RESEARCH ARTICLE

VEGF-C and VEGF-D Expression and its Correlation with Lymph Node Metastasis in Esophageal Squamous Cell Cancer Tissue

Zeng Yang[1]*, Yong-Gang Wang[2], Kai Su[2]

Abstract

Background: To explore vascular endothelial growth factor C (VEGF-C) and VEGF-D expression and its correlation with lymph node metastasis in esophageal squamous cell cancer (ESCC) tissue. Materials and Methods: Immunohistochemical methods were applied to detect the levels of VEGF-C and VEGF-D expression in 64 surgicall removal ESCC tissues, tissues adjacent to cancer and normal tissues, and the relationship between VEGF-C and VEGF-D expression and lymph node metastasis was analyzed. Results: Both VEGF-C and VEGF-D were expressed by varying degrees in esophageal cancer tissue, the tissue adjacent to cancer and normal tissue, and the positive expression rate went down successively. The positive expression rates of VEGF-C (59.4%) and VEGF-D (43.8%) in esophageal cancer tissue were significantly higher than in the tissue adjacent to cancer (34.4%, 15.6%) and normal tissue (20.3%, 12.5%), respectively, in which significant differences were manifested ($p<0.01$). Positive expression rates of VEGF-C and VEGF-D in esophageal cancers with lymph node metastasis were markedly higher than without such metastasis ($p<0.01$), while those in the tissue with TNM staging I~II were markedly lower than that with TNM staging III~IV ($p<0.01$). Conclusions: Both VEGF-C and VEGF-D are highly expressed in ESCC tissue, which may be related to the lymph node metastasis of cancer cells. Hence, VEGF-C and VEGF-D can be clinically considered as important reference indexes of lymph node metastasis in esophageal cancer.

Keywords: Esophageal SCC - vascular endothelial growth factor C - VEGF D - lymph node metastasis

Asian Pac J Cancer Prev, **16 (1)**, 271-274

Introduction

The important malignant behavior of esophageal cancer is lymph node metastasis of cancer cells through lymphatic ducts, whereas the lymph node metastasis directly affects treatment methods, local recurrence and long-term survival. In recent years, studies on lymphangiogenesis mechanism and regulatory factors in cancer tissue have been a hotspot in tumor research. Some studies have revealed that vascular endothelial growth factor C (VEGF-C) promotes the lymphangiogenesis by action on its corresponding receptor VEGFR-3 (Chang et al., 2014; Omoto et al., 2014). VEGF-D, a sort of expressive secretory dimer glycoprotein induced by C-FOS gene, is a lately-discovered growth factor of lymphatic ducts, which can promote tumor lymphangiogenesis and is intimately related to lymphatic metastasis of tumor cells (Sun et al., 2012; He et al., 2013). At present, there are few studies on VEGF-C and VEGF-D expression in esophageal cancer, especially on the lymph node metastasis of esophageal cancer. Hence, this study detected VEGF-C and VEGF-D expression in esophageal squamous cell cancer (ESCC) tissue and investigated its relationship with lymph node metastasis so as to provide new ideas for early diagnosis, blocking tumor cell infiltration and metastasis of esophageal cancer.

Materials and Methods

General materials

Sixty-four patients with complete clinical and pathological files who underwent ESCC radical operation in Cancer Hospital were collected from Jun., 2012 to Jun. 2014. Before operation, all patients were not given radiotherapy, chemotherapy and immunotherapy. There were 44 males and 20 females at the age of 42~74, with the median age of 65. Thirty cases encountered lymph node metastasis, 34 cases not. High, middle and low differentiation were 14, 33 and 17 cases, respectively. According to infiltration depth, there were 15, 17, 24 and 8 cases at T_1, T_2, T_3 and T_4, respectively. All samples were fixed by 10% neural formalin, dehydrated routinely, embedded by paraffin, cut into 4 μm slices and confirmed by pathological histology. Specific methods for collecting

[1]*Department of Thoracic Surgery, Beijing Tian Tan Hospital, Capital Medical University,* [2]*Department of Thoracic Surgery, Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China* **For correspondence: ttyyyz@sina.com*

materials: *i)* normal tissue: the incisal edge more than 5 cm far away from cancer lesion edge; *ii)* esophageal cancer tissue: the tissue from cancer lesion edge to the inside; *iii)* tissue adjacent to cancer: the tissue 2 cm away from cancer lesion edge to the outside.

