Vascular endothelial growth factor-D promotes growth, lymphangiogenesis and lymphatic metastasis in gallbladder cancer

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Abstract
Lymph node metastasis is a major prognostic factor for patients with gallbladder cancer (GBC), and greater understanding of the molecular mechanism of lymph node metastasis in GBC is needed to improve prognosis. VEGF-D has been implicated in the control of lymphangiogenesis in many carcinomas, but the biological function of VEGF-D in human GBC remains unclear. In this study, we analyzed the role of the VEGF-D in human GBC cells and addressed the functional role of VEGF-D using a xenograft mouse model. We examined the expression of VEGF-D in three human gallbladder cancer cell lines. A lentivirus-based effective VEGF-D siRNA vector was infected into GBC NOZ cells. The effect of VEGF-D siRNA on GBC NOZ cells was investigated by cell proliferation assay and invasion assay. Furthermore, we examined the role of VEGF-D-SiRNA on GBC NOZ cells in the mice of subcutaneous and orthotopic xenograft tumor. Our results are as follows: VEGF-D mRNA and protein were expressed in all three GBC cell lines (GBC-SD, NOZ, and SGC-996). We successfully selected D-3/siRNA as the most effective siRNA to silence VEGF-D expression after four VEGF-D siRNA plasmid transfection in NOZ cells. VEGF-D mRNA and protein expression were suppressed by lentivirus-mediated D-3/siRNA. D-3-RNAi-LV inhibited NOZ cells proliferation and invasion ability in vitro. D-3-RNAi-LV inhibited tumor growth and lymphangiogenesis in the NOZ cell subcutaneous xenograft model. D-3-RNAi-LV inhibited lymphangiogenesis and lymphatic metastasis in the NOZ cell orthotopic xenograft model. Furthermore, D-3-RNAi-LV inhibited tumor ascites and hepatic invasion in the NOZ cell orthotopic xenograft model. In conclusion, VEGF-D is involved and plays an important role in GBC progression, suggesting that VEGF-D may be a potential molecular target in the treatment of GBC.

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1. Introduction

Gallbladder cancer (GBC), a highly lethal disease, is the most common malignant neoplasm of the biliary tract and the seventh most common gastrointestinal cancer [1]. GBC has an extremely poor prognosis. The median survival for suspected carcinomas is 9.2 months, and for incidental carcinomas is 26.5 months [2]. Surgical resection is the only potentially curative therapy for GBC. Unfortunately, only a minority of patients with GBC are candidates for surgical treatment, mostly due to the high proportion of tumor that is advanced at the time of presentation [3]. Apart from surgery, all other treatment approaches are palliative. Adjuvant chemotherapy and/or radiotherapy also has not altered the dismal prognosis and survival of patients with GBC [4]. Local tumor growth, hepatic invasion and lymph node metastasis are the main prognostic factors in patients with GBC, especially the extent of lymph node...
metastasis. The overall rate of lymph node metastasis in GBC ranges from 54% to 64% [5]. Therefore, greater understanding of the molecular mechanism of lymph node metastasis in GBC is needed to improve prognosis.

Whereas the significance of angiogenesis for tumor growth and metastasis has been well understood, the molecular mechanisms regulating tumor-associated lymphangiogenesis and lymphatic metastasis has only recently started to be elucidated [6,7]. With the discovery of lymphatic vessel-specific markers such as lymphatic vessel hyaluronan receptor-1 (LYVE-1) [8]; the membrane mucoprotein, podoplanin [9]; prospero-related homeobox, Prox-1 [10] and monoclonal antibody directed against M2A antigen (D2-40) [11], which have provided valuable tools to discriminate between blood and lymphatic endothelium, lymphangiogenesis has accordingly become a focus of intense interest. It will be a new way that inhibit lymphangiogenesis and lymphatic metastasis of growing tumors.

