The role of intratumoral lymphovascular density in distinguishing primary from secondary mucinous ovarian tumors

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OBJECTIVE: Ovarian mucinous metastases commonly present as the first sign of the disease and are capable of simulating primary tumors. Our aim was to investigate the role of intratumoral lymphatic vascular density together with other surgical-pathological features in distinguishing primary from secondary mucinous ovarian tumors.

METHODS: A total of 124 cases of mucinous tumors in the ovary (63 primary and 61 metastatic) were compared according to their clinicopathological features and immunohistochemical profiles. The intratumoral lymphatic vascular density was quantified by counting the number of vessels stained by the D2-40 antibody.

RESULTS: Metastases occurred in older patients and were associated with a higher proportion of tumors smaller than 10.0 cm; bilaterality; extensive necrosis; extraovarian extension; increased expression of cytokeratin 20, CDX2, CA19.9 and MUC2; and decreased expression of cytokeratin 7, CA125 and MUC5AC. The lymphatic vascular density was increased among primary tumors. However, after multivariate analysis, the best predictors of a secondary tumor were a size of 10.0 cm or less, bilaterality and cytokeratin 7 negativity. Lack of MUC2 expression was an important factor excluding metastasis.

CONCLUSIONS: The higher intratumoral lymphatic vascular density in primary tumors when compared with secondary lesions suggests differences in the microenvironment. However, considering the differential diagnosis, the best discriminator of a secondary tumor is the combination of tumor size, laterality and the pattern of expression of cytokeratin 7 and MUC2.

KEYWORDS: Mucinous Ovarian Tumors; Ovarian Metastasis; Lymphatic Vascular Density; D2-40; Immunohistochemistry.

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INTRODUCTION

Malignant epithelial tumors account for 90% of all ovarian cancers and are the most lethal gynecological neoplasia (1). Primary mucinous tumors are not as frequent as serous tumors and account for 10-15% of all ovarian neoplasms. Approximately 80% are benign (adenomas), 10-12% are borderline and only 3-4% correspond to primary ovarian carcinomas (1). This last value was estimated after the recognition and exclusion of metastatic carcinomas simulating primary tumors in the ovaries (2,3). Secondary mucinous carcinomas in the ovaries can mimic primary ovarian carcinomas and even borderline tumors (4). The most common sources of secondary tumors are the colorectum, breast, stomach, endometrium, appendix, endocervix, pancreas and bile ducts (5). Primary mucinous ovarian carcinomas are therefore rare, generally unilateral and larger than 13 cm (4,6). Despite the refined diagnostic criteria and current ancillary techniques, particularly the coordinated expression of cytokeratins 7 and 20 (7), the problem of distinguishing primary from metastatic carcinomas persists in at least 15% of mucinous ovarian tumors.

Epithelial ovarian tumors present a variable stromal component that is particularly remarkable among those of the mucinous subtype. The most striking example is the Krukenberg tumor, which exhibits a unique cellular stroma.

No potential conflict of interest was reported.

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During cancer development, the stroma is remodeled to support cancer cell proliferation, migration, invasion, or metastasis. Lymphatic vessels are an important component of intratumoral stroma and are also responsible for creating conduits for tumor metastasis.

To the best of our knowledge, no studies have compared the intratumoral lymphatic vascular density (LVD) between primary and secondary mucinous ovarian tumors as a means of distinguishing between these tumors. Therefore, we proposed to investigate a potential role for LVD in the differential diagnosis of borderline and malignant mucinous ovarian tumors via the quantification of lymphatic vessels identified by podoplanin expression.

MATERIALS AND METHODS

Selection of cases

Cases of mucinous tumors in the ovary were identified from the surgical pathology files of the Division of Pathology of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (São Paulo/SP) and from Consultoria em Patologia (Botucatu/SP), which is a private reference pathology laboratory. We included cases with borderline and malignant mucinous histologies for which the following were available: information about the primary site, paraffin blocks and at least one representative histologic section taken from each centimeter of the tumor. A total of 124 cases from 1996 to 2005 (Universidade de São Paulo) and from 2004 to 2011 (Consultoria em Patologia) met the inclusion criteria. The distribution of these cases according to their diagnosis and the primary site is shown in Table 1. The age of this cohort ranged from 16 to 81 years (50.2 ± 15.8 years). From both the pathology report and the surgery description, we obtained information about laterality, tumor size and extraovarian extension, including the presence of pseudomyxoma. Slides from all cases were reviewed by two pathologists (BGLA and CRF). Doubtful cases were analyzed under a dual-head microscope by a third pathologist (FMC). The tumors were classified according to the presence and type of stromal invasion (infiltrative/destructive, multinodular and expansive/confluent) (Figure 1), cellular type (Mullerian, intestinal, pyloric, gastrointestinal, mixed Mullerian/intestinal and indeterminate) (Figure 2), histologic grade according to the Silverberg System (8), necrosis extension (focal, less than 50% or more than 50%) and peritumoral vascular involvement.

