Comparison of Lymphangiogenesis between Primary Colorectal Cancer and Corresponding Liver Metastases

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Abstract. *Background: Liver metastasis (LM) is the determining factor of poor prognosis in colorectal cancer (CRC). Peripheral lymphatico-venous communications have been discussed as a potential pathway of tumor cell dissemination for the development of LMs. In the current study, we investigated the clinical impact of the lymphangiogenic activity in CRCs and their corresponding LMs. Patients and Methods: In 47 patients with CRC, the primary tumors and the corresponding LMs were investigated. Lymphangiogenesis (LMVD), lymphovascular invasion (LVI), lymphatic vascular endothelial growth factor C expression (VEGF-C) were investigated Results: A significant correlation was observed between LMVD and LVI in CRCs (p=0.001) as well as in LMs (p=0.0001). LMVD in CRC correlated significantly with that in LMVD-LMs (p=0.026) and LVI in LMs (p=0.036). Survival analysis reveilled a significant difference in disease free and overall survival between patients with and without VEGF-C expression in LMs (p=0.0019 and p=0.0101, respectively). Conclusion: Our data provide evidence for an important role of lymphangiogenesis in liver metastasis of CRC and provide further support for a possible role of a lymphatico-venous metastatic pathway.*

Colorectal cancer (CRC) is the second most common cause of cancer-related mortality in the Western world (1). Primary sites of colorectal metastases are the lymph nodes followed by the liver, and approximately 50% of patients with CRC develop liver metastases (LM) at some point during the course of their disease (2, 3). The process of cancer metastasis is complex; tumor cells can spread directly into local tissue or migrate *via* blood and lymphatic vessels into distant organs (4).

Angiogenesis is known to play an important role in the development of tumor, its growth and metastasis. However, the role of tumor lymphangiogenesis is less clear and only recently has the significance of tumor lymphangiogenesis been described in various tumor entities (5-8).

In CRC, high levels of the specific lymphatic vascular endothelial growth factor C (VEGF-C) have been described to promote tumor lymphangiogenesis and metastasis and increased lymphangiogenic tumor activity (*e.g.* high levels of intratumoral VEGF-C expression and high degree of lymphangiogenesis) have been proven to be associated with reduced patient survival (9-11).

Peripheral lymphatico-venous (portal) communications have been discussed as a potential pathway of tumor cell metastasis to peritoneum and liver (12). In a recently published report on an animal model, fluorescently labeled tumor cells were shown to develop LMs *via* lymphatico-venous shunts after being infused into paracecal lymph nodes (13). The aim of this study was to investigate the clinical impact of the tumor lymphangiogenesis (the amount of lymphangiogenesis and VEGF-C expression) in CRCs and their corresponding LMs.

Patients and Methods

Patients and tissues. Forty-seven patients with CRC and the corresponding LMs were randomly selected for this study. Special care was taken to include only specimens with sufficient amounts of normal colorectal or liver tissue, respectively, directly adjacent to the invasive tumor.

Immunohistochemistry. Rabbit anti-human podoplanin IgG was raised against the recombinant human homologue of the rat 43-kDa glycoprotein podoplanin and purified as described previously (14). For immunohistochemical detection of VEGF-C protein expression, VEGF-C antibody (Zymed Lab., South San Francisco, CA, USA) was used.

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Immunohistochemistry was performed on 2 μm-thick serial paraffin sections. After deparaffinization in xylene, sections were rehydrated and microwave pre-treated in citrate buffer at 600 W for 10 min. After cooling for 15 min and washing in PBS, endogenous peroxidase was blocked by using 3% hydrogen peroxide for 15 min, followed by incubation with PBS containing 10% normal goat serum for 30 min. For immunostaining of podoplanin, specimens were incubated at 20˚C with the polyclonal rabbit antibody at a dilution of 1:2000 for 1 h. Detection of VEGF-C expression was performed on a separate, subsequent section. For this, the polyclonal anti-VEGF-C antibody was incubated with tissue at 20˚C at a dilution of 1:800 for 1 h. Positive staining was detected using biotinylated goat anti-rabbit IgG or horse anti-mouse IgG (both Vector Laboratories, Burlingame, CA, USA), respectively, for 30 min at 20˚C followed by a streptavidin-peroxidase complex, according to the manufacturer's instructions. Peroxidase reaction product was visualized by diaminobenzidine (Serva, Heidelberg, Germany). Slides were counterstained with haematoxylin.

