Tumor necrosis factor-α promotes the lymphangiogenesis of gallbladder carcinoma through nuclear factor-κB-mediated upregulation of vascular endothelial growth factor-C

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Key words
Gallbladder carcinoma, lymphangiogenesis, nuclear factor-κB, tumor necrosis factor-α, vascular endothelial growth factor-C

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Funding information
The National Natural Science Foundation of China (81272373). Key Project of Science and Technology Research Program in Fujian Province (2009Y0024). Key Project of Science Research in Fujian Medical University (09ZD017). The National Key Clinical Specialty Construction Project of China (201030402).

Received February 24, 2014; Revised July 30, 2014; Accepted August 10, 2014

Cancer Sci 105 (2014) 1261–1271
doi: 10.1111/cas.12504

Gallbladder carcinoma (GBC), which ranks sixth among gastrointestinal cancers, is the most common malignancy of the biliary system, representing 80–95% of biliary tract cancers worldwide.1-3 The gallbladder carcinoma, lymphangiogenesis, nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), a key inflammatory cytokine responding to chronic inflammation of GBC, has been reported to play a key role in the lymphangiogenesis of GBC.4-6 In the present study, the concentration of TNF-α and VEGF-C and the lymphatic vessel density (LVD) in the clinical GBC specimens were analyzed, and a linear correlation was found between the concentration of TNF-α and that of VEGF-C, the lymphatic vessel density (LVD); The transcription and protein level of VEGF-C in NOZ cell line were detected by real-time polymerase chain reaction (PCR) and enzyme linked immunosorbent assay (ELISA), and TNF-α enhanced the expression of VEGF-C in NOZ cell lines in a dose and time-dependent manner. Lymphatic tube formation in vitro was observed in a three-dimensional coculture system consisting of HDLECs and NOZ cell lines, and lymphatic vessels of GBC in nude mice model was detected by immunohistochemistry. TNF-α promoted the tube formation of lymphatic endothelial cells in vitro and the lymphangiogenesis of GBC in nude mice; The nuclear factor (NF)-κB binding site on the VEGF-C promoter was identified using Site-directed mutagenesis, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP). Taken together, TNF-α can upregulate the expression of VEGF-C and promote the lymphangiogenesis of GBC via NF-κB combining with the promoter of VEGF-C.
lead to cell death,(11,12) proliferation and migration,(13) its promoting(14,15) or inhibiting actions(16,17) in the tumor metastasis have also been reported in different tumors. Previous studies have showed that the expression of TNF-α in gallbladder carcinoma is higher than that in calculous cholecystitis, and a have showed that the expression of TNF-α has also been reported in different tumors. Previous studies we thought of the possibility that TNF-α promotes lymphangiogenesis www.wileyonlinelibrary.com/journal/cas  

| Factor                        | Concentration of VEGF-C (pg/mL) | P     | Concentration of TNF-α (pg/mL) | P     |
|-------------------------------|----------------------------------|-------|-------------------------------|-------|
|                               | >764                             | <764  | >609                          | <609  |
| Age                           |                                  |       |                               |       |
| <60                           | 5                                | 7     | 0.535                         | 4     |
| ≥60                           | 4                                | 4     | 0.658                         | 4     |
| Gender                        |                                  |       |                               |       |
| Male                          | 4                                | 5     | 0.025                         | 0     |
| Female                        | 5                                | 6     | 0.009                         | 1     |
| Clinical stage                |                                  |       |                               |       |
| I–III                         | 1                                | 7     | 0.246                         | 4     |
| IV–V                          | 8                                | 4     | 0.835                         | 8     |
| Lymph node metastasis         |                                  |       |                               |       |
| Negative                      | 1                                | 8     | 0.246                         | 4     |
| Positive                      | 8                                | 3     | 0.009                         | 1     |
| Histological grade            |                                  |       |                               |       |
| Poorly                        | 4                                | 3     | 0.835                         | 5     |
| Moderately                    | 4                                | 5     | 0.246                         | 3     |
| Well                          | 1                                | 3     | 0.025                         | 7     |
| Histological type             |                                  |       |                               |       |
| Adenocarcinoma                | 5                                | 7     | 0.835                         | 5     |
| Papillary carcinoma           | 2                                | 3     | 0.246                         | 4     |
| Others                        | 2                                | 1     | 0.025                         | 2     |

