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ORIGINAL ARTICLE

# VEGF-C in rectal cancer tissues promotes tumor invasion and metastasis

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## Summary

***Purpose:*** *To investigate the relationship between the expression of vascular endothelial growth factor-C (VEGF-C) in rectal cancer tissues and clinicopathological factors.*

***Methods:*** *The molecular expression of VEGF-C in rectal cancer tissue derived from 45 patients and normal colon tissue from 15 subjects was detected using the immunohistochemical streptavidin/biotin/peroxidase complex (SABC) three-step method. The expression of VEGF-C in rectal cancer and its relationship with clinicopathological factors were statistically analyzed via $x^2$ test or Spearman's rank correlation analysis or Wilcoxon rank sum test.*

***Results:*** *The positive expression rate of VEGF-C was 75.55% in rectal cancer tissues and 6.66% in normal tissues ($p<0.01$). The positive expression of VEGF-C was not related to patient gender, age and tumor diameter, but related to the grade of differentiation, depth of invasion, lymph node metastasis and Dukes stage ($p<0.05$). Positive intensity had no statistically significant difference with grade of differentiation and depth of invasion ($p>0.05$), but had statistically significant difference in lymph node metastasis and Dukes stage ($p<0.05$).*

***Conclusions:*** *1) VEGF-C is highly expressed in rectal cancer tissues; 2) The positive expression of VEGF-C is positively correlated with tumor invasion depth, lymph node metastasis and Dukes stage; 3) Detection of VEGF-C expression can be used as a prognostic marker in rectal cancer.*

***Key words:*** *metastasis, invasion, rectal cancer, VEGF*

## Introduction

Colorectal cancer is one of the common malignant tumors, and analysis of the World's cancer trend in 2000 showed that the global incidence rate of colorectal cancer ranked third and its mortality rate ranked fourth, especially in developed countries in the Northwestern Europe and North America [1]. In recent years, with the development of national economy and the continuous improvement of people's living standards in China, as well as the changes in lifestyle and dietary habits, the incidence rate of colorectal cancer has shown an obviously increasing trend year by year. Although great progress and gratifying achievements have been made in the diagnosis and surgical treatment of colorectal cancer, the overall 5-year survival rate after surgery is still 50-65%; local recurrence and distant metastasis are the main reasons for the failure of colorectal cancer treatment [2,3] with the liver metastasis rate up to 50%, while the liver metastasis rate is found to be as high as 71% in autopsies of patients with colorectal cancer [4]. About 90% recurrence and metastasis occur within 2 years after surgery. In recent years, ways on how to reduce the postoperative recurrence and metastasis rates after surgery of colorectal cancer have been studied from the basic experimental and clinical aspects.

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VEGF-C is the most specific lymphatic endothelial cell growth stimulating factor at present in the VEGF family [5], and the receptors of VEGF-C are VEGFR-2 and VEGFR-3. Although both receptors are expressed in blood and lymphatic vessels, VEGFR-3 is mainly located in lymphatic endothelial cells and is a specific regulator of lymphangiogenesis. After binding to VEGFR-3, VEGF-C causes lymphatic endothelial cell proliferation through MEK/ERK and PI3K/AKT pathway [6]. And the binding affinity of VEGF-C to VEGFR-3 is much higher than that to VEGFR-2, so VEGF-C is manifested in promoting lymphangiogenesis and expansion [7]. Many solid tumors can synthesize VEGF-C, different types of tumors have different expression capabilities of VEGF-C, and its expression intensity is related to the degree of lymph node metastasis in tumors [8].

Hypoxia inside rectal cancer tissues is the real microenvironment. Tumor growth and metastasis are closely related to the hypoxia-inducible factors, and lymphatic and hematogeneous metastases are key factors determining the prognosis of rectal cancer. VEGF is the most important growth factor stimulating the tumor angiogenesis. VEGF-C is a member of VEGF polypeptide growth factor family, which, as a specific marker of promoting lymphatic endothelial growth factor and tumor angiogenesis, gains extensive attention in tumor neovascularization, lymphangiogenesis and tumor metastasis.

Therefore, the expression of VEGF-C in human rectal cancer was detected using immunohistochemistry, its relationship with biological behaviors of rectal cancer was preliminarily analyzed and the theories of progression and metastasis of rectal cancer were investigated, so as to provide a theoretical basis for reducing the metastatic ability and improving the prognosis of rectal cancer clinically.

