Original Article

Detection and prognostic value of intratumoral and peritumoral lymphangiogenesis in colorectal cancer

Yanbin Zhang[1,2#], Yue Liu[1#], Danhua Shen[3], Hui Zhang[3], Hongyan Huang[2], Sha Li[2], Jun Ren[2]

[1]Department of Peritoneal Cancer Surgery, Beijing Shijitan Hospital, Capital Medical University, Beijing, China; [2]Department of Oncology, Capital Medical University Cancer Center, Beijing Shijitan Hospital, the Capital Medical University, Beijing, China; [3]Department of Pathology, Peking University People's Hospital, Peking University, Beijing, China

Contributions: (I) Conception and design: Y Zhang, J Ren; (II) Administrative support: H Huang; (III) Provision of study materials or patients: Y Liu, D Shen, H Zhang; (IV) Collection and assembly of data: Y Liu; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Jun Ren. Professor, Department of Oncology, Capital Medical University Cancer Center, Beijing Shijitan Hospital, the Capital Medical University, No. 10 Tieyi Road, Yangfangdian Street, Haidian District, Beijing, China. Email: jun.ren@duke.edu.

Background: Colorectal cancer (CRC) with lymphatic invasion is one of the critical prognostic factors in lymph node metastasis. Lymphangiogenesis has a significant effect on lymphatic metastasis and tumor progression. However, the significance of intratumoral and peritumoral lymphangiogenesis has been controversial in CRC. The aim of this study is to investigate the different role of introtumoral and peritumoral lymphangiogenesis in CRC progression and prognosis.

Methods: Lymphangiogenesis of 120 CRC specimens, as measured by lymphatic vessel density (LVD), was examined by immunostaining for podoplanin, a lymphatic vessel-specific marker. The mean number of lymphatic vessels of three hotspots was measured in intratumoral and peritumoral areas as intratumoral LVD (LVDit) and peritumoral LVD (LVDpt), respectively. The association of LVDit and LVDpt with the clinicopathological findings and prognosis was investigated.

Results: Compared to the peritumoral lymphatics, the intratumoral lymphatics were small, collapsed and irregular. The mean LVDpt was higher than the mean LVDit (P<0.001). LVDit was positively correlated with tumor size (P=0.009), tumor histologic grade (P=0.023), and overall survival (P=0.036). LVDpt was correlated with lymph node metastasis (P<0.001), tumor stage (P=0.004), and overall survival (P=0.016).

Conclusions: LVDpt plays a prominent role in lymph node metastasis, whereas LVDit is more closely correlated with tumor growth and histopathological differentiation. Both LVDpt and LVDit contribute to CRC progression and prognosis.

Keywords: Colorectal cancer (CRC); lymphangiogenesis; lymphatic vessel density (LVD); prognosis; lymphatic invasion

Submitted Feb 14, 2020. Accepted for publication Sep 12, 2020.
doi: 10.21037/tcr-20-1038
View this article at: http://dx.doi.org/10.21037/tcr-20-1038

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and is considered the third leading cause of cancer-related deaths worldwide. CRC with lymphatic invasion is one of the critical prognostic factors in lymph node metastasis (1). Given this, clarifying the molecular and cellular mechanisms underlying the process of metastasis, a major avenue of cancer research, is needed to help identify a new therapeutic target in the treatment of CRC.

It has been reported that there is a close relationship between increased lymphangiogenesis and metastatic spread

in studies of other human cancers (2-6). For example, in head and neck cancers, malignant melanoma, and prostate and breast cancer, increased intratumoral or peritumoral lymphatic vessel density (LVD) has been found to be associated with metastatic spread and poorer prognosis. It has also been suggested that high LVD in CRC is associated with lymphatic metastasis and poor prognosis (5,7,8). Nevertheless, the clinical significance of intratumoral or peritumoral lymphatics in CRC remains unclear in these studies, as is the case for studies of other tumors (9).