The cases were grouped as primary (Group 1) and secondary (Group 2) tumors. Primary tumors were borderline tumors without associated pseudomyxoma and adenocarcinomas without clinical or surgical suspicions of other neoplasia. Among the secondary tumors, we included borderline tumors associated with pseudomyxoma peritonei and adenocarcinomas with a known primary mucinous carcinoma at another site with a morphology similar to that of the ovarian tumor. Sixty-three tumors were classified as primary ovarian tumors and 61 tumors were classified as secondary ovarian tumors. We selected a representative area of the tumor for tissue microarray (TMA) construction and immunohistochemical study.

Tissue microarray construction

TMA construction was conducted at the Consultoria em Patologia (Botucatu, SP). Representative areas were identified on slides stained with hematoxylin and eosin and marked on paraffin blocks. Cylindrical tissues with a diameter of 2.0 mm were punched from the areas of interest of the donor paraffin block and mounted into the recipient block with 1.0-mm intervals between the cores using a precision microarray instrument (Beecher Instruments, Silver Spring, MD) positioned on a fixed sideboard. The cores were organized in lines and columns using the hepatic tissue for orientation in Position 1A. After a final configuration of the recipient blocks, they were heated at 60℃ for 10 minutes and sealed with the Paraffin Tape-Transfer System (Instrumedics, St. Louis, MO) for sectioning using the appropriate slides (Starfrost® slides) and a microtome at 3-μm intervals (Leica Instruments, Wetzlar, Germany). The first histological sections were stained with hematoxylin-eosin to identify losses for eventual study in whole sections.

Immunohistochemical analysis

Immunohistochemical detection of cytokeratin 7 (CK7), cytokeratin 20 (CK20), CA125, CDX-2, CA19.9, MUC2, MUC5AC and podoplanin was performed using slides from TMA blocks. The sources and dilutions of the antibodies as well as the epitope retrieval methods used are listed in Table 2. Bound antibodies were detected using Novolink® (Leica, Bannockburn, IL, USA). For all the markers, with the exception of podoplanin, any percentage of unequivocally positive neoplastic cells was scored as positive for the markers, although all the positive cases showed more than 10% stained cells. Identification of lymphatic vessels was established based on the presence of cells that were positive for podoplanin and that had a morphology consistent with vessel structure (Figure 3). In tumor sections that were negative for podoplanin staining, adjacent lymphatic endothelial cells that appeared normal served as positive internal controls.

Table 1 - Distribution of the mucinous ovarian tumors included in this study.

Diagnosis	Type/Primary Site		N (%)
Primary ovarian tumors	Borderline without pseudomyxoma peritonei		30 (24.19%)
	Adenocarcinoma		33 (26.61%)
Secondary ovarian tumors	Borderline associated with pseudomyxoma peritonei		8 (6.45%)
	Adenocarcinoma	colorectal	38 (30.64%)
		appendix	5 (4.03%)
		gastric	3 (2.41%)
		pancreatobiliary	3 (2.41%)
		breast	2 (1.61%)
		unknown (disseminated disease)	2 (1.61%)
Total			124

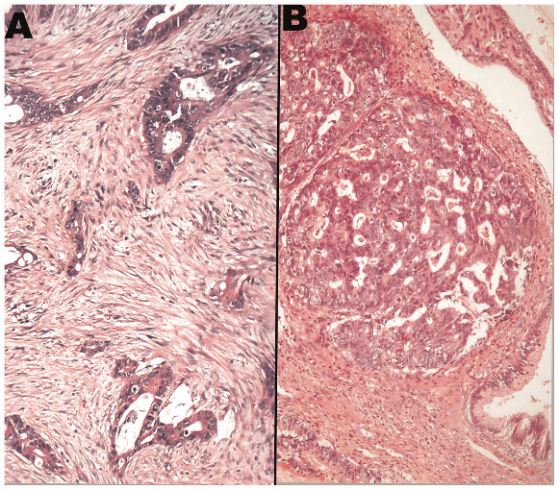

Figure 1 - Patterns of invasion in two mucinous adenocarcinomas: infiltrative/destructive (A) and confluent with nodular configuration (B).

