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Original Article

Lymphangigenesis in human epithelial ovarian cancer is related to the formation of ascites

Shouhua Yang, Rongwei Zhao, Jing Cai, Zehua Wang

Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei Province, PR China

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Abstract: Aims: To unveil the possible role of lymphangiogenesis in progression of human epithelial ovarian cancer (EOC). Materials and methods: By double immunohistochemistry for lymphatic endothelial cell marker D2-40 and cell proliferation-associated marker Ki-67, lymphatic vessel density (LVD), lymphatic vessel proliferation (LVP), and lymphatic vessel invasion (LVI) were investigated in ovarian specimens obtained from 45 patients with EOC and 10 controls in this study. Results: Although there was no significant difference of LVD between the EOC group and control group, LVP and LVI in the control group were rare. Lymphatic vessels were detected in 86.67% (39/45) EOC samples and in 90.00% (9/10) control samples, and LVD was significantly related to the volume of ascites, LVP and LVI. There were more LVP(+) cases in the group with severe ascites when compared to the group with less ascites ($P<0.05$). There was no significant difference between serous and non-serous, stage I-II and stage III-IV, well/moderate differentiation than poor differentiation ($P>0.05$, respectively). Conclusion: These findings indicated that the proliferation of lymphatic vessels in EOC possibly may induce the progression of EOC via the formation of ascites.

Keywords: Epithelial ovarian cancer, lymphangiogenesis, ascites

Introduction

Ovarian cancer is one of the leading causes of gynecological cancer-related deaths worldwide, and 90% of them are of epithelial origin [1, 2]. Most ovarian cancers are diagnosed at an advanced stage as the early detection is hampered by the lack of specific symptoms and effective screening procedures and the 5-year survival rate of ovarian cancer is only approximately 30% [1, 2]. Lymphatic spread is significant in aiding metastases in ovarian cancer [3], and the most important lymphangiogenic factor VEGF-C plays as an enhancer of ovarian cancer progression through autocrine and paracrine mechanisms [4]. The circulating VEGF-C can be considered as a clinically useful indicator for diagnostic and prognostic evaluation in ovarian cancer patients [5]. Lymphangiogenesis was involved in the dissemination of many solid tumors [6], and a model of ovarian cancer in mice was also identified substantial lymphangiogenesis and lymphatic remodeling, massive infiltration of macrophages and disseminated carcinomatosis in the mesentery and diaphragm, and progressive chylous ascites formation [7]. All these indicated that lymphangiogenesis should be an important role in the progress of EOC just like other solid tumors. However, lymphatic vessels density increased without significant changes in EOC based on the immunochemical study before [8], the possible role of lymphangiogenesis in the progression of EOC was still unknown. To address this issue, the proliferation of lymphatic vessels in 45 patients with EOC was analyzed by double immunohistochemistry for lymphatic endothelial marker D2-40 and cellular proliferation marker Ki-67 in this study.

Materials and methods

Samples

All cases examined in this study were pooled from the inpatients in Union Hospital, Tongji medical college, Huazhong University of Science and Technology, from Apr. 2006 to Apr. 2007. This work was directed by pathologists, and tumor tissues used in this study did not con-

tain any normal tissue or significant necrosis. Finally, 10 non-malignant ovarian samples and 45 pathologically verified EOC samples were studied, including 20 cases of serous cancer and 25 of non-serous cancer. 15 stage I to II, 30 stage III to IV based on the classification of FIGO (International Federation of Gynecology and Obstetrics); 17 of them were well-moderately differentiated and 28 of them with poor differentiation; the data of CA-125 antigen level of serum collected from every case. All of them had undergone cytoreductive surgery based bilateral salpingoophorectomy with total abdominal hysterectomy and omentectomy. The volume of ascites of each case was measured during operation. All patients were not subjected to any chemotherapy or radiotherapy before the surgery. The age rank of the 45 patients was 26-74 years with mean of 47.8 years. The 10 benign human ovarian tumor samples were collected as control. The collection procedure referred to the guidelines of the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Double immunohistochemistry of D2-40 and Ki-67