There is still debate concerning the effect of intratumoral or peritumoral lymphatics on the progression and prognosis of different tumors (10). In pancreatic ductal adenocarcinoma, in which early lymph node metastasis is common, neither intratumoral nor peritumoral lymphangiogenesis was present or detected (11). In gastric cancer, thyroid papillary carcinoma, and squamous cell carcinoma of the head, neck, and esophagus, intratumoral LVD (LVDit) was predictive of lymphatic metastasis (12-15). Meanwhile, peritumoral LVD (LVDpt) has been associated with lymph node metastases in cutaneous melanoma, breast cancer, prostate adenocarcinoma, and uterine cervix carcinoma (3-6). However, the function of different lymphatic vessels in CRC is still elusive and controversial. Some studies have suggested that LVDit is related to tumor progression and prognosis (16,17), whereas other studies have presented conflicting results (18-20).

Thus, the aim of the present study was to detect intratumoral and peritumoral lymphangiogenesis and explore the relationship between clinicopathological parameters, including LVDit or LVDpt, lymph node metastasis, pathological stage, and prognostic factors in CRC.

We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-1038).

Methods

CRC samples

The present study involved 120 primary CRC patients who underwent surgical resection at the Department of Gastroenterological Surgery, Peking University People's Hospital, Beijing, China, between September 2010 and April 2012. None of the patients had undergone preoperative radiotherapy and chemotherapy. Patients were followed up clinically for more than 5 years postoperatively. The time range of follow-up was 1–78 months, with an average of 53 months. All the samples were fixed using 10% formalin for 24 hours, and were then embedded with paraffin wax. After being cut into 4-μm sections, the samples were treated with hematoxylin and eosin (HE) staining and observed under a microscope by two experienced pathologists according to the criteria of the Union for International Cancer Control. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Ethics Committee of Peking University People's Hospital (No. 2020PBH006-01), and informed consent was taken from all the patients.

Immunohistochemical staining

The patients' biopsy sections were immunohistochemically stained using a streptavidin-peroxidase technique (Beijing Zhongshan Golden Bridge Biological Technology). Briefly, the embedded sections were deparaffinized in a graded series of ethanol and 100% xylene; 0.3% hydrogen peroxide in methanol was used for 10 minutes at room temperature to block endogenous peroxidase activity. After treatment with 10% normal rabbit serum for 10 minutes, sections were stained overnight with a mouse antihuman podoplanin monoclonal antibody (AngioBio) as the primary antibody at 4 ℃. Each slide was incubated with antimouse immunoglobulin G antibody for 10 minutes and streptavidin-biotinylated horseradish peroxidase complex for 5 minutes. Diaminobenzidine and Mayer's hematoxylin solution were used as a chromogen and nuclear counterstain, respectively. Phosphate-buffered saline (PBS) without a primary antibody was added for a negative control.

LVD assessment

LVD quantification was tested as previously described (21). A microvessel was considered to be a single endothelial cell or a cluster of endothelial cells positive for podoplanin, and was located around a visible lumen, which was easily separated from adjacent microvessels and from other connective tissue components. Intratumoral lymphatic vessels were defined as those within the tumor cell islets, and peritumoral lymphatic vessels as those in the periphery within 2 mm of tumors adjacent to the invasion front. Briefly, the three most vascularized areas examined by podoplanin were primarily visualized (so-called hotspots)

under a 40× field. Vessels in each of these areas were then detected under a 200× field. The mean values of three 200× field counts in this section were defined as the LVDpt or LVDit. The 120 cases were divided into two groups (LVDpt or LVDit group) in terms of the mean level of LVD.

Statistical analyses

All statistical analyses were carried out with SPSS version 22.0 software. Statistical comparisons were performed using unpaired two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) as appropriate. Overall survival curves were obtained by the Kaplan-Meier method, and the statistical significance of differences was evaluated by log-rank test. Univariate and multivariate analyses were carried out using the Cox proportional hazards model. Differences at P<0.05 were considered statistically significant.

Results

Intratumoral and peritumoral lymphatic vessels in CRC

The staining was specifically positive in lymphatic endothelial cells and negative in vascular endothelial cells, using podoplanin monoclonal antibody (*Figure 1A*,*B*). Intratumoral lymphatic vessels were usually small, collapsed, and irregular (*Figure 1C*,*D*). In contrast, the peritumoral lymphatic vessels were generally large and dilated, and were occasionally involved in tumor cell clusters (*Figure 1E*,*F*). Overall, the mean LVDpt was higher than the mean LVDit (18.99±6.89 *vs.* 9.91±4.25; P<0.001).