Material and Methods

Patients and tissue specimens. A total of 20 specimens of gallbladder carcinoma and the bile were randomly obtained from the patients admitted to the Affiliated Union Hospital of Fujian Medical University in China (from 2008 to 2012, background of clinical samples is shown in Table 1). Twenty specimens of “control bile” were obtained from the patients with cholesterol gallbladder polyps receiving surgical treatment, as it is practically infeasible to obtain pure normal bile in clinical practice. All patients enrolled in this study had not received any preoperative chemotherapy or radiotherapy. The tissues were collected according to the protocol approved by the Ethics Committee of the Medical Faculty of Fujian Medical University.

Cell culture. The human gallbladder carcinoma cell line NOZ was originally obtained from Health Science Research Resources Bank (HSRRB) in Japan. Human dermal lymphatic endothelial cells (HDLECS) was from Sciencell (San Diego, California, USA). NOZ cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Technologies Gibco, Carlsbad, California, USA). HDLECs were incubated in endothelial cell medium (ECM, Sciencell). Both of the cell lines were incubated at 37°C in a 5% CO₂ humidified incubator.

Immunohistochemistry and counting of lymphatic vessel. The lymph vessels of gallbladder carcinoma specimens were detected by immunohistochemistry as previously described.(23) The primary antibodies used in clinical specimens and nude mice specimens were mouse anti-human D2-40 rabbit monoclonal antibody (MAIXIN_BIO, Fuzhou, China) and goat anti-mouse LYVE-1 polyclonal antibody (R&D Systems, Minneapolis, MN, USA), respectively. Secondary antibodies used were goat anti-mouse and rabbit anti-goat IgHRP (ZSGB-BIO, Beijing, China). The specimens were visualized with stable 3, 3-diaminobenzidine (DAB, ZSGB-BIO, Beijing, China).

Sections were first examined at low magnification (×100) to identify areas with most intense staining and apparent highest density of microvessel (hotspot). Three areas of hotspots were selected by three pathologists who independently evaluated the slides for microvessel counting using 400× magnification (0.17 mm² field), without the knowledge of patient status. In the absence of hotspots, three or more randomly selected areas were counted. Intratumoral and peritumoral lymphatic vessel should be counted separately. Brown vessels without muscle layer or
red blood cells (RBCs) in their lumen were considered as lymphatic vessels. Single immunoreactive endothelial cells, or endothelial cell clusters separate from adjacent microvessels, were counted as a vessel. The highest number of vessels counted was recorded and used in the statistical analysis.

**Real-time RT-PCR.** Total RNA was extracted from NOZ cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer’s instructions. It was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo, Waltham, MA, USA) in accordance with the manufacturer’s recommendations. PCR reactions were performed with Fast Start Universal SYBR Green Master (Roche) and fluorescence was measured using the 7500 quantitative real-time thermocycler (Applied Biosystems). β-Actin served as an internal control. The primers for VEGF-C were: 5′-TGTGTGTCGGCTA CAGATGTG-3′ (forward) and 5′-TTCGAGAAGTGTG ATTGG-3′ (reverse). Each reaction was run in triplicate. All experiments had efficiencies between 95% and 105%, and the gene measure displayed normal melt curves. Fold changes were calculated by 2^−ΔCt, where ΔCt = Ct (target gene) − Ct (β-Actin) and Δ(ΔCt) = ΔCt (experimental group) − mean ΔCt.

**Enzyme linked immunosorbent assay.** Levels of VEGF-C and TNF-α in bile or cell culture media were assessed by double antibody ELISA. Enzyme linked immunosorbent assay was carried out on a Quantikine ELISA Kits from R&D Systems according to the manufacturer’s instructions. Standard curves were prepared before the antibody reaction. Bile samples were diluted twofold with Calibrator Diluent RD6U, and cell culture supernates were not diluted. The wells were read at 450 nm with a Model 550 Microplate Reader (Bio-Rad, Hercules, CA, USA). Each reaction was run in triplicate.