A block of breast cancer used in a previous study served as positive control for VEGF-C and podoplanin (15). For negative control, a slide was prepared from the same tissue block and pre-immune or irrelevant serum was used instead of the primary antibodies.

Morphometry. Determination of lymphatic microvessel density (LMVD) assessed by immunostaining for podoplanin in a defined examination area of 0.7386 mm^2 was performed as suggested by Weidner *et al*. (16, 17). In brief, an area of tissue with the greatest number of distinctly highlighted microvessels ('hot spot') was selected and LMVD was then determined by counting all immunostained vessels at a total magnification of ×200. Determination of the staining reaction was strictly confined to the hot spots. LVI was considered evident if at least one tumor cell cluster was clearly visible inside the podoplanin stained vascular space (18). The mean values of LMVD recorded by two independent observers, naive to the patient's pathologic and clinical status, were used for further calculations. In the case of inter-observer differences of >20% in microvessel count, the respective slides were re-investigated by both observers using a double-headed microscope (evident in <5% of cases).

Lymphatic vessel size (LVS) was evaluated semiquantitatively by categorizing the dominant lymphatic vessel appearance: LVS was graded as small, enlarged and ectatic vessels. The intensity of immunostaining in cancer cells was graded as strong, medium, or weak expression of VEGF-C as described previously (19).

Statistical analysis. Association of LMVD, LVI, LVS and VEGF-C with clinical and pathohistological parameters was investigated using Kruskal-Wallis test, Mann-Whitney test or Spearman's coefficient of correlation, as appropriate. Overall survival (OS) was assessed over the period from primary surgery until death of the patient. Death from a cause other than CRC, or survival until the end of the observation period, was considered a censored observation. Disease-free survival (DFS) was defined from the end of primary therapy until first evidence of progression of the disease. Univariate analysis of OS and DFS was performed as outlined by Kaplan and Meier (20). For all tests, a two-tailed *p*-value of <0.05 was considered significant.

Results

Clinical data. The study population comprised 47 patients, 21 (44.7%) women and 26 (55.3%) men, with CRC and their corresponding LM, treated at the University Hospital of Vienna between the years 1991 and 2004. The distribution of tumor localization was as follows: 15 (31.9%) were localized in the rectum and 32 (68.1%) in the colon. A summary of clinical and histopathological data are presented in Table I. The mean serum level of carcinoembryonic antigen (CEA) at the time of primary surgery was 91.76±12.15 ng/ml. The mean level of cancer antigen (CA) 19-9 was 63.7±32.1 ng/ml. Five (10.6%) patients had received neoadjuvant radiation therapy, in 2 (4.2%) patients neoadjuvant chemotherapy (Oxaliplatin-based) was administered. Twenty-five (53.2%) patients received no further adjuvant chemotherapy, whereas 6 (12.8%) patients received 5-fluorouracil (5-FU)-leucovorin, 5 (10.6%) received 5-FU-leucovorin-oxaliplatin and 11 (23.4%) received capecitabine. The mean patient age at the time of the colorectal surgery was 64 years (range 43–84 years). In 17 (36.2%) patients the LMs were metachronous and in 30 (63.8%) were synchronous. At the time of surgery for LM, the mean CEA level in the patients' sera was 654±1515 ng/ml, and the median serum level of CA 19-9 was 1659±3728 ng/ml. Twelve (25.5%) patients were diagnosed with steatosis hepatis and 5 (10.6%) had Child Pugh-A cirrhosis.

Table I. *Patients characteristics*.

