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Original Article

Clinical study on the correlation between lymph node metastasis and lymphangiogenesis in colon cancer

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Abstract: Objective: To investigate correlation between expression of vascular endothelial growth factor-C (VEGF-C), vascular endothelial growth factor-D (VEGF-D), and expression of lymphatic microvessel density (LMVD) in colon cancer tissues. Methods: A total of 100 cases with colon cancer (colon cancer group) and 100 healthy people (control group) were randomly selected in Qingdao Center Hospital from June 2015 to December 2017. Paraffin sections of colon cancer lesions, adjacent tissues, and normal colon tissues were collected. Expression levels of VEGF-C, VEGF-D, and LMVD were examined by immunohistochemistry. Analysis of the relationship between expression levels (VEGF-C, VEGF-D, and LMVD) in colon cancer tissues and clinicopathological parameters (age, sex, Duke's staging, differentiation, pathogenic site, as well as positivity and negativity of lymph node metastasis) was performed to explore the correlation between lymph node metastasis and lymphangiogenesis. Results: Positive rates of VEGF-C and VEGF-D in the colon cancer group were significantly higher than those in the control group. The positive rate of LMVD in colon cancer adjacent tissues was significantly higher than that in healthy colon tissue (all $P<0.05$). In the adjacent tissues, the expression of VEGF-C and VEGF-D was significantly higher than that in adjacent metastatic lymph nodes (both $P<0.05$). The LMVD level in adjacent tissues with positive VEGF-C was higher than that with negative VEGF-C, and the LMVD level in adjacent tissues with positive VEGF-D was higher than that with negative VEGF-D. Differences between expression of VEGF-C, VEGF-D, and differentiation of colon cancer and lymph node metastasis were statistically significant (all $P<0.05$). Expression of LMVD was related to Duke's staging, differentiation, and lymph node metastasis. Conclusion: Overexpression of VEGF-C and VEGF-D can promote lymphangiogenesis, increase expression of LMVD, and lead to lymph node metastasis of colon cancer.

Keywords: Colon cancer, lymph node metastasis, lymphatic vessel, correlation

Introduction

Colon cancer is a common clinical malignant tumor of the digestive tract [1, 2] and surgical resection is the main treatment method. However, studies have found that the postoperative recurrence rate of this disease is extremely high, mainly because colon cancer can be metastasized through lymphatic channels [3, 4]. Prevention of postoperative lymph node metastasis is important for the improvement of surgical effect and for avoiding postoperative recurrence [5-7]. Lymph node metastasis of colon cancer is a process including the deviation of tumor from the primary lesions, the invasion to the lymphatic system, and formation of lesions in the regional lymph nodes [8, 9]. Vascular endothelial growth factor-C (VEGF-C) and vascular endothelial growth factor-D (VEGF-D) are secreted glycoproteins and key factors of tumor metastasis. Lymphatic microvessel density (LMVD) refers to the number of lymphatic vessels, showing the formation of lymphatic vessels [10-12].

Recently, studies on lymphangiogenesis and lymph node metastasis of colon cancer have reached the molecular level, and biomarkers have been widely used, but studies have found that most biomarkers have low specificity and sensitivity, which affects the accuracy of research [13].

In this study, immunohistochemistry was used to detect the expression of VEGF-C, VEGF-D, and LMVD in colon cancer and adjacent tis-

sues. The correlation between lymph node metastasis and lymphangiogenesis was explored to provide a scientific basis for the treatment of colon cancer and the prevention of lymph node metastasis.

Materials and methods

Subjects

Related indicators of 100 patients with colon cancer (colon cancer group) and 100 healthy people (control group) in Qingdao Center Hospital from June 2015 to December 2017 were retrospectively studied. Colon specimens of each case in the colon cancer group included 1 colon cancer tissue and 1 adjacent tissue. Colon specimens in the control group were taken from colon tissue. In the colon cancer group, there were 54 males and 46 females, aged 24-67 years, with an average age of 50.42±5.91 years. In the control group, there were 50 males and 50 females, aged 27-69 years, with an average age of 52.54±6.03 years. Of all the patients with colon cancer, 38 were well-differentiated, 44 were moderately differentiated, and 18 were poorly differentiated. All patients were aware of the clinical protocol before the trial, and informed consents were obtained from all subjects. This study was approved by the Ethics Committee of Qingdao Center Hospital.

