

Effect of vascular endothelial growth factor-C expression on lymph node metastasis in human cholangiocarcinoma

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Abstract. The present study aimed to evaluate the expression of vascular endothelial growth factor C (VEGF-C) in human cholangiocarcinoma tissues and its role in metastasis *in vitro*. A total of 65 biopsy samples of cholangiocarcinoma, plus the FRH-0201 cell line, were investigated. The expression of VEGF-C in the human cholangiocarcinoma specimens was evaluated by immunohistochemistry (IHC). The effect of VEGF-C on tumor cell migration and proliferation was measured by MTT and Transwell assays in the FRH-0201 cell line. According to the IHC results, the biopsies of human cholangiocarcinoma were stained positively for VEGF-C, with a positive rate of 75.4% (49/65). Moreover, VEGF-C was expressed at a higher level in the patients with lymph node metastasis than in those without lymph node metastasis. *In vitro*, VEGF-C exhibited marked growth stimulation below the concentration of 5 ng/ml and was able to promote cholangiocarcinoma cell migration significantly. These findings suggested that VEGF-C may be a useful factor to predict lymph node metastasis in cholangiocarcinoma tissues and indicates that VEGF-C plays a significant role in proliferation and migration in cholangiocarcinoma cells.

Introduction

Cholangiocarcinoma is a highly malignant epithelial neoplasm arising from the bile duct epithelial cells or cholangiocytes of the intra- or extrahepatic biliary system. The incidence and mortality rates for cholangiocarcinoma are increasing worldwide. Due to the difficulty of forming an early diagnosis, more than two-thirds of cholangiocarcinomas have metastasis

and have no chance to benefit from surgical resection (1,2). Lymphatic metastasis plays a significant role in the process of cholangiocarcinoma metastasis, and the hepatic portal lymph node is the most commonly affected node. Tumor metastasis is a complex and multi-stage process in which a number of cytokines are now known to be involved, particularly the vascular endothelial growth factor (VEGF) family (3-5).

VEGF-C has been implicated as being a potent stimulator of angiogenesis and lymphangiogenesis (6). By binding with VEGFR-2 and VEGFR-3, VEGF-C exerts its biological functions in various cell types. Recent studies revealed that VEGF-C can be expressed in several cancer cell types, including ovarian carcinoma (7), lung adenocarcinoma (8), breast cancer (9), and head and neck squamous carcinoma (10) cells, and that it can promote lymphatic metastasis progression, thus promoting tumor malignancy. Ishikawa *et al* (10) reported that the expression of VEGF-C is negative in early-stage gastric cancers due to the extremely low level of nodal metastasis, and Yonemura *et al* (11) showed that VEGF-C expression significantly correlates with lymph node metastasis in advanced-stage gastric cancers. However, few studies have been reported on the correlation between VEGF-C expression and an invasive phenotype in cholangiocarcinoma tissues and cells.

The present study evaluated whether VEGF-C can be a useful factor in predicting lymph node metastasis in human cholangiocarcinoma tissues and the role played by VEGF in mediating proliferation in the cholangiocarcinoma FRH-0201 cell line.

Materials and methods

Tissue collection and immunohistochemistry (IHC). A total of 65 patients with cholangiocarcinoma who were diagnosed and treated with curative surgery using a standard lymph node dissection, in Qilu Hospital of Shandong University (Jinan, Shandong, China) between 2012 and 2015, were included in the study. Written informed consent was obtained from all patients, and the study was approved by the ethics committee of Qilu Hospital of Shandong University. As normal controls, 5 cholangiolar biopsies from patients (3 female and 2 male; age range, 64-74 years) with a normal histology who underwent laparotomies were assessed. Fragments of cholangiocarcinoma