Patient characteristic	n (%)
Gender	
Male	26 (55)
Female	21 (45)
Age (years)	64 (43-84)
UICC (primary tumor)	
I	6 (12.8)
II	4 (8.5)
III	7 (14.9)
IV	30 (63.8)
Grading (primary tumor)	
G1	2 (4.3)
G2	35 (74.5)
G3	10 (21.3)
Staging (primary tumor)	
T1	0
T2	12 (25.5)
T3	29 (61.7)
T4	6 (12.8)
Lymph node staging (primary tumor)	
pN0	26 (55.3)
pN1	12 (25.5)
pN2	14 (29.8)
Metastatic type (metastatic tumor)	
Metachronous	17 (36.2)
Synchnonous	30 (63.8)

Morphometry. Most lymphatic vessels were located within the tumor stroma at the border front of invasive tumor formations, as reported previously (21). In normal liver tissue lymphatic vessels were exclusively found in Glisson's

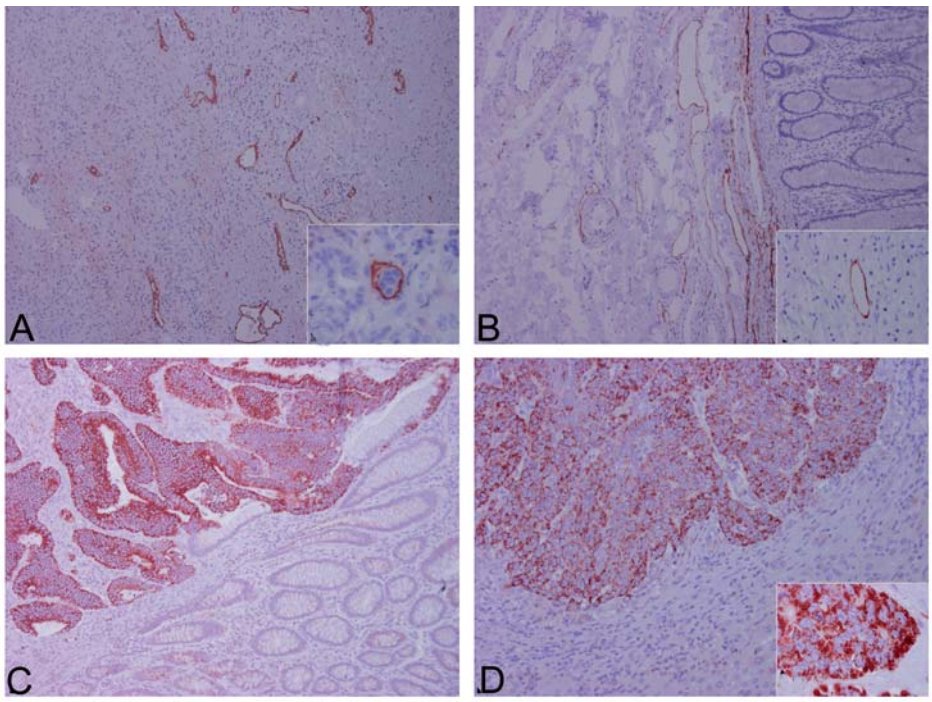

Figure 1. *Anti-podoplanin immunohistochemistry on a liver metastasis (A). The insert shows a typical lymphatic vessel invasion with a tumor cell cluster in a podoplanin-stained lymphatic vessel, and the corresponding primary colorectal cancer (B) shows stained lymphatic vessels with a podoplanin-stained ectatic lymphatic vessel (inset). Primary colorectal cancer (C) and the corresponding liver metastasis (D) with high VEGF-C expression. Note the absence of staining reaction in the surrounding normal tissue. The insert in D shows the typical cytoplasmatic VEGF-C staining reaction. Original magnification, main image, ×100, inset, ×400.*

system. No unequivocal communications between lymphatic vessels (podoplanin-stained) and blood vessels (no staining) were observed.

The median LMVD in CRC was 10 microvessels/field (range 0-41). The median LMVD in LMs was 10 microvessels/field (range 1-76). Details are shown in Figure 1 and Table II.

Patients with high LMVD in CRC had significantly more frequent LVI (p=0.001, Mann-Whitney test). A similar statistically significant association was found for LMs (p=0.0001, Mann-Whitney-test) (Figures 2A and B). In addition, there was a significant correlation between LMVD in CRC and LMs (p=0.006 and 0.026, for Pearson's coefficient of correlation and Spearman's coefficient of correlation, respectively), showing that patients with high lymphangiogenetic activity in the primary cancer also had a high degree of lymphangiogenesis in the corresponding LM (Figure 2C). LMVD in CRC correlated with LVI in LMs (p=0.036, Mann-Whitney test). A statistically inverse association was seen between LMVD in CRC and the size of lymphatic vessels. CRC with high LMVD had significantly smaller lymphatic vessels (p=0.008, Chi-Square test) (Figure 2D). Furthermore a significant correlation was observed between the presence of LVI and LMs: lymphatic vessels containing tumor cell clusters (LVI+) were significantly more often enlarged and ectatic (p=0.018, Chi-Square test). A further statistically significant association was found between LVI in CRC and positive lymphnode status at primary surgery (p=0.04, Spearman's coefficient of correlation). There was a