**Tube formation assay.** To determine whether TNF-α promoted the lymphangiogenesis of gallbladder carcinoma in vivo, we established a three-dimensional coculture system consisting of NOZ cell line and Dil (Beiyetome Institute of Biotechnology, Haimen, China)-labeled HDLECs by refering to Yiping Zeng’s method. A mixture of HDLECs (6 × 10^5 per well) with each NOZ cell line (3 × 10^5 per well) was seeded in 12-well plates with growth factor-reduced (GFR) Matrigel, and incubated in serum-free DMEM with 50 ng/mL of TNF-α. To assess the role of VEGF-C in the tube formation of HDLECs, we established a VEGF-C-RNAi-LV-transfected NOZ cell line, which was mixed-cultured with HDLECs. All tube formation experiments were observed by inverted fluorescence microscopy (Nikon, Japan), and images were digitally captured at 6 h after plating. The total length, area, and number of tube-like structures formed in each well were measured with Axiovision Rel 4.1 software (Carl Zeiss AG, Jena, Germany).

**Establishment of the orthotopic xenograft model.** Male athymic BALB/c nude mice in an age range of 4–6 weeks were obtained from Slaccas Laboratory Animal Co. (Shanghai, China). As Jan-Hendrik Egberts’ method described, after the anesthesia, about 4 × 10^6 cells were mixed with Matrigel (20 μL of aforementioned cell suspension in DMEM with 20 μL of reduced growth factor Matrigel). The gallbladder was exposed via abdominal midline incision (approximately 0.8–1.0 cm) and the bile in the gallbladder was extruded. Then 40 μL of cell suspension mixed with Matrigel was slowly injected into the gallbladder with a 29G insulin syringe (BD, Research Triangle Park, North Carolina, USA). The syringe was withdrawn from the gallbladder when the cell suspension became white because of solidification. Finally, the gallbladder and liver lobes were replaced and the abdominal wall was sutured. The physical condition of the mice was monitored every day in the first week and then every 3 days in the following 3 weeks. Four weeks later, the mice were euthanized by exposure to CO2 and primary tumors were dissected and excised.

**VEGF-C promoter luciferase constructs.** Genomic DNA from NOZ cells was extracted with DNeasy Tissue Kit (Bio Teke, Beijing, China) and used as a template for PCR amplification. The plasmid pGL3B-2000 with the VEGF-C promoter was constructed by ligation of the PCR-generated VEGF-C promoter (nucleotides -2000 +1, relative to the transcription start site) into the Xhol and HindIII (Thermo) cleaved sites of the luciferase reporter plasmid pGL3-Basic (Promega, Madison, WI, USA). Various VEGF-C promoter deletion constructs, which included pGL3B-1500 (nucleotides -1500 +1), pGL3B-1000 (nucleotides -1000 +1), pGL3B-600 (nucleotides -600 +1), pGL3B-487 (nucleotides -487 +1), pGL3B-332 (nucleotides -332 +1), and pGL3B-228 (nucleotides -228 +1), were also made similarly.

**Dual-luciferase reporter assay.** A total of 20 mg of cell lysate was used for the detection of intracellular luciferase activity in the Dual-Luciferase Reporter Assay System (Promega), in accordance with the manufacturer’s recommendations. Luminescence measurement was carried out on a luminometer (Orion II Microplate Lumimeter, Berthold Detection Systems, Prorzhem, Germany).

**RNA interference assay.** Vascular endothelial growth factor-C gene was knocked down by Lentiviral-mediated small interfering RNA (siRNA) in NOZ cell line, which had been constructed by Genechem (Shanghai, China) and stored in our laboratory. The target sequence for NF-κB (p65) siRNA was described by Chuan-Bian Lim et al. The siRNA duplexes, which included a negative control that had no homology with known human genes, were synthesized chemically by Biosune Company (Shanghai, China).