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and normal cholangiolar biopsies were processed for common histomorphology (hematoxylin-eosin and Masson's stains) and for IHC. For IHC, frozen cholangiocarcinoma tissue samples were stored at -80°C until use. Cryostat sections were fixed in 4°C acetone (Beijing Zhongshan Biological Technology Ltd., Beijing, China) for 20 min and air-dried for 30 min. Endogenous peroxidase activity was removed using 3% H_2O_2 in methanol (Beijing Zhongshan Biological Technology Ltd.) for 10 min. Subsequent to being washed three times with phosphate-buffered saline (PBS), the sections were incubated with an affinity-purified goat polyclonal IgG antibody against VEGF-C (catalog no. sc-7133; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; dilution, 1:100) overnight at room temperature. Tissue sections were rinsed in PBS and then incubated with a linked biotinylated polyclonal rabbit anti-goat immunoglobulin secondary antibody (catalog no. E046601; Dako, Glostrup, Denmark) for 15 min at room temperature. Subsequent to rinsing with PBS, the sections were incubated with DAB and counterstained with hematoxylin (Beijing Zhongshan Biological Technology Ltd.). Negative controls were processed by omitting the primary antibody. The sections were observed using an Olympus BX53 fluorescent microscope (Olympus Corporation, Tokyo, Japan). Cases in which $>5\%$ of the cells showed positive immunostaining for VEGF-C were judged to be positive.

Cell culture. The human hilar cholangiocarcinoma FRH-0201 cells were supplied by Dr Wu (Department of General Surgery, Qilu Hospital of Shandong University) and were maintained in Dulbecco's modified Eagle's medium (Hyclone, GE Healthcare, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 $\mu\text{g}/\text{ml}$ penicillin (Zhejiang Tianhang Biological Technology Ltd., Hangzhou, China) and grown at 37°C in 5% carbon dioxide. The cells were starved for 4 h in medium without fetal bovine serum and then stimulated with VEGF-C at concentrations of 1, 5, 10, 50 and 100 ng/m for 24, 48 and 72 h, respectively, at 37°C .

Proliferation assay. The effects of VEGF-C on the FRH-0201 cells were investigated by MTT assays (Promega Corporation, Madison, WI, USA). FRH-0201 cells at 85-100% confluence were harvested, and then 5×10^3 cells per well were seeded onto a 96-well plate. The cells were incubated in 200 μl medium in a humidified incubator. After 24 h, the cells were stimulated with VEGF-C (Sino Biological Inc., Beijing, China; catalog no. 10542-H08H) at 1, 5, 10, 50 and 100 ng/m for 24, 48 and 72 h, respectively, and the medium was changed daily. Next, 20 μl MTT solution was added and the cells were incubated for 4 h, solubilized with 200 μl DMSO (Amresco LLC, Solon, OH, USA) and agitated for 10 min. The optical density (OD) values were examined at 570 nm using a Safire 2 microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The cell survival percentages were calculated by dividing the mean OD of the VEGF-C-containing wells by that of the control wells.

Migration assay. Transwell chambers (8- μm , 24-well insert; Corning Inc. Life Sciences, Tewksbury, MA, USA) were used for the migration assay. Briefly, 600 μl 10% FBS-containing medium was added to the lower chamber and 1×10^5 cells

Table I. Summary of the clinicopathological features of 65 patients with cholangiocarcinoma.

Feature	Value
Age, years (mean \pm standard deviation)	55 \pm 8.5
Gender (male/female)	38/27
Tumor location, n	
Hilar / Intrahepatic bile duct	34
Extrahepatic bile duct	31
Histological differentiation, n	
Well	15
Moderate	19
Poor	31
Lymphatic invasion, n	
Present	42
Absent	23

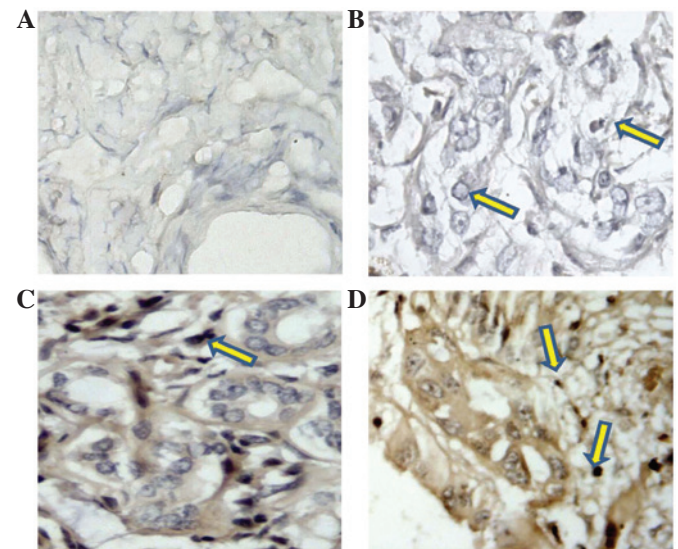


Figure 1. Immunohistochemical staining for vascular endothelial growth factor C (VEGF-C) in human cholangiocarcinoma. (A) Control specimen; (B) VEGF-C-negative cholangiocarcinoma specimen (arrows indicate VEGF-C was not observed); (C) cholangiocarcinoma specimen with weakly positive VEGF-C expression (arrow); and (D) cholangiocarcinoma specimen with strongly positive VEGF-C expression (arrows).

