Expression of VEGF-C/VEGFR-3 in Human Laryngeal Squamous Cell Carcinomas

Zhongliang Wang[1], Yao Chen[1], Ruixiang Li[1], Chuanyu Liang[2], Bin Zhou[3], Lin Zhang[3]

[1] Department of Human Anatomy, West China of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041
[2] Department of Otolaryngology, West China Hospital, Sichuan University, Chengdu 610041
[3] Department of Forensic Biology, West China of Preclinical and Forensic Medicine, Sichuan University, Chengdu 610041

Abstract **Objective** To study the expression of vascular endothelial growth factor C (VEGF-C)and Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) in laryngeal squamous carcinoma and its relationship to lymph node metastasis. **Methods** VEGF-C and VEGFR-3 gene expression in 30 cases of normal laryngeal mucosa tissue(NLM), primary laryngeal squamous cells carcinomas (PLC) and cervical lymph nodes were examined by reverse transcription polymerase chain reaction (RT-PCR). **Results** Expression of VEGF-C and VEGFR-3 was different among NLM, PLC and cervical lymph nodes in the same patient. In PLC, Expression of VEGF-C/VEGFR-3 was significantly higher in lymph node positive group than in lymph node negative group and associated with histological grade of differentiation(P<0.05); Expression of VEGF-C and VEGFR-3 were not associated with age, sex, site, T stages (P>0.05). **Conclusion** A close correlation was found between VEGF-C/VEGFR-3 expression and lymph node metastasis in PLC. thus, VEGF-C appeared to play a vital role in the metastatic process of laryngeal carcinoma.

Key Words VEGF-C; VEGFR-3; Laryngeal squamous cell carcinomas; Lymph node metastasis

Vascular endothelial growth factor (VEGF) family are a polypeptide growth factors that have mitogenic activity specific for endothelial cells, they have the functions to enhance existent ability of endothelial cell, to promote mitochysis and the ability to increase chemotaxis and vascular permeability1. Vascular endothelial growth factor C(VEGF-C), one of several members of the VEGF family, is a relatively specific lymphangiogenic growth factor , it induces hyperplasia of the lymphatic vasculature selectively, increases the metastasis of cancer cells [2]. Vascular endothelial growth factor receptors (VEGFRs) are expressed in a variety of normal and cancer tissues. VEGFR-3 is a VEGF-C receptor with expression restricted to lymphatic endothelial cells. Several studies had demonstrated that VEGF-C, together with its major receptor VEGFR-3, were expressed in many types of human cancers[3,4], and might to act a paracrine fashion to regulate lymphangiogenesis[5-8]. Whereas, there were little research that the expression of VEGF-C and VEGFR-3 in the cancer tissues of head and neck still, especially the cancers of laryngeal carcinomas. The goal of the present study was to investigate the possible role of VEGF-C/VEGFR-3 activation in cancer progression and other pathologies in humans. For this purpose, we investigated the expression of VEGF-C and VEGFR-3 in primary laryngeal squamous cell carcinomas (PLC) and their relation with clinic pathologic features, and to reveal the relationship between VEGF-C/VEGFR-3 and biologic behaviors of laryngeal carcinomas.

Address for correspondence: Chen Yao, PhD,
Fax: (0086-028) 85501220
e-mail: drwzl@163.com

MATERIALS AND METHODS

Patients and Fresh Tissue Samples

Fresh tissue samples were obtained from 60 patiens undergone major surgical resection for PLC at the department of otolaryngology, the West China Hospital, Sichuan University, from August 2002 to March 2003. There were 58 males and 2 females with a median age of 64 years old (range 54~75 yrs). The patients presented no detectable metastases in distant organs at the time of surgery. None of the patients has previously received preoperative chemotherapy or radiotherapy. In each case, the portion of cancer tissues from surgery excision were immediately snap-frozen and stored in liquid nitro-

gen until use. In 30 cases, tissue samples of cervical lymph nodes were also available for analysis, of them, 20 cases with metastatic lymph nodes and 10 cases without metastatic lymph nodes. Histologically normal laryngeal mucosal tissues (NLM) was obtained in 30 cases and used as controls.

RNA isolation and RT-PCR Assay

Tissue samples (30 cases) were immediately frozen in liquid nitrogen and stored at −70°C until needed. RNA from tissue sample was isolated according to the TRIzol reagent protocol (Invitrogen). The concentrati on of the isolated RNA was determined by ultraviolet (UV) absorption spectrophotometry at a wavelength of 260nm. RNA quality was checked on an 1.5% agarose gel containing 0.5 μg ethidium bromide/ml (Fig.1).

