Consecutive Observation of Dynamic Metastases of Human Ovarian Carcinoma to Grading Lymph Nodes in Mouse Model

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Research

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Abstract

Background: Lymphogenous metastasis, one of the most common dissemination routes for ovarian carcinoma, predicts a poor prognosis and relates to most cancer-related death. Now there were no effective therapy and control methods for ovarian carcinoma. Hence, it is necessary to build an animal model of lymph node metastasis in ovarian cancer to seek for the tools to find effective treatments. Our purpose of this study was to investigate the tumor cell dissemination of ovarian carcinoma to the gradient lymph nodes of nude mice and its possible mechanism.

Methods: The mice models of VEGF-D over-expressed were built, and the tumor growth and sentinel lymph nodes were evaluated weekly, while the visible lymph nodes and tumor masses were excised for histological examination using HE examination. Then Evan’s Blue was conducted to observe the unveil lymphatic network. Subsequently, immunohistochemistry (IHC) was performed to check the protein expression of relative genes. Meanwhile, microvessel counting was performed within the tumor tissues and measured using computer assisted morphometric analysis.

Results: The over-expression of VEGF-D promoted the tumor growth of ovarian carcinoma, facilitated the hyperplasia of tumor lymphatic and increased the intratumoral lymphatic vessel density. Besides, the up-regulation of VEGF-D induced the expression of MMP-2, which might be the underlying mechanism of the lymph metastasis in ovarian cancer.

Conclusion: It was a step-by-step progression from the inoculation of cancer cells to the proliferation of the primary tumors, and then to the lymphatic metastases, in which VEGF-D and MMP-2 played vital roles.

Introduction

Epithelial ovarian carcinoma (EOC) has by far the worst clinical outcome of all gynecological cancers, which is responsible for half of the deaths caused by female genital tract malignancy [12]. There were 295,414 patients newly diagnosed with EOC and 184,799 patients died of this cancer worldwide in 2018 [3]. Owing to the paucity of symptoms and insidious onset, more than 60% of patients are at advanced stages when they are diagnosed, and the prognosis is poor with an expected 5-year survival rate in the range of 10-20% [8,10,20]. Lymphatic metastasis is one of the main ways of tumor metastasis and is related to the prognosis of EOC patients. It has been reported that EOC patients has an incidence of 67.2% in Para-aortic and pelvic lymph node metastasis at advanced stage [9], while the rate of lymph metastasis reached to 10%-30% even at early stage [6,22]. Therefore, it is necessary to explore the underlying molecular mechanisms of lymph metastasis to improve the prognosis and prolong the overall survival of patients with EOC.

Lymphatic metastasis may occur at the early stage of most malignant tumors of the origin of the upper skin, which is associated with the special structure of the lymphatic vessel. It arises from the blind end of
the lymphatic capillaries, absorbs the liquid and macromolecular substance from the blood vessels, and returns to the blood vessels at last. The space between the endothelial cells of the lymphatic capillaries is large and protofibril combines the endothelial cells and extracellular matrix, which nearly has no heparan sulfate proteoglycan (HSPG) and fibronectin [17]. The increase of local matrix metalloproteinase degraded the internal and external substrate when the tissues became cancerous while the tumor cells were adhered to the fibronectin exposed to the endothelium, meanwhile the invasion and metastasis happened [21]. Lymph node metastasis is a multistep process, of which the mechanism remains unclear.

Vascular endothelial growth factor D (VEGF-D) is a member of VEGF family, which is the typical lymphangiopoiesis factor, and is often correlated with tumor metastasis and poor patient outcome [18]. Previous studies have proved that the abnormal expression of VEGF-D can act as a marker during the metastatic processes of various cancers [2,13,23]. Besides, VEGF-D may increase the diameter and the number of lymphatic vessels, thus providing increased surface area to facilitate lymphatic metastasis [14,21]. However, the observation of progression and morphology in animal models remains unclear.

