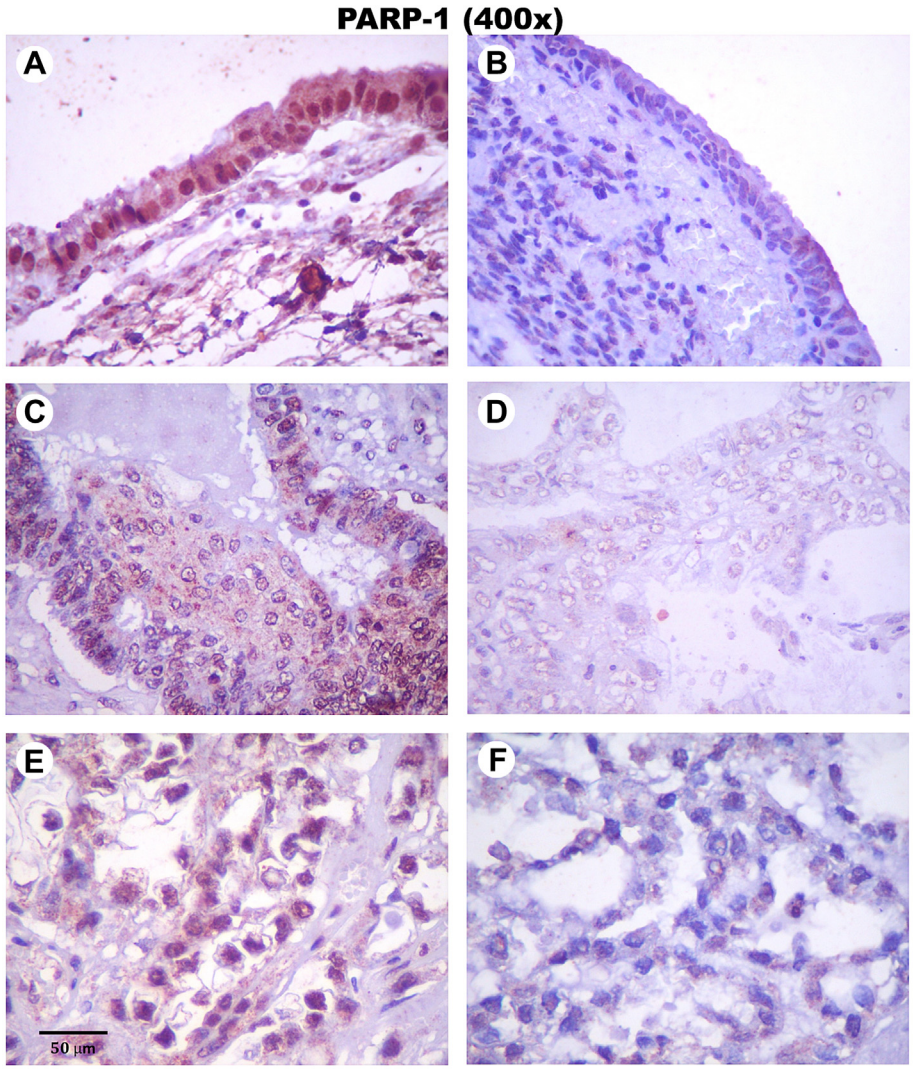

Fig. 4 PARP-1 immunohistochemical expression. PARP-1 immunohistochemical: positive nuclear expression with different number of stained cells and variable intensity of reaction. Ovarian endometriosis epithelium with strong and diffuse reaction in panel A and weak and focal in panel B; endometrioid carcinoma of the ovary with greater expression in panel C than in panel D; clear cell ovarian carcinoma with moderate and diffuse expression in panel E and weak and focal in panel F. All figure magnifications ×400 (bar corresponds to 50 μm).

the sections were then left to cool for 20 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Then, slides were incubated overnight with the primary antibodies at room temperature, followed by rinsing in phosphate-buffered saline (pH 7.6). This step was followed by the secondary biotin-conjugated antibody for 1 hour and finally the peroxidase-conjugated streptavidin for another hour. Diaminobenzidine tetrachloride (freshly prepared) was added for 25 minutes and then counterstained in Harris hematoxylin, followed by dehydration, clearing, and mounting. The positive control for VEGF and PD-L1 antibodies was the tonsil, the positive control for PARP-1 was brain tissue, and that for ARID1A was the kidney. The omission of the primary antibodies produced negative controls.