Vascular endothelial growth factor (VEGF)-C and -D, two novel members of the VEGF family, are known as lymphatic endothelial growth factors. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PIGF) [12]. VEGFs have a critical role in pathological conditions by binding to and activating VEGFRs, which includes VEGFR-1, VEGFR-2, and VEGFR-3. VEGF-C and VEGF-D, also known as c-fos-induced growth factor [13], are initially synthesized as preproteins that become proteolytically processed outside the cell to produce several polypeptides including a mature form [14,15]. This processing progressively increases the affinity for its receptors, VEGFR-2 and VEGFR-3, which are predominantly present on endothelium of blood vessels and lymphatic endothelial cells, respectively [16]. VEGF-C and VEGF-D have been implicated in the control of lymphangiogenesis in many carcinomas, such as human malignant melanoma [17], squamous cell carcinomas of the head and neck region [18], gastric adenocarcinoma [19] and breast cancer [20]. Our previous study [21] also demonstrated that expression of VEGF-C and -D correlated with evidence of lymphangiogenesis and lymph node metastasis in tissue samples of human GBC. However, the biological function of VEGF-D in human GBC have not been examined detailedly in vitro and in vivo so far. Thus, we analyzed the role of the VEGF-D in human GBC cells and addressed the functional role of VEGF-D using a xenograft mouse model in this study.

2. Materials and methods

2.1. Cell culture

Three cell lines established from human GBC were incubated at 37 °C under 95% air and 5% CO2. The GBC-SD cell line was purchased from Shanghai Institutes for Biological Sciences in China, and the SGC-996 cell line (from the primary mastoid adenocarcinoma of the gallbladder [22]) was provided by the Tumor Cytology Research Unit, Medical College, Tongji University, China. Both the cell lines were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% (v/v) calf bovine serum. The NOZ cell line (isolated from ascites derived from a 48-year-old female patient with GBC [23]) was obtained from the Health Science Research Resources Bank in Japan. The NOZ cell line was cultured in Williams E medium (GIBCO) supplemented with 10% fetal bovine (FBS).

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

PCR primers used for amplification of VEGF-D were 5’-CCA TCG CAC TAG GTT-3’ (forward sequence) and 5’-GTG TCT GGG TGA AAT AGC-3’ (reverse sequence), and it yielded a 684-bp product. PCR primers used for amplification of β-actin were 5’-GGC ATG GGT CAG AAG GAT TCC-3’ (forward sequence) and 5’-ATG TCA CGC ACG ATT TCC CGC-3’ (reverse sequence), and it yielded a 500-bp product. RT-PCR and the detection of the PCR products were carried out as described previously [24].

2.3. Western blot analysis

The appropriate diluted primary antibodies, including polyclonal goat anti-human VEGF-D antibody (1:1000; Santa Cruz), β-actin (1:1000; Santa Cruz) were used in this experiment. Western blot and detection of the blotted products were carried out as described previously [24].

2.4. VEGF-D siRNA plasmid construction and transfection

According to the siRNA design guidelines, four suitable siRNA target sequences (D-1, D-2, D-3, and D-4), from human VEGF-D gene GenBank accession no. NM_004469, were synthesized as follows: D-1 (GGG CTC CAG TAA TGA ACA TGG); D-2 (GCC AAT CAT ACA GGT TGT AAG); D-3 (GCT ATG GGA TAG CAA ATG); and D-4 (GCA GTT GTG AGT GCA AAG). A small hairpin RNA (shRNA) of human VEGF-D in a pGPU6 gene transfer vector and the negative control sequence were constructed by GeneChem Co., Ltd. (Shanghai, China). All the plasmids were verified by DNA sequencing. The process of transfection was carried out as described previously [24]. At the time of 72 h after transfection, cells were collected for detecting the VEGF-D expression by RT-PCR.

