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Clinic and biologic significance of angiogenesis and lymphangiogenesis in colorectal cancer

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Conflict of interest statement

None declared

Abstract

Background. Angiogenesis and lymphangiogenesis are essential for tumour development and progression. However, in colorectal cancer (CRC), the relationship between angiogenesis and clinic outcome is controversial, and the prognostic significance of lymphangiogenesis is not well examined because of the lack of specific marker for lymphatic vessels.

Aims. To evaluate blood microvessel density (MVD) following the proposed standard method for MVD assessment given by the first international consensus and lymph vessel density (LVD), and investigate their clinicopathologic and biologic significance in CRC.

Methods. MVD and LVD in primary tumours (n = 210), along with their corresponding adjacent normal mucosa (n = 105) and distant normal mucosa (n = 27) specimens, were immunohistochemically examined by using CD31 and D2-40 antibodies.

Results. Both MVD and LVD were higher in tumour compared with the corresponding normal mucosa. In tumours, MVD was positively related to particular interesting new cysteine-histidine-rich protein (PINCH) expression ($P = 0.006$), but not with clinicopathologic variables. LVD, in both intratumoural and peritumoural areas of tumours, was reversely related to Dukes' stage. There was no association between MVD or LVD and patients' survival ($P > 0.05$).

Conclusions. Angiogenesis and lymphangiogenesis occurred in CRC development, but were not related to CRC patient prognosis. PINCH may play a potential role in tumour angiogenesis.

Keywords: Angiogenesis; colorectal cancer; lymphangiogenesis

1. Introduction

The formation of new blood vessels (angiogenesis) and lymphatic vessels (lymphangiogenesis) is crucial for tumour development and progression of a variety of malignancies [1,2]. Previous studies on estimation of angiogenesis, most commonly by microscope quantification of microvessel density (MVD) on tissue immunostained with a variety of endothelial markers, have revealed the potential clinicopathologic significance of angiogenesis in a variety of cancers, predicting metastatic risk and patient prognosis. In colorectal cancer (CRC), some retrospective studies have demonstrated that an increased number of blood microvessels (high angiogenesis) often predicts locally aggressive, metastatic potential and unfavourable outcome [3-5], while others did not have the similar findings [6-8]. Recently, a meta-analysis of all published studies showed that high MVD predicted poor survival in patients with CRCs. However, the overall link between MVD and survival was rather weak, with a global risk ratio of 1.44 for overall survival [9]. Considering methodological differences among these studies including the variety of endothelial markers with difference in sensitivity (factor VIII, CD31 and CD34), the different cut off values and microvessel counting techniques as well as the difference of patient selection (such as tumour size, stage), the authors of the meta-analysis suggested that future studies on assessment the prognostic significance of angiogenesis should be carried out in a large series of patients with stratification of tumour stage, using specific antibody (CD31 or CD34), standard microvessel counting technique and multivariate regression survival analysis.

Lymphangiogenesis in tumour has been noticed, but its occurrence and regulation, especially its role in tumour development and metastasis has not well studied due to a lack of specific markers for identifying lymphatic vessels. Recent identification of novel antibodies to discriminate lymphatic vessels from the blood vessels has enabled the study of lymphatic

biology including lymphangiogenesis and its potential clinical implication [10,11]. Similarly, the clinicopathologic significance of lymphatic vessel density (LVD) in CRC remains controversial. Some studies demonstrated that LVD was an indicator of lymph node metastasis or patient survival [12-16], while others could not confirm the findings [17,18]. This may be also attributed to the difference in the various antibodies with different specificity, and the difference in the techniques of immunostaining and LVD quantification.

Angiogenesis is regulated by multiple pro- and anti- angiogenic molecules produced by various cell types, such as cancer, endothelial, and other stromal cells. It is generally thought that lymphangiogenesis is in an analogous fashion to angiogenesis. Identifying the factors that mediate angiogenesis and lymphangiogenesis in tumours could help us to determine appropriate anti-angiogenic and anti-lymphangiogenic therapy. Despite the members of the vascular endothelial growth factor (VEGF) as main regulators of angiogenesis and lymphangiogenesis, in particular, VEGF-A in angiogenesis, VEGF-C and VEGF-D in lymphangiogenesis, a growing number of pro- and anti-angiogenic molecules have been identified. Particular interesting new cysteine-histidine-rich protein (PINCH) was upregulated in the stroma of several common types of cancers including CRC [19] and expressed in a proportion of tumour-related endothelial cells stained by CD31 [20], suggesting that PINCH might be related to angiogenesis.