Inclusion criteria: Patients who were pathologically diagnosed as colon cancer, patients who did not receive drug therapy before surgery, patients with normal mental status, patients who cooperated with medical treatment and diagnosis, and patients who did not suffer from other intestinal malignant tumors. Exclusion criteria: Patients with serious injury in heart, liver, kidney or other important organs or patients who could not complete the entire study process.

Methods

Paraffin-embedded tissue specimens were processed into sections of 4 and 5 μm sections were processed for each specimen. One specimen was subjected to HE staining for the observation of histiocyte differentiation [14], whereas another one was used as a negative control and for elimination of background interference. The other three were used for immunohistochemical staining of VEGF-C, VEGF-D and LMVD, respectively.

Primary antibody: Rabbit anti-VEGF-C concentrated polyclonal antibody, rabbit anti-VEGF-D concentrated polyclonal antibody, rabbit anti-LMVD concentrated polyclonal antibody (all from Zhejiang Beyotime Biotechnology Co., Ltd.). The immunohistochemical staining kit contained a biotin-labeled secondary antibody working solution (from Zhejiang Beyotime Biotechnology Co., Ltd.). The staining and pathological diagnosis results were read separately by two pathologists in a single-blind state. In the colon cancer group, the data of Duke's staging, differentiation and pathogenic site of colon cancer were examined and recorded.

Observation index

Immunohistochemical diagnosis of VEGF: the positive expression was located in the cytoplasm and visualized as yellow granules. The scores were based on the degree of positive staining and the proportion of stained cells with the following scoring system: 0 point for no staining, 1 point for light staining, 2 points for moderate staining and 3 points for dark staining. The proportion of stained cells was scored as 0 point for stained cells less than 5%, 1 points for 5%-25%, 2 points for 25%-50%, 3 points for more than 50%. The final score = staining degree * proportion of stained cells, 0-1 point for negative, 2-3 points for weak positive (+), 4-6 points for moderately positive (++), scores greater than 6 points for strongly positive (+++) [15].

Determination of LMVD: Yellow stain appeared after immunohistochemical staining and the lymphatic vessels were composed of a single layer of endothelial-like cells with no basement membrane and smooth muscle cells. Lumens without erythrocytes were lymphatics. Thick areas of lymphatic vessels were selected through a low-power microscope (X20), and a high-power microscope (X40) was used to count lymphatic vessels; the average value of 5 continuous counting fields was recorded [16].

Statistical processing

SPSS 19.0 software was used for statistical analysis of the data. Measurement data are expressed as mean ± standard deviation (mean

Table 1. Comparison of general information between two groups

Data directory	Colon cancer group	Control group	t/χ^2	P
Gender (male/female)	54/46	50/50	0.294	0.619
Age (year)	50.42±5.91	52.54±6.03	0.432	0.864

Table 2. Comparison of VEGF-C, VEGF-D and LMVD between the two groups

Group	Cases	Positive rate (%)		
		VEGF-C	VEGF-D	LMVD
Colon cancer	100	50	60	12
Control	100	20	8	2
χ^2		7.892	9.783	11.211
P		0.032	0.021	0.012

Note: VEGF-C: vascular endothelial growth factor-C, VEGF-D: vascular endothelial growth factor-D, LMVD: expression of lymphatic microvessel density.

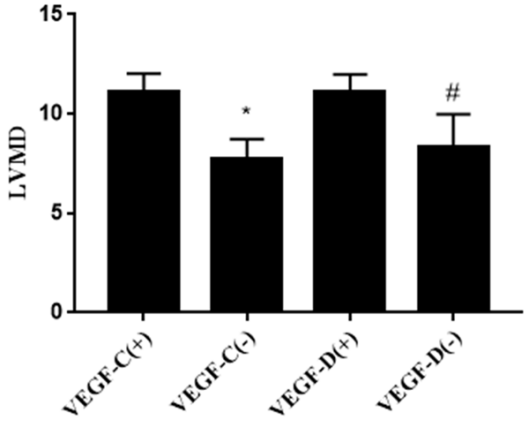

Figure 1. Comparison of LMVD levels in adjacent tissues with VEGF-C or VEGF-D. Positive (+), negative (-). Compared with positive VEGF-C, *P<0.05, and compared with positive VEGF-D, #P<0.05.

