INTRODUCTION

Lymphangiogenesis is the process of lymphatic vessels formation from preexisting postcapillary venules.1

1 Department of Obstetrics-Gynecology, 2 Department of Histology, Victor Babes University of Medicine and Pharmacy, Timisoara

Correspondence to:
Prof. Marius Raica, Victor Babes University of Medicine and Pharmacy, 2 E. Murgu Sq., Timisoara, Romania
Email: raica@umft.ro

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In normal conditions in the postnatal life, lymphangiogenesis is a quiescent process, but an active formation of new lymphatics was reported in various neoplastic and non-neoplastic human diseases. Although the lymphatic vessels invasion is unanimously accepted as a poor prognostic factor, the early steps of invasion by tumor cells and their clinical significance are still a matter of debate. Lymphangiogenesis is a complex multistep process, driven mainly by vascular endothelial growth factor-C and its cognate receptor.2 The binding of the ligand to the receptor stimulates endothelial cells of the postcapillary venules to acquire lymphatic phenotype and, finally, they lose the connection with blood vessels. The overexpression of Prox-1 gene is
mandatory in this step, and its nuclear expression is usually demonstrated in developing lymphatics.

The study of lymphangiogenesis is an acquisition of the last years, despite the fact that lymph node metastases were recognized as major indicator of prognosis and therapeutic strategy in patients with cancer. This apparent contradiction resides mainly in the lack of highly specific antibodies for the lymphatic endothelium. Recently, some specific antibodies were introduced in practice, that discriminate between blood vessels and lymphatic vessels (LVs) endothelium, like LYVE-1, podoplanin, Prox-1, desmoplakin, and vascular endothelial growth factor receptor. Using these antibodies, it was possible to count LVs in the peritumoral and tumoral area, procedure known as lymphatic microvessel density (LMVD). LMVD was calculated in almost all human tumors and particularly in carcinomas, and in most of the cases there was found a positive correlation between LMVD, lymph node metastases, and prognosis.

The presence of LVs in the tumor area was controversial for many years, but nowadays they were clearly demonstrated in squamous cell carcinoma of the head and neck, gastric carcinoma, breast cancer, or malignant melanoma. Few data is available about LVs and LMVD in ovarian tumors, and in the literature only few reports were published until now. Consecutively, results are controversial and the clinical significance of LMVD and lymphovascular invasion in ovarian carcinoma is virtually unknown. In the present work we investigated the presence and density of LVs in patients with advanced-stage ovarian cancer and we showed a correlation with the stage of the tumor, but not with the pathological form of carcinoma and grade.

**MATERIAL AND METHODS**

There were investigated 35 patients with ovarian cancer treated by open surgery in the last 4 years. From the 35 cases, 32 were primary tumors and in 3 cases a second look was performed. According to the clinical staging system, there were 11 cases in stage II, 14 cases in stage III, and 10 cases in stage IV. Abnormal peritoneal fluid was found during the surgical intervention in 22 cases and peritoneal spread was noticed in 19 patients. Chemotherapy was performed before surgery in 4 cases.

Archive paraffin blocks were collected, re-embedded and processed using the routine histological technique. Slides stained with haematoxylin-eosin were reviewed for the histological diagnosis and tumor grading. Based on the routine microscopic examination, there were 17 serous carcinomas, 6 mucinous carcinomas, 5 clear cell carcinomas, 3 endometrioid carcinomas and 4 with associated pathological forms. The grading was performed according the criteria of World Health Organization, and we found well differentiated tumors in 12 cases, moderately differentiated in 13 cases and poorly differentiated tumors in 10 cases.