## Methods

*Specimens and clinical data*

A total of 45 cases of paraffin embedded tissues confirmed as rectal adenocarcinoma *via* hematoxylin-eosin (H&E) staining in Qingdao Municipal Hospital from January 2005 to June 2009 were collected, and all cases had complete clinical medical records. Another 15 cases of normal intestinal wall tissues 5cm away from the tumor edge were taken as controls. This study was approved by the ethics committee of Qingdao Municipal Hospital. Signed written informed consents were obtained from all participants before the study.

*Inclusion criteria*

1: Patients with hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP);
2: Patients without diabetes mellitus or severe cardiovascular disease;
3: Patients who did not receive radiotherapy, chemotherapy, immunomodulatory and/or nonsteroidal anti-inflammatory drugs (NSAID) therapy before surgery.

Pathological grading was based on the grading criteria of WHO in 2000 (divided into Grade I: well differentiated group, Grade II: moderately differentiated group, and Grade III: poorly differentiated group), and the Dukes stage was according to the criteria developed jointly by the Union International Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC) in 2003.

*Immunohistochemistry*

Sections were dewaxed conventionally; 0.3% $H_2O_2$ was used to inactivate endogenous peroxidase at room temperature for 10 min, followed by washing with distilled water for 3 times. The sections were immersed in 0.01 M citrate buffer (pH 6.0) and microwave oven was used to heat them until boiling. After 10 min, this process was repeated twice; then, the sections were cooled at room temperature and washed twice with phosphate buffered saline (PBS) (pH 7.4); 5% bovine serum albumin (BSA) blocking solution was added at room temperature for 20 min and excess liquid was discarded. The rabbit anti-human HIF-2α, CCR7 and VEGF-C polyclonal antibodies (Cell Signaling, Danvers, MA, USA) (working concentration: HIF-2α: diluted at 1:100, CCR7: diluted at 1:100, VEGF-C: diluted at 1:100) were added for incubation at 37°C for 24 hrs, and then the sections were washed for 5 min × 3 times. Biotinylated goat anti-rabbit IgG (Cell Signaling, Danvers, MA, USA) working solution was added at 1:1000 dilution for incubation at 37°C for 20 min. After washing, SABC was added for incubation at 37°C for 20 min, followed by washing and color development *via* diaminobenzidine (DAB) (DAB color development kit, Boster, Wuhan, China) at room temperature for 5-30 min followed by washing with distilled water, hematoxylin re-staining dehydration *via* graded ethanol, transparency *via* xylene, sealing *via* neutral gum, and observation under light microscope. The primary antibody was replaced with PBS as negative control. The positive sections stained satisfactorily in pre-experiment were used as positive controls.

*Determination of results*

Following the double-blind method, two experienced pathologists analyzed the sections and the cells were re-counted when the difference between the two results was more than 10%. The positive staining of VEGF-C was manifested in the nucleus or cytoplasm as yellow, brown-yellow or yellowish-brown. The sum of staining intensity and positive percent was used for determination in 5 randomly-selected high power fields (×400): no staining: 0 point, weak staining (light yellow): 1 point, medium staining (brown-yellow): 2 points, and strong staining (yellowish-brown): 3 points. Cell positive rate ≤10%: 0 point, 10-25%: 1 point, 25-50%: 2 points, and >50%: 3 points. With both results added: 0 point: (-), 2 points: (+), 3-4 points: (++), and 5-6

points: (+++); the total score of 2 points and above indicated positive expression. In addition, a positive cell rate ≤10% was defined as negative (-).

*Statistics*

The Statistical Package for Social Sciences (SPSS) 13.0 was used for data statistics. Chi-square test was used for the comparison of expressions of VEGF-C in rectal cancer tissues and para-carcinoma normal tissues. Chi-square test was also used for the relationship between the expression of VEGF-C in rectal cancer tissues and clinicopathological parameters. Wilcoxon rank sum test was used to further analyze the difference in positive rates of the above correlated clinicopathological parameters. Significant level α=0.05 and two-tailed p<0.05 suggested that the difference was statistically significant.

## Results

*Basic patient data*

The basic statistical analysis was performed for the gender, age, diameter of tumor, differentiation grade, lymph node metastasis, depth of invasion and Dukes stage of patients enrolled (Table 1).