Quantification of lymphatic vascular density

The quantification of LVD was performed as previously described by our group (9-11). Briefly, stained TMA histologic sections were analyzed using standard light microscopy (Nikon, Eclipse 200). Under low magnification, the most vascularized intratumoral areas were identified. We counted the number of immunostained lymphatic vessels found in 10 ''hot spot'' areas at 400X magnification. The LVD for each case was expressed by the mean value (total number of vessels in 10 hot spot microscopic fields/10). The median of all the mean LVD values was the cutoff used to divide tumors into high or low LVD, as suggested by Hall et al. (12).

Statistical analysis

A *t*-test was used to compare the ages of patients in Group 1 (primary tumors) and Group 2 (secondary tumors) after confirmation of a normal distribution by the Kolmogorov-Smirnov test. The Chi-square test was used to evaluate the association of the categorical variables within the two groups. The odds ratio with a 95% confidence interval was calculated for these variables. LVD was analyzed either as a dichotomous variable or as a continuous variable. The median (0.8) value was the cut-off used to determine low or high LVD. Continuous LVD values were compared between primary and secondary tumors using the Mann-Whitney *U* test. The correlation between continuous LVD and tumor size was tested using Spearman's rank correlation. For multivariate analysis, the selected variables were analyzed with logistic regression using the stepwise method. Statistical analyses were performed using MedCalc for Windows (version 11.5.0.0; MedCalc Software, Mariakerke, Belgium), and *p*-values less than 0.05 were considered significant.

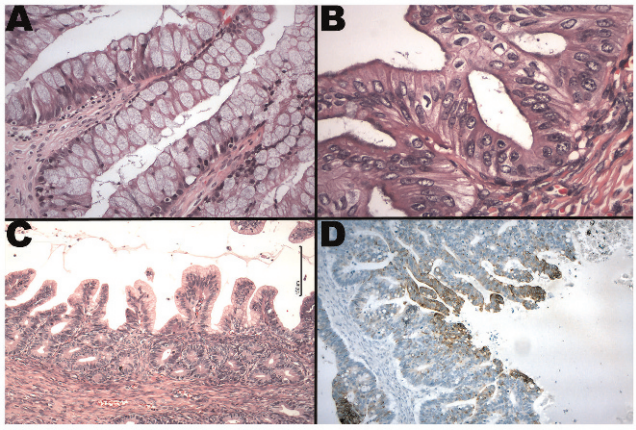

Figure 2 - Mucinous carcinomas with intestinal (A), Mullerian (B) and gastric (C) phenotypes. Focal cytokeratin 7 expression in a metastatic mucinous carcinoma is shown.

Ethics statement

This study was approved by the Scientific Committee of the Department of Pathology of the Faculdade de Medicina da Universidade de São Paulo and by the Ethics Committee for Research Projects of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (Comissão de Ética para Análise de Pesquisa - CAPPesq) (process number 1312/09).

RESULTS

Patients with primary tumors were younger than those with secondary tumors, with mean values of 46.3 years and 54.0 years, respectively ($p = 0.007$). Sixty-seven (54.03%) of the cases exhibited an intestinal phenotype and only 7 (5.6%) presented pure Mullerian differentiation. Other tumors presented the following morphological phenotypes: 10 (8.06%) pyloric, 19 (15.32%) pyloric and intestinal, 7 (5.64%) mixed Mullerian and intestinal and 14 (11.29%) indeterminate. All tumors with diffuse or partial Mullerian differentiation were primary. Among the 93 cases with a diffuse or partial intestinal pattern, 42 (45.1%) were primary and 51 (54.8%) were secondary. Peritumoral vascular invasion was observed in only three cases, all of which were secondary tumors. The LVD values ranged from 0 to 10.1 (1.6 ± 2.0). There was no correlation between tumor size and LVD (rho = 0.88; $p = 0.89$) or between age and LVD (rho = 0.04). The LVD was lower in secondary tumors (median 0.4 *vs.* 1.5; $p = 0.02$). The surgical-pathological characteristics of Groups 1 and 2 are presented in Table 3. Smaller and bilateral tumors, extensive tumoral necrosis and a surgical finding of extraovarian disease were associated with a higher probability of secondary ovarian involvement. High-grade invasive adenocarcinomas with multinodular patterns of ovarian parenchyma invasion had a higher probability of being metastatic. The comparative immunohistochemical study between primary and secondary tumors is shown in Table 3. Secondary tumors were mainly associated with the expression of CK20, CDX-2 and MUC2 and were negatively associated with a high LVD and the expression of CK7, CA125 and MUC5AC.