Additional sections from each case were stained for D2-40, the most specific marker of the lymphatic endothelium. Briefly, sections were dewaxed in benzene and hydrated in decreasing solutions of alcohols. Antigen retrieval was performed with microwave, using citrate buffer pH6 for 30 minutes. After blocking the endogenous peroxidase, slides were incubated with anti-D2-40 for 30 minutes (ready-to-use, Dako Cytomation, CA, USA). The working system was LSAB-HRP and the final product of reaction was visualized with diaminobenzidine (Dako, Glostrup, Denmark). Nuclei were stained with Lillie’s modified haematoxylin. The entire immunohistochemical procedure was performed with Dako Autostainer Plus (DakoCytomation, Denmark). Lymphatic microvessel density was counted at magnification x200, choosing three fields with maximum density in the peritumoral and intratumoral area. The arithmetical media was the final results in each case. The statistical analysis was performed with the commercially available SPSS version 17.0. Student test and Chi square were done to analyze the significance of D2-40 expression by tumor cells, and p<0.05 was considered as significant.

**RESULTS**

The staining for D2-40 was highly specific for the lymphatic endothelium and did not stain the blood vessel endothelium. (Fig. 1a) The final product of reaction was restricted to the cytoplasm of lymphatic endothelial cells, and in the normal ovarian tissue adjacent to the tumor no other cells were positive with this marker. In the ovarian medulla without tumor invasion the lymphatic microvascular density was less than 15 per field. In the tissue localized at a distance from the tumor the lymphatics have relatively large lumen, regular borders, without cellular content in the lumen. (Fig. 1b) The average density of lymphatics in this condition was 8.9/high power field x200 (HPF). In the peritumoral area lymphatic vessels were found in 32 cases, and in six cases, mostly from the group of clear cell carcinoma, there were tumor cells present in the lumen.

In the tumor area the lymphatic vessels were small, with irregular borders, narrow lumen (Fig.1c). The
maximum density of lymphatics in the intratumoral area was found in the serous adenocarcinoma (16/HPF). In 5 cases, in the lumen there were tumor cells, reflecting the lymphovascular invasion. No lymphatic vessels were detected in the intratumoral area in endometrioid, mucinous and in mix carcinomas containing mucinous component. The average density of lymphatics in the tumor area was 5.6/HPF.

A particular aspect found only in the intratumoral lymphatic vessels was the apposition of tumor cells to the outer side of the vessel wall, most probably reflecting an early stage of lympho-vascular invasion. (Fig. 1d) Definite lympho-vascular invasion was found in 8 cases and was characterized by clusters of tumor

Figure 1. D2-40-positive lymphatic vessels in the medulla of the normal ovary. Note that blood vessels are not stained (a). Peritumoral lymphatic vessels (b). Intratumoral lymphatic vessels (c). Lympho-vascular invasion (d). Attachment of tumor cells on the outer side of the lymphatic vessel (e). Buds of newly formed lymphatics in the tumor area (f). Original magnification, a-f, x400.
cells in the lumen. (Fig. 1e) In 9 out of 35 cases we noticed the presence of many D2-40 positive small structures without definite lumen, which may represent buds of new lymphatics. (Fig. 1f)

No overall correlation was found between the lymphatic microvessel density and pathological type of ovarian carcinoma or tumor grade (p = 0.23, and p < 0.12, respectively). A significant correlation was found between the intratumoral lymphatic microvessel density and the stage of the tumor (p < 0.0001). This is supported by the gradual increase in the number of D2-40-positive lymphatics with stage in both tumoral and peritumoral areas.

A particular aspect that was not mentioned in the literature is the expression of D2-40 by tumor cells. This aspect was found in 6 of 35 cases, and all were histologically serous adenocarcinomas. Notably, 5 of 6 positive cases were graded as poorly differentiated tumors. The final product of reaction had cytoplasmic granular pattern with heterogeneous distribution within the tumor mass. (Fig. 2a) Constantly, the reaction was significantly stronger in tumor cells from the front of invasion. (Fig. 2b) A strong correlation was found with the histological type and grade (p < 0.0002, and p < 0.0034, respectively).

**DISCUSSION**

Cancer is nowadays the most frequent disease with lethal potential in humans, and despite all efforts in the field of early diagnosis and adjuvant therapy, morbidity and specific mortality continue to increase. One of the most important factors with direct impact on prognosis and therapeutic strategy in various type of cancer is the lympho-vascular invasion and lymph node status. Although the relevance of this factor is well documented, the mechanisms by which tumor cells enter the lymphatic vessels and ultimately give rise to lymph node metastases are not completely understood. Lymphangiogenesis was less investigated in patients with ovarian cancer, on one hand due to the particular natural evolution of this tumor, and on the other, because few markers of the lymphatic endothelium are available.