A 5-μm section from one selected paraffin block per subject was stained immunohistochemically with commercially available monoclonal antibodies to D2-40 (Maxin, Fuzhou, China) and Ki-67 (Maxin, Fuzhou, China) sequentially with the double immunohistochemical kit (Maxin, Fuzhou, China). Sections were dewaxed and antigen retrieval was carried out by pressure cooker in citrate buffer (PH 6.0) at 121 Centi-degree for 2-3 min. Slides were incubated in phosphate buffered saline (PBS) with 5% human serum for five minutes. Peroxidase was quenched with methanol and 3% H_2O_2 for 15 minutes. The monoclonal antibody of human D2-40 (1:400) was applied at 4 Centi-degree overnight. After washing with PBS three times, sections were incubated with biotinylated secondary antibody and anti-biotin-alkaline phosphates for 10 minutes at room temperature and washed in PBS. The first color-black was developed by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NCIP/NBT) solution for 15 min. Slides were then incubated in phosphate buffered saline (PBS) with 5% human serum for five minutes again. Peroxidase was quenched with methanol and 3% H_2O_2 for 10 minutes. The monoclonal antibody of human Ki-67 (1:150) was applied at 4 Centi-degree for 1 hour. Sections were incubated with biotinylated secondary antibody and streptavidin- biotin- percxidase for 10 minutes at room temperature and washed in PBS. The second color-red was developed by incubation with 3-amino-9-ethylcarbozole (AEC) solution for 15 min and sections were weakly counterstained with haematoxylin. D2-40 was stained with black located on the membrane while Ki-67 was stained with red located at nucleus. Normal mouse IgG was substituted for primary antibody as the negative control and the invasive cervical squamous cancer section was used as the positive control.

Evaluation of LVD, LVI and LVP in EOC

By the immunohistochemistry of monoclonal antibody to human D2-40, the number and intensity of lymphatic vessels were evaluated. We defined the vessel which has endothelium with D2-40 positive staining and a vascular lumen as a lymphatic vessel. Lymphatic vessels in all the fields of each slide were facilitated by screening at low power, including within the tumor or at the periphery of the tumor. Single black-stained cells with a lumen were counted as individual lymphatic vessels. Larger arteries with thick smooth-muscle walls and widely distended venous sinuses were excluded. The vessels which diameter >8 red cell or the wall had muscular layer or fibrosclerosis were rejected. Lymphatic vessels were counted in at least three most vascularised fields ('hot spots') at higher power (HP) magnification (×400) for each individual case by two independent gynecological pathologists who did not know any detail regarding the patients' background. Lymphatic vessel density (LVD) was the average microvessel count per HP field. Lymphangiogenesis (lymphatic vessel proliferation, LVP) was revealed by existence of Ki-67(+) lymphatic vessels; Lymphatic vessel invasion (LVI) was defined as the existence of lymphatic vessels harboring cancer embolus.

Statistical analysis

LVD was expressed as mean ± SD and independent sample t-test was used to compare LVD between different groups. Fisher's exact test