2.5. Construction of lentiviral vectors and infection

In order to create better stable transfection for animal experiment, we used lentiviral-mediated siRNA targeting VEGF-D vector. Lentivirus vectors for human VEGF-D small hairpin RNA (shRNA) encoding a red fluorescent protein (RFP) sequence was constructed by Genechem Co., Ltd. (Shanghai, China). The lentivirus vectors containing VEGF-D shRNA were constructed by ligating the Age I/Eco I digests of pGCSIL-RFP and the VEGF-D shRNA PCR product and were verified by DNA sequencing. ShRNA for the negative controls was also provided by Genechem Co., Ltd. (Shanghai, China). NOZ cells (5 × 10^5 cells/well) were plated in 6-well plates with Williams E medium (GIBCO) containing 10% FBS overnight. At the time of infection, the cells were at approximately 30% confluency. The appropriate quantity of virus (the multiplicity of
infection (MOI) = 50) was mixed with 1 ml complete medium containing polybrene (8 mg/mL) and added to cells and incubated for 10 h at 37 °C. Then, the cells were incubated in fresh complete medium containing 10% FBS for 20 h. The infection efficiency was quantified by determining the percentage of cells that were RFP positive in every 100 cells with a microscope. The cells were collected for subsequent studies at the time of 72 h after infection.

2.6. Cell proliferation assay

Cell proliferation was assessed by MTT assay. NOZ cells were incubated in 96-well plates at a density of 8 × 10^3 cells per well with Williams E medium supplemented with 10% FBS for 48 h. The proliferative activity was determined by adding 10 μl of sterile MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml, Sigma) to each well and incubated for 4 h at 37 °C. Then 100 μl of dimethylsulphoxide (DMSO; Sigma) was added and thoroughly mixed for 10 min. The optical density (OD) value was obtained by measuring absorbance at a wavelength of 570 nm. The cell growth rate was calculated by following formula: Cell growth rate = (mean absorbance in all wells of the treatment group)/(mean absorbance in all wells of the cells control group) × 100%. Each growth assay was repeated three times.

2.7. Cell in vitro invasion assay

The in vitro cell invasive assay was done in matrigel invasion chambers (24-well) (BD Biosciences). The top and bottom of the cell invasion chamber were separated by a polycarbonate filter with pores of 8-μm, which was coated with matrigel matrix (20 μg/well) above and 5 μg Fibronectin (BD) below. Approximately 3 × 10^4 cells were suspended in 200 μl of Williams E medium with 2.5% FBS and were seeded onto the top chamber, and 600 μl of culture medium with 10% FBS was added to the lower chamber. The invasion chamber was incubated for 48 h at 37 °C and 5% CO2. The noninvading cells on the upper surface of the membrane were removed by gentle scrubbing with a cotton swab. Membranes were fixed in a stationary liquid (95% ethanol and 5% acetic acid) for 30 min and stained with H&E. The invasive activity of cancer cells was determined by counting the cells with a microscope at 200× magnification. Five random visual fields were counted for each well, and the average was determined.

2.8. Animal experiments

Four-to 6-week-old nu/nu nude mice were purchased from Shanghai SLAC Laboratory Animals Co. (Shanghai, China) and housed in the Experimental Center of the Fujian Medical University. All experiments were carried out in accordance with institutional guidelines and were approved by the Ethics Committee of the Medical Faculty of the Fujian Medical University. For induction of subcutaneous GBC tumors, three groups (blank control group, Con-RNAi-LV group and D-3-RNAi-LV group) of NOZ cells (1 × 10^6) in 0.1 ml serum-free Williams E medium were implanted subcutaneously into the left flank of mice (five mice/group). Tumor growth was monitored every 3 days and measured in two dimensions. Tumor volume was calculated using the formula width^2 (mm^2) × length (mm)/2, where width and length are the shortest and longest diameters, respectively. After 4 weeks mice were killed and primary tumor as well as axillary and inguinal lymph nodes were collected. All samples were fixed in 10% formalin solution for next immunohistochemical analysis. In order to induce orthotopic GBC tumors, we refer to the method which has been described previously [25]. Briefly, nude mice (five mice/group) were anesthetized and laparotomy was done. Three groups of NOZ cells (3 × 10^5) in 30 μl 50% precooling matrigel (BD Biosciences) were injected into the gallbladder taking caution to ensure that the tumor cells were not injected into the biliary tract. After waiting for approximately 30 s, the Matrigel solution was solid. The gallbladder and liver lobes were replaced and the abdominal wall closed using Vicryl 6/0. After 4 weeks mice were killed and abdominal and thoracic cavity as well as all visceral organs were macroscopically examined for tumor dissemination. Later, primary tumor as well as hilar and mesenteric lymph nodes were excised. All samples were fixed in 10% formalin solution for next immunohistochemical analysis.