Both VEGF-C and VEGF-D polyclonal antibodies were purchased from American Santa Cruz Company, trypsin and ready-to-use SABC immumohistochemical staining kits from Beijing Zhongshan Biotechnology Co., Ltd, the working solution of immumohistochemical secondary antibodies and DAB kits from Shanghai Changdao Biological Reagent Company.

Methods

Under the working concentration of 1:50, VEGF-C was conducted antigen retrieval at 37°C for 10 min through trypsin. Under the working concentration of 1:100, VEGF-D was repaired at high pressure for 5 min by 0.01 mol/L citrate buffer solution (pH=4.0). All primary antibodies stayed overnight at 4°C, and the working solution of secondary antibodies was incubated in an incubator at 37°C for 30 min. DAB and hematoxylin were respectively used for coloration and counterstaining. Phosphate buffer solution (PBS) was regarded as negative control instead of primary antibodies, and the known positive sections as positive control. Additionally, the staining procedures were performed according to SABC kit instructions.

Result evaluation

Evaluation on VEGF-C and VEGF-D immumohistochemical results: Under high power lens (×200), 5 visual fields were randomly observed in each piece of section. At first, the points were scored (0 point: hardly coloration; 1 point: canary; 2 points: claybank; 3 points: sepia) according to specific staining degrees emerging from the cytoplasm. Next, the points were scored again (0 point: colored cell number<1%; 1 point: colored cell number being 1%~10%; 2 points: colored cell number being 11%~50%; 3 points: colored cell number >50%) according to the percentage of colored cells occupying cell count. At last, the product of these two items was immumohistochemical point of this visual field, and the mean point of 5 visual fields was immumohistochemical score of one case, in which 0~2, 3~4, 5~7 and 8~9 points were respectively represented by (-), (+), (++) and (+++).

Statistical analysis

SPSS13.0 statistical software was applied to analyze the data statistically. The enumeration data and measurement data expressed by mean±standard deviation were compared respectively by x^2 and t tests. $p<0.05$ was considered to be statistically significant.

Results

Comparison of VEGF-C expression in three tissues

VEGF-C was expressed in esophageal cancer tissue, tissue adjacent to cancer and normal tissue by varying degrees. The positive granules of immumohistochemical staining were mainly located in tumor cell cytoplasm, and were from canary to claybank.

The positive expression rate of VEGF-C in esophageal cancer (59.38%) was markedly higher than in the tissue adjacent to cancer (34.37%) and normal tissue (20.29%), and the differences had statistical significance ($p<0.01$). In addition, the strongly-positive (+++) expression rate of VEGF-C in esophageal cancer tissue was similarly significantly higher than in the tissue adjacent to cancer and in the normal tissue ($p<0.05$ or $p<0.01$), and the moderately-positive (++) expression rate conspicuously higher than in normal tissue ($p<0.05$). However, there was no significant difference by comparison to its weakly-positive (+) expression rates in three groups ($p>0.05$) (Table 1).

Comparison of VEGF-D expression in three tissues

VEGF-D was expressed in esophageal cancer tissue, tissue adjacent to cancer and normal tissue to different extent. The positive granules of immumohistochemical staining were primarily distributed in the cytoplasm of tumor cells or normal epithelial cells, and were from canary to claybank.

The positive expression rate of VEGF-D in esophageal cancer (43.75%) was notably higher than in the tissue adjacent to cancer (15.36%) and normal tissue (12.50%), in which significant differences were presented ($p<0.01$). The strongly and moderately-positive expression rates of VEGF-D in esophageal cancer tissue were markedly higher than in the tissue adjacent to cancer and normal tissue ($p<0.01$), whereas there was no such significant difference by comparison to its weakly-positive expression rates in three groups ($p>0.05$) (Table 2).