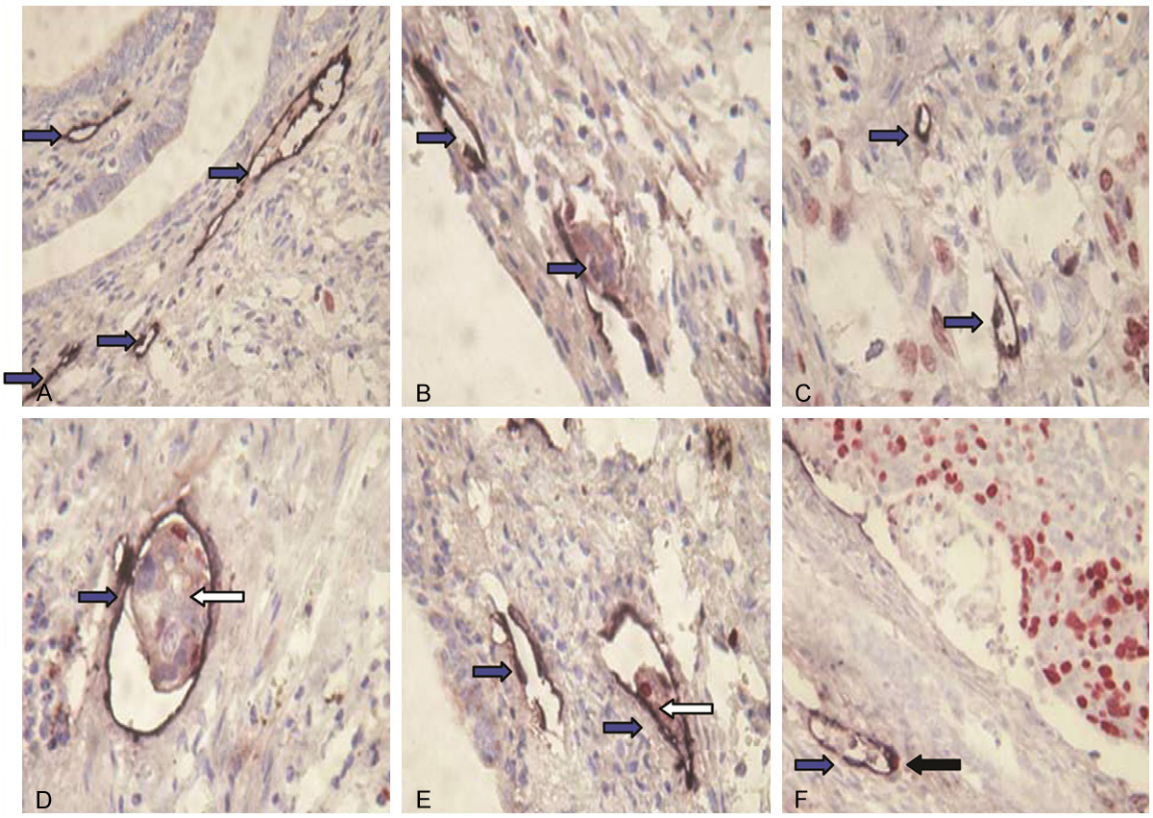

Figure 1. Representative results of double immunohistochemical labeling of D2-40 (black) and Ki67 (red) in epithelial ovarian cancer (EOC). The background was clear. The black ring-like structures (blue arrows in A-F) are lymphatic vessels. The white arrows in (D, E) point to lymphatic invasion, and the black arrow in (F) point a Ki-67(+) lymphatic vessel (lymphatic vessel proliferation) ×400 magnification.

was used to assess the difference in the frequency of lymphangiogenesis and LVI between groups. Analysis was performed by using Prism 5.0 on a Windows computer. A $P<0.05$ were considered to be statistically significant.

Results

Expression of D2-40/Ki-67 in EOC by double immunohistochemistry

As shown in **Figure 1**, the double immunohistochemistry for detection of D2-40 and Ki-67 in EOC was successful and the lymphatic vessels were outlined by black stained D2-40 positive lymphatic endothelial cells. D2-40(+) lymphatic vessels were detected in 86.67% (39/45) EOC samples and 90.00% (9/10) control samples. The absence of lymphatic vessels in EOC samples was not related to any clinical parameters (data not shown). Ki-67 positive staining (red) was mainly observed in nucleus of atypical cells. Meanwhile, red staining was also seen in some lymphatic endothelial cells, which indicated the presence of newborn lymphatic vessels or lymphangiogenesis.

LVD in EOC

As we showed before, LVD of each case was counted and analyzed, the LVD of EOC group wasn't much higher than that in control group (**Figure 2A**). We also subdivided cervical cancer group based the pathological types, cell differentiation, CA-125 level, and volume of ascites. There was no significant differences between serous and non-serous groups (**Figure 2B**), well-moderately and poor differentiated groups (**Figure 2C**), high and low CA-125 groups (**Figure 2D**). Interestingly, Patients with more ascites (≥400 ml) was significant higher than that of patients with less ascites (<400 ml) ((7.254±2.174) vessels *vs.* (5.159±3.399) vessels, $P=0.0153$) (**Figure 2E**). The detail of LVD was shown in **Table 1**.

