# 1. Study design

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 was performed to check the expression of relative genes. Meanwhile, microvessel counting was performed within the tumor tissues and measured using computer assisted morphometric analysis.

# 2. Sample size

|  |  |
| --- | --- |
| Groups | n |
| SR group | 12 |
| SV group | 12 |
| SC group | 12 |

# 3. Inclusion and exclusion criteria

# N/A.

# 4. Randomisation

# 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.

# 5. Blinding

# The blinding was not used at any of the steps outlined in the table above.

# 6. Outcome measures

# The following parameters were assessed: Tumor volume (mm3) = 0.52×length (mm) ×width (mm) ×height (mm); the hyperplasia of tumor lymphatic; the dynamic metastasis of lymph nodes; the expression of CA-125 and CD40; the intratumoral lymphatic vessel density.

# 7. Statistical methods

# 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.

# 8. Experimental animals

# Thirty-six Female athymic nude mice (BALB/c, 6-8-week-old, 18-20g) were used.

# 9. Experimental procedures

# Human serous cystadenocarcinoma cell lines SKOV-3 were cultured in RPMI-1640 medium (GIBCO) and transfected with VEGF-D cDNA (GenBank NM\_010216 GI: 6753873) or with the control vector pcDNA3.1 (+).Thirty-six Female athymic nude mice (BALB/c, 6-8-week-old, 18-20g) were used to build the mice model. Then the tumor volume was determined and Evan’s Blue was performed to observe the unveil lymphatic network. Immunohistochemistry was conducted to detect the expression of CA125, CD40, MMP-2 and CD31. Eventually, Microvessel counting was performed within the tumor tissues and measured using computer assisted morphometric analysis.

# 10. Results

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**Figure 1. The tumor growth in each group after tumor cell implantation.** In SR group, the tumor growth was much faster than that in SV and SC group.**\*** *P*<0.05, \*\*P<0.01, \*\*\*P<0.001.

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**Figure 2. The** **functional lymphangiography of lymph vessel using Evan’s Blue.** **Red blood vessel (*BV*)** indicated by arrows occurred in the tumor surface (***TS***) in SC group (A) and SV group (B) without perfused **functional lymphatic vessels (*LV*)** at 20-minute time point**. In the tumor surface of SR group** (C)there are dense perfused lymphatic vessel webs (***arrow***) meanwhile. Moreover, the lymphatic was distinctly expanded in SR group, while the lumen of lymphatic vessels was much narrow even not visible in SC and SV

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**Figure 3**. **Histological analysis of metastasis progression of tumor-draining gradient lymph nodes in nude mice after consecutive examination.** There weresome migrating tumor emboli (***arrow***) founded in the local dilated lymphatic capillaries among adjacent peritumoral connective tissue inSR group **after being inoculated for 15 days (A),** while only a few **isolated tumor cells** (***arrow***) **in enlarged marginal sinus of the first step tumor-draining LN (Sentinel lymph node) without apparent metastasis focus (B). After being inoculated for 30 days, small clusters of metastatic focus** (***arrow***) **were found in the cortical lymphatic sinus especially in SR mice (C), while** clumpy deposits of metastatic carcinoma (***arrow***) could be observed in **tumor-draining LN**s **(D)** after being inoculated for 45 days. Moreover, metastatic foci (***arrow***) occupied almost the entire LN **(E)** after being **inoculated for** 60 day**s**.In popliteal LNs, metastasis was found in SR group **30 days after inoculation, and** 45 day**s later its** metastasis rate reaches 36.1%, 60 day**s** reaches 81.9% (F). In inguinal LNs, SR group was found metastasis **30 days after inoculation, and its** 45 day**s** metastasis rate reaches 40.2%, 60 day**s later** reaches 75.1% (G). In parailiac LNs, metastasis was not found in SR group **on day 30 after inoculation, however,** metastasis rate reaches 19%, 60 day reaches 76.4% on day 45 (H). In renal hilum LNs, metastasis was also not found in SR group until 45 days after implantation. Metastasis rate on Day 45 reaches 12%, Day 60 reaches 48.7% (I).

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**Figure 4. Immunohistochemical analysis of** **metastasis** **progression of tumor- draining lymph nodes.** There were a few **isolated CA125-positive tumor cells** (***arrow***) **in enlarged marginal sinus of first step draining LN (Sentinel lymph node) without apparent metastasis focus** **15 days after inoculation** in SR group **(A). Typical metastases focus (B) and atypical metastases focus** (***arrow*) of third step draining LN (C) were positive for CA125 antibody 60 days after inoculation** in SR group**.** There were many **isolated CD40-positive tumor cells** (***arrow***) **in enlarged marginal sinus of third step draining LN without apparent metastasis focus** **30 days after inoculation** in SR group **(D). Typical metastasis focus** (**E. *arrow*) and atypical metastasis focus with osseous metaplasia /Calcification** (***arrow*) of third step draining LN (F) were positive for CD-40 antibody 45 days after inoculation** in SR group**.**

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**Figure 5. Intratumoral lymphatic vessel sprouting and blood vessel density of different groups via immunohistochemical analysis. There were sparse LYVE-1- positive neonatal lymphatic vessel (arrow) in the tumors of SC group (A) and SV group (B) after being inoculated for 60 days. However, rich nascent lymphatic microvessel sprouts with dilated lumina indicated by arrows were present among tumor cells of SR group (C). Representative sections for CD-31-positive blood microvessels (arrows) from tumor tissues of three groups were presented in SC group (D), SV group (E) and SR group (F). SR group displayed a little increased blood microvessel density after being inoculated for 60 days in compared with the controls.**

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**Figure 6. The microscopic column showed the expression of matrix metalloproteinase-2 (MMP-2) and VEGF-D of tumor tissues in different groups.** In comparison with the weak stromal but cellular staining (+) in SC group (A) and SV group (B), it was obviously stronger cellular staining (+++) in tumor tissue of SR group (C). A majority of tumor cells in SC (D) and SV (E) group as well as interstitum showed negative or only very weak staining (±) for VEGF-D. However, SR group showed much stronger staining (+++), indicating the overexpression of VEGF-D (F), which was properly corresponded with the expression of MMP-2.