± sd); t-test was used to compare the data with normal distribution between the two groups. Count data were expressed as percentages (%), tested by χ^2 and Fisher exact probability method, and denoted by χ^2. The difference was statistical significant when P<0.05.

Results

Comparison of general information between two groups

The gender and age of the two groups were compared, with no significant difference (both P>0.05). See **Table 1**.

Comparison of VEGF-C, VEGF-D and LMVD between the two groups

In patients with colon cancer, positive rates of VEGF-C VEGF-D, and LMVD were 50%, 60%, and 12%, respectively. In the control group, positive rates were 20%, 8%, and 2%, respectively. The positive rates of VEGF-C, VEGF-D, and LMVD in the colon cancer group were significantly higher than those in the control group (all P<0.05). See **Table 2**.

Comparing LMVD levels in adjacent tissues with VEGF-C or VEGF-D

The LMVD levels were 11.38±2.15, 7.34±1.15, 11.55±1.22 and 8.21±0.79 in adjacent tissues with positive VEGF-C, negative VEGF-C, positive VEGF-D and negative VEGF-D. The LMVD level in adjacent tissues with positive VEGF-C was higher than that with negative VEGF-C, and the level in adjacent tissues with positive VEGF-D was higher than that with negative VEGF-D, indicating that overexpression of positive VEGF-C and VEGF-D promoted lymphangiogenesis in the adjacent tissues. See **Figure 1**.

Expression of VEGF-C and VEGF-D in adjacent tissues and adjacent metastatic lymph nodes

In adjacent tissues, there were 56 cases of negative VEGF-C, 44 cases of positive VEGF-C; in adjacent metastatic lymph nodes, there were 4 cases of negative VEGF-C, and 14 cases of positive VEGF-C. The positive rate of VEGF-C in the adjacent tissues was significantly higher than that in the adjacent metastatic lymph nodes (P<0.05). There were 60 cases of negative VEGF-D and 40 cases of positive VEGF-D in adjacent tissues. There were 6 cases of negative VEGF-D and 16 cases of positive VEGF-D in adjacent metastatic lymph nodes. The positive rate of VEGF-D in adjacent tissues was obviously higher than that in adjacent metastatic lymph nodes with a statistically significant difference (P<0.05). See **Table 3**.

Relationship between expression of VEGF-C, VEGF-D and clinicopathological parameters in colon cancer tissues

The OR of VEGF-C expression and differentiation in colon cancer tissues was 1.432, and its 95% CI was 0.321-1.422; the OR of VEGF-C

Table 3. Expression of VEGF-C and VEGF-D in adjacent tissues and adjacent metastatic lymph nodes (%)

Tissue	VEGF-C		χ^2	P	VEGF-D		χ^2	P
	Negative rate	Positive rate			Negative rate	Positive rate		
Adjacent tissues	56	44	3.321	0.023	60	40	3.301	0.027
Adjacent metastatic lymph nodes	4	14			6	16		

Note: VEGF-C: vascular endothelial growth factor-C, VEGF-D: vascular endothelial growth factor-D.

Table 4. Regression analysis of risk factors for the expression of VEGF-C and VEGF-D in colon cancer tissues

	VEGF-C			VEGF-D		
Clinical pathological factors	P	OR	95% CI	P	OR	95% CI
Differentiation	0.021	1.432	0.321, 1.422	0.036	1.764	0.396, 1.499
Lymph node metastasis	0.032	1.632	0.421, 1.652	0.029	1.402	0.354, 1.732

Note: VEGF-C: vascular endothelial growth factor-C, VEGF-D: vascular endothelial growth factor-D, OR: odds ratio, CI: confidence interval.