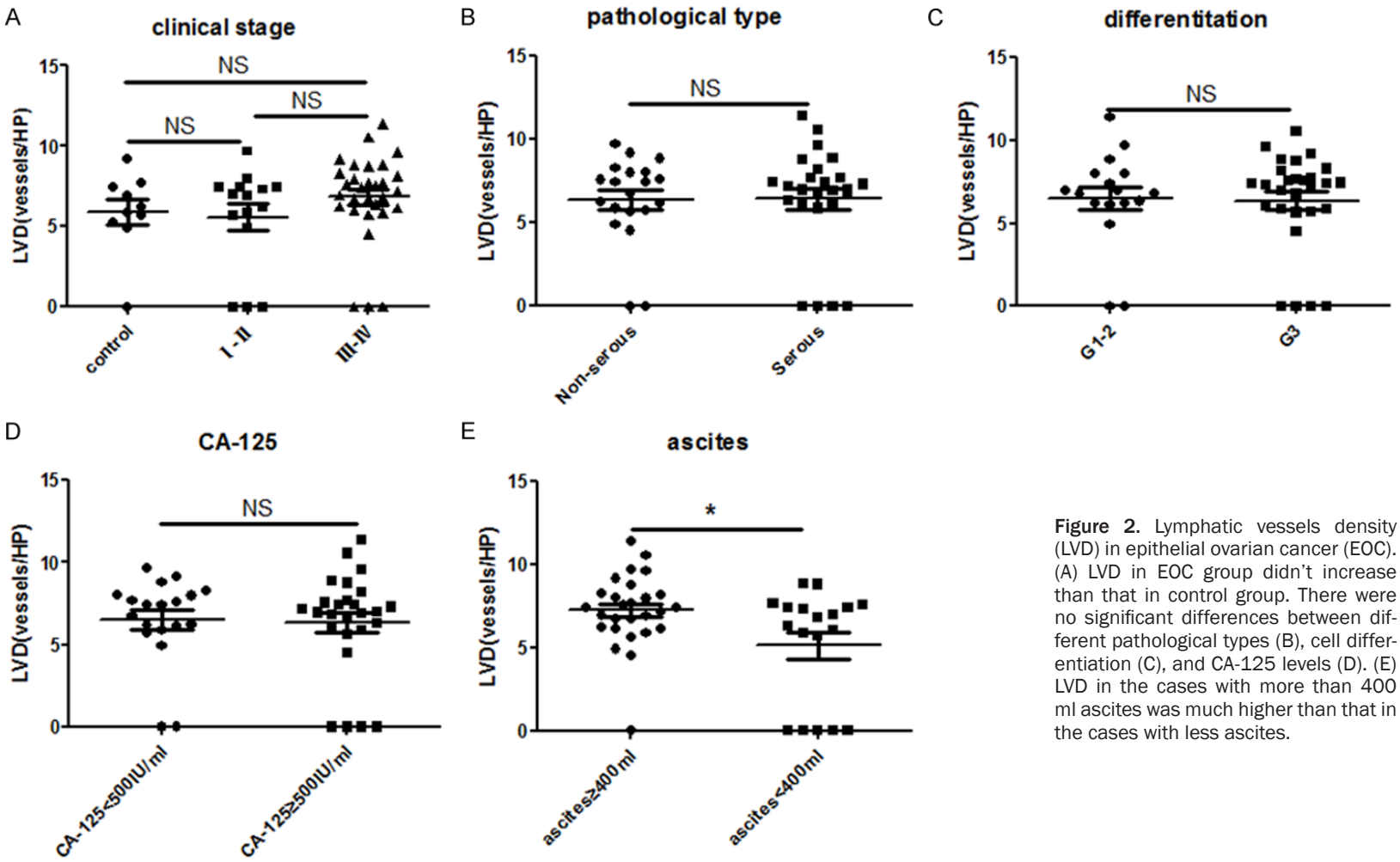

Figure 2. Lymphatic vessels density (LVD) in epithelial ovarian cancer (EOC). (A) LVD in EOC group didn't increase than that in control group. There were no significant differences between different pathological types (B), cell differentiation (C), and CA-125 levels (D). (E) LVD in the cases with more than 400 ml ascites was much higher than that in the cases with less ascites.

Table 1. LVD in 45 epithelial ovarian cancer tissues

Variable	Group	N	LVD** (vessels/HP)	*P**
Clinic	Controls	10	5.91±2.31	
Stage	I-II	15	6.12±3.37	>0.05
	III-IV	30	6.83±2.74	
Pathological	Serous	25	6.78±3.04	>0.05
Types	Non-serous	20	6.43±3.16	
Cell	G1-2	17	6.51±2.88	>0.05
Differentiation	G3	28	6.65±3.24	
Serum	<500 U/ml	19	6.52±2.61	>0.05
CA-125	≥500 U/ml	26	6.65±3.43	
Ascites	<400 ml	18	5.16±3.40	0.0153
	≥400 ml	27	7.25±2.17	

*P<0.05 was significant. **LVD: Lymphatic vessels density.