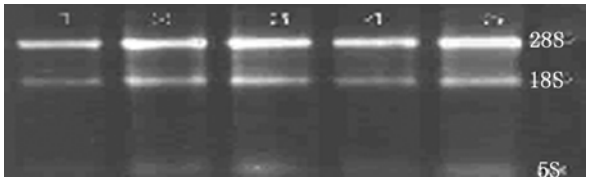

Fig.1 Representative results of mRNA in NLM, PLC and CLN(1.5% agarose gel eletrophoresis)
Note: 1. NLM; 2. PLC with lymphatic metastasis; 3. PLC without lymphatic metastasis; 4. LNM; 5. NLNM.

Primers pairs for studying expression of VEGF-C and its receptor VEGFR-3 were described (Table 1). Primer 1 for VEGFR-3 mRNA was used as reported by Shushanov et al [9], whereas other primers were designed using Primer Premier 5.0 Design software and checked against GenBank to avoid cross-reactivity with other known sequences. Subsequently, using the TaKaRa One Step RNA PCR Kit (AMV) protocol. A RT-PCR mixture was prepared in a total volume of 50μl. Reactive condition: reverse transcription at 50 ℃ for 30 min, inactivation of RTase at 94 ℃ for 2 min; denaturation at 94 ℃ for 30 sec, annealing at 56 ℃ for 30 sec, synthesis at 72 ℃ for 60 sec, cDNA were amplified over 32 cycles, then extension at 72 ℃ for 7min. After the amplification was completed, applied 3μl of the reactant for agarose gel electrophoresis to verify the amplified DNA fragment.

Statistical Analysis

Statistical analyses of the results by RT-PCR were performed using the chi-square test. $P<0.05$ was considered statistically significant between two groups.

RESULTS

The expression of VEGF-C and VEGFR-3 in 30 sample of human NLM, PLC and cervical lymph nodes were shown table 2 and table 3.

Form table 2, we found the expression of VEGF-C and VEGFR-3 was different in NLM and PLC (χ^2=11.14, χ^2=8.15, $P<0.01$), and also was different in NLM and cervical lymph nodes. (χ^2= 6.23, χ^2=6.53, $P<0.05$) which are significance in statistics; The expression of VEGF-C and VEGFR-3 was higher in LNM than in NLNM (χ^2=4.80, χ^2= 5.45, $P<0.05$), which is significance in statistics.

From table 3, we found the expression of VEGF-C was closely associated with lymph nodal metastasis and pathological types (χ^2=17.49, χ^2=9.46, $P<0.01$), and the expression of VEGFR-3 was associated with lymph nodal metastasis and pathological types also (χ^2=7.16, χ^2=5.44, $P<0.05$). But the expression of VEGF-C and VEGFR-3 was no associated with the age, sex, position and T stages respectively ($P>0.05$). The gene expression of VEGF-C and VEGFR-3 to see figure 2~5.

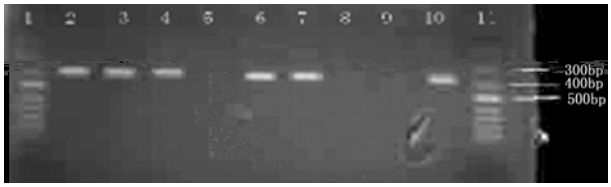

Fig.2 (Primer 1) amplification fragment at 380bp

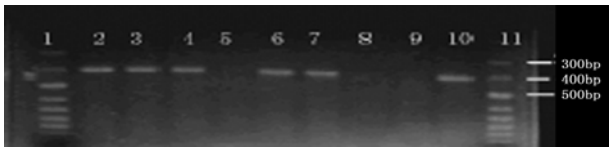

Fig3 (Primer 2) amplification fragment at 409bp

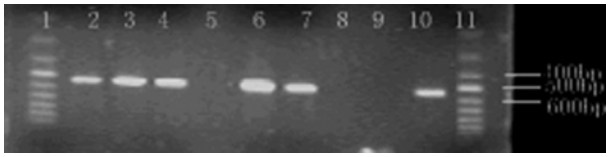

Fig.4 (Primer 3) amplification fragment at 557bp

Fig. 2~4 Representative results of RT-PCR analysis for the expression of VEGFR-3 in cases of NLM, PLC and CLN (1.5% agarose gel eletrophoresis)
Note: Lane 1, 11: Marker; Lane 2, 5, 8: NLM; Lane 3, 6, 9: PLC; Lane 4, 7, 10: CLN.