Relationship of LVDit and LVDpt with clinicopathological parameters in CRC

The correlations of LVDit and LVDpt with clinicopathological findings are summarized in *Table 1*. High LVDit was found to be significantly correlated with larger tumor size (P=0.009) and poor differentiation (P=0.023). In contrast, high LVDpt had a significant correlation with lymph node metastasis (P<0.001) and late tumor-node-metastasis (TNM) stage (P=0.004). No significant correlations were found between LVDit or LVDpt and other characteristics, including sex, age, tumor location, T-stage, and distant metastasis.

Survival analysis

Kaplan-Meier analyses were performed for the overall survival of LVDit or LVDpt. The survival rate of patients characterized with low LVDit (n=56) was remarkably higher than that of patients with high LVDit (n=64, 5-year survival rate: 66.9% *vs.* 50%, P=0.036, log-rank) (*Figure 2A*). Furthermore, the survival rate was considerably high in patients with low LVDpt (n=79) compared with patients with high LVDpt (n=41, 5-year survival rate: 66.1% *vs.* 42%, P=0.016, log-rank) (*Figure 2B*).

In the univariate analysis, decreased survival was associated with poor histopathological differentiation, lymph node metastasis, distant metastasis, advanced clinical stage, and high LVDit or LVDpt (*Table 2*). In the multivariate analysis, poor histopathological differentiation (P=0.042), lymph node metastasis (P=0.017), and distant metastasis (P<0.001) were still regarded as the crucial independent prognostic factors of a decreased overall survival rate (*Table 2*).

Discussion

The lymphatic vessel is the crucial metastasis pathway for the majority of cancers. Lymph node metastasis is a pivotal predictor of poor outcome, which implies the relevance of lymphatics to cancer biology (22). The discovery of novel markers for distinguishing blood and lymphatic vessels has facilitated the investigation of tumor-associated lymphangiogenesis and its potential function in tumor progression. Podoplanin is a special marker of lymphatic endothelial cells recommended for the evaluation of lymphangiogenesis in humans (21). In the present study, lymphatic vessels were immunostained with the podoplanin monoclonal antibody in CRC tissues, and we investigated the clinical significance of the podoplanin-positive lymphatic vessel counts. In the specimens used in our study, podoplanin expression was restricted to thin-walled lymphatic vessels with a single endothelial layer. Blood vessels with red blood cells failed to be stained, further demonstrating that podoplanin is a good lymphatic endothelial marker for the study of tumor-associated lymphangiogenesis.

It has been well established that lymphangiogenesis can occur in and around tumors (23). However, the functional significance of intratumoral and peritumoral lymphatics involved in the pathology of tumors remains controversial. In previous reports, lymphatic vessels were detected within the intratumoral area in gastric cancer (12), thyroid papillary carcinoma (13), and squamous cell carcinoma of the head, neck, and esophagus (14,15), and LVDit was found