Two investigators scored the immunohistochemistry analysis independently based on the percentage of cells and intensity of the reaction. Microphotographs of the 4 best fields at ×400 magnification were taken from each case, obtained through a 995 Nikon digital camera (Nikon Inc, Japan). The evaluation of the reactions was based on the cytoplasmic expression of VEGF, nuclear expression of PARP-1 and ARID1A, and membrane expression of PD-L1. We used the open-source image processing software ImageJ (Rueden, C. T.; Schindelin, J. & Hiner, M. C. et al. (2017), "ImageJ2: ImageJ for the next generation of scientific image data", BMC Bioinformatics 18:529, doi:10.1186/s12859-017-1934-z.) Version: ImageJ2. [12] to count the positive cells and the total number of cells per field to obtain the positive cell ratio in each case for each marker. For the score, we used the following: (*a*) intensity: 0, undetectable; 1, weak; 2, moderate; and 3, strong, and (*b*) percentage of cells, from 0 to 100%. Then a score (range, 0-300) was calculated by multiplying the intensity value by the percentage of cells [13]. For ARID1A immunohistochemistry evaluation, only the percentage of nonstained cells was recorded for the score (range, 0 to 100); ARID1A expression exhibits an all or nothing pattern, and the intensity of expression was not considered.

Table 1 Demographic characteristics according to the detection of endometriosis at histology review (n = 46)

	Endometriosis		*P*
	No	Yes	
Age (y), mean (SD)	59 (13)	56 (12)	.63 [a]
BMI (kg/m^2), mean (SD)	24 (4)	26 (6)	.31 [a]
No. of pregnancies, n (%)			
0	3 (30%)	16 (44.4%)	.32 [b]
≥1	7 (70%)	20 (55.6%)	
CA125 (U/mL), mean (SD)	776 (919)	1219 (2658)	.60 [a]
Menopause, n (%)			
Yes	6 (40%)	20 (55.6%)	.54 [b]
No	4 (60%)	16 (44.4%)	
Stage at diagnosis, n (%)			
I-II	3 (30%)	28 (77.8)	.008 [b]
III-IV	7 (70%)	8 (22.2)	
Histology, n (%)			
Clear cell	6 (60%)	16 (44.4%)	.30 [b]
Endometrioid	4 (40%)	20 (55.6%)	
ARID1A mutated (%), mean (SD) [c]	29 (35)	40 (39)	.43 [a]

Abbreviation: BMI, body mass index.
[a] *P* calculated with 2-sample, unpaired *t* test.
[b] *P* calculated with χ^2 or Fisher exact test.
[c] One missing case.

2.2. Statistical analysis

Initially, we reported each outcome descriptively. Continuous variables were compared using 2-sample *t* tests. Frequency distributions of categorical variables were compared using χ^2 tests or Fisher exact test where applicable. A receiver operator characteristic curve (ROC curve) was generated to pinpoint the optimal cutoff ARID1A percentage of unstained cells and for the score of every protein biomarker as related to disease progression and patient death. We used the cutoff point of ROC curves to separate every biomarker into 2 groups: (1) normal expression and (2) overexpression, and generated Kaplan-Meier curves for PFS and OS, as related to the stage of disease at diagnosis and the expression of ARID1A, VEGF, PD-L1, and PARP-1. The log-rank test was used to compare Kaplan-Meier curves. For statistical analysis, we divided stage at diagnosis into 2 groups: early stage (FIGO I-II) and advanced stage (FIGO III-IV). Women who were alive at their last follow-up or who had not developed a recurrence at their last follow-up were censored. All statistical calculations were 2-sided, and $P < .05$ was considered statistically significant. Statistical calculations were performed using the R Environment for statistical computing software (R Core Team [2017]. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

3. Results

After reviewing institutional databases and histologic diagnosis, we found 50 cases of clear cell and endometrioid ovarian carcinomas treated in the period from 1995 to 2016. In 46 cases, we could retrieve formalin-fixed, paraffin-embedded tissue samples archived at our pathology laboratory suitable for TMA construction. Clear cell ovarian carcinomas comprised 22 cases (47.8%),

Table 2 Comparison of expression of biomarkers between endometriosis and carcinomas in cases where it was possible to retrieve samples from both tissues

	Endometriosis, mean (SD)	EAOC, mean (SD)	*P*
VEGF (score; n = 24)	165 (84)	253 (59)	.0002
PD-L1 (score; n = 23)	50 (59)	97 (121)	.39
PARP (score; n = 21)	104 (115)	139 (114)	.29

NOTE. *P* calculated with 2-sample, unpaired *t* test.