**Western blotting.** Nucleoprotein was extracted from NOZ cells for measurement of the expression of NF-κB, separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with monoclonal anti- NF-κB p65 (1:1000; Abcam, Cambridge, UK), with Histone H3 as the internal protein control. Proteins were detected by addition of alkaline phosphatase (AP)-conjugated secondary antibody. Immuno-reactive proteins were visualized by addition of CDP STAR reagents (Roche Diagnostics, Germany). Fragment analysis was conducted using Phoretix 1D software.

**Identification of putative transcription factor binding sites.** Potential transcription factor binding site motifs were searched using TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html) and TESS (http://www.cbil.upenn.edu/tess) programs.
TGGAGCGG-3'; mutR: 5'-TTCCAGGAACCAGGGCGTATC-3' (mutation sites underlined). On step one, the pGL3-332 construct from the VEGF-C promoter was used as a template, and the PCR products (M1F, M1R, M2F and M2R) were respectively obtained with the primers of mutF/ NF-jBmut1R, NF-xBmut1F/mutR, mutF/ NF-xBmut2R and NF-xBmut2F/mutR. On step two, mutF/mutR were used as primers, and the templates were the mixture of M1F and M1R or the mixture

Fig. 1. Detection of tumor necrosis factor-α (TNF-α), vascular endothelial growth factor-C (VEGF-C) and lymphatic vessel density (LVD) in clinical specimens of gallbladder carcinoma (GBC). (a). The concentration of TNF-α in the GBC bile specimens and in control bile. (c). Lymphatic vessels (marked by D2-40 antibody) of the GBC were detected by immunohistochemistry. The brown tubular structures (indicated by red arrows) were positive lymphatic vessels. (b, d). Correlation between TNF-α concentration in the GBC bile specimens and VEGF-C level or LVD. r: Spearman’s correlation coefficient. (**P < 0.01; ***P < 0.001).

Fig. 2. (a) Tumor necrosis factor-α (TNF-α) promoted the transcription of vascular endothelial growth factor-C (VEGF-C). (b) TNF-α promoted protein level of VEGF-C. After TNF-α (10, 20, 50 and 100 ng/mL) acting on NOZ cells for 12, 24 and 48 h, the mRNA and protein level of VEGF-C, which were respectively detected by real-time polymerase chain reaction (PCR) and enzyme linked immunosorbent assay (ELISA), were dose and time-dependently improved. (**P < 0.01; ***P < 0.001).

Fig. 3. Downregulating vascular endothelial growth factor-C (VEGF-C) mRNA and protein expression by lentiviral-mediated VEGF-C siRNA in NOZ cell lines. (a) VEGF-C mRNA expression of NOZ cells was analyzed by realtime reverse transcription-polymerase chain reaction (RT-PCR), β-Actin served as a internal control; (b) VEGF-C protein expression of NOZ cells was detected by enzyme linked immunosorbent assay (ELISA). (**P < 0.01; ***P < 0.001).
of M2F and M2R. The PCR products of step two were ligated into the XhoI and HindIII cleaved sites of pGL3-Basic.

**Nuclear extraction and electrophoretic mobility shift assay.** Nuclear extracts (NE) were prepared from NOZ cells using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) and nuclear extraction and electrophoretic mobility shift assay (EMSA) was performed with the LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturers’ recommendations. A biotin-labeled oligonucleotide probe (5'-biotin-GAGGGAAACGGGGAGCTCCAGGGAG-3'), which contained -315 to -306 nt, was used to confirm the DNA binding of NF-κB. For competition analysis, we used 100-fold excess of unlabeled competitor probe including cold probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') and mutational cold probe (5'-AGTTGAGGAAACTTCCAGGC-3', mutation sites underlined). Fragment analysis was conducted using Phoretix 1D software.