Table 1 Sequence of primer and site of segment

Gene			Sequence of primer	site of segment
VEGFR-3	Primer 1	Sense	5′-CCCACGCAGACATCAAGACG-3′	380bp
		Antisense	5′-TGCAGAACTCCACGATCACC-3′	
	Primer 2	Sense	5′-GCGGACTCCAACCAGAAG -3′	409bp
		Antisense	5′- AGGCTTCCACCACCTTCC -3′	
	Primer 3	Sense	5′- CCTGAAAGCATCTTCGAC -3′	557bp
		Antisense	5′- GAGCCTTTGTAGGTCGTT -3′	
VEGF-C		Sense	5′-CATGAACACCAGCACGAG -3′	464bp
		Antisense	5′-ATTGGCTGGGGAAGAGTT -3′	

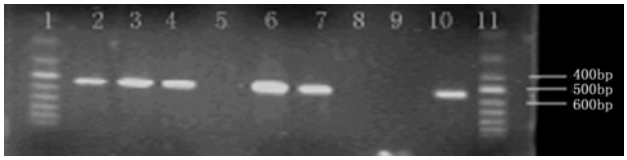

Fig.5 amplification fragment at 464bp

Fig.5 Representative results of RT-PCR analysis for the expression of VEGF-C in cases of NLM, PLC and CLN (1.5% agarose gel eletrophoresis)
Note: Lane 1, 11: Marker; Lane 2, 5, 8: NLM; Lane 3, 6, 9: PLC; Lane 4, 7, 10: CLN.

Table 2 The expression of VEGF-C and VEGFR-3 in NLM, PLC and CLN

Tissue type	Cases	VEGF-C		VEGFR-3	
		Positive	Negative	Positive	Negative
NLM	30	10	20	8	22
PLC	30	23	7	19	11
CLN					
metastasis	20	16	4	12	8
No metastasis	10	4	6	10	0

Note: CLN: cervical lymph nodes

Table 3 The expression of VEGF-C and VEGFR-3 in PLC

Item	VEGF-C		VEGFR-3	
	Positive	Negative	Positive	Negative
Sex/gender				
men	22	6	18	10
women	1	1	1	1
Age				
≤60	3	1	3	1
>60	20	6	16	10
Position				
over glottis category	12	3	9	6
glottis category	5	2	5	2
cross glottis category	6	2	5	3
T stages				
T_1 stages	3	1	2	2
T_2 stages	8	2	6	4
T_3 stages	12	4	11	5
Pathological Types				
well-differentiated	1	3	0	4
moderately-differentiated	5	2	5	2
poorly-differentiated	17	2	14	5
Lymph node				
metastasis	20	0	16	4
no metastasis	3	7	3	7

DISCUSSION

VEGF family is only growth factor specific to vascular endothelial cell, which consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PIGF [10-12]. VEGF-C is the chief lymphangiogenic factor, it promotes lymphatic endothelial cell proliferation and lymphatic vessels hyperplasia through activating VEGFR-3 or VEGFR-2 on the membrane of endothelial cell. Overexpression of VEGF-C in the skin of transgenic mice induces lymphatic proliferation and vessel enlargement[15]. In adults, it become weakened distinctly that the combination of VEGF-C and VEGFR-2 promoting lymphatic endothelial cell proliferate activity. Thus, mostly through VEGFR-3, VEGF-C can enhance proliferation of lymphatic vessels. VEGFR-3 is a regulate factor of lymphatic vessels, and mostly expressed in lymphatic endothelium of human adult tissues[17]. Currently, people had commonly considerd that VEGF-C could activate VEGFR-3 to induce lymphogeosis.

VEGF-C had been detected in variety human cancers and associated closely with lymph nodal metastasis and lymphogenesis [13-15]. However, few people had studied the expression of VEGF-C in cancer tissue of head and neck especially in human PLC. and so far, there were no reports on the investigation of VEGFR-3 in PLC.

Our study showed that VEGF-C and VEGFR-3 could selectively expressed in the PLC, expression of VEGF-C and VEGFR-3 were significantly higher in lymph node positive group than in lymph node negative group and associated with histological grade of differentiation. It suggested, in PLC, the lymph node metastasis and differentiate degree of cancers both were important prognosis factor. Our results had proved that there wer-C/VEGFR-3 and the ratio of lymphtic metastasis.