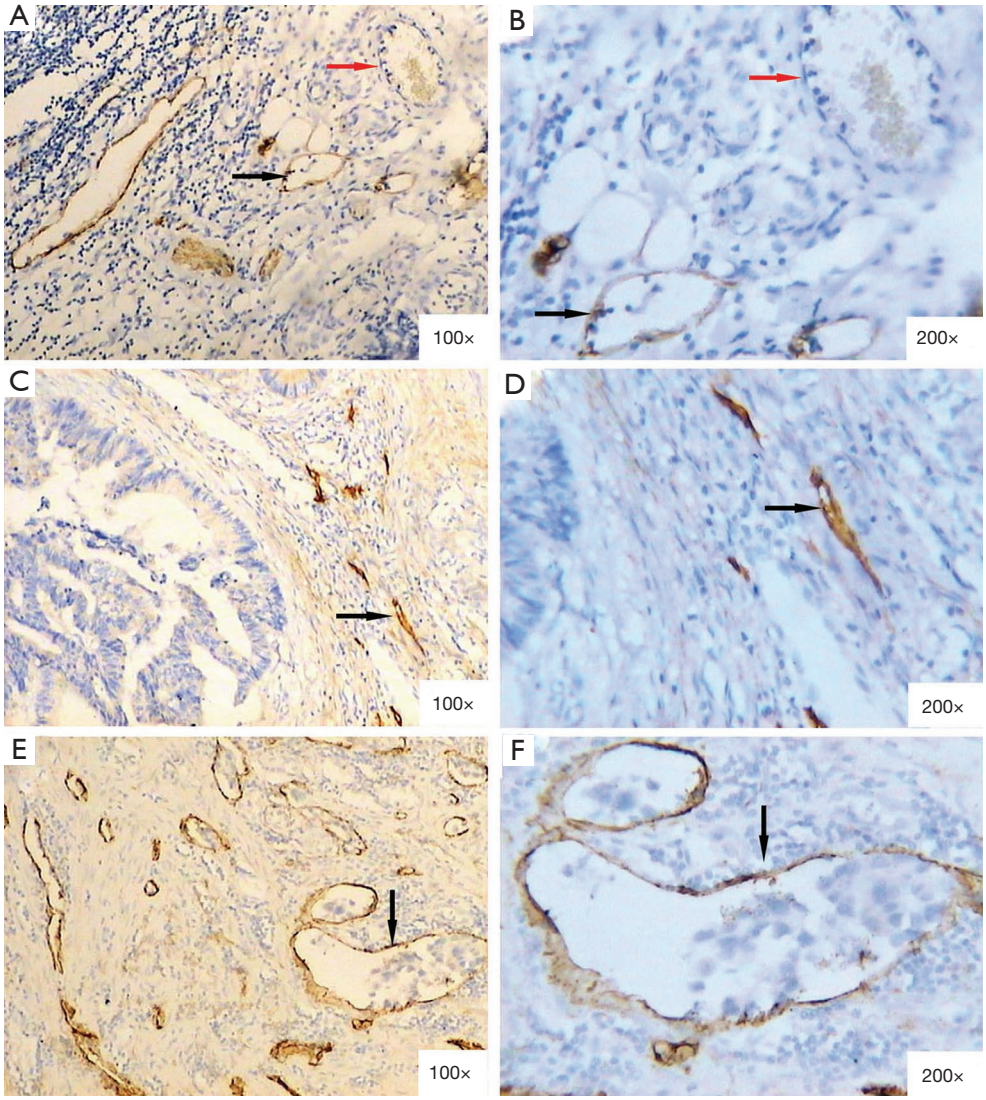

Figure 1 Streptavidin-peroxidase technique was used for immunostaining of lymphatic vessels (black arrows) by podoplanin monoclonal antibody. (A) Magnification 100× and (B) magnification 200×: blood vessels containing red blood cells were podoplanin-negative (red arrows); (C) magnification 100× and (D) magnification 200×: intratumoral lymphatic vessels; (E) magnification 100× and (F) magnification 200×: peritumoral lymphatic vessels, occasionally containing tumor cell clusters.

to be significantly associated with lymph node metastasis and a poorer prognosis compared to LVDpt. However, some other studies noted that high LVDpt was predictive of lymphatic involvement and poor prognosis in cutaneous melanoma, breast cancer, prostate adenocarcinoma, and uterine cervix carcinoma (3-6). Saad *et al.* reported that LVDit in CRC was related to both lymph node and liver metastases (16). Barresi *et al.* found that lymphangiogenesis was mostly located in the peritumoral area of CRC tissues, and LVDpt, rather than LVDit, was associated with lymph node metastasis in early CRC (18). Longatto-Filho *et al.* noted that LVDpt was correlated with hepatic metastasis, but not lymph node metastasis, in colon cancer, although LVDpt was lower than LVDit (20). These controversial findings might the result of a selective bias and a smaller number of samples. Most studies have mainly focused on a specified group of CRC patients with either early or late TNM stage. Based on our results from 120 CRC cases, spanning all TNM stages, we found that LVDpt, rather than LVDit, was substantially associated with lymph node