2.9. Immunohistochemical analysis

Immunohistochemical staining was performed using the standard immunoper-oxidase staining procedure. In brief, serial 4-μm-thick sections were cut from formalin-fixed and paraffin-embedded tumor blocks, dewaxed in xylene, rehydrated in alcohol, and then incubated with fresh 3% hydrogen peroxide (H2O2) for 20 min at room temperature. After washing with phosphate-buffered saline (PBS), the tissue sections were antigen-retrieved by heating the sections in a microwave for 13 min in a citric acid buffer solution (pH 6.0). Sections were blocked with appropriate normal serum in PBS. VEGF-D polyclonal goat anti-human antibody (1:80, Santa Cruz Biotechnology), LYVE-1 polyclonal goat anti-mouse antibody (1:150, R&D Systems), CD31 polyclonal goat anti-mouse antibody (1:200, Santa Cruz Biotechnology) and anti-pan-cytokeratin (CK) antibody (1:100, Maixin-bio, Fuzhou, China) were diluted and placed on the sections overnight in humidified boxes at 4 °C. The sections were then washed with PBS for 5 min followed by an incubation with an UltraSensitive S-P kit (Maixin-bio, Fuzhou, China) according to the manufacturer’s instructions. After exposure to stable 3,3-diaminobenzidine for 5–10 min, slides were counterstained with hematoxylin, dehydrated, and mounted. For a negative control, PBS was used instead of the primary antibody. The expression of VEGF-D was evaluated by using digital microscopic imaging system and Quantity One image analysis software (Bio-Rad Inc., USA). Three slices were randomly selected for each group, and then five fields were selected for each slice. VEGF-D expression in each group was semiquantitatively evaluated based on the mean of positive area and optical density in fifteen fields respectively. The average of CD31-positive and LYVE-1-positive vessels were assessed according to the method described by Takammi I [26]. Each slide was first scanned at 100× magnification to determine three “hot spots” defined as
areas with the maximum number of positive vessels. The positive vessel density was determined by counting all the immunostained vessels at a 400× magnification and the mean number of positive vessels was calculated in the five selected areas for each case.

2.10. Statistical analysis

All statistical analyses were performed using the SPSS (the Statistical Package for Social Science) software (version 17.0). Data were analyzed by the χ² test and Student’s t-test. Results are expressed as means±SE and were considered to be statistically different at P<0.05.

3. Results

3.1. Expression of VEGF-D in human GBC cells

To determine the expression of VEGF-D in human GBC cells, we analyzed different GBC cell lines (GBC-SD, NOZ, and SGC-996) in our laboratory. All three gallbladder cancer cell lines constitutively expressed VEGF-D mRNA by RT-PCR, and the express intensity of VEGF-D mRNA in the laboratory. All three gallbladder cancer cell lines constitutively expressed VEGF-D mRNA by RT-PCR, and the express intensity of VEGF-D mRNA in our laboratory. All three gallbladder cancer cell lines constitutively expressed VEGF-D mRNA by RT-PCR, and the express intensity of VEGF-D mRNA in our laboratory.

3.2. Expression of VEGF-D mRNA after siRNA plasmid transfection

In order to find an effective siRNA to silence VEGF-D expression, we designed four suitable siRNA target sequences from human VEGF-D gene GenBank accession no. NM_004469 according to the siRNA design guidelines. DNA sequencing results verified that four VEGF-D siRNA plasmid construction was successful. We used RT-PCR to detect the VEGF-D expression at the time of 72 h after transfection in NOZ cells. RT-PCR indicated a decrease in VEGF-D mRNA levels in siRNA transfected cells, while mRNA levels of the housekeeping gene β-actin remained relatively unchanged. The D-3/siRNA vector resulted in a greater suppression of VEGF-D mRNA expression when compared to the other vectors (D-1/siRNA, D-2/siRNA, and D-4/siRNA), while mock transfection or the D–N/siRNA vector had no effect on VEGF-D mRNA expression (Fig. 2a). The corresponding densitometric ratios in the D-1/siRNA, D-2/siRNA, D-3/siRNA, D-4/siRNA, and D–N/siRNA transfected groups were 68.7, 48.7, 28.0, 68.0 and 86.0, respectively. There were distinct differences between D-3/siRNA and mock transfected siRNA groups (P<0.05; Fig. 2b).