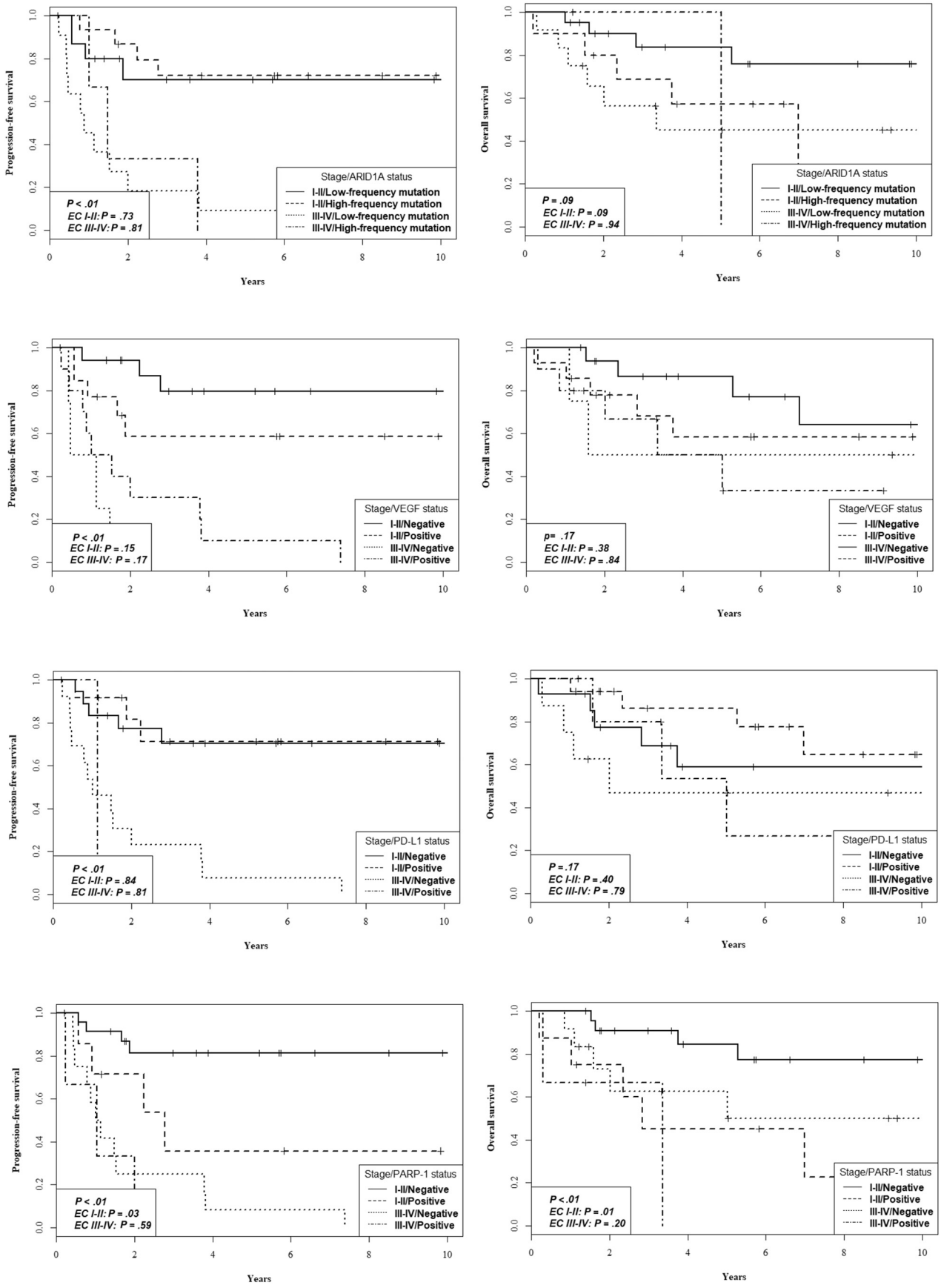

Fig. 5 ARID1A, VEGF, PD-L1, and PARP-1 expression effect in PFS and OS.

and endometrioid ovarian carcinomas comprised another 24 cases (52.2%). Thirty-six of the 46 cases had the diagnosis of endometriosis associated with the carcinomas, and 24 cases had endometriosis tissue suitable for immunohistochemical analysis. Figs. 1 to 4, respectively, show different ARID1A, VEGF, PD-L1, and PARP biomarker expressions in positive/negative examples for illustrative purposes.

