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Vascular endothelial growth factor C (*VEGF-C*) in esophageal cancer correlates with lymph node metastasis and poor patient prognosis

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Abstract

Background: The diagnosis of lymph node metastasis in esophageal cancer by the presence and number of metastatic lymph nodes is an extremely important prognostic factor. In addition, the indication of non-surgical therapy is gaining more attention. Vascular endothelial growth factor C (*VEGF-C*) is potentially lymphangiogenic and selectively induces hyperplasia of the lymphatic vasculature. In this study, we investigated the expression of *VEGF-C* and whether it correlated with various clinico-pathologic findings.

Methods: KYSE series of esophageal cancer cell lines and 106 patients with primary esophageal squamous cell carcinomas who had undergone radical esophagectomy were analyzed. *VEGF-C* mRNA expression was determined by quantitative RT-PCR.

Results: High expression of *VEGF-C* was detected in most of the KYSE cell lines, especially KYSE410, yet, in an esophageal normal epithelium cell line, Het-1A, *VEGF-C* was not detected. In the clinical specimen, the expression of *VEGF-C* in the cancerous tissue was higher than in the corresponding noncancerous esophageal mucosa ($p = 0.026$). The expression of *VEGF-C* was found to be higher in Stage2B-4A tumors than in Stage0-2A tumors ($p = 0.049$). When the patients were divided into two groups according to their expression levels of *VEGF-C* (a group of 53 cases with high expression and a group of 53 cases with low expression), the patients with high *VEGF-C* expression had significantly shorter survival after surgery than the patients with low expression ($p = 0.0065$). Although univariate analysis showed that high expression of *VEGF-C* was a statistically significant prognostic factor, this was not shown in multivariate analysis. In the subgroup of patients with Tis and T1 tumors, the expression of *VEGF-C* was higher in N1 tumors than in N0 tumors ($p = 0.029$). The survival rate of patients from the high expression group ($n = 10$) was lower than that in the low expression group ($n = 11$), and all the patients in the low *VEGF-C* expression group survived.

Conclusions: The expression of *VEGF-C* correlates with lymph node metastasis and poor prognosis. In patients with Tis and T1 esophageal tumors, the expression of *VEGF-C* may be a good diagnostic factor for determining metastasis of the lymph node.

Background

Esophageal cancer is a disease with poor prognosis. Of many prognostic factors, the metastatic lymph nodes are one of the most significant. To avoid highly invasive surgery, endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), chemoradiotherapy, and

their combinations have been suggested for patients with early esophageal cancer. When applying these non-surgical treatments, preoperative diagnosis of tumor invasion and lymph node metastasis becomes especially important. Unfortunately, computed tomography (CT) and positron emission tomography (PET) are unable to diagnose lymph node metastasis accurately. In order to develop plans for new diagnoses and treatment, it is essential that the biological behavior of esophageal cancer be understood. Recent studies have revealed that several

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genes and molecules are involved in the origin and/or progression of esophageal cancer, including *TP53* [1,2], *deleted in esophageal cancer 1(DEC1)* [3], *deleted in colorectal cancer (DCC)* [4], *deleted in lung cancer 1(DLC1)* [5], *cyclinD1* [6,7], *transforming growth factor-beta receptor type II (TGFBRII)* [8], *adenomatous polyposis coli (APC)* [9,10], *survivin* [11], and *murine double minute 2 (MDM2)* [12]. However, the precise mechanisms that underlie the development and progression of esophageal squamous cell cancer (ESCC) are far from clear. VEGF-C has been characterized as a lymphangiogenic and angiogenic growth factor and has been shown to signal through the receptors VEGFR-3 (also called Flt-4) and VEGFR-2 [13]. In this paper, we report the relationship between the expression of VEGF-C, the clinico-pathological factors, and the prognosis of patients with ESCC.

