

Expression of vascular endothelial growth factor C in human esophageal squamous cell carcinoma

Hongxin Zhang¹, Lan Zhang¹, Kuisheng Chen¹, Dongling Gao¹, Fucheng He², Yunhan Zhang^{1,*}

¹Department of Pathology, The First Affiliated Hospital, Zhengzhou University; Henan Key Laboratory of Tumor Pathology, Zhengzhou, Henan 450052, China; ²Department of Clinical Laboratory, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, China

Received August 20, 2006

Abstract

To examine the expression of vascular endothelial growth factor C (VEGF-C) in human esophageal squamous cell carcinoma (ESCC) and to clarify its role in lymphatic metastasis in ESCC. Esophageal carcinoma EC9706 cells and samples of 49 patients with primary ESCC were investigated by using S-P immunohistochemistry (IHC), semi-quantitative RT-PCR *in situ* and hybridization (ISH) technology for VEGF-C expression, respectively. VEGF-C positive expressions were found in EC9706 cells through ICH, ISH and RT-PCR, respectively. Positive IHC for VEGF-C was observed in 36 of 49 cases of ESCC. The brown staining granules for VEGF-C were identified in the cytoplasm of carcinoma cells. There was significantly difference between the expression of VEGF-C in the group with lymph node metastasia and the group without lymph node metastasis ($\chi^2 = 4.7, P < 0.05$). Positive ISH for VEGF-C was observed in 23 of 49 cases of ESCC. The blue-purple staining granules for VEGF-C were identified in the cytoplasm of carcinoma cells. There was a significant difference between the expression of VEGF-C in lymph node group and group without lymph node metastasis ($\chi^2 = 31.3, P < 0.01$). The expression of VEGF-C protein level was significantly higher in group with lymph node metastasia than in group without lymph node metastasis. Of 49 ESCC, VEGF-C gene expression was observed by RT-PCR in 29 cases. There was a significant difference between the expression of VEGF-C in group with lymph node metastasia and group without lymph node metastasis ($\chi^2 = 23.3, P < 0.01$). VEGF-C mRNA expression was significantly higher in group with lymph node metastasia than in group without lymph node metastasis. The expression of VEGF-C mRNA level was not significantly associated with age, gender, or pathological grade. There was a correlation between VEGF-C mRNA expressions by RT-PCR and ISH ($\chi^2 = 18.5, P < 0.01$) in ESCC, but no significant difference between the two methods. VEGF-C expression may induce lymphangiogenesis in human ESCC. There must be a close correlation between VEGF-C expression and lymph node metastasis. VEGF-C may be serve as a useful prognostic factor in ESCC. [Life Science Journal. 2007;4 (1):24-28] (ISSN: 1097-8135).

Keywords: esophageal squamous cell carcinoma; EC9706 cells; VEGF-C; lymphatic metastasis

1 Introduction

Esophageal carcinoma is a common cause of death throughout the world including China especially in the Taihang mountain range. The lymphatic system is the primary pathway of metastasis for esophageal squamous cell carcinoma (ESCC) and the extent of lymph node involvement is a key prognostic factor for the outcome of patients. However, the mechanism of lymphatic metastasis remains unclear.

Lymphangiogenesis, the development of new lymph

vessels, is a relatively new area of clinical investigations. Recent studies show that vascular endothelial growth factor C (VEGF-C) has been identified as a new member of the VEGF family, and is believed to be the only lymphangiogenesis factor in that gene family^[1]. It activates both vascular endothelial growth factor receptor 2 (VEGFR-2) and VEGFR-3^[2]. However, little investigation was on VEGF-C expression in ESCC. The purpose of this study was to detect the expression of VEGF-C and its association with lymph node metastasis in ESCC with immunohistochemistry (IHC), *in situ* hybridization (ISH) and RT-PCR technique. The relationship of VEGF-C to clinicopathological features was investigated furthermore.

*Corresponding author. Tel: 86-371-6665-8175 (Office); Email: yhzhang@zzu.edu.cn

2 Materials and Methods

2.1 Materials

2.1.1 Reagents. Trizol reagent, TaKaRa One Step RNA PCR Kit (AMV), PCR primers for VEGF-C and β -actin, probe for VEGF-C, VEGF-C rabbit anti-human polyclonal antibody, and S-P immunohistochemical staining kit (SP9001) were purchased from Invitrogen Corporation (USA); TaKaRa Biotechnology (Dalian) Co. Ltd Beijing AuGCT Biotechnology Co. Ltd (Beijing, China); Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd, respectively.