D2-40 recognizes the formalin-insensitive epitope of podoplanin, which is one of the most specific markers of the lymphatic endothelium. As we have shown in the present study on ovarian tissue, D2-40 discriminates between lymphatics and blood vessels and allows an accurate count that is referred to as lymphatic microvessel density. Podoplanin/D2-40 belongs to the family of type-1 transmembrane sialomucin-like glycoproteins. A C-type lectin-like receptor-2 (CLEC-2) was identified as an endogenous receptor of podoplanin on platelets. Recombinant CLEC-2 has inhibited platelet aggregation induced by podoplanin-expressing tumor cells and lymphatic endothelial cells. These findings suggest that CLEC-2 is a physiological target protein of podoplanin and the interaction between podoplanin and CLEC-2 may regulate tumor invasion and metastasis. This is supported by our results on the expression of D2-40 in six cases of high-grade, advanced-stage serous adenocarcinoma that strongly suggest a role for podoplanin in the invasion and spreading of tumor cells.

In the normal ovary, primary and secondary ovarian follicles show strong podoplanin expression and the reaction becomes negative in the luteal

**Figure 2.** Heterogeneous expression of D2-40 in the cytoplasm of tumor cells in the serous adenocarcinoma (a). Stronger intensity is noticed predominantly at the front of proliferation (b). Original magnification, a-b, x400.
the conventional anti-tumor therapeutic options. In the same study, the podoplanin expression in ovarian tumor cells was found in 4 of 4 cases with dysgerminoma, and in one of 3 cases of granulosa cell tumors. All other investigated ovarian tumors were negative. Contrary to these findings, we report D2-40-positive tumor cells in serous adenocarcinoma, but on the other hand, germ cell tumors were not included in the present study.

Recently, a role for podoplanin was proposed in invasion and metastasis.14-15 This hypothesis is mainly based on the observation that high expression of podoplanin is consistently correlated with the presence of metastasis. The invasion of tumor cells correlates with significant changes of adhesion molecules and regulatory proteins. The molecular phenotype of cells in the invasion front is frequently different from that of cells in the tumor core.16 It was reported that podoplanin-expressing cells are found at the invasion front in more than 80% human squamous cell carcinomas, but currently there are no data regarding ovarian tumors as reported in our results.17

Based on its specificity for the lymphatic endothelium, D2-40 is nowadays the most used method to quantify the density of lymphatic vessels. Peritumoral lymphatics are significantly larger than in the intratumoral tissue, but associated with a significant lower density.18 Peritumoral lymphatics are large, irregular, and sometimes packed with tumor cells in the tumor periphery, as found in the present study. An increased lymphatic microvessel density in the peritumoral area was found even in early stages of the squamous cell carcinoma of the uterine cervix, but similar data were not reported in ovarian cancer.19 Intratumoral lymphatics were found in a large variety of tumors and usually they are small, flattened and irregular, and occasionally contain tumor cells. Several authors found podoplanin-positive vessels within the stroma in ovarian and cervical malignant tumors.20-22 We have found lymphatic vessels not only in the stroma, but also in the tumor area, and lymphovascular invasion was detected in five cases. Moreover, in our study, a strong correlation was found between the intratumoral lymphatic microvessel density and the stage of the ovarian cancer.

In the last years, the molecular profile of malignant tumors seems to play a crucial role in the management of patients with cancer and in the individual therapeutic strategy. Our data suggest that newly formed lymphatic vessels and D2-40-positive tumor cells could be an important target for therapy in aggressive ovarian tumors that skipped the conventional anti-tumor therapeutic options.

CONCLUSIONS

The evaluation of lymphatic vessels in 35 patients with ovarian carcinoma showed a significant correlation between their density and the stage of the tumor, but not with the pathological type and grade. Immunohistochemical expression of D2-40 by tumor cells was found in six cases with advanced-stage serous adenocarcinoma. Our results suggest that these parameters could be used as indicator of invasion and bad prognosis.

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