In order to investigate the relationship of MVD and LVD with clinicopathologic and biologic variables in CRCs, we assessed angiogenesis according to the first international consensus on methodology and criteria of evaluation of angiogenesis quantification in solid human tumours [21] using monoclonal antibody CD31 and lymphangiogenesis using monoclonal antibody D2-40, a novel monoclonal antibody specific for lymphatic vessels, in a series of 210 primary CRCs and their corresponding normal mucosa specimens.

2. Materials and Methods

2.1. Materials

Both blood vessels and lymphatic vessels were immunohistochemically examined in paraffin-embedded primary CRCs (n = 210), along with their corresponding distant normal mucosa (from the distant margin of resections, n = 27) and adjacent normal mucosa (normal mucosa adjacent to the primary tumour, n = 105) specimens. The samples were consecutively collected from CRC patients who underwent surgical resection at Linköping University Hospital, Linköping, and Vrinnevi Hospital, Norrköping, Sweden, between 1974 and 2001. The distant normal mucosa specimens were histological free from pretumour and tumour. The clinicopathologic characteristics of the patients, including gender, age, tumour location (colon and rectum), Dukes' stage, growth pattern (expansive and infiltrative) and differentiation (better and worse) (Table 1), were obtained from surgical and pathological records. The data of PINCH expression determined by immunohistochemistry [20] were taken from our previous study carried out at our laboratory. Among the 210 patients included in this study, 172 had radical surgical resection, 14 had radiotherapy alone, 16 had chemotherapy alone, 5 had a combination of radiotherapy and chemotherapy, 157 had no any adjuvant therapy, and 18 had no information of radiotherapy or chemotherapy. The patients were followed up until April 2006, and 73 patients died of CRC by that time. The median follow-up time was 56 months (range, 0.07-280 months) and mortality was 35% during the follow-up period. There was no information available concerning tumour location in three patients, Dukes' stage in five, growth pattern in nine, and PINCH expression in 104. The study was approved by the ethical committee at the Faculty of Health Sciences, Linköping University, Sweden.

Table 1. MVD and LVD in tumours in relation to clinicopathologic and biologic variables

Category	No.	MVD (Range)	<i>P</i>	LVD (Range)	<i>P</i>
Gender			0.988		0.283
Male	114	104.17 (13.00-372.17)		12.67 (0-36.00)	
Female	96	104.67 (16.33-248.83)		13.00 (0-127.67)	
Age (year)			0.987		0.745
≤ 71	86	103.17 (15.83-372.17)		12.25 (0-127.67)	
>71	124	105.58 (13.00-288.33)		13.00 (0-90.17)	
Tumor location			0.959		0.209
Colon	109	106.00 (13.00-372.17)		12.67 (0-127.67)	
Rectum	98	101.42 (15.83-372.17)		13.33 (0-39.83)	
Unknown	3				
Dukes' stage			0.494		0.012
A	32	106.50 (16.67-372.17)	0.218*	14.00 (0-35.83)	0.002*
B	80	105.92 (15.83-372.17)		15.83 (0-38.33)	
C	60	104.75 (13.00-237.17)		10.50 (0-90.17)	
D	33	95.50 (16.33-201.67)		9.67 (0-127.67)	
Unknown	5				
Growth pattern			0.678		0.416
Expansive	110	101.25 (13.00-248.83)		12.67 (0-39.83)	
Infiltration	91	105.83 (15.83-372.17)		13.17 (0-90.17)	
Unknown	9				
Differentiation			0.153		0.398
Better	147	105.67 (13.00-372.17)		13.17 (0-76.00)	
Worse	63	91.33 (15.83-372.17)		12.33 (0-127.67)	
PINCH			0.006		0.559
Weak	56	88.42 (13.00-372.17)		11.00 (0-127.67)	
Strong	50	121.17 (45.50-372.17)		11.08 (0-35.83)	
Unknown	104				