We had also detected that the expression of VEGF-C was significantly higher in lymph node tissue with Cancer cell infiltration than that without Cancer cell infiltration, it show that VEGF-C was secreted from cancer cells; whereas the expression of VEGFR-3 was significantly lower in lymph node tissue with metastasis cancer cell than that no metastasis, the mechanism maybe was the cancer cells destroyed lymphatic cell of lymph node tissue. In 20 cases of PLC with lymph nodal metastasis, the results of whole VEGF-C expression were positive, this was similar as reported by Yang et al[16], the results revealed that there were correlated with the levels of VEGF-C expression and lymphatic metastasis of carcinomas. In the process of cancer growth and metastasis, cancer cells could secreted VEGF-C. We found a prominently correlation between the levels of VEGFR-3 and VEGF-C expression in primary cancers tissues, this had also been demonstrated in gastric cancers [19]. It suggests that VEGF-C possible through bonding VEGFR-3 to increase invasive ability of cancer cells, and through araeosis lymphatic endothelial cell to make the bonding sites multiply of cancer cells and lymphatic vessels, Thus, cancer cells easy infiltrating lymphatic vessels and infiltrating lymph nodes eventually.

Our results revealed that there were closely relationship between VEGF-C/VEGFR-3 and cancer cells infiltration and lymphatic metastasis, namely, VEGF-C/VEGFR-3 could be an important factor regulating the mutual paracrine relationships between cancer cells and endothelial cells in lymphatic vascular metastases, participated in regulating hyperplasia and permeability of lymphatic vessels and influencing metastatic fashion of cancer cells. This research would provide a new thought to early diagnosis and theraphy of laryngeal carcinomas.

REFERENCES

1. Veikkola T, Alitalo K. VEGFs receptors and angiogenesis. Scmin Cancer Biol, 1999, 9(3): 211-220.
2. Wang Zhongliang, Chen Yao, Li Ruixiang. Advanced in the researches for Vascular Endothelial Growth Factor (VEGF)-C and Vascular Endothelial Growth Factor Receptor (VEGFR)-3. China J Cancer Prev Treat, 2002, 9(Suppl 5): 454-457.
3. Pajusola K, Aprelikova O, Korhonen J, et al. FLT4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. Cancer Res, 1992, 52(20): 5738-5743
4. Salven P, Lymboussaki A, Heikkila P, et al.Vascular endothelial growth factors VEGF-B and VEGF-C are expressed in human cancers. Am J Pathol, 1998, 153(1): 103-108
5. Jeltsch M, Kaipainen A, Joukov V, et al. Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. Science, 1997, 276(5317): 1423-1425.
6. Oh SJ, Jeltsch MM, Birkenhager R, et al. VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. Dev Biol, 1997, 188(1): 96-109.
7. Kaipainen A, Korhonen J, Mustonen T, et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl

Acad Sci U S A, 1995, 92(8): 3566–3570.

8. Kukk E, Lymboussaki A, Taira S, et al.VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. Development, 1996, 122(12): 3829–3837.
9. Shushanov S, Bronstein M, Adelaide J.VEGFc and VEGFR3 expression in human thyroid pathologies. Int J Cancer, 2000, 86 (1): 47–52.
10. Joukov V, Pajusola K, Kaipainen A, et al. A new vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4(VEGFR-3) and KDR (VEGFR–2) receptor tyrosine kinases. EMBO J. 1996; 15(2): 290–298.
11. Eriksson U, Alitalo K. Structure expression and receptor–binding properties of novel vascular endothelial growth factors. In: Claesson-Welsb L, editor. Vascular growth factors and angiogenesis. Berlin: Springer–Verlag, 1999, 41.
12. Ogawa S, Oku A, Sawano A, et al. A novel type of vascular endothelial factor, VEGF–E(NA–7 VEGF), preferentially utilizes KDR/Flt–1 receptor and carries a potent mitotic activity without beparin–binding domain. J Biol Chem.1998; 273(47): 31273–31282.
13. Fellmer PT, Sato K, Tanaka R, et al. Vascular endothe–lial growth factor-C gene expression in papillary and follicular thyroid carcinomas. Surgery, 1999, 126(6): 1056–1061.
14. Tsurusaki T, Kanda S, Sakai H, et al. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer, 1999, 80(1–2): 309–313.
15. WANG Zhong–liang, CHEN Yao, ZHOU Bin, et al. Expression of Vascular Endothelial Growth Factor C in Laryngeal Squamous Carcinoma and Its Role in Lym phatic Metastasis. J Sichuan univ (Med Sci Edi). 2004, 35(1): 47–49.
16. Yang yan, Ge Rong ming. Expression of VEGF and VEGF-C in laryngeal carcinoma and their clinical significance. J Clin Otorhinolaryngol (China), 2002, 16 (12): 650–652.
17. Yonemura Y, Fushida S, Bando E, et al. Lymphangiogenesis and the vascular endothelial growth factor receptor (VEGFR)–3 in gastric cancer. Eur J Cancer, 2001, 37(7): 918–923.