in 200 μl serum-free medium were added to the upper chamber. The cells were incubated for 48 h at 37°C , and the non-invading cells were then scraped off. Finally, the insert membranes were stained with 0.1% crystal violet (Amresco LLC) and the permeating cells were counted, with images captured under an Olympus CKX41 inverted microscope (Olympus Corporation). The migrated cells were counted in 10 random fields at $\times 200$ magnification. At least three randomly selected fields were counted, and the average number of cells was calculated per field.

Statistical analysis. Statistical analysis was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean \pm standard deviation, and

Table II. Correlation between VEGF-C expression and pathological features.

Feature	n	VEGF-C expression, n		P-value
		Positive	Negative	
Age, years				P>0.05
≥60	30	21	9	
<60	35	28	7	
Gender				P>0.05
Male	38	28	10	
Female	27	21	6	
Tumor location				P>0.05
Hilar/Intrahepatic	34	26	8	
Extrahepatic	31	23	8	
Tumor size, cm				P>0.05
≥2	21	18	3	
<2	44	31	13	
Differentiation				P>0.05
Well/moderate	34	25	9	
Poor	31	24	7	
Lymphatic invasion				P<0.01
Present	42	36	6	
Absent	23	13	10	

VEGF-C, vascular endothelial growth factor C.

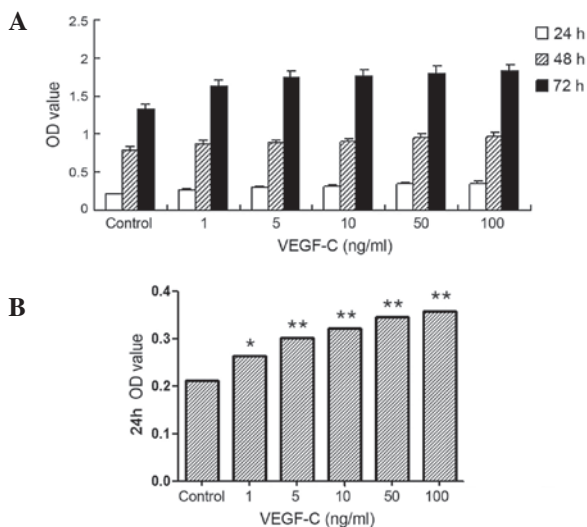


Figure 2. Effect of vascular endothelial growth factor C (VEGF-C) on the proliferation of FRH-0201 cholangiocarcinoma cells treated for different times, assessed by MTT assay. (A) The proliferation of cholangiocarcinoma cells treated with VEGF-C at concentrations of 1, 5, 10, 50 and 100 ng/ml for 24, 48 and 72 h, respectively; (B) The proliferation of cholangiocarcinoma cells treated with VEGF-C at concentrations of 1, 5, 10, 50 and 100 ng/ml for 24 h. Cell proliferation in the VEGF-C group was significantly different compared with that in the control group (*P<0.05, **P<0.01 vs. control), but the rate of increase in cell proliferation slowed when VEGF-C concentrations exceeded 10 ng/ml.

differences between groups were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

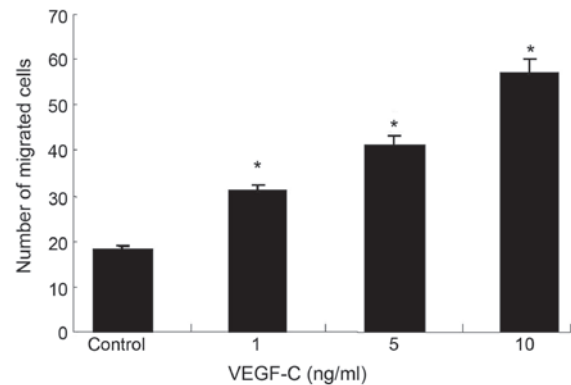


Figure 3. Effect of vascular endothelial growth factor C (VEGF-C) on the migration of FRH-0201 cells, as determined by Transwell chamber assay. *P<0.05 vs. control.