*Dukes' stage A+B versus C+D

2.2. Immunohistochemistry

Five-micrometer paraffin-embedded sections were deparaffinised in xylene, rehydrated with a graded series of ethanol to water. The sections were cooked with Tris-EDTA buffer (pH 9.0) in a calibration bath (Julabo TW8) at 99°C for 40 min for CD31 or in a high pressure-cooker for 8 min for D2-40. Following preincubation in methanol with 0.3% H₂O₂ for 20 min, the sections were incubated with protein block (Dako, Carpinteria, CA) for 10 min and then mouse monoclonal anti-CD31 antibody (1:40; Dako) or mouse monoclonal D2-40 antibody (prediluted; Abcam, Cambridge, UK) at room temperature for 30 min. After washing in phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with a secondary antibody (Dakocytomation EnVision+ System-HRP Labelled Polymer anti-mouse, Dako) at room temperature for 25 min. Subsequently, the sections were subjected to 3,3'-diaminobenzidine tetrahydrochloride for 8 min and then counterstained by haematoxylin. Sections known positive staining for the proteins as positive control, and as negative control replacing the primary antibodies by IgG₁ were included in each run.

2.3. Assessment of microvessel density

Microvessel density was assessed by counting microvessels immunostaining for CD31 or D2-40 antigen under light microscope based on the proposed standard method for MVD assessment given by the first international consensus [21] and expressed as the number of vessels per 200x field, corresponding to an optical field of 0.74 mm². Briefly, the CD31 or D2-40 stained sections were initially scanned at low power (40x and 100x) and the areas of specimens having the highest number capillaries and small venules stained by CD31 or D2-40 (hot spots) were selected. Subsequently, microvessel counting was performed in three fields

of the hot spots, at 200x magnification (20x objective lens and 10x ocular lens) and the mean value of the three fields at 200x for each case was used for further analysis. Any brown-staining endothelial cells or cell cluster clearly separated from adjacent microvessels, tumour cells and other connective tissue elements were considered as a single countable vessel.

Neither vessel lumens nor the presence of red blood cells were needed to define a microvessel. Large vessels with a muscular layer (artery) and microvessels in the areas with necrosis, ulceration or intense inflammation (because vascularisation in these areas could be related to the inflammatory reaction and not to the presence of a tumour) within tumours was excluded and no cutoff calibre size was used for small microvessels and venules.

The microvessel count was performed by two of the authors (Gao J and Knutsen A), using a double-headed light microscope simultaneously, and agreeing on the identification of each countable microvessel. Both the authors had no any knowledge of the clinical outcomes and clinicopathologic features. In the case of discrepancy (16%), a recount was taken to reach an agreement.

2.4. Statistical analysis

Student's t-test was used to compare the difference in MVD or LVD between normal mucosa and tumour. Speraman rank correlation test was used to assess correlation between MVD and LVD, and the Mann Whitney U-test was used to analyze the association of clinicopathologic and biologic variables of the patients with MVD or LVD. Cox's Proportional Hazard Model was used to estimate relationship between MVD or LVD and patients' survival in univariate and multivariate analyses. Two-sided p-values of less than 5% were considered as statistical significance.

3. Results

3.1. Microvessel density of angiogenesis and lymphangiogenesis in normal mucosa and tumour

Blood vessels determined by CD31 were observed in the stroma of all distant normal mucosa (Fig. 1A), adjacent normal mucosa and tumour specimens (Fig. 1B). Lymphatic vessels stained by D2-40 were observed in stroma of 26 of 27 distant normal mucosa (Fig. 1C), 99 of 105 adjacent normal mucosa and 194 of 210 tumour specimens (Fig. 1D). Normal epithelial and tumour cells were not stained by either CD31 or D2-40 antibody.

In normal mucosa, blood vessels were found between crypts with regular lumens of vessels (Fig. 1A) while lymphatic vessels were confined to mucosa adjacent to the muscularis mucosae (Fig. 1C). In tumours, both blood (Fig. 1B) and lymphatic vessels (Fig. 1D) were heterogeneously distributed in the stroma, mostly in the peritumoral areas near the margin of invasion. On the same section, blood vessels next to lymphatic vessels were not stained with D2-40 (Fig. 1 C), confirming the specificity of D2-40 antibody as a lymphatic endothelial marker.