Lympangiogenesis and lymphatic invasion in EOC

The existence of LVI and LVP in control group was rare in this study. LVI was relative common which was observed in 37.78 % (17/45) EOC cases (**Figure 1D**, **1E**), and LVP in 17.78% (8/45) EOC cases (**Figure 1F**). The presence of LVI was not positively related to clinical stage, pathological types, cell differentiation, level of CA-125, while LVP was related to severe ascites only (P<0.05). As we showed in **Figure 3**, LVD of LVI(+) group was significant greater than that of LVI(-) group ((8.052±2.732) vessels *vs.* (5.422±3.055) vessels, P=0.0021), LVD of LVP(+) was also significant greater than that of LVP(-) group ((8.359±2.176) vessels *vs.* (5.996±2.871) vessels, P=0.0341).

Discussion

The study of lymphatic vessels had been hampered with difficulty due to the overlapping morphological features between blood and lymphatic endothelial cells. Several markers of lymphatic endothelial cell such as podoplanin (D2-40), LYVE-1, Prox-1, and VEGFR-3 had been reported in the past decade, lymphatic vessels and lymphangiogenesis have received great attention owing to their putative implications in terms of metastatic dissemination [9], but none of them was absolutely restricted to lymphatic endothelial vessel [10]. By Immunochemistry, lymphangiogenesis had been reported in many solid tumors, not only in peritumoral but also intratumoral [5]. Podoplanin (D2-40), originally detected on the surface of podocytes, belonged to the family of type-1 transmembrane sialomucin-like glycoproteins, was a specific marker for lymphatic endothelial cell. The expression of D2-40 was induced by the homeobox gene Prox-1 and a specific endogenous receptor was identified on platelets [11]. Anti-D2-40 antibody was identified as the first commercially available antibody for the specific staining of a defined lymphatic marker in archival human tissue sections, thereby enabling more widespread studies of tumor lymphangiogenesis in human cancers [12]. In ovarian cancer, D2-40 was also a useful diagnostic marker in testicular germ cell tumors ovarian germ cell tumors [13] and it might have utility as a marker for ovarian clear cell carcinoma [14]. It can be used as a marker of lymphatic endothelial cells in epithelial ovarian cancer [15, 16]. In our study, the D2-40-stained endothelial cells with a lumen were defined as individual lymphatic vessels, as shown in **Figure 1**, we have get high quality images to analysis the lymphatic vessels in EOC.

Lymphatic vessels spread may be significant in aiding metastases in ovarian cancer but requires other biological factors to act in conjunction, as it does not have clear-cut prognostic significance [2]. The most important lymphangiogenic factor VEGF-C was closely related to invasive phenotype and affected the patient's survival in ovarian carcinomas [4], and the overexpression of Her-2/NEU via activating NF-kappa B in EOC can induce VEGF-C increasing to regulate tumor lymphangiogenesis and malignant ascites formation [17]. Another lymphangiogenic factor VEGF-D was also reported as an essential role in tumoral lymphangiogenesis and lymphatic spread, the expression VEGF-D and intratumoral lymphatics vessel density may be clinically useful indicators for prognostic evaluation in patients with ovarian cancer [15, 18]. Recently, VEGF expression was found to be significantly correlated to survival, and a prognostic factor independent of the stage of disease and residual tumor status. Angiogenic evaluation of patients with EOC may play a role in predicting a subgroup of patients with aggressive disease [19]. Lymphangiogenesis in EOC was statistically significant as a prognostic factor for progression-free and overall survival, however, lymphangiogenesis