*Expression of VEGF-C in colon cancer tissues*

Yellow or brown-yellow staining in the cytoplasm of tumor cells and lymphatic endothelial cells indicated positive protein expression of VEGF-C, especially significant in the edge of cancer nest and interstitial infiltration. The positive expression rate of VEGF-C was 75.55% in rectal cancer tissues, and 6.66% in para-carcinoma normal tissues. The positive expression of VEGF-C was statistically significant between the two groups (p<0.01) (Table 2).

*Relationship between expression of VEGF-C and clinicopathological factors*

There was no statistically significant difference in the positive expression of VEGF-C between patients with different age, gender and tumor size (p>0.05). However, difference statistically significant was observed between patients with different grades of differentiation, lymph node metastasis, depth of invasion and Dukes stage (p<0.05) (Table 3).

For four types of clinicopathological factors related to positive expression of VEGF-C, Wilcoxon rank sum test was used to analyze whether the positive intensity of VEGF-C was related to these factors. The results showed that grade of differentiation and the depth of invasion were not correlated with positive intensity of VEGF-C (p>0.05) (Table 4), while nodal metastasis and Dukes stage were correlated with positive intensity of VEGF-C (p<0.05) (Table 4).

**Table 1.** Patient and disease characteristics

| *Characteristics* | *n* | *%* |
|---|---|---|
| Gender | | |
| Male | 25 | 55.6 |
| Female | 20 | 44.4 |
| Age (years) | | |
| <60 | 19 | 42.2 |
| ≥60 | 26 | 57.8 |
| Tumor diameter (cm) | | |
| <5 | 17 | 37.8 |
| ≥5 | 28 | 62.2 |
| Differentiation grade | | |
| High | 11 | 24.4 |
| Medium | 20 | 44.4 |
| Low and no | 14 | 31.1 |
| Lymph node metastasis | | |
| No | 18 | 40 |
| Yes | 27 | 60 |
| Depth of invasion | | |
| T1, T2 | 31 | 68.9 |
| T3, T4 | 14 | 31.1 |
| Dukes stage | | |
| A | 4 | 8.9 |
| B | 14 | 31.1 |
| C | 18 | 40.0 |
| D | 9 | 20.0 |

**Table 2.** Expressions of VEGF-C in colon cancer tissues and normal tissues

| *Group* | *n* | *VEGF-C expression* | | $x^2$ | *p value* |
|---|---|---|---|---|---|
| | | *Negative* | *Positive* | | |
| Cancer tissues | 45 | 11 | 34 | 21.966 | <0.001 |
| Normal tissues | 15 | 14 | 1 | - | - |

**Table 3.** Relationship between VEGF-C and clinicopathological features of colon

| *Features* | *Cases* | *VEGF-C expression* | | $x^2$ | *p value* |
|---|---|---|---|---|---|
| | | *Negative (n)* | *Positive (n)* | | |
| Gender | | | | | |
| Male | 25 | 8 | 17 | 0.265 | >0.5 |
| Female | 20 | 5 | 15 | | |
| Age (years) | | | | | |
| <60 | 19 | 3 | 16 | 2.747 | >0.1 |
| ≥60 | 26 | 10 | 16 | | |
| Tumor diameter (cm) | | | | | |
| <5 | 17 | 4 | 13 | 0.862 | >0.5 |
| ≥5 | 28 | 11 | 19 | | |
| Differentiation grade | | | | | |
| High and medium | 31 | 13 | 18 | 8.256 | <0.01 |
| Low and no | 14 | 0 | 14 | | |
| Lymph node metastasis | | | | | |
| No | 18 | 9 | 9 | 6.508 | <0.05 |
| Yes | 27 | 4 | 23 | | |
| Depth of invasion | | | | | |
| T1, T2 | 31 | 13 | 18 | 8.256 | <0.01 |
| T3, T4 | 14 | 0 | 14 | | |
| Dukes stage | | | | | |
| A, B | 18 | 10 | 8 | 10.385 | <0.01 |
| C, D | 27 | 3 | 24 | | |