In present study, we established the metastasis model of ovarian carcinoma and observed the process and morph of lymph node metastasis in nude mice for two months. Then the types of metastasis morphology were described, while the underlying molecular mechanisms of lymphatic spread were preliminarily discussed. The importance of the diagnose at earlier stages and to seek routinely realistic effective screening program were considered as important and our finding might verify the clinical significance of VEGF-D as a promising target gene in the treatment of EOC.

Results

**VEGF-D was stably expressed in transfected cells**

To detect the quality of the transfection in SKOV-3 cells, QT-PCR analysis was implemented to check the expression of VEGF-D. As a result, the expression of VEGF-D was stably expressed in cells transfected with pcDNA3.1 (+)/VEGF-D, while it was undetectable in the cells transfected with pcDNA3.1 (+) (Fig 1. P<0.05). The data indicated that the transfected cells were stable and VEGF-D was stably expressed.

**The tumor growth in each group**

To explore the tumor growth, the tumor volume was measured at the 0, 20th, 40th and 60th day after cell inoculation in each group. The results was shown in Fig 2, the tumor growth began to accelerate from the 15th day after tumor cell implantation in SR group. Moreover, the tumor growth rate was quicker than that in SV and SC group at every subsequent point of observation since the 20th day after inoculation (P<0.05, Fig. 2). What's more, the tumor growth was obviously expedited since the 25th day. Nevertheless, there was no significant difference between SV group and SC group. These data demonstrated that the over-expression of VEGF-D promoted the tumor growth of ovarian carcinoma.

**The hyperplasia of tumor lymphatic**
To investigate the hyperplasia of tumor lymphatic, Evan’s Blue assay was implemented. The result manifested that the most lymphatic in draining areas and popliteal lymph nodes from mice in SR group were perfused with blue at the 20-minute time point, while infused functional lymphatic were hard to be found in the SV and SC groups (Fig. 3A-C). Furthermore, we checked the proliferation of tumor lymphatic. Consequently, the lymphatic was distinctly expanded in SR group, while the lumen of lymphatic vessels was much narrow even not visible in SC and SV groups (Fig. 3D-F). The data demonstrated that the expansion and proliferation of lymphatic could be enhanced by the over-expression of VEGF-D.

The dynamic metastasis of lymph nodes

To research the metastasis of lymph nodes after tumor inoculation, consecutive observation for gradient lymph nodes of the mice in three groups was conducted for two months, including inspection and palpation of the popliteal and inguinal lymph node (sentinel lymph node). As consequence, the inguinal lymph nodes of two mice in SR group were palpable in two weeks after inoculation, while the popliteal lymph node of one mouse was touched after four weeks of inoculation in SC group. Moreover, HE assay manifested that the nodal involvement occurred from the lower grading lymph nodes to the upper ones step by step (Fig. 4A-E). What’s more, the difference of the metastasis rates of gradient lymph nodes between SC and SV group wasn’t significant. However, the metastasis of lymph nodes was dramatically quicker in SR group compared to that in SC or SV group after being inoculated for 45 day (Fig. 4F-I).

The distinction of nodal metastasis morph

Although it is easy to confirm the typical metastasis focus using H&E staining, the atypical nodal involvement is hard to estimate. Herein, we analyzed the atypical nodal involvement via checking the expression of CA-125 and CD40 with immunohistochemical (IHC) staining. The data displayed that there were various types of atypical nodal involvement according to detect the expression of CA-125 (Fig. 5A-C) and CD40 (Fig. 5D-F) in comparison to the primary tumor sections.

VEGF-D increases the intratumoral lymphatic vessel density

To know whether VEGF-D played a role in blood vessel sprouting, IHC analysis was performed to detect the expression of LYVE-1 and CD31. Immunostaining with an antibody against LYVE-1 indicated that the lymph vessels density was obviously elevated while the immunostaining of CD31 showed that the density of microvessels was distinctly increased in SR group compared to that in SC and SV group (Fig. 6). These results suggested that the over-expression of VEGF-D could promote the proliferation of lymph vessels and the formation of tumor blood vessels.