**Chromatin immunoprecipitation assay.** The Chromatin immunoprecipitation (ChIP) assay was performed with an EZ-Magna ChIP kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer’s instructions. *In vivo* cross-linking was performed after 1 day with and without TNF-α (50 ng/mL) treatment in the culture of NOZ cells, and then the cell lysates were sonicated to shear genomic DNA. For immunoprecipitation,

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**Fig. 4.** Tumor necrosis factor-α (TNF-α) promoted the tube formation of human dermal lymphatic endothelial cells (HDLECs). (a). MTT assay were used to observe the growth of HDLECs from 6 to 72 h. (b). When cultured alone, HDLECs formed a few tubes with 2–6 h after seeding. Compared with the 6th hour, the tube number of the 10th hour did not increase (P>0.05) (c). The total length, area, and number of tubes formed by HDLECs in the assays of cocultured tube formation without or with the treatment with TNF-α; (d). Dil-labeled HDLECs (emit red fluorescence) were cocultured with three NOZ cell lines and treated with TNF-α (50 ng/mL) for 6 h, the tube formation of HDLECs was observed under an fluorescence microscopy. (*P<0.05).
tion, an antibody against NF-κB p65 and the control normal rabbit immunoglobulin G (IgG) were used. The primers for PCR were: 5'-GACAGGGCGGGGAGGGAGA-3' (forward) and 5'-CTCAGCTCTCCCTCGGAAAGCGTCTC-3' (reverse), which amplified the area (-389 to -278 nt) including the NF-κB binding site. PCR was performed using personal thermal cycler (Bio-Rad). Digital gel image analysis using Phoretix 1D software.

Statistics. The results were presented as mean ± SEM. Statistical analyses were done with the SPSS software 17.0 (using
Results

Detection of clinical specimens. Tumor necrosis factor-α and VEGF-C level in the bile specimens from 20 GBC patients and 20 patients with cholesterol gallbladder polyps were assessed by ELISA, and lymphatic vessels density (LVD) of 20 GBC specimens were detected with D2-40 antibody by immunohistochemistry. The concentration of TNF-α in GBC bile specimens was significantly higher than that in control bile (609.0 ± 43.43 vs 193.3 ± 13.74, P < 0.001, Fig. 1a). A linear correlation was found between the concentration of TNF-α and that of VEGF-C in the bile of GBC patients (P < 0.01, Fig. 1b). A significant correlation was also found between TNF-α level of the GBC bile and LVD of the GBC tissue (P < 0.01, Fig. 1c,d).

The correlation between clinicopathological factors and concentration of VEGF-C and TNF-α in GBC were also analyzed (Table 1). The data showed that clinical stage and lymph node metastasis of GBC were related with the concentration of VEGF-C and TNF-α.

TNF-α promotes the expression of VEGF-C in vitro. NOZ cells were incubated in cell culture plates and treated respectively with different doses of TNF-α (10, 20, 50 and 100 ng/mL) for 12, 24 and 48 h, compared with control group without TNF-α. Relative mRNA of VEGF-C was detected by real-time PCR, and protein expression of VEGF-C was assayed by ELISA. As shown in Figure 2, TNF-α promoted the transcription and protein expression of VEGF-C in NOZ cells in a dose and time-dependent manner, and the peak effect appeared when the dose was 50 ng/mL and the time was 24 h or 48 h.

TNF-α promotes the tube formation of HDLECs in vitro. To observe the role of VEGF-C gene in the the lymphangiogenesis of GBC, we structured NOZ cell line, of which VEGF-C was knocked down by Lentiviral-mediated siRNA. As shown in Figure 3, the mRNA and protein expression of VEGF-C in NOZ/VEGF-C siRNA group (NOZ cells transfected with lentiviral-mediated VEGF-C siRNA) sharply decreased (P < 0.01), compared with NOZ and NOZ/Ctrl group (NOZ cells transfected with empty vector). Before exploring whether TNF-α could enhance tube formation of HDLECs, we firstly observed the effects of TNF-α on the cell number...
of HDLECs by MTT assay. As shown in Figure 4(a), 50 ng/mL of TNF-α had no effect on the growth of HDLECs in 6–48 h (P > 0.05), and the number of cells reduced after the treatment with TNF-α for 72 h (P < 0.05). In the experiment of tube formation, the HDLECs cultured alone had formed a few tubes 2–6 h after seeding, but the tube number did not increase with the formation of small tight clusters 10 h after seeding (Fig. 4b). So we decided to observe the tube formation for 6 h in the following mixed-culture experiment. HDLECs were separately cocultured with three cell lines: NOZ, NOZ/Ctrl and NOZ/VEGF-C siRNA on GFR Matrigel. In the treatment with TNF-α, the tube number (10.89 ± 1.10 vs 6.82 ± 0.62, P < 0.05), tube length (10.26 ± 1.034 mm vs 6.14 ± 0.74 mm, P < 0.05) and tube area (0.86 ± 0.04 mm² vs 0.65 ± 0.06 mm², P < 0.05) of HDLECs increased obviously, and with the knockdown of VEGF-C, the tube number of treated group (4.11 ± 0.35) was still higher than that of non-treated group (3.44 ± 0.29) (P < 0.05), but the increase rate of VEGF-C siRNA group (39.27% ± 5.60%) was lower than that of control (71.14% ± 5.15%) (P < 0.05). As for the tube length and tube area, there was no significant differences between the treated group and non-treated group (P > 0.05). (Fig. 4c,d).