Results

Expression of VEGF-C in cholangiocarcinoma tissues. The clinical and pathological characteristics of the 65 patients are summarized in Table I. The expression of VEGF-C was measured by IHC in the cholangiocarcinoma tissues and normal human bile duct specimens. Strong immunoreactivity was present for VEGF-C in the cholangiocarcinoma specimens, but minimal immunoreactivity was observed in the normal specimens (Fig. 1A and B). In the normal bile duct specimens, moderate- to low-level expression was only observed in 5% of the normal bile duct specimens. By contrast,

high-level VEGF-C expression was observed in the majority of cholangiocarcinoma specimens, and the positive rate was 75.4% (49/65) (Fig. 1C and D).

Association between the expression of VEGF-C and clinicopathological data. The association between clinicopathological data and the expression of VEGF-C is shown in Table II. No significant difference in tumor size was observed among the positive and negative expression types. There was also no significant difference between the expression of VEGF-C and age, gender and tumor location. However, VEGF-C was expressed at a significantly higher level in the patients with lymph node metastasis than in those without lymph node metastasis ($P < 0.01$).

Effect of VEGF-C on the proliferation of FRH-0201 cells. The cell proliferation activities of the FRH-0201 cells were monitored by MTT assay. The results showed that VEGF-C exhibited marked growth stimulatory effects, compared with the control group, at concentrations of 1 ng/ml ($P < 0.05$) and ≥ 5 ng/ml ($P < 0.01$). However, when the concentrations of VEGF-C exceeded 10 ng/ml, the rate of increase in cell proliferation slowed, and this increase was abolished by 100 ng/ml (Fig. 2).

VEGF-C promotes tumor cell migration. The effect of VEGF-C on FRH-0201 cell migration, a key determinant of malignant metastasis, was further examined. As shown in Fig. 3, ectopic high expression levels of VEGF-C led to significantly increased abilities of cell migration compared with the control cells ($P < 0.01$), while more migrated cells were observed in the VEGF-C group compared with the control group ($P < 0.05$).

Discussion

Tumor metastasis, an important factor in determining patient survival in cancer, occurs via the blood vascular and lymphatic systems to transport carcinogenic fluid and cells. A number of cytokines are now known to be involved in the process of tumor metastasis. VEGF-C is one of these factors, and is an essential predictor of tumor growth, lymphangiogenesis and metastatic behavior, as well as a variety of other significant pathologies (12,13). However, there have been few studies on the expression of VEGF-C in cholangiocarcinoma, and the association between VEGF-C and lymph node metastasis has not been made entirely clear.

The present study clearly demonstrated the close association between VEGF-C expression and lymph node metastasis. Tumors with high expression levels of VEGF-C exhibited more remote lymph node involvement than those with low VEGF-C expression levels. The IHC study showed that VEGF-C was overexpressed in the cholangiocarcinoma tissues (positive ratio, 75.4%), but the normal bile duct tissues were negative for this cytokine. Moreover, the concrete expression of VEGF-C was associated with lymphatic metastasis. *In vitro*, VEGF-C at 1 to 5 ng/ml exhibited marked growth stimulation compared with the control group. In Transwell chamber assays, VEGF-C led to significantly increased cell migratory ability. This is compatible with previous studies

and suggests that VEGF-C induces tumor cell metastasis in cholangiocarcinoma (14,15).

In the present study, certain clinically lymph node-negative cholangiocarcinoma specimens also showed VEGF-C expression. It is possible that the VEGF-C-positive cholangiocarcinoma patients without clinical lymph node metastasis in the present study may have exhibited lymph node metastasis at a microscopic level. Nevertheless, more importantly, the expression of VEGF-C was significantly higher in the lymph node-positive patients than in lymph node-negative patients, suggesting that the examination of VEGF-C expression in cholangiocarcinoma tissues may be an indicator of the presence of lymph node metastasis.

Taken together, these findings suggested that VEGF-C could promote proliferation and migration significantly in cholangiocarcinoma cells, and that there is a significant correlation between the expression of VEGF-C and lymphatic invasion. Hence, the targeting of VEGF-C may be a useful therapeutic strategy for certain patients with cholangiocarcinoma.

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