

RESEARCH ARTICLE

Comprehensive Analysis of Vascular Endothelial Growth Factor-C Related Factors in Stomach Cancer

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Abstract

Background: Vascular endothelial growth factor-C (VEGF-C), which contributes to lymphatic metastasis (LM) in malignant disease, is one of the most important factors involved in physical and pathological lymphangiogenesis. Some VEGF-C related factors such as sine oculis homeobox homolog (SIX) 1, contactin (CNTN) 1 and dual specificity phosphatase (DUSP) 6 have been extensively studied in malignancies, but their expression levels and associations have still to be elucidated in stomach cancer. **Methods:** We detected their expression levels in 30 paired stomach cancer tissues using quantitative real-time reverse transcription-PCR (qRT-PCR). The expression and clinical significance of each factor was analyzed using Wilcoxon signed rank sum test. The correlation among all the factors was performed by Spearman rank correlation analysis. **Results:** The results suggest that VEGF-C and CNTN1 are significantly correlated with tumor size, SIX1 with the age and CNTN1 also with the cTNM stage. There are significant correlations of expression levels among VEGF-C, SIX1, CNTN1 and DUSP6. **Conclusions:** There exists an important regulatory crosstalk involving SIX1, VEGF-C, CNTN1 and DUSP6 in stomach cancer.

Keywords: Stomach neoplasms - VEGFC - SIX1 - CNTN1 - DUSP6 - crosstalk

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Introduction

Stomach cancer, the fourth most common cancer worldwide in men and the fifth in women, is one of the most fatal diseases (Guggenheim and Shah, 2012). VEGFC correlates with lymphatic metastasis (LM) in many malignancies, including stomach cancer (Achen and Stacker, 2008; Wang et al., 2012). VEGFC is proteolytically processed, binds VEGFR-3 and induces tyrosine autophosphorylation of VEGFR-3 and VEGFR-2 (Joukov et al., 1996; Joukov et al., 1997). VEGFC/VEGFR-3 axis plays an important role in cancer metastasis (Su et al., 2006; Su et al., 2007; Achen and Stacker, 2008; Cao, 2008; Peng et al., 2011), and is significantly related to prognosis in diverse malignant disease, like breast cancer (Zhu et al., 2011; Wang et al., 2012), prostatic cancer (Jennbacken et al., 2005) and so on.

SIX protein family, with 6 members, is highly conserved through various organisms that range from flatworms to human (Kumar, 2009). SIX1 is one of the members implicated in carcinogenesis through various mechanisms among which VEGFC is involved (Christensen et al., 2008; Kumar, 2009; Wang et al., 2012). Wang (Wang et al., 2012) et al demonstrated that both in vitro and in vivo SIX1 could directly activate VEGFC transcription by binding to specific DNA sequences in the VEGFC promoter.

Besides molecules inducing VEGFC expression, there are many proteins can be induced by VEGFC/VEGFR3 axis such as CNTN1, which is increased by activation of the VEGFC/VEGFR3 axis and is required for the axis-mediated cell mobility and metastasis in different types of cancer cells (Su et al., 2006).

Moreover, VEGFC can be induced by MAKP pathways (Tsai et al., 2003). Among all the members involved in MAKP pathways, phosphorylated extracellular signal-regulated kinase (ERK) 1/2 is critical for insulin-like growth factor-1-induced VEGF-C upregulation (Zhu et al., 2011). As a negative regulator of MAKP pathways, DUSP6 can directly inhibit the phosphorylation of ERK1/2 (Maillet et al., 2008; Cejudo-Marin et al., 2012), which can transcriptionally activate DUSP6, that constitutes a feedback regulatory loop between ERK 1/2 and DUSP6 (Furukawa et al., 2008). Intriguingly, SIX1 can also regulate the ERK1/2 pathway by directly controlling DUSP6 transcription (Le Grand et al., 2012). According to previous reports, there are complicated regulatory networks among VEGFC, SIX1, CNTN1 and DUSP6 as summarizing in Figure 1. Given the confirmed importance of VEGFC and the lack of study about VEGFC related factors mentioned above in stomach cancer, we firstly perform this study to evaluate the significance of VEGFC and its related factors (SIX1, CNTN1 and DUSP6) in stomach cancer.