Table 1 shows the variables in the study in women with clear cell and endometrioid ovarian carcinomas according to the presence or absence of an association with endometriosis. Patients whose carcinomas were associated with endometriosis were diagnosed at significantly earlier stages than those without ($P = .008$).

Table 2 compares the expression of the protein biomarkers between endometriosis and carcinomas in those 24 cases where it was possible to retrieve endometriosis and carcinoma areas from the same surgical sample. The results show the tendency of increased expression of the biomarkers in cancer tissue. However, only the VEGF increased in a statistically significant way ($P = .0002$).

We used ROC curves to generate optimal cutoff points to separate overexpression and normal expression of VEGF, PD-L1, and PARP-1 for disease recurrence and patient death. For ARID1A, the lack of expression was considered as altered. The values defined by the curves were as follows: ARID1A/progression, 32.5% (range, 0-100); ARID1A/death, 71 (range, 0-100); VEGF/progression, VEGF/death, score 273 (range, 0-300); PD-L1/progression, 47 (range, 0-300); PD-L1/death, 17.5 (range, 0-300); and PARP-1/progression = PARP-1/death, 205 (range, 0-300). Then, Kaplan-Meier curves of PFS and OS according to negative/positive biomarker expression and stage of disease were generated (Fig. 5). Results demonstrated that the PARP-1 overexpression was related to an early progression ($P = .03$) and worst OS ($P = .01$) for clear cell and endometrioid ovarian carcinomas at stages I-II. For all other groups, there was no difference in PFS and OS related to the expression of the biomarker.

4. Discussion

We found that more than 60% of the cells in endometriosis foci and 40% of the cells in EAOC exhibited some loss of ARID1A gene expression. We also assessed the expression of VEGF, PD-L1, and PARP-1 protein biomarkers. We found that endometriosis and EAOC expressed all of these protein biomarkers, with VEGF expression being increased in carcinomas compared with endometriosis. In this study, we also verified the earlier recurrence and worst OS of women with EAOC in the PARP-1 overexpression group. The OS expectation in the group of cases of EAOC with the overexpression of PARP-1 diagnosed in the initial stages was similar to the OS expectancy of cases of EAOC diagnosed at advanced stages.

The loss of ARID1A expression was evaluated in both endometriosis and EAOC. Several studies have linked the loss of expression of the proto-oncogene ARID1A to clear cell, and endometrioid ovarian carcinomas [8,9,14]. They also detected the loss of ARID1A expression in typical and atypical endometriotic tissue [8]. Our study results align well with those results from previous studies because the immunohistochemical analysis showed that more than 40% of EAOC cells had a loss of ARID1A expression. Other studies showed that the loss of ARID1A expression was linked to promoting the development of EAOC [6], although the increased percentages of mutated cells were not related to differences in cancer prognosis, a finding that was also described in our study. Previous studies demonstrated that the loss of immunohistochemical expression of the BAF250a protein coded by the ARID1A gene correlates well with mutations in this gene [8,9]. Also, ARID1A gene mutations are difficult to study as many are point mutations, deletions, or base exchange, and even those mutations led to a complete loss of BAF250a expression [9]. We assume that this fact was responsible for the all or nothing pattern of ARID1A expression status observed in this study.

Current pieces of evidence show that the origins and the prognosis of EAOC (type I) differ from those of type II ovarian carcinomas [1]. However, the basis for the treatment of both groups is the same for the first and subsequent lines of therapies [10]. The microenvironments of endometriosis and EAOC are known to stimulate angiogenesis, be proimmunogenic, and be promutagenic, all 3 pinpoints for targeted therapy [5,15-17]. However, there is limited knowledge about the expression of biomarkers and the use of targeted therapies for the treatment of EAOC. Some targeted therapies are more effective when tumor cells express its protein biomarkers, as is valid for small cell lung cancer and anti–PD-L1 drugs [18,19]. Moreover, some targeted therapy protein biomarkers, when overexpressed, are associated with a worsening of prognosis, as detected when PARP-1 is overexpressed on some breast cancers [20]. Those facts demonstrated the importance of studying the diverse targeted therapy biomarkers on different types of cancers.