2.1.2 Clinical data. The resected specimens from 49 cases of ESCC were obtained from the Anyang Tumor Hospital, Henan, China, between September to November 2004. The carcinoma tissues and normal tissues were immediately placed in liquid nitrogen and pooled at -80°C until use for RT-PCR. Of 49 cases of ESCC, 25 were male, 24 were female, with a mean age of 58.3 years old, arranging from 44 to 76 years. All had not received any radiotherapy or chemotherapy. All the specimens were clearly identified by experienced pathologists. Routine pathological diagnosis showed that grade I, II and III were 14, 23 and 12 cases respectively. Among them, 20 cases presented lymph node metastasis, and 29 cases had no lymph node metastasis.

2.1.3 Cell culture. Human esophageal carcinoma EC9706 cell line was kindly provided by the Chinese Academy of Medical Sciences. The cell line was grown in monolayer culture containing humidified 50 ml/L CO_2 and 950 ml/L air at 37°C . It was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin (pH 7.2–7.4).

2.2 Methods

2.2.1 RNA isolation and RT-PCR. The total RNA was extracted from ESCC tissue samples stored at -80°C and EC9706 cell line by using Trizol reagent according to the procedures described in the kit. The quality of the isolated RNA was determined by gel electrophoresis, and the concentration and purity of RNA were determined by A260/280 ratios. Amplification of VEGF-C and β -actin as an internal control in each reaction was carried out by PCR with the following primer described previously^[3]. The primers of VEGF-C that yield 229bp and the sequences are 5'-AAG GAG GCT GGC AAC ATA AC-3' (forward) and 5'-CCA CAT CTG TAG ACG GAC AC-3' (reverse). The primers of β -actin that yield a 302 bp product as follows: 5'-TCC TCC CTG GAG AAG AGC TA-3' (forward), 5'-TCA GGA GGA GCA ATG ATC TTG-3' (reverse).

2.2.2 In situ hybridization (ISH). Sections ($6\ \mu\text{m}$) of the tissue and the prepared cell slides for hybridization were stained according to Chen *et al*^[4]. The probe

oligonucleotide for VEGF-C sequence was 5'-GTC ATG GAA TCC ATC TGT TGA GT-3' as described previously^[5]. Biotin-labeled VEGF-C cDNA anti-sense probe was modified by sulphur.

2.2.3 Immunohistochemistry (IHC). ESCC tissues were sectioned at the thickness of $4\ \mu\text{m}$. After deparaffinization with xylene and dehydration with graded ethanol, the tissue section and cell slides were incubated in PBS containing 30 ml/L H_2O_2 to remove endogenous peroxidases and then in PBS containing 0.1 mol/L citrate to saturate the nonspecific binding sites. After incubation with VEGF-C rabbit anti-human polyclonal antibodies at 1:150 dilution, the sections were treated with instant S-P immunohistochemical reagents and then incubated in a buffer solution containing 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 for visualization, followed by dehydration and mounting procedures. Microscopic examination of the sections was then performed. Omission of primary antibodies was used as a negative control.

2.2.4 Statistical analysis. All statistical calculations were carried out using SPSS 10.0 (SPSS Inc., Chicago, USA). Each data is presented as mean \pm SD. The Chi-square test or Student's *t* test was used to analyze data. *P* value of 0.05 or less were considered statistically significant.

3 Results

3.1 Expression of VEGF-C in EC9706 cell line

EC9706 cells expressed VEGF-C by immunohistochemical staining, ISH, and RT-PCR, respectively. The brown staining granules for VEGF-C were identified in the cytoplasm of EC9706 cells by IHC. VEGF-C mRNA (blue-purple granule) was located in cytoplasm of EC9706 cells by ISH (Figure 1).

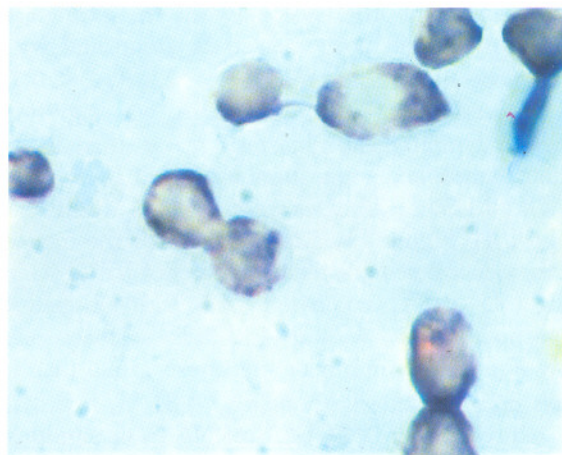


Figure 1. Expression of VEGF-C mRNA in EC9706 cells. There were blue-purple granules in cytoplasm. (ISH, $\times 1000$)