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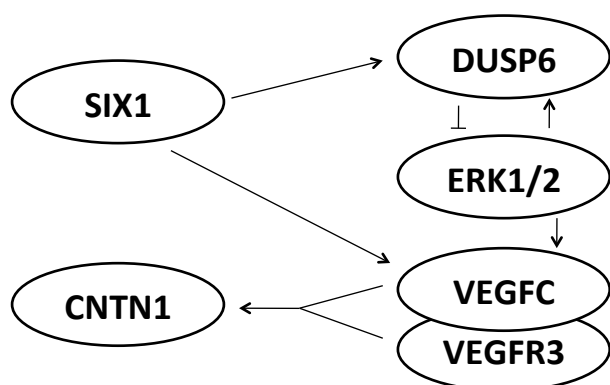


Figure 1. The Regulatory Network among VEGFC and its Related Factors. The regulatory network among VEGFC and its related factors is presented as above. Arrow head represents stimulatory effect. Hammer head represents inhibitory effect

Materials and Methods

Patients

All paired pathological samples were collected from 30 stomach cancer patients from 2012.11 to 2013.7 in Fudan university affiliated Huashan Hospital. Each sample pair included a piece of cancer specimen and a piece of normal tissue. They were all confirmed as cancer or normal tissue respectively by two pathologists. All samples were stored in liquid nitrogen immediately within 30 minutes after stomach resection until RNA extraction.

All 30 tumor tissues were identified as adenocarcinoma. Totally, there were 19 male patients and 11 female patients, with the age of 35 to 77, and on an average of 57.5. One was classified as stage 0, 5 as stage I, 8 as stage II, 15 as stage III and 1 as stage IV in accordance with cTNM Cancer Staging Manual released by American Joint Committee on Cancer. One was well differentiated, 5 were moderate and 24 were poor according to their differentiation status.

All patients enrolled in this study were informed and consent was given.

Total RNA extraction

Total RNA was extracted using the TRizol reagent (Invitrogen, CA) as described previously (Liu et al., 2011). RNA quantity and quality were determined by Nanodrop2000.

Reverse transcription and qRT-PCR

Reverse transcription was performed using the PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). The cDNA template was amplified by qRT-PCR using the SYBRORPremix EX Taq™ II kit (TaKaRa). The thermal cycling conditions were as follows: 95°C for 60 s followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. After amplification, the products were subjected to an increasing temperature gradient from 60 to 95°C. Plates were held for 1 s and read every 0.4°C to create a melting curve. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was used as an internal control to normalize input mRNA level.

Table 1. Significances of Deregulated VEGFC and Its Related Factors

Factors	No of down regulated	No of up regulated	<i>p</i> value
SIX1	20 (66.7%)	10 (33.3%)	0.553
VEGFC	19 (63.3%)	11 (36.7%)	0.0858
CNTN1	24 (80.0%)	6 (20.0%)	0.0614
DUSP6	17 (56.7%)	13 (43.3%)	0.7558

In each specimen pair (cancer and normal tissue from the same patient), the expression level of cancer comparing with normal tissue which is more than 1 is considered upregulated, or it is downregulated. All experiments were performed in triplicate. *P* value <0.05 was considered statistically significant

Table 3. Significances of Deregulated VEGFC and its Related Factors in Terms of Clinical and Pathological Characteristics

Items	<i>p</i> value					
	Gender	Age	Tumor size	cTNM	Differen- tiation	Lymphatic metastasis
SIX1	0.9499	0.008	0.1432	0.5094	0.5548	0.7145
VEGFC	0.4109	0.2622	0.0191	0.2828	0.4015	0.5826
CNTN1	0.2801	0.6304	0.0003	0.0419	0.1141	0.2677
DUSP6	0.8176	0.2095	0.3063	0.4292	0.8169	1

Expression levels were analyzed in terms of clinical and pathological characteristics using Wilcoxon signed rank sum test. *P* value <0.05 was considered statistically significant