Materials and methods

Cell lines and tissue samples

Samples were obtained from 106 patients (87 males and 19 females) with ESCC who had undergone radical esophagectomy at the Department of Surgery II, Nagoya City University Hospital, between 1996 and 2005. The study design was approved by the Institutional Review Board of our university, and written consent was obtained from all patients. Tumors were classified according to UICC[14]. All samples were frozen immediately in liquid nitrogen and stored at -80°C until use. Characteristics of the 106 patients with ESCC are shown in Table 1. The SV40-immortalized esophageal cell line Het-1A was purchased from the American Type Culture Collection (Manassas, VA, USA). KYSE series was obtained from the DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). KYSE esophageal cancer cells were plated in tissue culture dishes and grown in RPMI-1640 medium (Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (JRH Bioscience, Kansas, USA), at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Het-1A cells were grown in LHC-9 serum-free medium (Biofluids, Rockville, MD, USA) in tissue culture dishes at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

RNA extraction and RT-PCR analysis

Total RNA was extracted from esophageal cancer tissue, and from corresponding noncancerous esophageal mucosa taken from apparently normal mucosa as far away from the tumor as possible, using an Isogen kit (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Total RNA was extracted from the cell lines in the same way. The concentration of total RNA was adjusted to 200 ng/ml using a spectrophotometer. The reverse transcription reaction was performed using 1

Table 1: Relationship between clinicopathological factors and mRNA expression of VEGF-C

		VEGF-C expression			
		case	mean	± sd	p-value
age	^65	44	-0.074	± 0.6	0.73
	< 65	62	0.16	± 0.66	
gender	male	87	0.066	± 0.65	0.06
	female	19	0.037	± 0.63	
Tfactor	Tis	5	-0.021	± 0.14	0.8
	T1	12	0.11	± 0.34	
	T2	11	-0.098	± 0.42	
	T3	33	-0.038	± 0.7	
	T4	17	0.218	± 1.0	
Tis, T1 vs T2-T4					0.8
Nfactor	N0	29	-0.049	± 0.37	0.28
	N1	77	0.1	± 0.72	
Stage	Stage0	6	-0.23	± 0.14	0.049
	Stage1	6	-0.072	± 0.35	
	Stage2A	13	-0.09	± 0.31	
	Stage2B	17	0.061	± 0.47	
	Stage3	30	0.085	± 0.66	
	Stage4	11	-0.19	± 1	
	Stage4A	23	0.34	± 0.73	
Stage0-2A vs Stage2B-4A					0.049
Histological Type					0.34
	well	41	0.092	± 0.57	
	moderate	56	0.053	± 0.75	
	poor	9	-0.087	± 0.19	
well vs moderate · poor					0.34
lymphatic invasion					0.61
	positive	69	0.056	± 0.72	
	negative	37	0.07	± 0.47	
vein invasion					0.22
	positive	54	0.024	± 0.78	
	negative	52	0.098	± 0.47	

The expression of VEGF-C is higher in Stage2B-4A patients than in Stage0-2A patients

μg of total RNA, 0.5 μg of oligo (dT) primer and Superscript II enzyme (Gibco BRL, Gaithersburg, MD, USA), for 60 min at 37°C, followed by 10 min 90°C and 10 min at 70°C.

TaqMan gene expression assay

Gene expression in all samples was measured by quantitative RT-PCR using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR was performed in a 20 μl reaction mixture containing 10 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 80 nM of each primer, 2 nM of probe, and 2 μl of cDNA sample. The thermal cycling conditions included an initial denaturation step of 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and annealing at 60°C for 30 seconds. Relative mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). PCR primers and fluorogenic probes for the target gene and endogenous controls were purchased from Applied Biosystems. The assays were supplied as a 20 \times mix of PCR primers and TaqMan minor groove binder 6-FAM dye-labeled probes with a non-fluorescent quencher at the 3'-end of the probe. The assay numbers for *GAPDH* and *VEGF-C* were as follows: Hs99999905_m1 (*GAPDH*), Hs01099206_m1 (*VEGF-C*).

Statistical analysis

Relative mRNA expression levels ($\log_{10} \text{VEGF-C}/\text{GAPDH}$) were calculated from quantified data relative to the expression level of *GAPDH*. Data is expressed as the mean \pm SD. Statistical analysis was performed using the Stat-View software package (Abacus Concepts, Berkeley, CA, USA). Mann-Whitney U test was used to analyze the association between mRNA expression levels and the clinical histopathological parameters of the patients. The survival of patients with ESCC after surgery was examined using the Kaplan-Meier method, and the survival times were compared using the log-rank test. Univariate analysis and multivariate analysis was performed using the Cox's regression model. P-values were considered significant at $p < 0.05$.