3.2 Relationship between the expression of VEGF-C and pathological features of ESCC

Positive staining was defined as the presence of VEGF-C immunoreactivity in at least 30% of tumor cells^[6]. Positive IHC for VEGF-C was observed in 36 of 49 cases of ESCC (Figure 2). Most of the metastatic lymph nodes showed positive staining for VEGF-C 18 of 20 (90.0%), compared with the cases of non-metastatic lymph node 18 of 29 (62.1%). There was a significant difference between the expression of VEGF-C in group with lymph node metastasis and group without lymph node metastasis ($\chi^2 = 4.7, P < 0.05$). The expression of VEGF-C in ESCC was significantly higher in lymph node metastasis group than in group without lymph node metastasis by IHC.

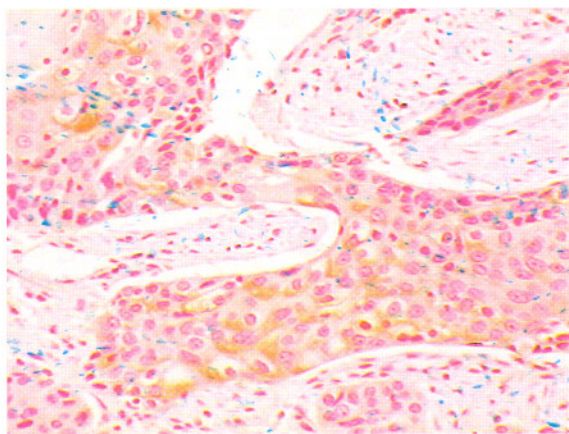


Figure 2. Expression of VEGF-C in ESCC. The brown stained granules for VEGF-C were identified in the cytoplasm of carcinoma cell (IHC, $\times 200$).

29 out of 49 cases of ESCC, VEGF-C mRNA was detected in tumor tissues by RT-PCR (Figure 3). There was a significant difference between the expression of VEGF-C in lymph node metastasis group and without lymph node metastasis group ($\chi^2 = 23.3, P < 0.01$). The expression of VEGF-C mRNA was significantly higher in lymph node metastasis group than in group without lymph node metastasis by RT-PCR.

Of 49 ESCC, 23 cases were detected VEGF-C mRNA by ISH (Figure 4). Most cases with the metastatic lymph node showed positively staining of VEGF-C by 19 of 20 (95.0%), compared with the non-metastatic lymph node group, 4 of 29 (13.8%). It was significantly higher in lymph node metastasis group than in no lymph node metastasis group ($\chi^2 = 31.3, P < 0.01$). The expression of VEGF-C mRNA was significantly higher in lymph node metastasis group than in non-metastasis lymph node group by ISH method. The expressions of VEGF-C were not significantly associated with age, gender, and pathological grade. Comparison

of VEGF-C positive expression levels in ESCC between clinicopathological features is shown in Table 1.

3.3 Correlation between the expression of VEGF-C by RT-PCR and ISH in ESCC

In present study, the detection results of VEGF-C mRNA by RT-PCR and by ISH were correlated ($\chi^2 = 18.5, P < 0.01$), but not different with each other ($\chi^2 = 2.5, P > 0.05$) (Table 2).

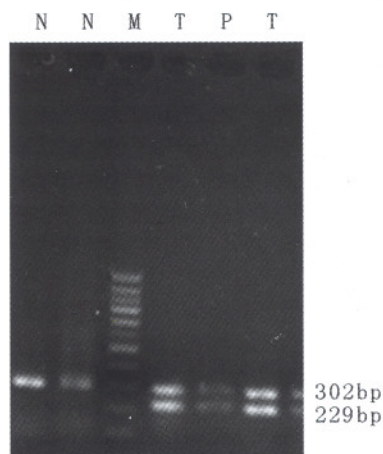


Figure 3. Expression of VEGF-C mRNA in ESCC tissues by RT-PCR. T: tumor; N: normal tissue; P: tissue adjacent to tumor; M: 100bp DNA marker.