The primer sequences are as follows: SIX1-forward-5'-AGGAAAGGGAGAACACCCGAAA-3', reverse-5'-CCCTTCCAGAGGAGAGAGTTG-3'; VEGFC-forward-5'-TCATTCCATTATTAGACGTTCCCTG-3', reverse-5'-TGTTGAGTCATCTCCAGCAT-3'; CNTN1-forward-5'-GCCAATCAATACCATTTATCCAG-3', reverse-5'-AACAAGGTTTCCTCCTACCATAC-3'; DUSP6-forward-5'-CCTCGTCCTTGAGCTTCTTGA-3', reverse-5'-GCTGTGGCACCACAGT-3'; GAPDH-forward-5'-TCGACAGTCAGCCGCATCTTCTTT-3', reverse-5'-ACCAAATCCGTTGACTCCGACCTT-3'.

All the experiments were performed according to the provided protocol. The relative expression fold change mRNAs were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All reactions were performed in triplicate.

Statistical Analysis

All data were analyzed by SAS 8.0. The expression and clinical significance of each factor was analyzed using Wilcoxon signed rank sum test. The correlation among all the factors was performed by Spearman rank correlation analysis. *P*-value<0.05 was considered statistically significant.

Results

Deregulation of VEGFC, SIX1, CNTN1 and DUSP6

We found that mRNA levels of VEGFC, SIX1, CNTN1 and DUSP6 varied a lot among 30 paired stomach cancer tissues. All the factors were not significantly deregulated (Table 1). VEGFC, SIX1, CNTN1 and DUSP6 were upregulated in 36.7%, 33.3%, 20.0% and 43.2% of 30 stomach cancer cases respectively (Table 1).

Table 2. The Expression Levels of VEGFC and its Related Factors in Terms of Clinical and Pathological Characteristics

Items	No of cases	SIX1		VEGFC		CNTN1		DUSP6	
		↑	↓	↑	↓	↑	↓	↑	↓
Gender									
Male	19	7	12	9	10	5	14	8	11
Female	11	3	8	2	9	1	10	5	6
Age (years)									
≤65	22	9	13	9	13	5	17	11	11
>65	8	1	7	2	6	1	7	2	6
Diameter(cm)									
≤4	18	9	9	8	10	5	13	8	10
>4	12	1	11	3	9	1	11	5	7
cTNM									
0	1	0	1	1	0	0	1	1	0
I	5	2	3	2	3	2	3	2	3
II	8	3	5	3	5	1	7	4	4
III	15	5	10	5	10	3	12	6	9
IV	1	0	1	0	1	0	1	0	1
Differentiation									
Well	1	0	1	1	0	0	1	1	0
Moderate	5	2	3	3	2	1	4	2	3
Poor	24	8	16	7	17	5	19	10	14

↑represents in each specimen pair (cancer and normal tissue from the same patient), the expression level of cancer comparing with normal tissue which is more than 1 is considered upregulated, or it is downregulated and represented by ↓. All experiments were performed in triplicate

Table 4. The Correlation among VEGFC and its Related Factors

Items	rs (p value)			
	SIX1	VEGFC	CNTN1	DUSP6
SIX1		0.66274 (<0.0001)	0.53281 (0.0024)	0.15729 (0.4065)
VEGFC	0.66274 (<0.0001)		0.73170 (<0.0001)	0.48966 (0.0060)
CNTN1	0.53281 (0.0024)	0.73170 (<0.0001)		0.07764 (0.6834)
DUSP6	0.15729 (0.4065)	0.48966 (0.0060)	0.07764 (0.6834)	

The correlation among all the factors was performed by Spearman rank correlation analysis. The rs and p value was as presented. P-value<0.05 was considered statistically significant

The association between expression level with clinical and pathological characteristics

The clinical and pathological characteristics were presented as Table 2. The expression of SIX1 was significantly correlated with patients' age (Table 3). Both VEGFC and CNTN1 were profoundly correlated with the tumor size (Table 3). CNTN1 was also correlated with patients' cTNM staging (Table 3).