The MVD and LVD in normal mucosa and tumour were calculated from the mean value of three hot spots in each entire specimen regardless of the location of hot spots, further LVD in tumour was examined in three hot spots either in intratumoural or in peritumoural areas. Intratumoural lymphatic vessels (ITLs) were defined as those within the tumour cell islets, and peritumoural lymphatics (PTLs) as those located at the peritumoural region near tumour invasion margin. In tumours, MVD positively correlated with LVD ($r = 0.24$, $P = 0.0004$). The mean MVD (114.32 ± 56.23) and LVD (14.82 ± 14.26) in tumours were significantly higher than those in distant (MVD, 67.72 ± 44.98 ; LVD, 4.22 ± 2.61) or adjacent normal mucosa

(MVD, 63.15 ± 44.07 ; LVD, 4.22 ± 3.47) ($P < 0.0001$, Table 2). There was no difference of the mean MVD or LVD between distant and adjacent normal mucosa ($P > 0.05$, data not shown).

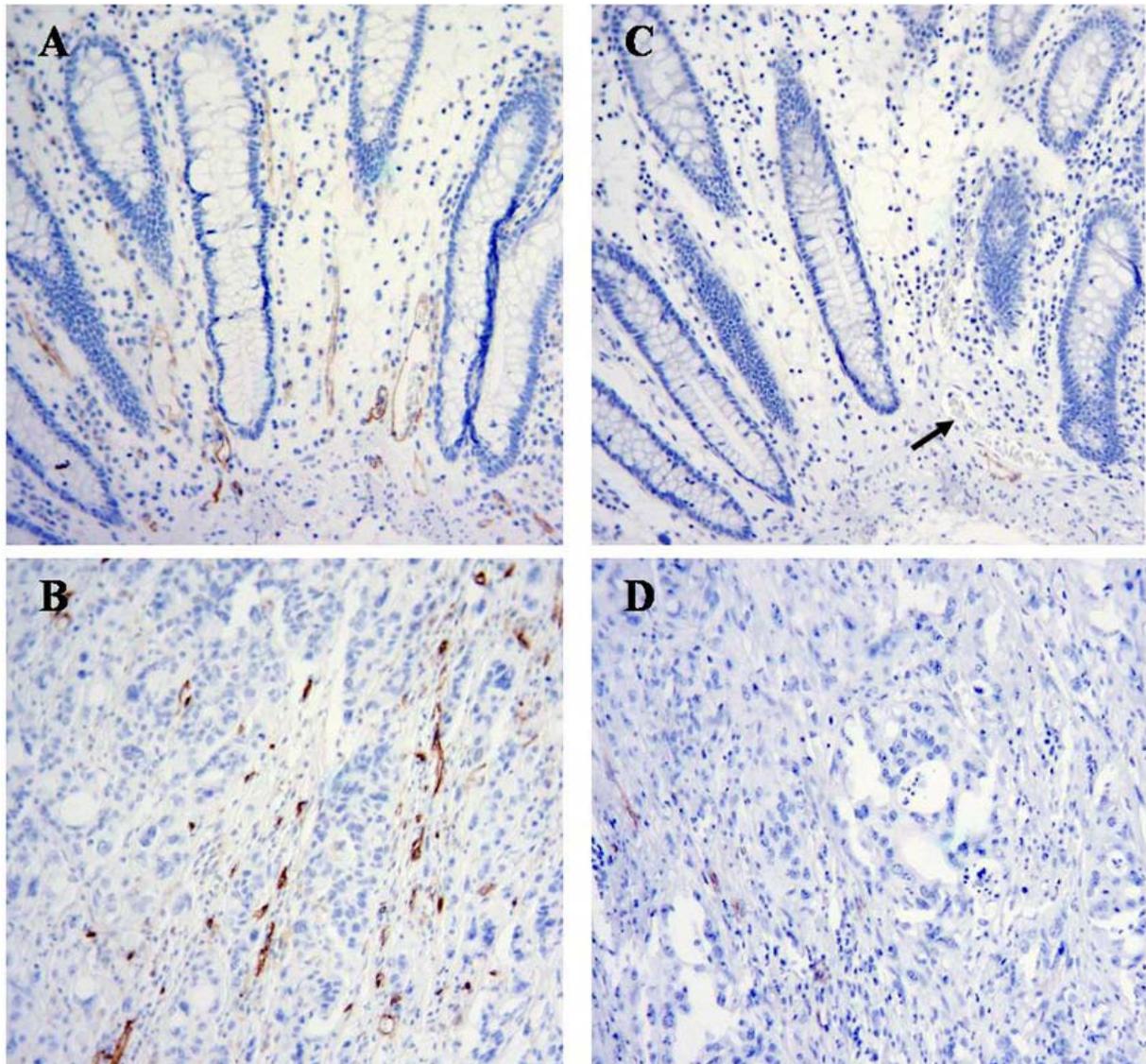


Fig. 1. Blood vessels and lymphatic vessels immunohistochemically stained by CD31 and D2-40 in normal mucosa and primary colorectal cancer. In normal mucosa, blood vessels were found between crypts with regular lumens of vessels (A) while lymphatic vessels were confined to mucosa adjacent to the muscularis mucosae (C). Blood vessels (arrow) next to lymphatic vessels were not stained with D2-40 (Fig. 1 C), confirming the specificity of D2-40 antibody as a lymphatic endothelial marker. In tumor, both blood (B) and lymphatic vessels (D) were heterogeneously distributed in the stroma.

Table 2. MVD and LVD in normal mucosa and colorectal cancer

	Distant normal mucosa	Adjacent normal mucosa	Tumour	<i>P</i>
MVD				
Range	3.33-177.50	5.00-231.83	13.00-372.17	