4 Discussion

VEGF gene family is the only growth factor that is specific for vascular endothelial cells, and consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF. Among them, VEGF-C is a potent stimulator of lymphangiogenesis (the growth of lymphatic vessels) both *in vitro* and *in vivo*. Recently, it has been reported that VEGF-C is expressed in several solid tumors including gastric cancer^[5], lung cancer^[6], breast cancer, thyroid carcinoma, prostate cancer, cervical cancer and colorectal carcinoma^[7]. The over-expression of VEGF-C was relevant to lymphatic spread.

VEGF-C has been implicated in the regulation of tumor lymphangiogenesis and enhancement of lymphatic invasion. It was initially identified as a ligand of VEGF receptor-3 (VEGFR-3), which at the time was an "orphan" receptor that showed sequence similarity to VEGFR-2 and VEGFR-3. Because expression of VEGFR-3 is largely restricted to lymphatic endothelium, the major function of VEGF-C appears to be the regulation of lymphatic vessel growth^[1]. It is thought that VEGF-C plays a role in the maintenance of lymphatic endothelium, and over-expression of VEGF-C has been found to induce lymphatic endothelial proliferation in the skin of transgenic mice^[8]. These results indicate that VEGF-C

is a lymphangiogenic factor. Over-expression of VEGF-C transgenes in lab revealed a direct correlation between lymphangiogenesis and lymph-node metastasis^[9]. The majority of clinical studies showed a strong positive relationship between the expression of VEGF-C and lymph node metastasis. VEGF-C could promote growth of tumor cells, which was correlated with the growth of lymphatic vessels around tumors and the intralymphatic spread of cancer. The expression of VEGF-C in tumor cell is closely associated with lymph node metastasis^[10].

In the current study, VEGF-C could be positively detected by IHC, ISH and RT-PCR. The results correlated with highly metastatic EC9706 cell line's invasive character^[11]. In addition, the result demonstrated a positive correlation of VEGF-C expression with lymph node metastasis in ESCC. A strong correlation was found between VEGF-C protein and mRNA expression and metastasis in ESCC by IHC, ISH, and RT-PCR. The result was consistent with the previous reports^[6-10]. The expressions of VEGF-C were not significantly associated with age, gender, or pathological grade, which was different from Onogawa's research^[12] in gastric carcinoma and Hanrahan's report^[7] on col-

orectal cancer. It may be that VEGF-C expresses differently in different tissues. The cases that express VEGF-C are possibly a pre-clinical status. In contrast, cases with lymph node metastasis without VEGF-C expression are likely to belong to selective expressing of VEGF-C.

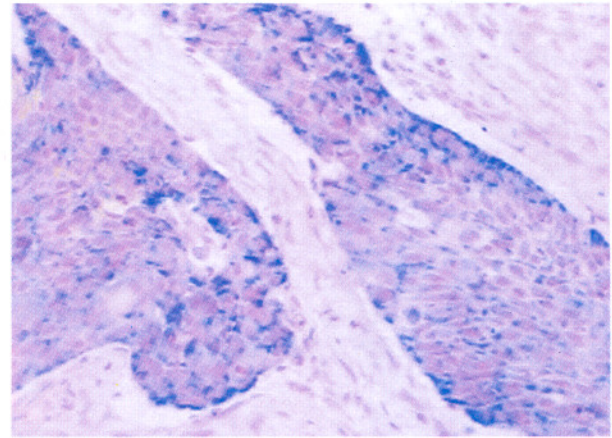


Figure 4. Expression of VEGF-C mRNA in ESCC. There were blue-purple granules in cytoplasm of carcinoma cells. (ISH, $\times 100$)

Table 1. Correlation between clinicopathological factors and the VEGF-C expression by 3 methods in ESCC

Characteristics	n	IHC			RT-PCR			ISH		
		(+)	(-)	P	(+)	(-)	P	(+)	(-)	P
Age (years)		64.5 \pm 4.4	61.8 \pm 4.6	>0.05 [□]	61.4 \pm 3.8	60.6 \pm 5.6	>0.05 [□]	60.8 \pm 4.4	59.6 \pm 5.7	>0.05 [□]
Gender				>0.05 [#]			>0.05 [#]			>0.05 [#]
M	25	20	5		16	9		13	12	
F	24	16	8		13	11		10	14	
Histological grade				>0.05 [#]			>0.05 [#]			>0.05 [#]
I	14	10	4		10	4		8	6	
II	23	17	6		12	11		10	13	
III	12	9	3		7	5		5	7	
Lymph node metastasis				<0.05 [#]			<0.01 [#]			<0.01 [#]
Positive	20	18	2		20	0		19	1	
Negative	29	18	11		9	20		4	25	