Table II. *Lymphangiogenic factors in CRC and LMs.*

	Primary tumor, n (%)	Metastases, n (%)
Lymphatic microvessel density		
microvessels/field (range)	10 (0-41)	10 (1-76)
Lymphatic vessel invasion (LVI)		
LVI+	10 (21.3)	9 (19.1)
LVI–	37 (78.7)	38 (80.9)
Lympahtic vessel size		
Small	32 (69.6)	27 (57.4)
Enlarged	11 (23.9)	15 (31.9)
Ectatic	3 (6.4)	5 (10.6)
Lymphnode involvement		
pN-	26 (55.3)	-
pN+	26 (55.3)	-
VEGF-C expression		
Weak	13 (27.7)	11 (23.4)
Medium	23 (48.9)	23 (48.9)
Strong	11 (23.4)	13 (27.7)

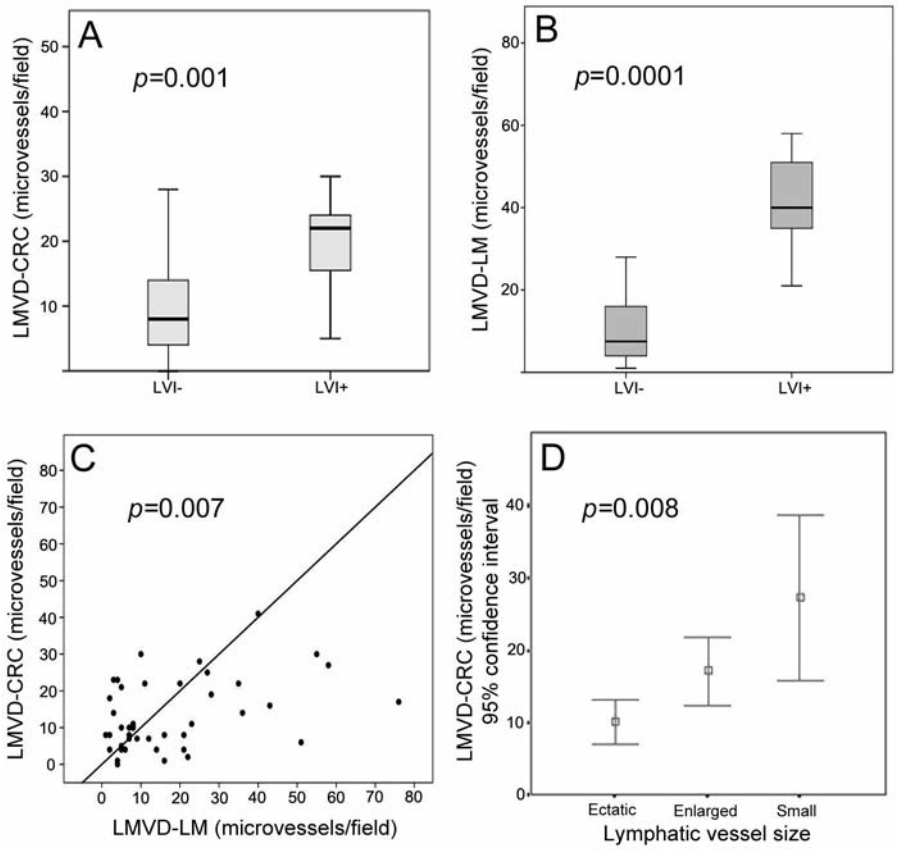

Figure 2. *A: Box-blot showing lymphatic microvessels/field (LMVD) in primary colorectal cancer (CRC) with (LVI+) and without (LVI-) lymphovascular invasion (p=0.001). B: Box-blot showing LMVD in corresponding liver metastases (LM) with and without LVI (p=0.0001). C: Dot-blot showing the significant association between the LMVD of primary colorectal cancer (LMVD-CRC) and the corresponding liver metastases (LMVD-LM) (p=0.026, Spearman's coefficient of correlation). D: Errorbar showing the statistically significant association between LMVD-CRC (95% confidence interval of LMVD) and lymphatic vessel size (LVS) (small versus enlarged versus ectatic, p=0.008, Chi-square test.*