**TNF-α promotes the lymphangiogenesis of GBC in vivo.** To study the effect of TNF-α on lymphangiogenesis in vivo, we established three orthotopic xenograft models of GBC in nude mice, which were respectively inoculated the abovementioned three NOZ cell lines: NOZ, NOZ/Ctrl and NOZ/VEGF-C siRNA in nude mice. Two weeks after inoculation, a daily dose of TNF-α (2 μg/kg) was injected into the abdominal cavity for 2 weeks. The lymphatic vessels of tumors were observed by immunohistochemistry. Since the D2-40 antibody against lymphatic vessel marker podoplanin does not recognize the murine antigen, mouse lymphatic vessels were detected using LYVE-1. (31) The intratumoral lymphatic vessels of the

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Fig. 7. Nuclear factor (NF-κB) binding sites in vascular endothelial growth factor-C (VEGF-C) promoter. (a) Effect of mutation of the NF-κB binding sites on the activity of the VEGF-C promoter. Mutants, indicated as black rectangle, were depicted schematically on the left. A total of 0.2 μg of mutants together with 10 ng of pRL-TK was co-transfected into NOZ cells. pGL3-Basic served as the negative control. The luciferase units were obtained (right) by comparison with pGL3-332. Each transfection was performed in duplicate and the data were expressed as mean ± SEM of three separate experiments. (**P < 0.01). (b1) Electrophoretic mobility shift assay (EMSA) of NF-κB. The 5'-biotin end-labeled probe was incubated in the absence (lane 0) or presence (lane 1-4) of nuclear extracts from transfected NOZ cells. Unlabeled cold probe (lane 1) and cold mutated probe (lane 2) were used as competitors. The 5'-biotin end-labeled probe and nuclear extracts were incubated without (lane 3) or with (lane 4) tumor necrosis factor-α (TNF-α). (b2). The densitometric value for lane 0-4. TNF-α improved the gray value of lane 4 (***, P < 0.01), which indicate TNF-α enhanced the combined effect of the probe and the binding site. (c1) ChIP assay. Chromatin from NOZ cells was immunoprecipitated with the anti-NF-κB. The total extracted DNA (Input) and the immunoprecipitated samples were PCR-amplified using primers specific to a region that spanned -389 to -278 nt (containing the NF-κB binding sites) of the VEGF-C promoter. The normal rabbit IgG or pre-blocked protein A/G (no antibody control) was also performed for control purpose. Another experiment group was treated with 50 ng/mL of TNF-α (bottom row). (c2). The densitometric value for treated and non-treated groups. Relative expression was represented by value of anti-NF-κB/value of input (***P < 0.01).
tumors in the nude mice models were hardly to be observed, so all of the lymphatic vessels are from peritumor. As shown in Figure 5, TNF-α increased the LVD \(14.33 \pm 1.35 \text{ vs } 9.89 \pm 0.80, P < 0.05\) of orthotopic xenograft tumors. In the VEGF-C siRNA group, TNF-α also increased the LVD \(7.44 \pm 0.44 \text{ vs } 5.89 \pm 0.29, P < 0.05\) of the tumors, but the increase rate \(26.37\% \pm 4.85\%\) was lower than that of control \(44.19\% \pm 3.85\%\) \((P < 0.05)\). Meanwhile, the lymph node metastasis rates of orthotopic xenograft tumors were increased by TNF-α \((P < 0.01)\) (Table 2).