Table 1 Relationship of LVD with clinicopathological parameters in CRC

Characteristics	N	LVDit (mean ± SD)	P value	LVDpt (mean ± SD)	P value
Age at presentation (years)			0.314		0.875
<65	62	10.29±4.17		18.89±6.72	
≥65	58	9.51±4.32		19.09±7.12	
Sex			0.062		0.668
Male	74	10.48±4.34		19.20±7.20	
Female	46	8.99±3.96		18.64±6.42	
Site of tumor			0.156		0.488
Colon	75	10.33±4.25		19.33±7.03	
Rectum	45	9.20±4.18		18.42±6.99	
Size of tumor			0.009*		0.516
>5 cm	35	11.49±4.23		19.63±7.54	
≤5 cm	85	9.26±4.11		18.73±6.63	
Histologic grade (differentiation)			0.023*		0.452
Well, moderate	21	11.81±3.73		16.04±7.41	
Poor, mucinous	99	9.51±4.26		19.14±7.06	
Tumor status			0.327		0.960
T2	19	10.12±3.56		18.65±6.69	
T3	94	9.77±4.49		19.02±7.04	
T4	7	11.29±2.25		19.48±6.19	
Lymph node metastasis			0.194		<0.001*
Negative	57	9.38±4.21		16.47±5.12	
Positive	63	10.39±4.26		21.27±7.50	
Distant metastasis			0.061		0.101
Negative	98	9.55±4.26		18.47±6.64	
Positive	22	11.36±3.97		21.06±7.60	
TNM stage			0.205		0.004*
I	11	9.58±3.07		16.03±4.26	
II	43	8.88±4.43		16.65±5.27	
III	44	10.39±4.29		20.64±7.59	
IV	22	11.12±4.06		21.73±7.59	

*, P<0.05. LVD, lymphatic vessel density; CRC, colorectal cancer; LVDit, intratumoral LVD; LVDpt peritumoral LVD; SD, standard deviation.

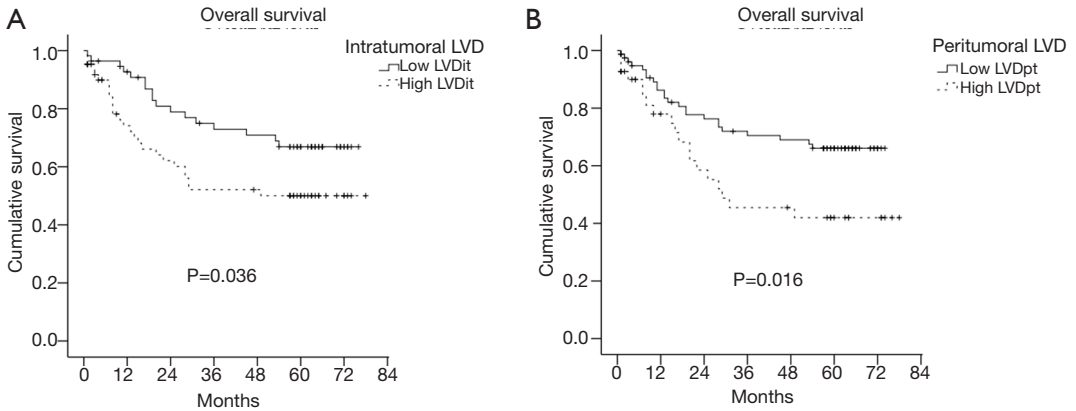

Figure 2 Survival analyses according to LVD, as determined by podoplanin staining. (A) Overall survival according to LVDit; (B) overall survival according to LVDpt. LVD, lymphatic vessel density; LVDit, intratumoral LVD; LVDpt, peritumoral LVD.

Table 2 Significant prognostic factors by univariate and multivariate analyses (Cox proportional hazards model)

Variables	Hazard ratio (95% CI)	P value
Univariate analysis		
Histologic grade (differentiation)	2.379 (1.168–4.847)	0.017
Well, moderate *vs.* poor, mucinous		
Lymph node metastasis	5.300 (2.527–11.113)	<0.001
Negative *vs.* positive		
Distant metastasis	13.303 (6.906–25.625)	<0.001
Negative *vs.* positive		
TNM stage	5.154 (2.381–11.161)	<0.001
I, II *vs.* III, IV		
LVDit	1.897 (1.028–3.501)	0.041
Low *vs.* high		
LVDpt	2.061 (1.126–3.771)	0.019
Low *vs.* high		
Multivariate analysis		
Histological grade (differentiation)	2.144 (1.029–4.467)	0.042
Well, moderate *vs.* poor, mucinous		
Lymph node metastasis	5.520 (1.364–22.341)	0.017
Negative *vs.* positive		
Distant metastasis	11.593 (5.224–25.730)	<0.001
Negative *vs.* positive		