Mean±S.D	67.72±44.98	63.15±44.07	114.32±56.23	< 0.0001
Median	59.00	54.42	104.67	
LVD				
Range	0-10.00	0-16.17	0-127.67	
Mean±S.D	4.22±2.61	4.22±3.47	14.82±14.26	<0.0001
Median	3.67	3.67	12.67	

P value for MVD or LVD difference in cancer versus distant/adjacent normal mucosa

3.2. Microvessel density of blood and lymphatic vessels in tumours in relation to clinicopathologic and biologic variables

Table 1 shows the relationship of MVD or LVD in tumours with clinicopathologic and biologic variables. MVD was positively related to PINCH expression ($P = 0.006$). LVD was reversely associated with Dukes' stage ($P = 0.012$). LVD was significantly lower in tumours with later stage (Dukes' stage C+D) than in earlier stage (Dukes' stage A+B), even in tumours with lymph node metastasis when compared with tumours without lymph node metastasis ($P = 0.002$, data not shown). Besides, MVD or LVD was not related to other variables examined in the Table 1 ($P > 0.05$).

To analyze impact of MVD or LVD on prognosis, we divided the patients into a low and a high group of the density by using the median value as the cut-off point. Univariate analyses showed that Dukes' stage, growth pattern and differentiation were related to patients' survival, but MVD or LVD in the tumours (Fig. 2A, $P = 0.97$, and Fig. 2B, $P = 0.17$) or in the non-neoplastic tissue surrounding the primary tumour was not ($P > 0.05$). Stratification

analyses on tumour stage (Dukes' stage A, B, C or D) or multivariate analysis after adjusting for Dukes' stage, growth pattern, differentiation and adjuvant therapy showed that MVD or LVD was not related to patients' survival ($P > 0.05$, data not shown).