**Activity analysis of VEGF-C promoter.** To further investigate the mechanism by which TNF-α increased the expression of VEGF-C, we analyzed the promoter of VEGF-C. A series of 5'-deletion constructs of the VEGF-C gene promoter (products of PCR amplification shown in Fig. 6a) were transiently transfected into the NOZ cell line. Cells transfected with pGL3B-2000, pGL3B-1500, pGL3B-1000, pGL3B-600, pGL3B-487 and pGL3B-332 plasmids displayed higher relative luciferase activities than pGL3B-228 plasmid constructs \(P < 0.01\), with the input DNA, whereas the normal IgG control and no antibody control did not result in the immunoprecipitation of DNA fragments detectable by PCR amplification. In line with the results by EMSA, TNF-α enhanced the intensity of the anti-NF-kB band. Taken together, these results demonstrate that NF-kB transcription factor can bind directly to their corresponding consensus binding site in the VEGF-C promoter region, and TNF-α can improve the combined effect by 32.6% ± 6.5% \((P < 0.05)\) (Fig. 7c).

**Upregulation of VEGF-C promoter by TNF-α/NF-kB.** To determine the effects of TNF-α/NF-kB signaling pathway on the VEGF-C promoter, we used pGL3B-332 as a luciferase reporter plasmid, which was treated with TNF-α or co-transfected with NF-kB (p65) siRNAs into NOZ cells. TNF-α was demonstrated to enhance the expression of NF-kB \((P < 0.01)\) (Fig. 8a) and increase the luciferase activity of VEGF-C promoter when compared with the control non-mutated construct (pGL3B-332). Meanwhile, the mut2 binding site \((-271 \text{ nt to } -262 \text{ nt})\) had no effect on the promoter activity. So mut1 binding site was further confirmed by EMSA. The results demonstrate that the nuclear extract can combine with biotin-labeled probe (Fig. 7b1, lane 3). In addition, a competition assay showed that preincubation with a 100-fold molar excess of cold probe (Fig. 7b1, lane 1) diminished the intensity of the bands, but not with the cold mutated probe (Fig. 7b1, lane 2). Meanwhile, TNF-α enhanced the combined effect of the probe and the binding site by 89.7% ± 7.84% \((P < 0.01, \text{Fig. 7b1, lane 4 and } b2)\). To determine whether the NF-kB transcription factor was associated with VEGF-C promoter in vivo, we performed ChIP assay with specific antibody and PCR using the primers against the regulatory elements of the VEGF-C promoter. As RelA/p65 is a key active subunit in NF-kB transcription in several cell types, it was used for the antibody reactions. As shown in Figure 7c1, a 112-bp DNA fragment covering the NF-kB binding site was amplified by chromatin immunoprecipitation with an anti-NF-kB antibody. The same band was obtained with the input DNA, whereas the normal IgG control and no antibody control did not result in the immunoprecipitation of DNA fragments detectable by PCR amplification.
promoter (P < 0.01, Fig. 8c), consequently leading to an increase in VEGF-C protein level (P < 0.001, Fig. 8d). In contrast, when the expression of NF-κB in NOZ cells was knocked down by the addition of siRNA (P < 0.01, Fig. 8b1, b2), the upregulation of TNF-α on the luciferase activities (75.4% ± 5.7% vs 47.6% ± 5.2%, P < 0.05) and the protein expression (101.7% ± 7.2% vs 24.8% ± 3.2%, P < 0.001) of VEGF-C were reduced (Fig. 8c,d).

Discussion

In this study we firstly found that TNF-α in GBC specimens was significantly correlated with the expression of VEGF-C and lymphatic vessel density, which is consistent with CHA’s finding in the study of rheumatoid synovocytes (20). It is noteworthy that we obtained the results in gallbladder carcinoma, a kind of malignancy that mostly metastasizes through lymph channels. This result suggests that TNF-α may promote the lymphangiogenesis of gallbladder carcinoma via enhancing the expression of VEGF-C. In the subsequent in vitro and in vivo experiments, we demonstrated that TNF-α strongly enhanced the transcription and protein expression of VEGF-C, and elevated the lymphangiogenesis of gallbladder carcinoma in nude mice. Further study on the molecular mechanism showed that TNF-α-stimulated promoter activation of VEGF-C depended on NF-κB.