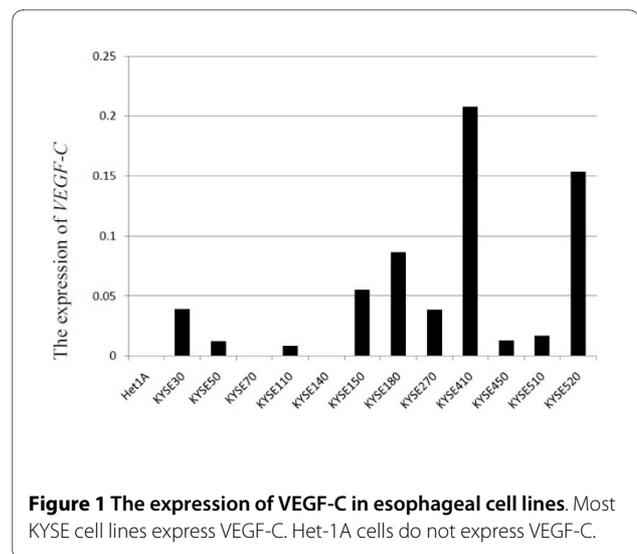
Results

Quantitative RT-PCR of *VEGF-C* in cell lines

We first investigated the expression of *VEGF-C* in 12 esophageal cancer cell lines (KYSE30, KYSE50, KYSE70, KYSE110, KYSE140, KYSE150, KYSE180, KYSE270, KYSE410, KYSE450, KYSE510, KYSE520), and in the Het-1A cell line. In most of the KYSE series of cell lines, especially KYSE410, high levels of *VEGF-C* were detected, yet in Het-1A, *VEGF-C* was not detected at all (Fig. 1).

Quantitative RT-PCR of *VEGF-C* in clinical specimens

We next examined *VEGF-C* expression in 106 pairs of resected ESCC tumors and in corresponding noncancer-



ous esophageal mucosal tissue specimens. Our data reveals that *VEGF-C* expression in cancerous tissue is higher than in corresponding noncancerous esophageal mucosa (Fig. 2a). We also examined the relationship between the clinico-pathological factors and the expression of *VEGF-C* in ESCC. The expression of *VEGF-C* was found to be higher in Stage2B-4A tumors than in Stage0-2A tumors (Table 1, Fig. 2b). We also examined the relationship between the expression of *VEGF-C* and the survival data. The patients were divided into two groups according to the expression of *VEGF-C*. The cut off value was median expression of *VEGF-C* (high expression group of 53 cases and a low expression group of 53 cases). The patients in the high *VEGF-C* expression group had significantly shorter survival after surgery than the patients in the low expression group ($p = 0.0065$ by log-rank test; Fig. 3). Univariate analysis showed that, among the clinico-pathological factors, the extent of the primary tumor, lymph node metastasis, and high expression of *VEGF-C* were all statistically significant prognostic factors (Table 2). Multivariate analysis showed that the extent of the primary tumor and lymph node metastasis were independent prognostic factor (Table 3).

We next analyzed a subgroup of patients with Tis and T1 tumors (Table 4). In this subgroup, we examined the relationship between the clinico-pathological factors and the expression of *VEGF-C* in ESCC. The expression of *VEGF-C* was found to be higher in N1 tumors than in N0 tumors (Table 4, Fig. 4). The expression of *VEGF-C* was found to be higher in T1 and Stage2A, 2B tumors than in Tis and Stage0-1 tumors (Table. 4). We also examined the relationship between the expression of *VEGF-C* and the survival data. The patients were divided into two groups according to the expression of *VEGF-C*. The cut off value was median expression of *VEGF-C* (a high expression group of 10 cases and a low expression group of 11 cases). The survival rate of the patients in the high expression