3.3. Expression of VEGF-D mRNA and protein after D-3/siRNA lentiviral vectors infection

Next, we used lentiviral-mediated D-3/siRNA targeting VEGF-D vector to create better stable transfection for next experiment. DNA sequencing results verified that D-3/siRNA lentiviral vectors construction was successful. Transfection efficiency was quantified by counting the cells under a fluorescent microscope 72 h after infection, and the efficiency of two groups (Con-RNAi-LV and D-3-RNAi-LV) were greater than 80% (Fig. 3). The RT-PCR results indicate that endogenous VEGF-D mRNA expression was significantly inhibited at 72 h after infection in NOZ cells. Compared with the Con-RNAi-LV group, the D-3-RNAi-LV group showed relatively lower quantities of VEGF-D mRNA (Fig. 4a). The semiquantitative analysis revealed that D-3-RNAi-LV suppressed VEGF-D mRNA levels to 37.91% when normalized to the blank control, and there were significant differences between blank control group and D-3-RNAi-LV group (P<0.05; Fig. 4b). In accordance with this, western blotting showed that VEGF-D protein expression was suppressed in the D-3-RNAi-LV group compared with the Con-RNAi-LV group in NOZ cells (Fig. 4c). The corresponding densitometric ratios in the D-3-RNAi-LV and Con-RNAi-LV groups were 23.28 and 97.66, respectively. There were distinct differences between D-3-RNAi-LV and blank control groups (P<0.05; Fig. 4d).

3.4. VEGF-D promotes NOZ cell proliferation and invasion in vitro

To experimentally address the biological function of VEGF-D in vitro, we analyzed the effect of lentivirus-mediated VEGF-D siRNA on NOZ cell proliferation and invasion by MTT assay and cell in vitro invasion assay, respectively. MTT assay results show that compared to blank control and Con-RNAi-LV groups, the cell growth of the D-3-RNAi-LV group was slower (Fig. 5a). The cell viability of D-3-RNAi-LV was reduced to 41.3% when compared with blank control group (P<0.01). The data from the cell in vitro invasion assay indicated that the number of cells that penetrated the basal membrane from the D-3-RNAi-LV group (35.6±4.0) was significantly less than the blank control group (46.8±5.3; P<0.01), which was similar to the number of cells from the Con-RNAi-LV group (44.8±3.9; P<0.05) (Fig. 5b).

3.5. VEGF-D induces tumor growth and increases lymphangiogenesis in the mice of subcutaneous xenograft tumor

To then address the impact of VEGF-D expression on GBC tumor biology in vivo, three groups (blank control group, Con-RNAi-LV group and D-3-RNAi-LV group) of NOZ cells were subcutaneously xenografted onto nude mice. As shown in Fig. 6, at the end of the 4-week experimental period, the xenograft tumor sizes are as follows (mean±SE): (2229.6±942.5) mm³ in the blank control group (1909.8±896.1) mm³ in the Con-RNAi-LV group, and (571.0±126.7) mm³ in the D-3-RNAi-LV group. There were distinct differences between D-3-RNAi-LV group and Con-RNAi-LV group (P<0.01). Compared with Con-RNAi-LV group, delayed tumor growth was significant in the D-3-RNAi-LV group and was evident from 2-week after starting xenograft until the day the mice were sacrificed. Next, we analyzed the expression of VEGF-D by immunohistochemistry, and its effect on lymphangiogenesis and angiogenesis was also determined by immunohistochemical analysis using anti-LYVE-1 and anti-CD31 antibodies respectively. Compared with the Con-RNAi-LV and blank control groups, the expression level of VEGF-D was significantly decreased in the D-3-RNAi-LV group (Fig. 7a). Typical results and quantitative data from microlymphatic density (MLVD) and the microvessel density (MVD) analysis are also shown in Fig. 7b and C, respectively. Most lymphatic vessels are located in the peritumoral xenograft tumor, but little in intratumoral xenograft tumor. A statistically significant decrease in MLVD was noted in D-3-RNAi-LV group tumors (12.0±2.0) when compared to Con-RNAi-LV (20.6±2.5) or blank control groups tumors.
(20.2 ± 1.7) \( (P < 0.01) \). However, there are no statistical significance in MVD between D-3-RNAi-LV group tumors (34.3 ± 6.2) and Con-RNAi-LV (34.6 ± 6.4) or blank control groups tumors (35.4 ± 3.2) \( (P > 0.05) \). These data suggest that inhibition of VEGF-D in xenograft tumors by D-3-RNAi-LV decreases tumor growth and lymphangiogenesis.