Table 1. Comparison of VEGF-C Expression in three Tissues [n(%)]

Groups	-	+	++	+++	Positive rate (%)
Esophageal carcinoma tissue	26 (40.63)	7 (10.94)	18 (28.13)#	13 (20.31)*##	59.38**##
Tissue adjacent to carcinoma	42 (65.63)	6 (9.36)	12 (18.75)	4 (6.25)	34.37
Normal tissue	51 (87.50)	4 (6.25)	7 (10.94)	2 (3.13)	20.29

Compared with the tissue adjacent to cancer, *$p<0.05$, **$p<0.01$; Compared with normal tissue, #$p<0.05$, ##$p<0.01$

Table 2. Comparison of VEGF-D Expression in three Tissues [n(%)]

Groups	-	+	++	+++	Positive rate (%)
Esophageal carcinoma tissue	36(56.25)	6(9.36)	13(20.31)**##	9(14.06)**##	43.75**##
Tissue adjacent to carcinoma	54(84.38)	6(9.36)	3(4.69)	1(1.56)	15.63
Normal tissue	56(87.50)	5(7.81)	2(3.13)	1(1.56)	12.50

Compared with the tissue adjacent to cancer, **$p<0.01$; Compared with normal tissue, ##$p<0.01$.

Table 3. Correlation Between VEGF-C, VEGF-D Expression and Lymph Node Metastasis [n(%)]

Clinical pathological parameters		n	Positive VEGF-C	Positive VEGF-D
Lymph node metastasis	Yes	30	25 (83.33)**	21 (70.00)**
	No	34	13 (38.24)	7 (20.59)
TNM staging	I~II	44	21 (47.73)##	13 (29.55)##
	III~IV	20	17 (85.00)	15 (75.00)

*Compared with the tissue without lymph node metastasis, **p<0.01; Compared with phase III~IV tissue, ##p<0.01

Correlation between VEGF-C, VEGF-D expression and lymph node metastasis

The positive expression rates of VEGF-C and VEGF-D in the tissue with lymph node metastasis were notably higher than that without lymph node metastasis, while those in the tissue with TNM staging being I~II markedly lower than that with TNM staging being III~IV. Both differences had statistical significance (p<0.01) (Table 3).

Discussion

As one of clinical commonly-encountered malignant tumors, esophageal cancer threatens the human health seriously. Lymphatic drainage directly enters into thoracic ducts through regional lymph nodes because there is no serosa, but abundant lymphatic drainage in esophageal walls, which makes esophageal caner occur lymph node metastasis at an early stage. A lot of studies indicated that the survival rate of patients with lymph node metastasis was evidently lower than those without lymph node metastasis (Feng et al., 2013; Hsu et al., 2013; Sui et al., 2014). Therefore, it is of great importance to research the lymphatic metastasis mechanism of cancer cells.

It is found that lymphangiogenesis is related to lymphatic metastasis with further studies made. In recent years, the regulatory factor VEGF which is associated with lymphangiogenesis has been a hotspot. As one member of platelet-derived growth factor family, VEGF is the most important vascular endothelial growth stimulating factor in the process of tumor angiogenesis. There are a few studies that have demonstrated that the growth factors of lymphatic ducts (VEGF-C and VEGF-D) and its generation are related to cancer metastasis and poor prognosis (Jiang et al., 2012; Karaman et al., 2014). By expressing VEGF-C and VEGF-D, tumor cells can induce lymphangiogenesis and promote lymphatic metastasis. VEGF-C, a new member of VEGF family, is expressed in most of tumors. It can promote endothelial cell proliferation and increase vascular permeability. As a key factor of tumor angiogenesis, infiltration and metastasis, VEGF-C has a special function in enhancing lymphangiogenesis (Kostis et al., 2014). Being highly homologous with VEGF-C, VEGF-D overexpression in dermal keratinocytes has the effect of lymphangiogenesis (Trompezinski et al., 2004). With the action of paracrine mode, VEGF-D makes receptors autophosphorylation by VEGF-D/VEGFR-3 pathways, stimulates lymphatic endothelial cell proliferation and migration of tumors and promotes lymphangiogenesis through the signal transduction in cytoplasm, consequently increasing the incidence of lymphatic metastasis of tumors. The research confirmed that VEGF-D could not only induce lymphangiogenesis in tumor tissues, but also cause the tumor cell diffusion to regional lymph nodes (Kawai et al., 2003; Harris et al., 2013).