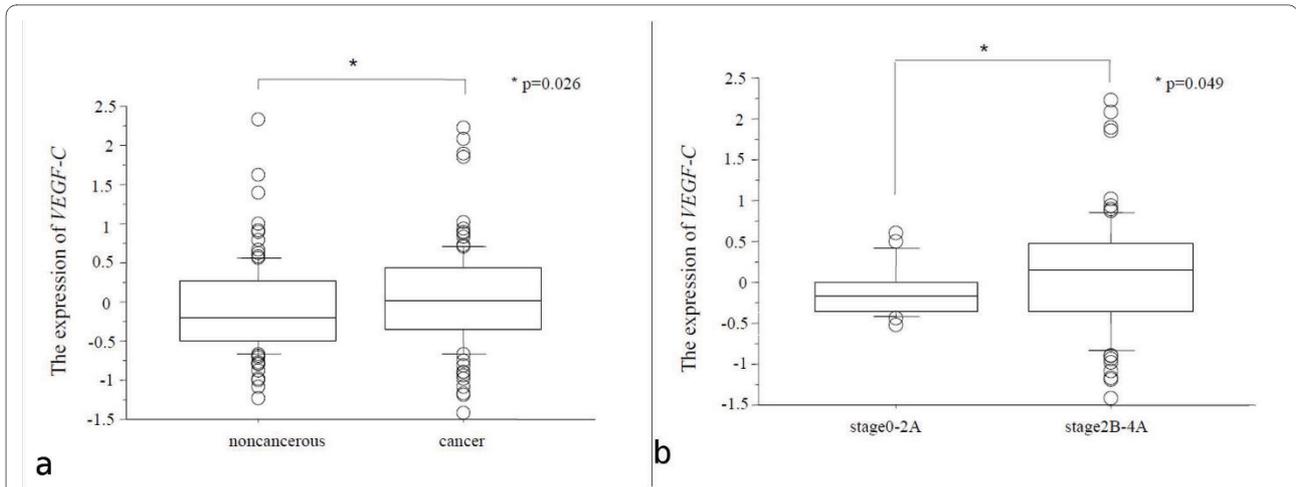


Figure 2 Comparison of mRNA expression of *VEGF-C* in cancer and corresponding noncancerous esophageal mucosa (a) and in Stage0-2A patients and Stage2B-4A patients (b). The *VEGF-C* expression in ESCC tumors is significantly higher than in the corresponding noncancerous esophageal mucosa (a). The *VEGF-C* expression is higher in Stage2B-4A patients than in Stage0-2A patients (b).

group was clearly lower than that in the low expression group, and all the patients in the low *VEGF-C* expression group were survived (data not shown).

Discussion

The vascular endothelial growth factor (*VEGF*) gene family, which encodes five polypeptides, *VEGF-A*, *-B*, *-C*, *-D*, and *-E*, is particularly important because of its angiogenic and lymphangiogenic properties [15]. *VEGF-C* has been shown to signal through the receptors *VEGFR-3* (also called *Flt-4*) and *VEGFR-2* [13]. *VEGFR-3* has also been shown to be important in determining the potential for a lymphangiogenic response. Recent studies have indicated

Table 2: Univariate analysis for clinicopathologic variables and mRNA expression of *VEGF-C*

parameter	Riskratio	95% ^a CI	p-value
Primary tumor			
Tis, T1	1	2.11-16.13	< 0.001
T234	5.85		
Lymph node metastasis			
N0	1	1.66-6.9	< 0.001
N1	3.38		
Lymph Invasion			
Negative	1	0.98-3.11	0.056
Positive	1.75		
Vein invasion			
Negative	1	0.96-2.72	0.067
Positive	1.62		
<i>VEGF-C</i> expression			
Low expression	1	1.2-3.4	0.0085
High expression	2.02		

^aCI; confidence interval

Univariate analysis shows that, among the clinico-pathological factors, the extent of the primary tumor, lymph node metastasis, and high expression of *VEGF-C* are all statistically significant prognostic factors

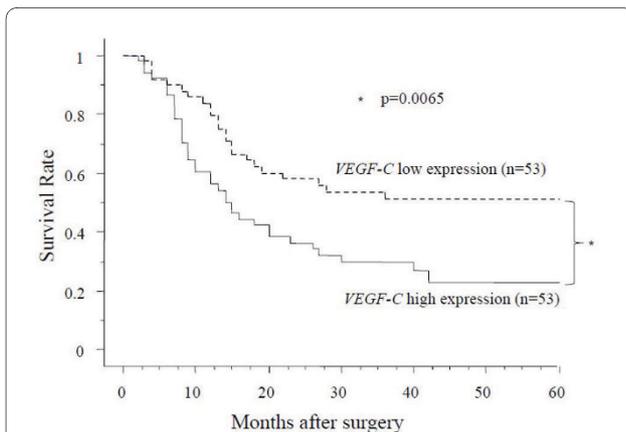


Figure 3 Survival rate of patients with ESCC according to the mRNA expression of *VEGF-C*. Patients with high expression of *VEGF-C* have significantly shorter survival after surgery ($p = 0.0065$ by log-rank test). The cut off value was median expression of *VEGF-C*