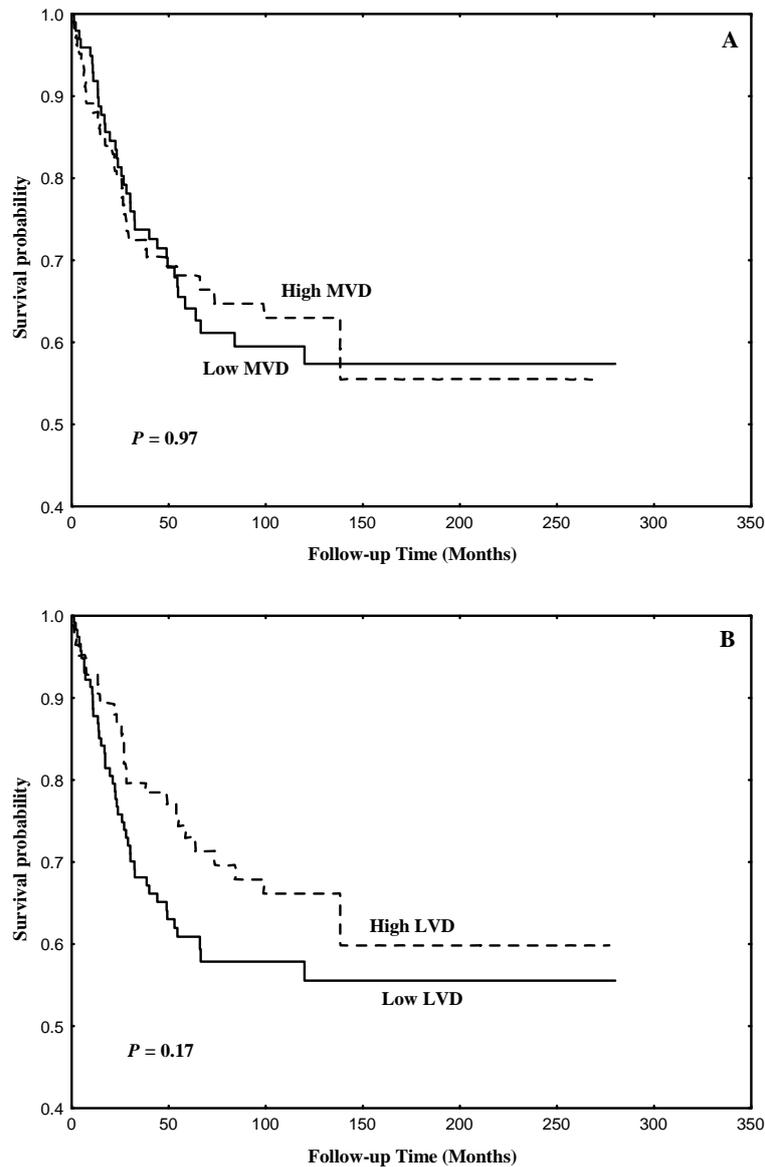


Fig. 2. Microvessel density (MVD, A) and lymphatic vessel density (LVD, B) in relation to prognosis of the patients with colorectal cancer.

We further examined the precise location of lymphatic vessels and assessed their relationships with clinicopathologic variables. Similarly, both ITLs ($P = 0.06$) and PTLs ($P =$

0.07) seemed to be lower in tumours with later stage (Dukes' C+D) than those in earlier stage (A+B), and did not associate with patients' survival ($P > 0.05$).

Even when combining MVD with LVD, we could not find any association of the combined MVD and LVD with clinicopathologic variables including gender, age, tumour location, growth pattern, Dukes' stage, differentiation, and patients' survival ($P > 0.05$, data not shown).

4. Discussion

MVD has been found to be higher in primary tumour compared with the corresponding normal colorectal tissue [22,23]. However, the significance of MVD in prediction of tumour aggressive behaviour and patients' prognosis remains controversial, probably due to the difference in selection of endothelial markers or technique of MVD quantification. In the present study, we assessed MVD with a marker CD31 in 210 consecutive Swedish CRCs, strictly following the first international consensus report on the methodology and criteria of the evaluation of angiogenesis quantification in solid tumours. Consistent with previous studies [22,23], we found a significant increase of MVD in CRCs compared with their corresponding normal mucosa specimens. We did not see a correlation between MVD and tumour stage or patient's survival, either in the entire group or in the subgroups stratified by tumour stage, which is in agreement with other reports in which MVD was also quantified using CD31 marker [6-8]. It is generally accepted that angiogenesis is essential for tumour growth, invasion and metastasis; however, the view that malignant tumour growth is angiogenesis-dependent is currently questioned. Recent histomorphological studies indicate that some tumour may be vascularised without significant angiogenesis, probably by using existing vessels, a process later described as vascular co-option, or even by forming vascular channels on their own through a non-endothelial cell process called "vascular mimicry" [24]. It has been recognized that human cancers consist of heterogeneous population of tumour cells with diverse biochemical properties, in which abnormal growth is derived by a minority, pathological cancer stem cells. A more recently study has provided direct evidence that glioblastoma stem-like cancer cells could mediate tumour growth by co-opting the host vasculature without any sign of angiogenesis, and that invasion and angiogenesis were two

independent strategies for tumour progression [25]. Thus, the significance of angiogenesis in cancer progression and patients' survival requires to be re-adjusted in the future.