This study adopted immunohistochemical method to detect VEGF-C and VEGF-D expression in esophageal cancer tissue, tissue adjacent to cancer and normal tissue, and the results showed that both VEGF-C and VEGF-D were expressed differently in esophageal cancer tissue, tissue adjacent to cancer and normal tissue. The positive reaction of VEGF-C primarily occurred in cytoplasm of tumor cells, while that of VEGF-D mainly in cytoplasm of tumor cells or normal epithelial cells. Both positive and strongly-positive expression rates of VEGF-C in esophageal cancer were markedly higher than in the tissue adjacent to cancer and normal tissue, and moderately-positive expression rate conspicuously higher than in normal tissue and slightly higher than in the tissue adjacent to cancer, indicating that VEGF-C is highly expressed in esophageal cancer tissue, and expression intensities (moderately and strongly positive) are higher than in the tissue adjacent to cancer and normal tissue. The positive, strongly and moderately-positive expression rates of VEGF-D in esophageal cancer were significantly higher than in the tissue adjacent to cancer and normal tissue, illustrating that VEGF-D is highly expressed in esophageal cancer tissue, and expression intensities (moderately and strongly positive) are higher than in the tissue adjacent to cancer and normal tissue. Additionally, the positive expression rates of VEGF-C and VEGF-D in the tissue with lymph node metastasis were notably higher than that without lymph node metastasis, while those in the tissue with TNM staging being I~II markedly lower than that with TNM staging being III~IV, suggesting that high expression of VEGF-C and VEGF-D may be related to the lymph node metastasis and clinical staging of esophageal cancer.

To sum up, both VEGF-C and VEGF-D are highly expressed in esophageal cancer tissue, which may be associated with the lymph node metastasis and clinical staging of esophageal cancer. Hence, high expression of VEGF-C and VEGF-D can be considered as important reference indexes of lymph node metastasis in esophageal cancer, whereas it clinically manifests to be related to TNM staging.

References

Chang YW, Su CM, Su YH, et al (2014). Novel peptides suppress VEGFR-3 activity and antagonize VEGFR-3-mediated oncogenic effects. *Oncotarget*, **5**, 3823-35.

Feng JF, Zhao Q, Chen QX (2013). Prognostic value of subcarinal lymph node metastasis in patients with esophageal squamous cell carcinoma. *Asian Pac J Cancer Prev*, **14**, 3183-6.

Harris NC, Davydova N, Roufail S, et al (2013). The propeptides of VEGF-D determine heparin binding, receptor

heterodimerization, and effects on tumor biology. *J Biol Chem*, **288**, 8176-86.

He L, He J, Zhao X (2013). Expression of VEGF-D in epithelial ovarian cancer and its relationship to lymphatic metastasis. *Asia Pac J Clin Oncol*, [Epub ahead of print].

Hsu PK, Huang CS, Wang BY, et al (2013). Survival benefits of postoperative chemoradiation for lymph node-positive esophageal squamous cell carcinoma. *Ann Thorac Surg*, **97**, 1734-41.

Jiang JT, Zhang LF, Zhou B, et al (2012). Relationships of uPA and VEGF expression in esophageal cancer and microvascular density with tumorous invasion and metastasis. *Asian Pac J Cancer Prev*, **13**, 3379-83.

Karaman S, Detmar M (2014). Mechanisms of lymphatic metastasis. *J Clin Invest*, **124**, 922-8.

Kawai Y, Takeda A, Gershenwald JE (2003). Effects of vascular endothelial growth factor D on the signaling cascade of sentinel lymphatic endothelial cells from melanoma patients undergoing sentinel lymphadenectomy. *Gan To Kagaku Ryoho*, **30**, 2141-4.

Kostis G, Ioannis L, Helen K, et al (2014). The expression of vascular endothelial growth factor-C correlates with lymphatic microvessel density and lymph node metastasis in prostate carcinoma: an immunohistochemical study. *Urol Ann*, **6**, 224-30.

Omoto I, Matsumoto M, Okumura H, et al (2014). Expression of vascular endothelial growth factor-C and vascular endothelial growth factor receptor-3 in esophageal squamous cell carcinoma. *Oncol Lett*, **7**, 1027-32.

Sui P, Hu P, Zhang T, et al (2014). High expression of CXCR-2 correlates with lymph node metastasis and predicts unfavorable prognosis in resected esophageal carcinoma. *Med Oncol*, **31**, 809.

Sun JJ, Jing W, Ni YY, et al (2012). New model of in-situ xenograft lymphangiogenesis by a human colonic adenocarcinoma cell line in nude mice. *Asian Pac J Cancer Prev*, **13**, 2823-8.

Trompezinski S, Berthier-Vergnes O, Denis A, et al (2004). Comparative expression of vascular endothelial growth factor family members, VEGF-B, -C and -D, by normal human keratinocytes and fibroblasts. *Exp Dermatol*, **13**, 98-105.