We constructed a logistic regression model including the patients' age, tumor size and laterality as predictors of a secondary tumor. The age of the patient was excluded from the model. Next, we tested a model with tumor size, laterality and all the immunohistochemical markers (CK7, CK20, CDX-2, CA125, CA19.9, MUC2 and MUC5AC). The variables that remained in the model were tumor size, laterality, CK7 and MUC2. Finally, we tested the LVD with these variables in a new model; however, LVD was

Table 2 - Reagents and methods used for immunohistochemical analyses.

Antigen	Clone/source	Dilution	Epitope retrieval method
CK7	OV-TL 12/30 MOUSE IgG1/DAKO	1/1000	PT LINK 20 minutes, low pH
CK20	KS20.8 Ks20.8 MOUSE IgG2a/ZETA	1/800	PT LINK 20 minutes, low pH
CA125	OC 125 MOUSE IgG1/ZYMED	1/800	PT LINK 20 minutes, low pH
CDX-2	DAK-CDX-2 MOUSE IgG1/DAKO	1/800	PT LINK 20 minutes, high pH
CA19.9	SPM110 MOUSE IgG1/NEOMARKERS	1/1600	PT LINK 20 minutes, low pH
MUC2	Ccp 58 MOUSE IgG1/NOVOCASTRA	1/100	PT LINK 20 minutes, high pH
MUC5AC	CLH2 MOUSE IgG1/NOVOCASTRA	1/200	PT LINK 20 minutes, high pH
Podoplanin	D2-40 MOUSE IgG1/DAKO	1/200	PT LINK 20 minutes, low pH

excluded. The final model identified a tumor size of 10.0 cm or less (OR 9.4; 95% CI 1.2-69.2), bilaterality (OR 51.5; 95% CI 7.1-370.2) and CK7 negativity (OR 64.8; 95% CI 9.4-447) as predictors of a secondary deposit. The probability of a secondary tumor in this model is reduced if MUC2 is negative (OR 0.1; 95% CI 0.01-0.6). This model allows 90.0% of all cases to be classified correctly, including 92.2% of primary tumors and 86.2% of metastases.

DISCUSSION

Ovarian metastases commonly present as the first sign of many adenocarcinomas, including (but not limited to) gastrointestinal adenocarcinomas, with the primary tumor remaining undiagnosed (13). When compounded by the fact that some metastatic carcinomas can stimulate primary tumors, this presentation can lead even experienced pathologists to incorrectly diagnose a secondary deposit as a primary neoplasm, causing delays and the implementation of incorrect therapeutic approaches, with serious consequences for the patient. In such situations, the use of morphological criteria can often be helpful; however, none of these criteria are pathognomonic of metastasis (4,6,13,14). The main characteristics indicating that a deposit is secondary are a small tumor size; bilaterality; multiple nodules on the cut surface; a microscopic pattern of stromal invasion with a nodular, heterogeneous and infiltrative/destructive phenotype; surface implants; lymphatic or blood vessel invasion, especially if conspicuous; and the presence of signet-ring cells and neoplastic cells floating in mucin pools (14,15). The pattern of ovarian parenchyma involvement can suggest a secondary neoplasm. In our cases, we observed that all secondary tumors had a multinodular pattern of invasion. In a retrospective study of 104 cases comparing expansive *versus* infiltrative invasion, the authors found a worse prognosis and a higher probability of lymph node metastasis among individuals with the infiltrative pattern (16). A simple algorithm based on tumor size and laterality has been previously determined and can correctly classify 84-90% of the cases (6,15). In addition, immunohistochemistry may help to identify the profile determined by coordinated CK7 and CK20 expression. For example, a CK7-/CK20+ immunoprofile suggests a colorectal origin, whereas a CK7+/CK20- profile favors the ovary as the primary site. However, this ancillary technique has limitations in this setting because primary mucinous ovarian tumors may express CK20 focally or, at times, diffusely (17). Similarly, large bowel adenocarcinomas can show focal or multifocal staining for CK7, which happens more frequently in poorly differentiated tumors and in those localized in the right colon and rectum (18). Additionally, the CK7+/CK20- immunophenotype is also observed in adenocarcinomas originating in the pancreas, breast, stomach, lung, bile ducts and female genital tract (19). Due to this overlap, it is not advisable to use immunohistochemistry alone when investigating whether a mucinous ovary tumor is primary or secondary; rather, this approach should always be utilized in conjunction with clinical and morphologic data. In the last few years, new immunohistochemical markers have been used in combination with the cytokeratins (CK7 and CK20) to increase the accuracy of the test. Immunohistochemical markers, such as Cdx-2, β-catenin, p504, Dpc4, MUC1, MUC2, MUC5AC and Hep Par, can be useful. However, despite all the available approaches, there will be cases in which doubt will remain. In this study, we included the most common predictors of metastatic nature: surgical findings (such as tumor size, laterality and extraovarian spread) and immunohistochemical markers. Although our study was limited by the use of TMA for immunohistochemical reactions, our results agreed with the published data. Interestingly, when multivariate analysis was performed only on tumors smaller than 10.0 cm, bilaterality and negative CK7 remained as predictors of metastasis. Our model accurately predicted 86.2% of metastasis cases; thus, a correct diagnosis could not be obtained for least 15% of cases.