MMP-2 was up-regulated by the over-expression of VEGF-D

Furthermore, we explored that whether the promoting effects of VEGF-D on the tumor lymph nodes metastasis was correlated with the enhanced expression of MMP-2. IHC analysis exhibited that MMP-2 were all positive expressed in three groups. Moreover, MMP-2 in SC and SV groups was weakly positive expressed (+) in tumor stroma instead of tumor cells (Fig. 7A, B), while more than 90% tumor cells and...
stroma of SR group showed much stronger staining for MMP-2 (+++), which was properly corresponded with the over-expression of VEGF-D (Fig. 7C). All above, the tumor lymph metastasis was intensified by the over-expression of MMP-2 induced by VEGF-D.

**Discussion**

Ovarian carcinoma is the fifth leading cause of death among all gynecological malignancies in developing countries \[24\]. Due to the lack of typical clinical symptoms and available precise biomarkers, 70\% patients have widespread metastasis at advanced stages when diagnosed \[15\]. Although the overall survival of patients with ovarian carcinoma has greatly improved along with the advances in clinical diagnosis and treatment, the effective treatment and control method still lack for patients at advanced stage, especially for those with lymph metastasis. Therefore, it is central to uncover the underlying mechanism of tumor metastasis so as to develop effective therapeutic strategy. Abundant investigations have been done on angiogenesis and vascular relevant molecules and much progress have been made, while with respect to the lymphatic spread, much smaller number of researches has been done.

The observation and definition of nodal invasion is the basement for estimate the lymphatic spread, which is the first step of tumor dissemination. To our knowledge, there are no previous reports about the determination of metastasis. In our study, we established the lymphogeneous high metastasis animal model with SKOV-3 cells transfected with VEGF-D \[9\], and explored the underlying mechanisms of the lymph metastasis.

VEGF-D, an angiogenic and lymphangiogenic glycoprotein, can facilitate tumor growth and distant organ metastasis \[18\]. Accumulated evidences have proved that the expression of VEGF-D is usually connected with tumor metastasis and poor patient outcome. For instance, Wei et al. found that VEGF-D was involved and played an important role in gallbladder cancer progression, which indicted that VEGF-D was a potential molecular target in the treatment of gallbladder cancer \[16\]. VEGF-D was highly expressed and could predict the lymph node metastasis in patients with urothelial carcinoma of the bladder at the time of radical cystectomy, which combined with the low MMP-2 serum levels \[2\]. Moreover, the up-regulation of VEGF-D was significantly correlated with CXCR4, CCR7 and VEGF-C in the lymph node metastasis of patients with cervical cancer \[4\]. Besides, we had verified that VEGF-D could promote the lymph metastasis \[9\]. Our study revealed that the over-expression of VEGF-D was positive to the tumor growth, indicating that VEGF-D could raise the proliferative capability of tumor cells. It was reported that the lower the degree of differentiation of tumor cells, the stronger the ability of cell proliferation and invasion, the stronger the sex, the more malignant it was \[1\]. However, whether VEGF-D could increase the malignancy of the tumor via strengthening the tumor cell proliferation needs further studies.

Sentinel lymph node (SLN) has been demonstrated to become functional blood vessel-enriched and lymph vessel/sinus-enriched before metastasis formation, and there are reorganizations of the lymphatic channels and the vasculature before the establishment of metastasis in the node \[19\]. The dilation of the
lymph sinuses is correlated with the primary tumor weight and the proliferation rate of the endothelial cells is significantly increased \cite{11}. In our study, we found that there were a few isolated tumor cells in enlarged marginal sinus of SLN in SR group before apparent metastasis focus was established, which might be related to the dilation of lymph sinuses. In addition, we discovered that it was not a very short interval from formation of primary tumor mass to nodal involvement, comprising four steps. Firstly, presence of increased lymphatic sinus in size. Secondly, invasion of single or scattered cancer cells via sinus. Thirdly, formation of cancer cell clusters in the sinus or into the parenchyma. Finally, cancer cells defeated the lymphocytes and proliferated in the cortex. What we have found suggested and emphasized that it was vital to diagnose at earlier stages and to seek routinely realistic effective screening program.