It has been a long time since the effect of VEGF expression in cancer and endometriosis and the use of targeted therapies that block angiogenesis have been under scrutiny [21]. Increased levels of VEGF in the peritoneal fluid of women with ovarian carcinomas were related to ascites [22]. However, studies have failed to show any relationship between the VEGF expression and survival [23]. Despite this, many types of solid tumors exhibit overexpression of VEGF. Therefore, anti-VEGF drugs are already in use as first- and second-line therapy treatments for many such tumors, sometimes with significant PFS and OS benefit [6-24]. In this study, there was no association between VEGF overexpression and prognosis, but almost all our carcinomas samples had moderate to high VEGF expression. This suggests that the use of anti-VEGF drugs in combination with platinum chemotherapy could improve survival in EAOC.

The faulty immune response against cancerous and precancerous lesions was described a long time ago, but sound knowledge about its causes has evolved only in the last decade [25]. Endometriosis and EAOC seem to be diseases that may

respond to treatments that boost the immune response. In both cases, lesions are surrounded by a rich proimmunogenic environment that is ineffective in identifying and clearing these diseases [15,17]. Anti–PD-L1 treatments are the prototype of modern therapy that uses the immune system of patients to fight cancer [26]. PD-L1 is a recognized immune checkpoint expressed at the cell membrane of tumors. PD-L1 binds with the PD-1 membrane protein in T cells and signals to those lymphocytes to ignore cancer. When PD-1/PD-L1 interaction is blocked by anti–PD-L1 drugs, the immune response of T cells is reactivated, leading it to recognize and destroy cancer cells. In fact, anti–PD-L1 drugs are already in use to treat some lung cancers that express PD-L1 in their cell membranes [26]. Our study shows that not all cases of endometriosis and EAOC exhibit membrane expression of PD-L1, and we could not verify any effect of PD-L1 expression over PFS and OS. Also, among the protein biomarkers analyzed by us, PD-L1 was the less expressed, suggesting that these carcinomas escape from the immune system by another mechanism, like findings found in other studies [19].

Poly(adenosine diphosphate-ribose) polymerase (PARP) enzymes have been known since about the mid-1960s [27]. They are a group of molecules that are important for the repair of DNA recombination errors. PARP overactivation occurs in the presence of other defects linked to DNA recombination, for example, when there are ARID1A/B and BRCA/B mutations [28]. The inhibition of PARP is known to cause an effect called "synthetic lethality," where the inhibition of 2 or more DNA repair pathways leads to catastrophic cell events and apoptosis. PARP inhibitors are already in clinical use for some cancers [29]. In ovarian cancer, PARP inhibition is used in second-line maintenance therapy for platinum-sensitive recurrent tumors [30]. Several studies have assessed the effect of PARP-1 overexpression in breast cancer prognosis; these studies found that patients whose tumors overexpress PARP-1 fare worse than those whose tumors do not [20]. In our study, PARP-1 overexpression was associated with a worse prognosis (reduced PFS and OS) in early-stage EAOC patients.

The use of TMA could be seen as a limitation of our study owing to the possibility of heterogeneity in biomarker expression within the tumor and endometriosis. However, previous studies have validated the TMA technique for use in ovarian carcinomas with up to 95% of concordance of the findings in TMAs with whole section samples [31]. We tried to overcome such limitations by the use of large 2-mm core and 2 samples per case. We also think that TMA has the advantage of allowing for the evaluation of multiple cases under the same immunohistochemical conditions.

In conclusion, our study found convergent PD-L1 and PARP-1 expression, and ARID1A loss of expression among EAOC and endometriosis-related benign ovarian lesions. However, in malignant tissues, VEGF expression was significantly increased. We also found that PARP-1 overexpression was associated with earlier recurrence and worse OS. These findings encourage further studies on these histologic types of ovarian carcinomas, aiming to seek new treatment protocols that may eventually increase the survival of women with EAOC.

CRediT authorship contribution statement

Amilcar Barreta: Conceptualization, Methodology, Investigation, Data curation, Writing - original draft, Resources, Supervision. **Luís Otávio Sarian:** Formal analysis, Resources, Supervision. **Amanda Canato Ferracini:** Investigation, Data curation, Writing - original draft. **Larissa Bastos Eloy Costa:** Investigation. **Priscila Gava Mazzola:** Writing - review & editing, Supervision. **Liliana de Angelo Andrade:** Methodology, Investigation, Writing - review & editing, Supervision. **Sophie Derchain:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Resources, Supervision.

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