Therefore, the search for a new tool that may help to accurately determine whether a mucinous tumor is primary or metastatic continues.

The lymphatic vasculature is one important route of neoplastic dissemination for most carcinomas. Additionally, conspicuous lymphovascular invasion is one of the characteristics indicative of metastases of ovarian tumors (14,15). On the other hand, the stromal component, which includes

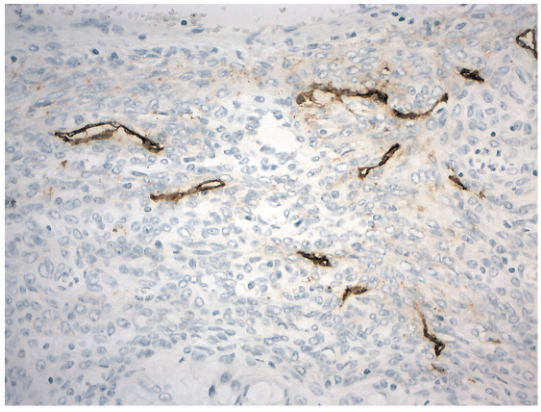

Figure 3 - Mucinous ovarian tumor showing ten lymphatic vessels identified by podoplanin staining using the D2-40 antibody (original magnification 200X).

Table 3 - Surgical-pathological variables and immunohistochemical characteristics of 124 cases of mucinous ovarian tumors.

Feature	Categories	Primary (n = 63)	Metastatic (n = 61)	OR (95% CI)	*p*
Tumor size	>10 cm	48 (76.19%)	21 (34.42%)	5.9 (1.9-18.8)	0.002
	≤10 cm	5 (7.93%)	13 (21.31%)		
	unknown	10 (15.87%)	27 (44.26%)		
Bilaterality	yes	6 (9.52%)	20 (32.78%)	5 (1.8-13.6)	0.001
	no	57 (90.47%)	38 (62.29%)		
	unknown	0 (0%)	3 (4.91%)		
Patterns of stromal invasion *	infiltrative	15	31	1.5 (0.6-3.7)	0.32
	multinodular	0	11	17.3 (0.9-304)	0.05
	expansive/ confluent	18	13		
Histologic grade*	1	16	15		
	2	16	24	1.6 (0.6-4.1)	0.33
	3	1	16	13.1 (1.6-104.4)	0.01
Extra-ovarian disease	yes	8 (12.69%)	36 (59.01%)	9.9 (4-24.3)	<0.0001
	no	55 (87.30%)	25 (40.98%)		
Necrosis	<50%	32 (50.79%)	43 (70.49%)	8.9 (1.1-72.2)	0.04
	>50%	1 (1.58%)	12 (19.67%)		
	unknown	30 (47.61%)	6 (9.83%)		
CK7	positive	58 (92.06%)	18 (29.50%)	0.04 (0.01-0.1)	<0.0001
	negative	5 (7.93%)	42 (68.85%)		
	unknown	0 (0%)	1 (1.63%)		
CK20	positive	37 (58.73%)	52 (85.24%)	4.6 (1.8-11.2)	0.0009
	negative	26 (41.26%)	8 (13.11%)		
	unknown	0 (0%)	1 (1.63%)		
CDX-2	positive	45 (71.42%)	57 (93.44%)	7.6 (2.1-27.4)	0.002
	negative	18 (28.57%)	3 (4.91%)		
	unknown	0 (0%)	1 (1.63%)		
CA125	positive	38 (60.31%)	7 (11.47%)	0.08 (0.03-0.2)	<0.0001
	negative	24 (38.09%)	53 (86.88%)		
	unknown	1(1.58%)	1 (1.63%)		
CA19.9	positive	49 (77.77%)	37 (60.65%)	0.4 (0.2-0.9)	0.04
	negative	13 (20.63%)	23 (37.70%)		
	unknown	1(1.58%)	1 (1.63%)		
MUC2	positive	34 (53.96%)	56 (91.80)	11.5 (3.7-35.7)	<0.0001
	negative	28 (44.44%)	4 (6.55%)		
	unknown	1(1.58%)	1 (1.63%)		
MUC5AC	positive	50 (79.36%)	25 (40.98%)	0.2 (0.08-0.4)	<0.0001
	negative	13 (20.63%)	35 (57.37%)		
	unknown	0 (0%)	1 (1.63%)		
LVD	high (>0.8)	36 (57.14%)	23 (37.70%)	0.4 (0.2-0.9)	0.02
	low (≤0.8)	26 (41.26%)	38 (62.29%)		
	unknown	1(1.58%)	0 (0%)		