clear trend towards a higher LMVD in CRC in patients with high VEGF-C expression in primary tumors (p=0.056, Wilcoxon rank test). The same borderline significance was observed between VEGF-C and LMVD in LMs (p=0.059, Wilcoxon rank test).

Survival analysis. The mean observation time was 36 months (range 0.5±145 months). During this time, 37 (78.7%) patients developed recurrent disease and 29 (61.7%) died. In univariate survival analysis, a significant difference was found between patients with tumors expressing high amounts of VEGF-C compared to those with none or low VEGF-C expression, in both CRC and LMs (p=0.0391 and 0.0101 respectively, log rank test). In addition, a statistical significance was found for shortened DFS in patients with tumors with high VEGF-C compared to those with absent or low VEGF-C expression in LMs (p=0.009, log rank test) (for LMs see Figure 3). A clear trend towards diminished DFS was seen for positive VEGF-C expression in CRC (p=0.070, log rank test). Furthermore, a significant reduction of DFS, and a clear trend for a decreased OS were found in patients with LVI in CRC compared to patients without LVI (p=0.0148 and p=0.141, respectively, log-rank test).

Discussion

Lymphatic microvascular endothelial cells are very difficult to distinguish from those of blood vessels since both express a similar set of markers such as CD34, CD31, von Willebrand factor (vWF), *etc*. (22-24). Numerous studies have demostrated clinically relevant correlations between microvessel counts and patient survival for various tumor entities (18, 21, 25-27). Recently, the microvessel count in patients with LMs from CRC was shown to be of clinical significance, with diminished prognosis for patients with high numbers of CD34-positive vessels (28). However, as the authors admited, performing microvessel assessment after staining with the panendothelial marker CD34 will always

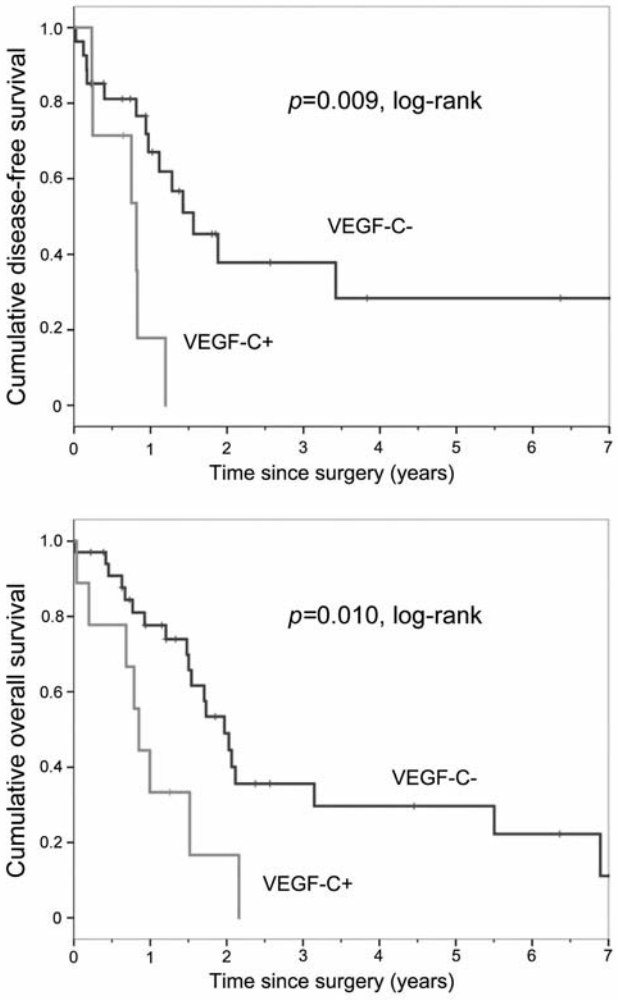

Figure 3. *Kaplan-Meier curves comparing disease-free survival (DFS, upper graph) and overall survival (OS, lower graph) of patients with (VEGF-C+) and without (VEGF-C–) VEGF-C overexpression in colorectal liver metastases, respectively (p=0.01, and p=0.008, respectively, both log-rank test).*