Table 3: Multivariate analysis for clinicopathologic variables and mRNA expression of VEGF-C

parameter	Riskratio	95% ^a CI	p-value
Primary tumor			
Tis, T1	1	1.62-12.7	0.004
T234	4.52		
Lymph node metastasis			
N0	1	1.14-4.85	0.02
N1	2.36		
VEGF-C expression			
Low expression	1	0.97-2.78	0.065
High expression	1.64		

^aCI; confidence interval

Multivariate analysis shows that, among the clinico-pathological factors, the extent of the primary tumor and lymph node metastasis are statistically significant prognostic factors

that VEGFR-3 is expressed in a variety of human malignancies [16]. The expression of VEGF-C and VEGFR-3 has been significantly and negatively correlated to the progression of gastric cancer [17], cervical cancer [18], colorectal cancer [19], and head and neck squamous cell carcinoma [20]. In esophageal cancer, few studies have dealt with the relationship between VEGF-C expression and tumor progression or prognosis. Ishikawa et al investigated the expression of VEGF-C in esophageal carcinoma, dysplasia, and normal mucosa by immunohistochemistry. The authors reported that all

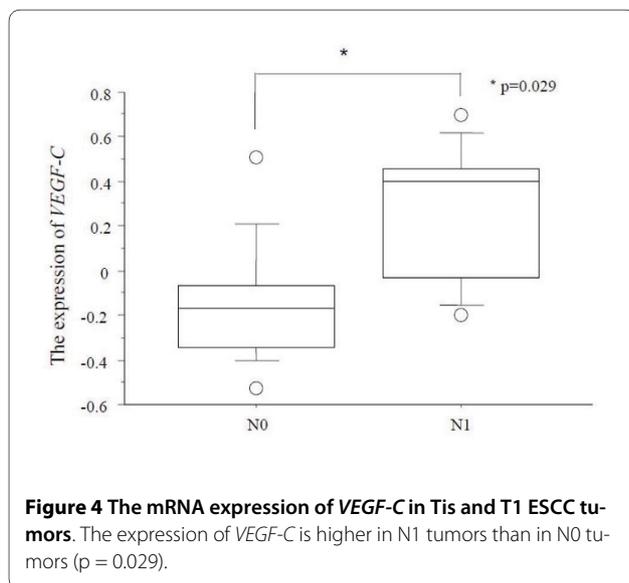


Figure 4 The mRNA expression of VEGF-C in Tis and T1 ESCC tumors. The expression of VEGF-C is higher in N1 tumors than in N0 tumors ($p = 0.029$).

Table 4: Relationship between clinicopathological factors and mRNA expression of VEGF-C with Tis, T1 tumors

		VEGF-C expression			
		case	mean	\pm sd	p-value
age	≥ 65	8	-0.11	\pm 0.34	0.15
	< 65	13	0.12	\pm 0.33	
gender	male	19	0.06	\pm 0.35	0.28
	female	2	0.25	\pm 0.24	
Tfactor	Tis	6	-0.02	\pm 0.14	0.029
	T1	15	0.13	\pm 0.35	
Nfactor	N0	12	-0.15	\pm 0.27	0.003
	N1	9	0.27	\pm 0.3	
Stage	Stage0	6	-0.23	\pm 0.14	0.014
	Stage1	6	-0.072	\pm 0.35	
	Stage2A	1	-0.09		
	Stage2B	8	0.31	\pm 0.29	
Stage0,1 vs Stage2A,2B					
Histological Type	well	4	0.45	\pm 0.18	0.69
	moderate	14	-0.1	\pm 0.29	
	poor	3	0.092	\pm 0.36	
well · moderate vs poor					
lymphatic invasion					
	positive	7	0.006	\pm 0.39	0.77
	negative	14	-0.04	\pm 0.34	
vein invasion					
	positive	3	0.053	\pm 0.51	> 0.99
	negative	18	0.025	\pm 0.33	