*Evaluated only among cases of non-borderline morphology.

the lymphovascular spaces, is an important component of epithelial ovarian tumors (20,21). Therefore, we decided to investigate the potential role of the intratumoral LVD in the characterization of a mucinous tumor as primary or secondary. Published studies examining LVD in patients with ovarian tumors were generally designed to evaluate the behavior of primary carcinomas but not to help identify secondary tumors. For example, intratumoral and peritumoral LVD have been studied in borderline ovarian serous tumors in an attempt to determine their role in nodal metastasis (22). No association between LVD and nodal metastasis could be demonstrated, perhaps because the nodal tumor deposits observed in borderline tumors do not occur via tumoral lymphatics (22). Lymphangiogenesis was also investigated by Sundar et al. in 108 ovarian tumors, including 12 mucinous type tumors. In that study, lymphatic vessel density was statistically significant in a multivariate analysis of overall survival and progression-free survival. However, lymphatic counts did not impact the survival curves. The authors suggested that lymphatic spread might act in conjunction with other biological factors to cause metastasis (23).

In our previous experience with cancers of the vulva, cervix and endometrium, we found an inverse correlation between intratumoral LVD and lymph node metastasis and prognosis (9-11). To explain our previous results, we hypothesized that the intratumoral lymphatic vessels were non-functional and therefore had the potential to disturb local drainage, including the transport of neoplastic cells outside the tumor. Another hypothesis to be considered is that the intratumoral lymphatics are important in the early steps of neoplastic progression, just prior to the transport of cells outside of the tumor. Once the dissemination has begun, intratumoral lymphangiogenesis is no longer necessary. In this study, we aimed to determine a possible role for LVD in the definition of primary or secondary origin. Although primary tumors showed higher LVDs, this difference did not offer any advantage over the classical features (tumor size, laterality and CK7 expression) with respect to the diagnosis. In routine surgical pathology, 90%

of the cases can be reliably categorized using these predictors (size ≤ 10.0 cm, bilaterality and lack of CK7 expression). Our deceptive results with the LVD did not exclude the possibility of other differences between the stroma of primary and secondary tumors, which may or may not involve the lymphatic vasculature. On the other hand, recent studies have advocated a change in the paradigm with regard to what concerns an ''extraovarian origin'' of epithelial ovarian cancer (either from tubal fimbria or from endometriosis) (24,25). Because all epithelial ovarian tumors might be secondary, this could explain why no great differences were detected in LVD between our two study groups.

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AUTHOR CONTRIBUTIONS

Almeida BG collected the data, reviewed all the histological samples, interpreted the immunohistochemical analyses and performed the LVD counting. Ferreira CR collected some of the data, reviewed the original slides and contributed to the manuscript drafting. Bacchi CE carried out the immunohistochemical reactions and assisted in their interpretation. Carvalho JP collected the surgical samples and contributed to the design, coordination and drafting. Carvalho FM conceived the study, participated in its design, contributed to the review of the slides, performed the statistical analysis and wrote the manuscript. All of the authors have participated sufficiently in the work to take public responsibility for the appropriate portions of the content. Additionally, all of the authors have read and approved the final manuscript version.

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