PINCH is a family of cell-extracellular matrix adhesion proteins involved in regulating cellular proliferation, differentiation and survival by interaction with integrin-linked kinase, participating in integrin mediated intracellular and growth factor signalling pathways. It has been shown in *vitro* and in *vivo* that integrin-linked kinase is implicated in the promotion of tumour angiogenesis by stimulating VEGF expression [26]. Overexpression of PINCH protein has been seen in tumour-associated stroma in several common types of cancers [19], and was associated with advanced stage and a poor prognosis in CRCs [20]. In the present study, we further found that strong expression of PINCH was associated with high MVD. In addition, PINCH protein expression has been seen in tumour-associated myofibroblasts [20]. It is likely that PINCH is involved in angiogenesis through activation of fibroblasts in response to tumour. PINCH protein is also expressed in a proportion of tumour endothelial cells stained by CD31 [20] and its gene expression is upregulated in microvascular endothelial cells [27], indicating that PINCH was a likely regulator of angiogenesis. The potential role of PINCH in angiogenesis needs to be further studied.

Lymphatic vessel is one the major route of tumour spread. Growing evidence has shown that tumour dissemination seems to depend on both pre-existing lymphatics and lymphangiogenesis within the tumour or at tumour periphery [2]. ITLs could provide more direct route and extensive interface for lymphatic invasion than PTLs, although ITLs are more easily destroyed or compressed and collapsed with proliferating cells and high intratumoural pressure, proposed as non-functional in numerous experimental tumour models. Indeed, intratumoural lymphangiogenesis appears as an increased risk factor for lymph node metastasis development and correlating with poor survival in various human cancers.

Meanwhile, many studies in experimental models or humans have also indicated that PTLs are critical for spreading tumour cells to regional lymph nodes [2].

In CRCs, intratumoural lymphatics were detected and an increase of LVD in tumour, compared to normal tissue, has been observed [12,17]. However, the correlation of LVD in intratumoural areas with lymph node metastasis or prognosis versus the correlation of LVD in peritumoural areas with lymph node or prognosis remains controversial. LVD in the intratumoural areas, but not in peritumoural areas, was found to be related to lymph node metastasis or prognosis [12-14], whereas other studies showed that LVD in peritumoural areas was related to advanced tumour stage or lymph node metastasis [15,16]. In addition, the presence of intratumoural proliferating lymphatics was detected immunohistochemically by double-staining technique using D2-40 and Ki-67 monoclonal antibody, and both LVD and proliferating activity of lymphatic vessels were significantly increased in CRCs compared with their normal counterparts, but there was no correlation between degree of lymphangiogenesis and lymph node metastasis, Dukes' stage, or patients' prognosis [17]. In the present study, we also observed an increase of LVD in CRCs with respect to their corresponding normal mucosa specimens. However, high LVD, either in the intratumoural areas, peritumoural areas or in the entire specimens, was reversely related to lymph node metastasis, tumour Dukes' stage, and not related to patients' survival. Our results indicate that lymphangiogenesis may be involved in the earlier stage but not later stage of CRC development. The discrepancies regarding the correlation of LVD with lymph node metastasis and prognosis among those studies might be attributed to the problem in methodology such as different antibodies used and the lack of standardization on estimation of lymphangiogenesis. Further, we would speculate that, with cancer progression, cancer cells may cause a lymphatic destruction to significantly reduce the number of ITLs and PTLs, leading them indiscernible, thus their impact on patients' outcome could not be adjusted. On the other hand, although we

examined the clinicopathologic significance of LVD in intratumoural and peritumoural areas, the method used in the present study could not distinguish newly proliferating lymphatic vessels from pre-existing lymphatic vessels. A further study is needed to clarify the clinicopathologic significance of proliferating versus nonproliferating lymphatic vessels in intratumoural versus peritumoural areas, by double immunostaining with D2-40 and Ki-67 monoclonal antibodies.

In conclusion, angiogenesis and lymphangiogenesis occurred in CRC development, but were not related to patients' prognosis. PINCH may play a potential role in tumour angiogenesis.

Practice Points

- MVD and LVD were higher in tumour compared with the corresponding normal mucosa.
- MVD was not related to clinicopathologic variables and survival.
- MVD was positively related to PINCH expression.
- LVD, in both intratumoural and peritumoural areas of tumours, was reversely related to Dukes' stage, but there was no association between LVD and patients' survival.

Research agenda

- Comparison of MVD and LVD between normal mucosa and primary tumour
- Analysis of correlation between MVD or LVD and clinicopathologic and biologic variables

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