**Table 4.** Correlation between VEGF-C positive intensity and pathological factors

| *Factors* | *VEGF-C positive intensity* | | | *Mean* | *Z* | *p value* |
|---|---|---|---|---|---|---|
| | + | ++ | +++ | | | |
| Differentiation grade | | | | | | |
| High and medium | 9 | 5 | 4 | 6 | 0.727 | 0.394 |
| Low and no | 4 | 6 | 4 | 4.5 | | |
| Lymph node metastasis | | | | | | |
| No | 4 | 3 | 2 | 2 | 5.538 | 0.019 |
| Yes | 6 | 7 | 10 | 6.5 | | |
| Depth of invasion | | | | | | |
| T1, T2 | 8 | 6 | 4 | 6.33 | 1.123 | 0.289 |
| T3, T4 | 3 | 4 | 7 | 4.33 | | |
| Dukes stage | | | | | | |
| A, B | 4 | 2 | 2 | 2 | 5.586 | 0.018 |
| C, D | 7 | 8 | 9 | 6.5 | | |

## Discussion

Angiogenesis plays an important role in local tumor infiltration and metastasis. VEGF family controls the angiogenesis, lymphangiogenesis, vascular permeability and endothelial cell survival. VEGF is currently the most potent angiogenetic stimulating factor, which increases the vascular permeability to cause plasma protein and fluid exosmosis, causing extracellular matrix changes, promoting angiogenesis and new matrix formation, and providing a basis for the tumor growth, invasion and metastasis [9]. The currently known members of mammalian cell VEGF growth factor family are: VEGF-A, -B, -C, -D, PIGF and the newly found -E. VEGF-C is a recently found ligand to VEGFR-3. The synthetic precursor protein becomes mature *via* protein lysis, whose affinity with VEGFR-3 is increased 400-fold and it can also bind to VEGF-2. VEGF-C, as promoting lymphatic endothelial growth factor and a specific marker of tumor angiogenesis, plays an important role in neovascularization, lymphangiogenesis and tumor metastasis [10].

In physiological or pathological conditions, VEGF-C promotes the increase of vascular permeability, enhances the effect of stimulating factors on endothelial cells, promotes the exosmosis of plasma protein and forms a temporary matrix of neovascularization, which contributes to angiogenesis [11]. It has been found that VEGF-C and its receptors are highly expressed in papillary thyroid carcinoma [12], breast cancer [13], bladder cancer [14], colorectal cancer [15], cervical cancer [16], gastric cancer [17], and prostate cancer [6]. Their binding can stimulate vascular endothelial cell proliferation and induce neovascularization, providing blood supply for tumor growth, promoting the uncontrolled proliferation of tumor cells, and participating in the tumor cell growth, invasion and metastasis.

In this study, the expression of VEGF-C was found significantly expressed in the edge of cancer nest and interstitial infiltration site, and the positive rate in rectal cancer tissues was significantly higher than that in normal tissues ($p<0.01$). VEGF-C was closely correlated to the grade of differentiation of rectal cancer, depth of tumor infiltration and Dukes stage ($p<0.05$). The positive expression rate of VEGF-C in patients with lymph node metastasis was significantly higher compared with patients without such metastasis and the difference was statistically significant ($p<0.05$), suggesting that the local invasion capacity of rectal cancer and even tumor growth and distant metastasis may require nutrition *via* blood vessels. The higher the grade of differentiation and the lower the infiltration stage, the more VEGF-C will be expressed in rectal cancer tissues, indicating that the abnormally high angiogenesis-promoting capacity may be an important mechanism causing intense invasion, rapid progression, lymph node metastasis, as well as distant metastasis. Moreover, Furudoi et al. [15] used immunohistochemistry to analyze the expression of VEGF-C in colon cancer, and the results showed that the positive expression of VEGF-C was positively correlated with tumor pathological stage, lymphatic invasion and metastasis, venous invasion, liver metastasis, Dukes stage and angiogenesis. Rank sum test was performed for the assessment of the relationship of positive intensity of VEGF-C with the grade of differentiation, lymph node metastasis, depth of invasion and Dukes stage and the results showed that the positive intensity of VEGF-C was not related to the grade of differentiation and depth of invasion, but related to the lymph node metastasis and Dukes stage.

## Conclusions

VEGF-C is highly expressed in rectal cancer tissues, and its positive expression is correlated with tumor invasion depth, lymph node metastasis and Dukes stage.

## Conflict of interests

The authors declare no conflict of interests.

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