3.6. VEGF-D increases lymphangiogenesis and promotes lymphatic metastasis in the mice of orthotopic xenograft tumor

Since the positive lymph nodes are not found in the mouse model of subcutaneous xenograft tumor, we try to induce the mouse model of orthotopic GBC tumors according to the method which has been described previously [25]. We have successfully established the mouse model of orthotopic GBC tumors by using NOZ cells, and also found that hepatoduodenal ligament could seen enlarged lymph nodes, which were mostly positive by detected human cytokeratin positive GBC cells using an anti-human pancytokeratin antibody (Fig 8c and d). Three groups (blank control group, Con-RNAi-LV group and D-3-RNAi-LV group) of NOZ cells were orthotopicly xenografted into the gallbladder of nude mice and allowed to form tumors for 28 days. When mice were sacrificed, 4 out of 5 mice of Con-RNAi-LV/blank control groups tumors had developed ascites, whereas none of the animals with D-3-RNAi-LV group tumors had ascites at the time of autopsy (Fig. 8a and Table 1) \( (P < 0.05) \). Hepatic invasions were macroscopically detected in 5 out of 5 livers (100%) from mice of Con-RNAi-LV/blank control groups tumors compared to 0 out of 5 livers from mice of D-3-RNAi-LV group tumors (Fig. 8b and Table 1) \( (P < 0.001) \). We also analyzed the expression of VEGF-D,
lymphangiogenesis and angiogenesis by immunohistochemical analysis. Compared with the Con-RNAi-LV and blank control groups, the expression level of VEGF-D was significantly decreased in the D-3-RNAi-LV group \((P < 0.01)\) (Fig. 9a). A statistically significant decrease in MLVD was noted in D-3-RNAi-LV group tumors \((14.8 \pm 2.1)\) when compared to Con-RNAi-LV \((49.1 \pm 3.0)\) or blank control groups tumors \((51.5 \pm 3.8)\) \((P < 0.01)\) (Fig. 9b). However, there are no statistical significance in MVD between D-3-RNAi-LV group tumors \((55.0 \pm 1.6)\) and Con-RNAi-LV \((56.4 \pm 2.4)\) or blank control groups tumors \((56.4 \pm 1.9)\) \((P > 0.05)\) (Fig. 9c). Importantly, the inhibition of lymphangiogenesis was paralleled by an inhibition of lymphatic spread. Metastases were found in 0 out of 5 \((0\%)\) lymph nodes from mice of D-3-RNAi-LV group tumors compared to 5 out of 5 \((100\%)\) lymph nodes from mice of Con-RNAi-LV/blank control groups tumors \((P < 0.05)\) (Fig. 8c, d and Table 1).

4. Discussion

GBC has an extremely poor prognosis because of metastasis. Lymphatic spread is a major component in the GBC metastasis [5], and it is also the most important prognostic factor in patients with GBC. Thus, the therapeutic method of inhibiting lymphatic spread would be predicted to convey a major clinical benefit in GBC. But how lymphatic metastasis arises from the primary tumor is obscure. Recent studies have demonstrated a principal role of molecules in the mechanism of lymphogeneous metastasis,
such as VEGF-D and VEGFR-3 [27]. VEGF-D (also known as c-fos-induced growth factor or FIGF), which is similarly to VEGF-C, has been identified as a kind of essential agent for the growth and establishment of lymphatic vessels in many human tumors, such as gastric carcinoma [19], hepatocellular carcinoma [27], lung cancer [28], colorectal cancer [29] and pancreatic cancer [30]. In human GBC, our previous study [21] has demonstrated that the protein expression of VEGF-D in tumor tissues was significantly higher than that in normal gallbladder away from the tumor, and it also correlated with evidence of lymphangiogenesis and lymph node metastasis in tissue samples of human GBC. In order to further study VEGF-D, we next explored detailedly their biological function in vitro and in vivo.