The expression of VEGF-C is higher in T1, N1 and Stage2A, 2B tumors than in Tis, N0 and Stage0,1 tumors

esophageal carcinomas clearly expressed VEGF-C. In esophageal dysplasia, 82% of the cases expressed VEGF-C. In contrast, none of the esophageal normal mucosa expressed VEGF-C [21]. In the study by Ming-Xing Ding, the expression of VEGF-C mRNA was higher in esophageal carcinoma than in normal tissue [22]. In our study,

most of the KYSE cell lines expressed *VEGF-C*, the SV40-immortalized esophageal cell line Het-1A did not express *VEGF-C* mRNA, and the expression of *VEGF-C* in cancerous tissue was higher than in corresponding noncancerous esophageal mucosa. This suggests that *VEGF-C* may play an important role in tumor progression. Okazawa et al. reported that *VEGF-C* expression correlated with the depth of tumor invasion, lymphatic invasion, and lymph node metastasis in esophageal cancer. They also claimed that the prognosis was significantly worse for patients with tumors positive for *VEGF-C* than for those with tumors negative for *VEGF-C*, and that *VEGF-C* expression was an independent prognostic determinant [23]. The discrepancy between their report and present study may be from methodology. They investigated 100 tumors by immunohistochemistry, and treated 43% of *VEGF-C* positive cases.

Esophageal carcinoma most likely metastasizes in lymph node, which correlates with the prognosis of the patients. In this study, the expression of *VEGF-C* mRNA correlates with lymph node metastasis, and the patients with high *VEGF-C*-expressing tumors have a poorer prognosis than those with low *VEGF-C*-expressing tumors. To avoid highly invasive surgery, EMR, ESD, chemoradiotherapy, and their combinations have been indicated for patients with early esophageal cancers. In the non-surgical treatment of early esophageal cancer, a high rate of local recurrence and lymph node metastasis is evident [24]. For non-surgical treatment, particularly ESD and EMR, preoperative diagnosis of lymph node metastasis is essential. However, the accuracy of diagnosis of lymph node metastasis by computed tomography is reported to be 11-38%, endoscopic ultrasound 75-76%, and positron emission tomography 30-52% [25-28]. The sensitivity of endoscopic ultrasound is high, yet it does not detect distant metastases [26]. For the decision of non-surgical treatment, the sensitivity is just not high enough. Our study shows that expression of *VEGF-C* correlates with lymph node metastasis, and negatively correlates with survival in early squamous cell carcinoma. If early esophageal cancer expresses high *VEGF-C*, the patients have increased risk of lymph node metastasis and thus, a poor prognosis. Hence, the expression of *VEGF-C* may assist in the diagnosis of lymph node metastasis for esophageal superficial carcinoma. Although the precise molecular mechanisms of up-regulated *VEGF-C* expression need to be clarified, our data suggests that *VEGF-C* is a good candidate as a molecular prognostic marker as well as a molecular target for the development of effective treatment for patients with esophageal cancer.

Conclusions

The expression of *VEGF-C* correlates with lymph node metastasis and poor prognosis. In patients with Tis and T1 esophageal tumors, the expression of *VEGF-C* may be

a good diagnostic factor for determining metastasis of the lymph node.

List of abbreviations

VEGF: vascular endothelial growth factor; *VEGF-C*: vascular endothelial growth factor C; *EMR*: endoscopic mucosal resection; *ESD*: endoscopic submucosal dissection; *CT*: computed tomography; *PET*: positron emission tomography; *DEC1*: *deleted in esophageal cancer 1*; *DCC*: *deleted in colorectal cancer*; *DLC1*: *deleted in lung cancer 1*; *TGFBR2*: *transforming growth factor-beta receptor type II*; *APC*: *adenomatous polyposis coli*; *MDM2*: *murine double minute 2*; *ESCC*: esophageal squamous cell cancer; *VEGFR-2*: Vascular endothelial growth factor receptor 2; *VEGFR-3*: Vascular endothelial growth factor receptor 3

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT carried out most of experiments, participated in the design of the study, performed the statistical analysis and drafted the manuscript. HI, YF and HT participated in the design of the study and helped to draft the manuscript. YK participated in its design and coordination. MK, AM, TK, MS and YN assisted the experiments. All authors read and approved the final manuscript.

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