provide a result representing a mixture of lymphatic and blood microvessels (28). A great deal of progress in this research field has been initiated by the discovery of lymphatic endothelial-specific markers and the lymphangiogenic VEGF-C and -D and their corresponding receptors (14, 29). Evidence is now growing that these molecules are involved in tumor-induced lymphangiogenesis and lymphatic dissemination of tumor cells *via* stimulation of their receptor VEGFR-3 and that this metastatic system might offer targets for antilymphangiogenic therapies (30). Several reports have documented a correlation between expression levels of the lymphangiogenic factor VEGF-C and lymphatic metastasis in cancer patients and have linked VEGF-C overexpression to worse patient outcome for various cancer entities (31). In our cohort, we observed a significant correlation between the lymphangiogenic potential (lymphatic microvessel count) of the primary tumor (LMVD and LVI in CRC) and that one in the corresponding LMs. Furthermore a significant correlation between the degree of lymphangiogenesis (LMVD) and LVI in primary tumors was detected, a finding that also applies to the corresponding LMs where we found a statistically significant correlation between high LMVD and LVI. This can likely be explained by the increased number of newly formed lymphatic capillaries providing tumor cells with more opportunities to enter the lymphovascular system and start the metastatic process. After tumor cell invasion into the lymphatic microvessels (*via* LVI), the locoregional lymph nodes are the first organs where tumor cells can establish themselves. Therefore it is logical that patients with proven LVI had significantly more frequent lymph node metastasis at primary surgery in our patient collective. Intrahepatic lymphatic invasion has previously been reported as a pathway of intrahepatic spread in patients with intrahepatic cholangiocarcinoma and was associated with worse prognosis (32). Recently it was shown that intrahepatic lymphatic invasion constitutes a negative prognostic factor after liver LMs of CRC, additionally predicting the tumor cell involvement of hepatic pedicle lymph nodes (33, 34). Our findings that patients with high amounts of VEGF-C expression in LMs have diminished OS and DFS, are in good correlation with these observations, thus confirming the involvement of VEGF-C in lymphangiogenesis and lymphovascular invasion. Venous invasion is considered to be closely related to the development of LMs, providing a direct path from the primary tumor location (2). However, occasionally unequivocal venous invasion of tumor cells within the surgical specimens of patients with LMs cannot be identified and therefore the significance of blood vessel invasion has been debated (35). In gastric cancer, for example, one of the risk factors with the greatest significance for the development of LM is the presence of lymph node metastasis, suggesting a direct association between the lymphatic metastatic process and the development of LMs (36). The phenomenon of lymphatico-venous communication was primarily reported in the late 1970s (37). These communications were thought to reach significance when cancer cells metastasize to a lymph node or lymphatic vessel and obstruct the flow of the lymphatic fluid, changing the intravascular pressure. More recently the possibility of LMs occurring *via* a lymphatic route was supported by the findings of a study applying a 'mesenteric lymphatic obstruction model' in rats (12). In this study, it was shown that tumor cell obstruction (from CRC) in lymphatic vessels led to the establishment of LM *via* the opening of lymphocapillary anastomosis. Although, our experimental setting is not appropriate to determine if there is a clinically

evident mechanism of LM *via* a lymphatic route in CRC, our data provide indirect evidence for the clinical importance of lymphangiogenesis in CRC with particular reference to LMs. Furthermore our findings indirectly support the theory of clinically relevant lympho-venous anastomosis in the development of LMs. Taken together, our data provide evidence for an important role of lymphangiogenesis LMs of CRC and provide further support for a possible lymphatico-venous metastatic pathway with clinical relevance in this tumor entity. Targeting VEGF-C and its receptor VEGFR-3 may be therapeutically significant and, in particular, new drugs blocking the VEGF-C/VEGFR-3 signaling pathway may provide useful anticancer therapeutics by mechanisms other than the blockage of lymphangiogenesis.

Conflict of Interest

The Authors declare no disclosure of any commercial interest

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