To our knowledge, this study is the first to demonstrate the role of VEGF-D in promoting growth, lymphangiogenesis and lymphatic metastasis in GBC. In this study, we used RT-PCR and Western blot techniques to analyze the mRNA and protein expression of VEGF-D in three different GBC cell lines (GBC-SD, NOZ, and SGC-996). The mRNA and protein levels of VEGF-D were detected in all the three cell lines, which indicated that VEGF-D is one of the important molecules in participating in process of GBC metastasis. The mRNA expression levels of VEGF-D was relatively consistent in three cell lines, but NOZ cells expressed relatively higher levels of VEGF-D protein when compared to the other cell lines. This may be due to different mechanisms of protein translation in the different cells. The results were similar to our previous research [24], in which found NOZ cells expressed relatively higher levels of VEGF-C compared to the relatively lower mRNA levels in NOZ cells.

Fig. 6. Suppression of tumor growth by lentivirus-mediated VEGF-D siRNA in NOZ cell xenografts. Three groups (blank control group, Con-RNAi-LV group and D-3-RNAi-LV group) of NOZ cells were subcutaneously xenografted onto nude mice. The size of the primary tumors was measured every 3 days. Mice were sacrificed after 4 weeks. Points, mean tumor volume in each group; bars, SE. *Significantly different from Con-RNAi-LV (P < 0.01).

Fig. 7. Effects of lentivirus-mediated VEGF-D siRNA on lymphangiogenesis and angiogenesis in NOZ cell subcutaneous xenograft tumor. Tumors were harvested and immunostained with VEGF-D, LYVE-1 and CD-31 antibody to assess lymphangiogenesis and angiogenesis, as described in Section 2. (a) VEGF-D expression in tumor cells. Shown are quantitative evaluation of the results (right panel) and representative VEGF-D staining on blank control, Con-RNAi-LV and D-3-RNAi-LV tumors (left panel). Bars represent the mean OD ± SEM. (b) LYVE-1-positive microlymphatic vessel. Shown are quantitative evaluation of the results (right panel) and representative LYVE-1 staining on blank control, Con-RNAi-LV and D-3-RNAi-LV tumors (left panel). Bars represent the mean MLVD ± SEM. *Significantly different from Con-RNAi-LV (P < 0.01).

(c) CD-31-positive microvessel. Shown are quantitative evaluation of the results (right panel) and representative CD-31 staining on blank control, Con-RNAi-LV and D-3-RNAi-LV tumors (left panel). Bars represent the mean MVD ± SEM. *Significantly different from Con-RNAi-LV (P < 0.01).
Based on the protein level, we used NOZ cells to next further study.

SiRNA technology is the most widely used technique of gene silencing, and provides a novel strategy for investigating gene expression and function [31]. Compared with the traditional genesilencing technology (such as antisense oligo-nucleotides), SiRNA allows us to target any gene with greater specificity, stability and efficiency [32–34]. In addition, SiRNA plays an important role in the study of gene function and has the potential for therapeutic applications in human cancer diseases [35,36]. Thus, we used siRNA to assess the effect of suppressing the VEGF-D gene in GBC in vitro and in vivo. In this study, we successfully selected D-3/siRNA as the most effective siRNA to silence VEGF-D expression after four VEGF-D siRNA plasmid transfection in NOZ cells. VEGF-D mRNA and protein expression was suppressed by lentivirus-mediated D-3/siRNA. In vitro, downregulation of VEGF-D by siRNA technology inhibited the proliferation and invasion of NOZ cells. Our results were similar to previous research, in which Tanaka et al. [37] found transfection with VEGF-D increased in vitro cell growth of human gastric carcinoma cells KKLS cells as a result of an autocrine role of VEGF-D in gastric carcinoma. Goodyear et al. [38] also found that the VEGF-D leads to increased cell migration and invasion in primary prostate cell lines. The results suggest that VEGF-D can affect the biological characteristics of tumor cells in GBC.