CI, confidence interval; TNM, tumor-node-metastasis; LVD, lymphatic vessel density; LVDit, intratumoral LVD; LVDpt, peritumoral LVD.

metastasis, suggesting that LVDpt is more important in lymph node metastasis than LVDit.

The main location of lymphangiogenesis might vary in different types of tumors. In pancreatic ductal adenocarcinoma, lymphangiogenesis was not detected in either intratumoral or peritumoral areas (11). However, both LVDit and LVDpt increased in cutaneous melanoma (12). In another study, the majority of lymphatic vessels were located in intratumoral areas in squamous cell carcinoma of the head, neck, and esophagus (15); however, it was found to be the opposite for uterine cervix carcinoma (6). In our study, podoplanin-positive lymphatic vessels were observed both within the tumor mass and around the tumor periphery, and LVDpt was significantly higher than LVDit. Compared to intratumoral lymphatic vessels, peritumoral lymphatic vessels were generally large and dilated, thus further verifying the findings that in peritumoral areas of the tumor, both lymphangiogenesis and lymphatic vessel remodeling occurred, facilitating the entry of tumor cells into the lymphatics, and had functional importance in the spread of cancer (22). In addition, high LVDit was positively associated with larger tumor size and poor histopathological differentiation, indicating that intratumoral lymphangiogenesis may play a critical role in tumor growth and differentiation. These findings suggest that both intratumoral and peritumoral lymphangiogenesis contribute to CRC progression, but in a different manner.

In their study, Gao *et al.* found no relationship between LVD and other prognostic parameters, such as survival, in CRC (24). Longatto-Filho *et al.* (20) noted that LVDpt was correlated with CRC poor outcome markers, but not with significantly poor survival, while Matsumoto *et al.* (17) suggested that LVD was an independent prognostic factor of CRC. However, none of these studies analyzed the prognostic value of LVDpt or LVDit. In the present study, the survival curves demonstrated that both LVDit and LVDpt were associated with the overall survival of patients with CRC. The significance of LVDit and LVDpt for CRC prognosis was in agreement with that for gastric cancer (25).

There were several limitations in our study. Firstly, this was a retrospective study of post-surgical samples in a local medical institution and selection bias could not be avoided as the distribution of clinical characteristics of CRC patients. The results in this study needs to be validated in multicenter institutions prospectively. Secondly, further studies are required to elucidate the mechanisms underlying tumor associated lymphangiogenesis in CRC although it is true that lymphangiogenesis plays an important role in the progression of CRC.

In conclusion, our study showed that both intratumoral and peritumoral lymphangiogenesis occurs in CRC. Peritumoral lymphangiogenesis might have a more important role in lymph node metastasis compared with intratumoral lymphangiogenesis, while intratumoral lymphangiogenesis was found to be more correlated with tumor growth and histopathological differentiation. In addition, both high LVDit and LVDpt were predictive of poor prognosis in CRC. Considering the significance of intratumoral and peritumoral lymphangiogenesis contributing to CRC progression and prognosis, antilymphangiogenesis could be a valuable and reliable treatment for CRC.

Acknowledgments

Funding: The present study was supported by Beijing Postdoctoral Research Foundation (grant No. 2015422-54).

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/tcr-20-1038

Data Sharing Statement: Available at http://dx.doi.org/10.21037/tcr-20-1038

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr-20-1038). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This research was approved by the Ethics Committee of Peking University People's Hospital (No. 2020PBH006-01), and informed consent was taken from all the patients.

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Cite this article as: Zhang Y, Liu Y, Shen D, Zhang H, Huang H, Li S, Ren J. Detection and prognostic value of intratumoral and peritumoral lymphangiogenesis in colorectal cancer. Transl Cancer Res 2020;9(10):6189-6197. doi: 10.21037/tcr-20-1038