[□]Student's *t*-test; [#]Chi-square test

Table 2. Relationship between the expression of VEGF-C by RT-PCR and ISH

Variable	ISH (+)	ISH (-)	Total
RT-PCR(+)	21	8	29
RT-PCR(-)	2	18	20
Total (n)	23	26	49

Tumor tissues consist of multiple cell types including tumor cells and host, stromal, endothelial and infil-

trating cells. mRNA isolated by RT-PCR from bulk tissues represents the average amount of mRNA for all the cells in the sample and cannot determine if a specific mRNA is derived from normal or tumor cells. RNA from a few contaminating cells may be amplified during RT-PCR and obscure tumor specific alterations. RT-PCR analysis cannot reveal any site-dependent differential expression of VEGF-C. In contrast, ISH and IHC can identify the cellular source as well as reveal intratumor heterogeneity in expression. Comparison with the

two ways detecting VEGF-C mRNA, there was a correlation between RT-PCR and ISH, but no statistically difference between the two methods. So our finding suggests that the best way for detecting VEGF-C mRNA expression in ESCC tissue might be ISH.

In conclusion, this study has demonstrated that vascular endothelial growth factor-C may play a key role in tumor cell lymphatic metastasis. There is an association with expression of VEGF-C and lymph node metastasis in ESCC. VEGF-C may become a target for the treatment of ESCC by many methods^[15]. Furthermore the examination of VEGF-C may be useful in predicting lymph node metastasis of ESCC.

Acknowledgments

The authors wish to express their gratitude to Dr. Sanshen Zhang and his group members of Anyang Tumor Hospital for assistance in tissue collections.

References

1. Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK, Alitalo K. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* 1997; 276:1423-5.
2. Nathanson SD. Insights into the mechanisms of lymph node metastasis. *Cancer* 2003; 98:413-23.
3. Liu XE, Sun XD, Wu JM. Expression and significance of VEGF-C and FLT-4 in gastric cancer. *World J Gastroenterol* 2004; 10:352-5.
4. Chen KS, Zhang L, Tang L, Zhang YH, Gao DL, Yan L, Zhang L. Expression of heparanase mRNA in anti-sense oligonucleotide-transfected human esophageal cancer EC9706 cells. *World J Gastroenterol* 2005; 11:4916-7.
5. Yonemura Y, Fushido S, Bando E, Kinoshita K, Miwa K, Endo Y, Sugiyama K, Partanen T, Yamamoto H, Sasaki T. Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)-3 in gastric cancer. *Eur J Cancer* 2001; 37: 918-23.
6. Arinaga M, Noguchi T, Takeno S, Chujo M, Miura T, Uchida Y. Clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in patients with nonsmall cell lung carcinoma. *Cancer* 2003; 97: 457-64.
7. Hanrahan V, Currie MJ, Gunningham SP, Morrin HR, Scott PAE, Robinson BA, Fox SB. The angiogenic switch for vascular endothelial growth factor (VEGF)-A, VEGF-B, VEGF-C, and VEGF-D in the adenoma-carcinoma sequence during colorectal cancer progression. *J Pathol* 2003; 200:183-94.
8. Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K. Signaling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001; 20:1223-31.
9. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Jo. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001; 61:1786-90.
10. Stacker SA, Williams RA, Achen MG. Lymphangiogenic growth factor as markers of tumor metastasis. *APMIS* 2004; 112:539-49.
11. Han YL, Wei F, Xu X, Cai Y, Chen BS, Wang J, Xia SH, Hu H, Huang XP, Han YS, Wu M, Wang MR. Establishment and comparative genomic hybridization analysis of human esophageal carcinomas cell line EC9706. *Chin J Med Genet* 2002; 19:455-7.
12. Onogawa S, Kitadai Y, Amioka T, Kodama M, Cho S, Kuroda T, Ochiuni T, Kimura S, Kuwai T, Tanaka S, Chayama K. Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in early gastric carcinoma: correlation with clinicopathological parameters. *Cancer Lett* 2005; 226:85-90.
13. Stacker SA, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J* 2002; 16: 922-34.
14. Achen MG, McColl BK, Stacker SA. Focus on lymphangiogenesis in tumor metastasis. *Cancer Cell* 2005; 7:121-7.
15. Chen ZT, Varney ML, Backora MW, Cowan K, Solheim JC, Talmadge JE, Singh RK. Down-regulation of vascular endothelial growth factor-C expression using interfering RNA vectors in mammary spontaneous metastasis and enhances survival. *Cancer Res* 2005; 65:9004-11.