In our in vivo model of GBC, we similarly observed that downregulation of VEGF-D by siRNA inhibited growth of subcutaneous xenograft tumor, consistent with our in vitro study. In addition to the induction of tumor growth, downregulation of VEGF-D by siRNA also significantly decreased lymphangiogenesis in the GBC subcutaneous xenografts. Our results are consistent with study of the function of VEGF-D in other tumor entities [39–41], which indicated that VEGF-D played a key role in an induction of lymphangiogenesis. For instance, VEGF-D stimulated lymphangiogenesis when overexpressed in hepatocellular carcinoma [27] and promoted the development of lymphatics and lymph node metastasis in experimental tumors [30]. Since the positive lymph nodes were not found in the mouse model of subcutaneous xenograft tumor, we also induced the mouse model of orthotopic GBC tumors. In order to in line with human tumor natural

Table 1

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<th>Con-RNAi-LV</th>
<th>D-3-RNAi-LV</th>
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<td>Animals with ascites (%)</td>
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<tr>
<td>Lymph node metastasis (%)</td>
<td>5/5(100%)</td>
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*a Significantly different from Con-RNAi-LV (P < 0.05).
metastasis in vivo, Fidler [42] proposed the tumor cell suspension was injected directly into the corresponding organ recipient in animal (i.e., orthotopic transplantation), which could improve tumor spontaneous metastasis. In the orthotopic transplantation model, the graft site is better to the growth of tumor, and the tumor can display preferably their biological characteristics, so it can be similar to the way the human tumor invasion and metastasis. In this study, we have successfully established the mouse model of orthotopic GBC tumors by using NOZ cells, and also found that hepatoduodenal ligament could seen enlarged lymph nodes. Downregulation of VEGF-D by siRNA can decrease significantly lymphangiogenesis and lymphatic metastasis in the mice of orthotopic xenograft tumor. This indicated that VEGF-D can induce tumor growth, increase lymphangiogenesis and promote lymphatic metastasis in the GBC. However, downregulation of VEGF-D by siRNA cannot decrease angiogenesis in the mice of subcutaneous and orthotopic xenograft tumor. Their results are in agreement with previous research, in which [43] VEGF-D expression did not increase or even reduce angiogenesis in transgenic approaches. Apart from less lymphangiogenesis and lymph node metastasis, downregulation of VEGF-D by siRNA in the experimental GBC tumors reduced ascites and hepatic invasions. This might be associated with an decreased tumor cells proliferation and invasion.

In this study, VEGF-D mRNA and protein were expressed in all three GBC cell lines (GBC-SD, NOZ, and SGC-996). We successfully selected D-3/siRNA as the most effective siRNA to silence VEGF-D expression after four VEGF-D siRNA plasmid transfection in NOZ cells. VEGF-D mRNA and protein expression was suppressed by lentivirus-mediated D-3/siRNA. D-3-RNAi-LV inhibited NOZ cells proliferation and invasion ability in vitro. D-3-RNAi-LV inhibited tumor growth and lymphangiogenesis in the NOZ cell subcutaneous xenograft model. D-3-RNAi-LV inhibited lymphangiogenesis and lymphatic metastasis in the NOZ cell orthotopic xenograft model. Furthermore, D-3-RNAi-LV inhibited tumor ascites and hepatic invasion in the NOZ cell orthotopic xenograft model.

Taken together, our results firmly link the expression of VEGF-D to lymphangiogenesis and increased lymphatic node metastasis in GBC xenograft models as well as to the induction of tumor growth, ascites and hepatic invasions, indicating an important role for VEGF-D in GBC progression. All this suggest that VEGF-D is involved in the progression of GBC and represents a potential molecular target in the treatment of GBC.

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