Table 5. Relationship between LMVD and pathological factors of colon cancer

Group	Cases	LMVD expression	t/χ^2	P
Age (year)				
<60	20	10.26±0.72	0.765	0.823
>60	80	10.09±0.79		
Gender				
Male	54	10.07±0.97	0.875	0.793
Female	46	9.79±0.54		
Duke stage				
A	14	1.02±0.14	9.654	0.015
B	44	4.38±0.57		
C	26	15.87±0.79		
D	16	18.84±0.87		
Differentiation				
Well-differentiated	38	5.68±0.76	5.432	0.032
Moderately differentiated	44	9.91±0.98		
Poorly differentiated	18	17.02±0.59		
Pathogenic site				
Straight left hemicolon	64	10.03±0.81	0.213	0.654
Right hemicolon	36	9.81±0.92		
Lymph node metastasis				
Positive	64	44.94±0.73	8.653	0.021
Negative	36	15.26±1.08		

Note: LMVD: expression of lymphatic microvessel density.

expression and lymph node metastasis was 1.632, and its 95% CI was 0.421-1.652. The OR of VEGF-D expression and differentiation in colon cancer tissues was 1.764, and its 95% CI was 0.396-1.499; the OR of VEGF-D expression and lymph node metastasis was 1.402 with, and its 95% CI was 0.354-1.732. See **Table 4**.

Relationship between LMVD and pathological parameters of colon cancer

The expression of LMVD at Duke's Stage A, B, C, and D was 1.02±0.14, 4.38±0.57, 15.87±0.79, and 18.84±0.87, respectively. The expression level of LMVD in well differentiation, moderate differentiation, and poor differentiation was 5.68±0.76, 9.91±0.98, and 17.02±0.59, respectively. The expression of LMVD in negative lymph node metastasis was 15.26±1.08, and the expression of LMVD in positive lymph node metastasis was 44.94±0.73. See **Table 5**.

Univariate logistic regression analysis of LMVD

Expression of LMVD was not related to gender, age and pathogenic site, but was related to Duke's staging, differentiation and lymph node metastasis. See **Table 6**.

Discussion

As a malignant tumor of the digestive tract, colon cancer has shown an increasing incidence. This disease greatly impacts on patients'

Table 6. Univariate logistic regression analysis of LMVD

Factor	P	OR	95% CI
Age	0.0923	0.984	0.235, 1.256
Gender	0.0823	0.132	0.374, 1.321
Duke stage	0.0231	0.112	0.493, 1.926
Differentiation	0.0156	0.923	0.294, 1.243
Pathogenic site	0.1243	0.102	0.205, 0.985
Lymph node metastasis	0.0231	0.132	0.420, 1.854

Note: LMVD: expression of lymphatic microvessel density, OR: odds ratio, CI: confidence interval.

life and work, with a low survival rate and high recurrence rate during treatment, so treatment for colon cancer has attracted clinical attention [17]. At present, the disease is mainly treated by surgery, however, high rate of recurrence and metastasis appears after the resection of lesions. Relevant studies have shown that the survival rate of the patients decreases with the increase of lymph node metastasis, so prevention of lymph node metastasis is needed to improve the operation effects and to prolong survival time of patients [18, 19]. Therefore, lymph node metastasis is an important research topic for the treatment of colon cancer. Lymphangiogenesis can occur in malignant tumors, lymphedema, inflammation and other pathological conditions, thus, it is speculated that there is a correlation between lymphangiogenesis and lymph node metastasis [20]. Previous studies did not find any specific markers that could be used for labeling lymphatic vessels, therefore, there were few studies about lymphatics in malignant tumors. Currently, with in-depth study of lymphatic vessels, multiple specific markers of lymphatic endothelial cells have been discovered, which provides technical support for research of lymphatic vessels [21, 22]. In this study, immunohistochemical methods were used to determine the indexes of lymphangiogenic factors and lymph node metastasis in colon cancer tissues, so as to determine whether there was correlation between them or not from the perspective of molecular biology and morphology. Also, we studied the relationship between lymphangiogenic factors and clinicopathological parameters of colon cancer from the perspective of lymphatic survival. The above findings could provide a strong guide regarding the significance for the prognosis of colon cancer treatment and the prevention of lymph node metastasis.