Gallstones and subsequent inflammatory changes are believed to be important risk factors for gallbladder carcinoma, and the macrophage is the key player of the chronic inflammatory response. This is because it releases a great number of bioactive products, such as TNFα-stimulated production of a proinflammatory cytokine cascade and is thus considered to be a “master regulator” of proinflammatory cytokine production. (36,37) So we can speculate that TNF-α plays an important role in the evolution from gallstones and chronic inflammation to GBC development. In the present research, we detected 20 clinical bile specimens, and observed that the concentration of TNF-α in the GBC bile specimens was higher than that of controls. These results suggest that the promoter polymorphisms may not fully reflect the physiological environment of the gallbladder carcinomas, or gene and protein levels may not be consistent in the gallbladder carcinoma.

Although TNF-α has been documented to induce a variety of biological effects, its role in tumor progression remains controversial. TNF-α can trigger pro-cancer or anti-cancer signaling under different circumstances (38) Pei-Wen Tsai reported that TNF-α was not capable of upregulating VEGF-C in human breast cancer cells (39) but the present study showed that TNF-α promoted the transcription and protein level of VEGF-C within the dose range of 10–50 ng/μL in a dose-dependent manner. However, when the concentration of TNF-α was increased to 100 ng/μL, this pro-expression action dropped, the cell number of NOZ reduced, and the cell activity seemed to decline. These data suggest that the conflicting pro-cancer and anti-cancer effects may also coexist in this particular carcinoma.

Previous studies have demonstrated that the dual action of TNF-α in cancers is due to the different signaling pathways. TNF-α trimer binds to TNFR1. The latter can activate the transcription factor NF-κB and evokes the transcription of pro-inflammatory and survival genes through a complex signaling pathway. (37,40,41) TNFR1 can also initiate pro-inflammatory, survival and cell death signals by activating apoptosis signaling kinase 1 (ASK1), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase. These kinases, in turn, activate various forms of the heterodimeric transcription factor AP-1. Alternatively, TNFR1 can initiate the formation of a death-inducing signaling complex (DISC) that can trigger cell death through apoptosis or through necrosis. (11,32,38) In addition, TNF-α binding to TNFR2 can regulate cell-cell interactions and signal cell migration (13,38). On the basis of these research findings, we further investigated whether TNF-α could upregulate VEGF-C through the NF-κB signaling pathway and validated this hypothesis experimentally. It should be noted that the knockdown of NF-κB did not completely block the promotion effect of TNF-α on VEGF-C, which may be due to the involvement of AP-1 or other transcription factors binding with the VEGF-C promoter.

Although some growth factors have been found to regulate the expression of VEGF-C, (39,42) we demonstrated for the first time that TNF-α can upregulate VEGF-C in the gallbladder carcinoma cell line, which is dependent on NF-κB. In addition, we identified the core activity area of VEGF-C promoter and the specific binding site of NF-κB on the VEGF-C promoter. The interconnected signaling networks of biological factors are very complicated, and the regulation of VEGF-C cannot be completely explained by our experiment results, but the role of NF-κB was defined in this regulation.

Above all, our research documents that TNF-α can upregulate the expression of VEGF-C via NF-κB combining with the promoter of VEGF-C and upregulating the activity of it, which reveals, at least in part, the molecular mechanism by which TNF-α promotes lymphangiogenesis of gallbladder carcinoma. Moreover, the study suggests that TNF-α, a key cytokine in chronic inflammation, is an important accelerator in the progression and metastasis of gallbladder carcinoma.

Acknowledgments

This study was supported by the grants from The National Natural Science Foundation of China (No. 81272373), Key Project of Science and Technology Research Program in Fujian Province (No. 2009Y0024) and Key Project of Science Research in Fujian Medical University (No. 09ZD017).

Disclosure Statement

No conflicts of interest exist for either of the authors of this manuscript.

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