Moreover, we investigated the morphology of the invaded nodes which displayed that some types of atypical morph of metastatic tumor cells were occurred in the involved lymph nodes. Herein, we speculated this phenomenon might occur in clinical cases. Many papers have revealed that the poor patient survival of unsuitable treatment was caused by missing microinvasion. Consistent with this, we assumed missing atypical metastases also resulted in the worse outcome of patients. It should be argued that immunostaining for CA125 or CD31 might be a promising approach to tell the atypical lymphatic metastasis, which deserved further investigations.

Furthermore, the role of VEGF-D and the underlying mechanisms of ovarian tumor metastasis were investigated. A high tendency of earlier and dilated lymph node appeared in SR group, and enlargement, fixation and harder feature (osseous metaplasia) of lymph nodes seemed to be the indicators of metastases.

Finding that microscopic blood vessels were increased in VEGF-D overexpression tumors was not anticipated, which might be another reason for tumorigenesis and tumor growth caused by VEGF-D. It may be due to the activation between VEGF-D and up-regulated VEGFR-3 on blood vessels within tumors, and to the increased microscopic vessel density caused by strong MMP-2 expression. It could not be excluded the possibility that VEGF-D bind to the VEGFR-3-VEGFR-2 heterodimers \cite{5,7}. Currently, we used Evan's blue dye to explore tumor-draining lymph flux. More rapid dye perfusion into the SR lymph nodes was observed, both in the popliteal and iliac nodes (more central lymph nodes), which indicated an accelerated lymph flow both into and out of lymph nodes. We deduced that VEGF-D-induced lymphangiogenesis and enlarged lymphatic sinus might facilitate tumor cell transport via accelerate lymph flow and finally act as a stimulator of lymphatic spread.

**Conclusions**

In conclusion, our study observed the morphology and progress of tumor cell metastasis, and further investigated the correlation among tumorigenesis, lymphangiogenesis, angiogenesis and VEGF-D expression, which showed that in addition to lymphatic expansion stimulated by VEGF-D we have demonstrated before, enlargement of lymphatic sinus in LNs and increased lymph flux also ease cancer cell invasion. Original idea did not indicate mouse VEGF-D, which was ligand to VEGFR-3, would promote
angiogenesis. Our study verified that cancer spread via lymphatic was a step-by-step progression, and suggested that the treatment against any phase of this progression would be promising, but what was the most important factor for the diagnosis of ovarian carcinoma at an earlier stage. Atypical metastases as well as microinvasions are both barriers for appropriate treatment selection. Immunostaining for CA125 or CD31 may be a promising approach to tell the missable lymphatic metastasis. We hope that our findings will ease the experimental research on metastasis later. More studies and consideration should be performed in this field.

**List Of Abbreviations**

| Full name                                         | abbreviation |
|---------------------------------------------------|--------------|
| Epithelial ovarian carcinoma                       | EOC          |
| Vascular endothelial growth factor D              | VEGF-D       |
| Lymph vessel                                      | LV           |
| Matrixmetallo proteinase-2                        | MMP-2        |
| Carbohydrate antigen-ca 125                       | CA-125       |
| Sentinel Lymph Node                               | SLN          |

**Methods**

**Cell lines and culture**

Human serous cystadenocarcinoma cell lines SKOV-3 were obtained from American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI-1640 medium (GIBCO) or in the medium with 400µg/ml G418-RPMI-1640 medium, supplementing with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 100 microg/ml streptomycin. Then the cells were maintained in a 37°C-humidified incubator with 5% CO₂ atmosphere.

**Cell transfection**

A vector containing a mouse VEGF-D cDNA (GenBank NM_010216 GI: 6753873) or with the control vector pcDNA3.1 (+) was synthesized by Invitrogen (Carlsbad, CA) and transfected into the cells using Lipofectamin2000 (Invitrogen) according to the operation instruction.