There are various receptors of VEGF-C and VEGF-D *in vivo*, and the main ones are vascular endothelial growth factor receptor-2 and vascular endothelial growth factor receptor-3 (VEGFR-3). The two growth factors bind to VEGFR-3 in normal body to promote the proliferation and migration of endothelial cells, thus, angiogenesis and proliferation of lymphatic endothelial cells can be promoted, and the growth of tumor cells can be regulated. Lymphatic vessels, formed by the promotion of VEGF-C and VEGF-D, have features of high permeability, no pericytes and having smooth muscle cells. The endothelial cells of lymphatic vessels are not tightly connected, showing a laminar shape and a deletion of basement membrane, which leads to the above lymph become vulnerable to tumor cells. LMVD is an important factor of lymph node metastasis. Overexpression of LMVD indicates the increase of lymphatic vessels, which promotes the contact probability of tumor cells and lymphatic endothelium as well as the possibility of tumor cells entering the lymphatic vessels [23, 24].

In this study, immunohistochemistry was used to observe the morphology of lymphatic vessels, with the principle of enzymatic histochemical staining, it could better identify the lymphatic capillary. There is 5'-Nase activity on the wall of capillary lymphatic vessels, but there is no such enzyme activity in normal capillaries, so the type of capillary can be determined by discriminating 5'-Nase. This method, with strong specificity and accuracy, was applied to the determination of lymphangiogenic growth factors in this study [25].

We found that expression of LMVD in the adjacent tissues of patients with colon cancer was higher than that in colon tissues of healthy people, and its expression levels were higher in the positive VEGF-C tissues and positive VEGF-D tissues than in the negatives. LMVD increases the area of lymphatic vessels at the tumor edge as well as the contact area of the tumor cells and the lymphatic vessel wall, hence, the likelihood of tumor cells invading lymphocytes is increased, indicating that LMVD can promote lymph node metastasis. While exploring LMVD and clinicopathological parameters, we found that LMVD was related to differentiation, Duke's staging, and lymph node metastasis. The expression of LMVD in Duke's stage D and C was higher than in the other two stages. LMVD expression in a poorly differentiated phase was

higher than that in well-differentiated and moderately differentiated phases. The expression level of LMVD increased with differentiation. Expression of LMVD in positive lymph node metastasis was significantly higher than that in the negative phase, with statistical significance (all $P<0.05$). The results were consistent with reported findings [26, 27]. The above results suggest that LMVD is an important indicator of lymph node metastasis of colon cancer, and excessive expression may increase the possibility of lymph node metastasis.

In the investigation of the relationship between the expression of VEGF-C, VEGF-D and clinicopathological parameters in colon cancer tissues, we found that the expression of VEGF-C and VEGF-D was related to differentiation and lymph node metastasis with significant differences (all $P<0.05$), indicating that the above two factors could promote lymph node metastasis of colon cancer. Therefore, drug therapy can be used to reduce the growth of lymphangiogenic factors and the expression of VEGF-C, VEGF-D, to avoid postoperative lymph node metastasis, or to inhibit the secretion of VEGF-C and VEGF-D receptors, leading to less formation of lymphatic vessels in colon cancer. The results of this study were similar to the study of Dai [28].

This study only revealed the correlation between lymphangiogenesis and lymph node metastasis. The relationship between the expression levels of lymphangiogenic factors VEGF-C, VEGF-D and the number of metastatic lymph nodes has not been statistically analyzed. So, further studies are needed. In addition, it is necessary to further explore the inhibition of the secretion of VEGF-C and VEGF-D, so as to provide an effective way for the prevention of lymph node metastasis after colon cancer surgery.

In summary, the expression of VEGF-C, VEGF-D, and LMVD in colon cancer patients was significantly higher than those in healthy people. The above three factors were closely related to lymphangiogenesis and lymph node metastasis of colon cancer. These data provide scientific support for the prevention of lymph node metastasis and the inhibition of lymphangiogenesis of colon cancer.

Disclosure of conflict of interest

None.

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