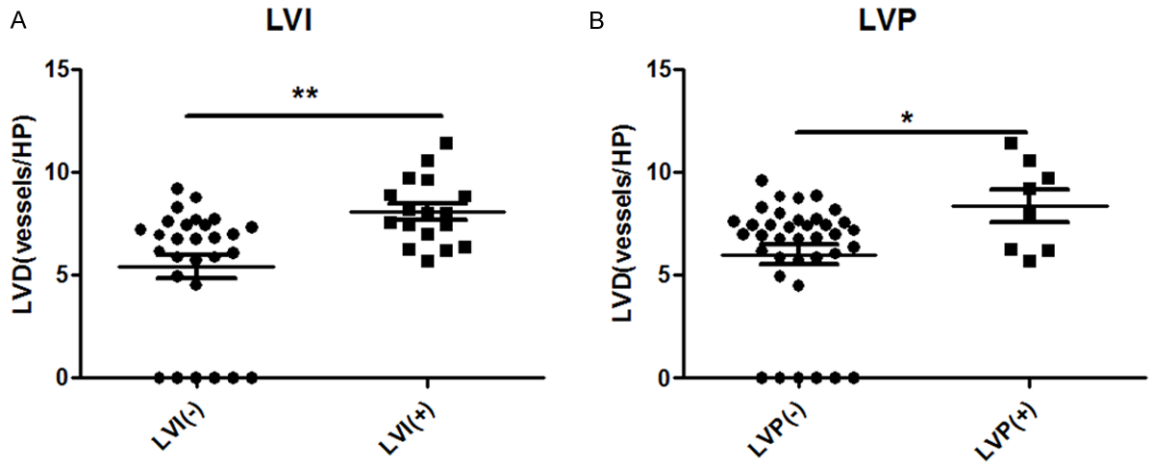

Figure 3. The relationship lymphatic vessels density (LVD) and lymphatic vessel invasion (LVI) or lymphatic vessel proliferation (LVP). A. LVD of LVI positive group was much higher than LVI negative group; B. LVD of LVP positive group was significantly increased than LVI negative group.

did not impact on survival curves, no correlation was found between lymphangiogenesis and age, histological subtype, stage or residual disease [7]. While, another report found the formation of lymphatic vessels has no influence on the progression of epithelial ovarian cancer [16]. In this study, we also found that lymphatic vessels density in epithelial ovarian cancer was not related to clinical stage, pathological types, cell differentiation of tumor cell and CA-125 level, while lymphatic vessels density was positive related to LVI, LVP and the volume of ascites. These results indicated that the newborn of lymphatic vessels was related to lymphatic invasive and formation of ascites. As a special solid tumor, there was not much peritumoral tissue around the focus of advanced EOC, which was the hot area of lymphangiogenesis in many tumors.

Lymphatic endothelial cell proliferation is measured by a double immunostaining of tumor sections with antibodies directed at a lymphatic endothelial cell marker and a marker of proliferating cells [9]. Lymphatic vessels containing proliferating nuclei have been observed in some tumor types such as breast cancer [20], endometrial cancer [21], melanoma [22, 23] and head and neck cancer [24]. The study of lymphatic endothelial cell proliferation on EOC hadn't been reported before. Here, we detected the lymphatic endothelial vessel proliferation (LVP) by double immunostaining of D2-40 and Ki-67 in EOC, and found that the presence of LVP was related to severe ascites. Actually, many advanced stage ovarian cancer patients present with rapid growth of intraperitoneal tumors along with abdominal distention as a result of accumulation of ascites fluid in the peritoneal cavity [25]. The formation of ascites occurs as malignant cells secrete proteins, growth factors and cytokines that cause neovascularization, angiogenesis, increased fluid filtration and/or lymphatic obstruction, resulting in the buildup of serum-like fluid within the abdomen [26].

The presence of ascites correlated with both the intraperitoneal and also the retroperitoneal tumor spread in EOC. Once ascites develops, tumor grade seems to be important for larger ascites volumes. Neither the presence of ascites or its volume nor the cytologic positivity was an independent predictor of survival [27]. Severe ascites is a hallmark of advanced EOC, yet the underlying mechanism that creates an imbalance between peritoneal vascular leakage and lymphatic drainage is unknown. The lymphatic system is best known for draining interstitial fluid from the tissues and returning it to the blood circulation. The dysfunctional of newborn lymphatic vessels increased permeability endothelium, the connection between endothelial cells is not continuous, interstitial fluid containing tumor cell and macromolecule leak to abdomen. In advanced ovarian cancer mice model, lymphangiogenic factors secreted by CD11b(+) macrophages can induced dys-

functional lymphangiogenesis, the combined blockade of VEGF-C/D and VEGF-A signaling with soluble VEGF receptor-3 and VEGF-Trap can markedly inhibited chylous ascites formation [6].

In all, the relationship between lymphatic vessels and severe ascites indicated the proliferation of lymphatic vessels in EOC possibly induced the progress of advanced epithelial ovarian cancer via the formation of ascites. Lymphatic endothelial cell may be the therapeutic target to ameliorate chylous ascites formation in patients with EOC.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zehua Wang, Department of Obstetrics and Gynecology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, Hubei Province, PR China. Tel: +86-2785351649; Fax: +86-2785351649; E-mail: zehuawang@163.net

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