**The establishment of the mice model and hematoxylin and eosin (HE) examination**

Female athymic nude mice (BALB/c, 6-8-week-old, 18-20g) were provided by Sichuan University Animal Center, and housed in a pathogen-free animal facility, fed with the irradiated mouse chow and autoclaved reverse-osmosis treated water. Before inoculation, mice were randomly assigned to three groups (12
mice/group): recombination VEGF-D experimental group (SR group), empty vector group (SV group) and not transfection group (SC group). Tumor cell inoculation was performed according to the protocols we had done before [9]. Briefly, the cells were harvested and suspended in serum-free medium. Then 50 μl suspension liquid was injected subcutaneously into the left hind-footpads of the mice. Tumor growth and sentinel lymph nodes were evaluated weekly. Tumor volumes and the weight of the mice were evaluated consecutively for two months. The tumor volume was determined by the following formula: tumor volume (mm³) = 0.52×length (mm) ×width (mm) ×height (mm). All studies involving mice were approved by the Institute’s Animal Care and Use Committee. Subsequently, three mice of each group were severally sacrificed on the 15 th, 30 th, 45 th and 60 th day. The visible lymph nodes and tumor masses were excised for histological examination using HE examination.

**Evan's Blue Injection and Analysis of Lymph Flux**

Evan's Blue was performed to observe the unveil lymphatic network. Evan's Blue was injected into the left footpads of the mice in each group using subcutaneous injection until lymph vessel (LV) draining to the inguinal lymph nodes were marked. At the timepoints of 20 and 30 minutes after injection, the popliteal nodes (SLN) were examined to determine the relationship between infusion speed and the increased lymph flow. Besides, the iliac lymph nodes on the lymph draining way from popliteal nodes were also assessed.

**Immunohistochemistry**

Deparaffinized sections were placed into 0.01M citrate buffer (pH 6.0) and heated in an autoclave for 5 min for antigen retrieval. Then endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 10 min. Subsequently, nonspecific binding sites were subsequently blocked with homoeotypic non-immunoglobulin of the secondary antibody at 37°C for 20 minutes. Sections were then incubated with the primary antibodies: mouse anti-human CA125 monoclonal antibody (Zhongshan -Golden Bridge, Beijing, China), anti-CD40 polyclonal antibody (ab13545, Abcam, Cambridge, MA), anti-mouse VEGF-D monoclonal antibody (R&D Systems) (Santa Cruz Biotechnology, CA, USA), anti-MMP-2 antibody (ab86607; Abcam), rabbit anti-LYVE-1 (ab14917, Abcam) and anti-CD31 (cat.#ab28364, Abcam) at 4°C overnight in a humidified chamber. After being washed for three times, the sections were incubated with the secondary antibody for 40 minutes at 37°C, sequential incubation with streptavidin-biotin-peroxidase complex followed. The immunoreactions were visualized with diaminobenzidine (DAB) solution as chromogen, and the crimson precipitates were identified as positive staining. Counterstaining was performed with hematoxylin for 30 seconds. For the negative control, all of the reagents except for the primary antibody were used. Finally, the sections were detected by a light microscope (CHS, Olympus).

**Computer-Assisted Morphometric Analysis**

Microvessel counting was performed within the tumor tissues and measured using computer assisted morphometric analysis. Five fields with abundant blood vessels and the highest microvessel density
(MVD-hot spots) were first selected, which were captured by Spot digital camera at high-power field (HPF) and then parameters were evaluated using IP-Lab computer aided image analysis software.

**Statistical Analysis**

Statistical analysis were conducted by the SPSS 22.0 software and all data were presented as Mean±SD. The Chi-Square test was used to analyze the Evan's Blue perfusion of lymph nodes. The difference between two groups was compared by students’ t test, while that among three groups or above was analyzed by One-way ANOVA analysis. Differences with a P value below 0.05 were considered statistically significant.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the society for the Ethics Committee of Animal Experiments of Shandong University.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Data sharing was not applicable to this article as no datasets were generated or analysed during the current study.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

Licheng Du and Fenghua Kong performed the experiment and were major contributors in writing the manuscript. Qing Yang, Peikai Ding, Guoxiang Ding and Xuemei Wang participated in the experiment and collected the data. Bing Kan and Yiping Wang analyzed the data. Xingsong Tian and Yueran Zhao who were the joint corresponding author reviewed and edited the manuscript. All authors read and approved the final manuscript.

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