The association among each VEGFC related factors

The expression of SIX1, CNTN1 and DUSP6 were all significantly correlated with that of VEGFC (Table 4). Besides, SIX1's expression was significantly correlated with that of CNTN1 (Table 4). There was no obvious correlation between SIX1 and DUSP6 (Table 4).

Discussion

There was no factor significantly deregulated in our study, we thought that is because of the limited cases and the lack of follow-up information. Yet the results still provided us with some interesting implications. VEGFC is known for its irreplaceable role in lymphangiogenesis (Alitalo and Carmeliet, 2002), which is one of the mechanisms contributing to LM (Achen and Stacker,

2008). In our study, VEGFC was upregulated in 36.7% (Table 1) patients, which is in accordance with Arigami and Amioka's reports (Amioka et al., 2002; Arigami et al., 2009). But in our samples, not all the VEGFC-upregulated cases had metastatic lymph nodes, meanwhile not all the lymphatic metastatic cases had upregulated VEGFC expression (Table 2). So we assumed that because there are lots of pathways contributing to LM, and lymphangiogenesis is just one of them, VEGFC cannot account for all the LM mechanisms in stomach cancer and in these cases lymphangiogenesis played a less important role in LM. This may also be the reason that other VEGFC related factors in our study were not significantly correlated with LM (Table 3). As for CNTN1, which is correlated with cTNM staging (Table 3), there is much to do to reveal its role in stomach cancer LM.

When it comes to clinical and pathological characteristics, first, besides the positive result about CNTN1 mentioned above, SIX1 is positively correlated with age which made us speculate SIX1 has something to do with aging. Second, we found that VEGFC and CNTN1 were both correlated with the tumor size (Table 3). As reported, VEGFC can stimulate the growth of stomach cancer cells (Kodama et al., 2008), which means the role of VEGFC in stomach cancer is far beyond stimulating

lymphangiogenesis. As for CNTN1, which is reported to be involved in VEGFC/VEGFR3 pathway, it can be induced when VEGFC activates VEGFR3 downstream effectors (Su et al., 2006; Liu et al., 2011) (Figure 1). It would be reasonable to believe that as a downstream factor of VEGFC, CNTN1 could also play critical role in stomach cancer carcinogenesis more than stimulating lymphangiogenesis, which can also be supported by the result that the expression level of VEGFC and CNTN1 were significantly correlated (Table 4).

In our study we found a positive correlation between VEGFC and SIX1 (Table 4), especially in these cases with upregulated VEGFC expression. Wang's results (Wang et al., 2012) showed that SIX1, an upstream molecular of VEGFC, has several binding domains in genetic promoter region of VEGFC and SIX1 can activate the expression of VEGFC (Figure 1). These results provided underlying mechanism for our finding of the expression association between VEGFC and SIX1. Besides, SIX1 is also correlated with CNTN1 (Table 4), which made us speculate that there is indeed a pathway in stomach cancer involving SIX1/VEGFC/CNTN1 (Figure 1). Some molecular biological research is needed to demonstrate this hypothesis thoroughly.

As we know, MAPK pathway is implicated in stomach cancer (Wu et al., 2010). VEGFC can be induced when this pathway is activated (Tsai et al., 2003; Zhu et al., 2011) (Figure 1). DUSP6 services as the inhibitor of MAK pathway. So it is assumed that DUSP6 should have reverse correlation with VEGFC expression, however, we saw a positive correlation between DUSP6 and VEGFC in our study (Table 4). Given that there is a complicated negative regulatory feedback loop between ERK1/2 and DUSP6, which also can be transcriptionally regulated by SIX1 (Le Grand et al., 2012) (Figure 1), we thought that the complicity in these important pathways cannot be outlined by single molecule, it is a network that counts.

In conclusions: Our study implied that VEGFC and its related factors, like SIX1, CNTN1 and DUSP6, may play critical role in stomach cancer. There exists an important regulatory crosstalk involving SIX1, VEGFC, CNTN1 and DUSP6 in stomach carcinogenesis and aggressiveness.

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