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Molecular Mechanisms of Lymphatic Metastasis

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. Considering the high rate of incidence and mortality of CRC it is critical to determine the mechanisms of its dissemination. Although one of the better characterised tumours the prognosis of patients decreases dramatically when lymphatic metastasis occurs. In addition, the main important prognostic factor of CRC is the stage of tumour at the time of diagnosis, which is defined by the TNM system from the American Joint Committee on Cancer and the International Union Against Cancer. Therefore, during surgical treatment not only the primary tumour but also the draining lymph nodes have to be removed. From multivariate analysis it is known, that the number of examined lymph nodes is an independent prognostic factor. In this context, a prognostic relevance has been demonstrated not only for N0-, but also for N1- and N2-status. Adjuvant chemotherapy is recommended for stage UICC III colon cancer. It has been shown to reduce tumour recurrence and improve overall survival (Schmiegel, Reinacher-Schick et al. 2008). The five-year survival rate drops significantly from the UICC stage I to IV (Table 1). Patients with an early stage tumour (UICC I) have an excellent prognosis and a five-year survival rate of 90%, compared to those with advanced tumours and lymph node metastasis, who have a five-year survival rate of 30-60%. Patients with distant metastasis have a five-year survival rate below 10%.
Thus the prognosis of CRC is significantly influenced by the occurrence of lymph node metastasis and in addition to its value as a prognostic indicator it also affects the therapeutically management of patients. The understanding of molecular mechanisms involved in lymphatic metastases may open the door for future treatment strategies.

2. The lymphatic system

2.1 Development of the lymphatic system

Aspects of the lymphatic fluid and the associated transport system were already mentioned by the ancient Greeks, but it was poorly considered until the 17th century. In 1622 the Italian physician Gasparo Asselli re-identified lymphatic vessels as "milky veins" in the gut of a

Stage			5-year survival rate
UICC	TNM	Dukes	
I a b	T1N0M0 T2N0M0	A	>90%
II a b	T3N0M0 T4N0M0	B	60-80%
III a b c	T1/2N1M0 T3/4N1M0 T1-4N2M0	C	30-60%
IV	T1-4N1-2M1	D	<10%

Table 1. UICC stage and 5-year cancer related survival of patients with CRC.

dog. The embryonic origin of lymphatic vessels remains further unclear. Since the beginning of the 20th century, two developmental theories -the centrifugal and the centripetal- have been controversial debated. The centrifugal theory by Sabin based upon dye and ink injection experiments in pigs. According to her view, lymphatic vessel formation occurs early during embryonic development from isolated primitive lymph sacs that originate from endothelial cells that bud from the veins. The peripheral lymphatic system originates from these primary lymph sacs by endothelial sprouting into the surrounding tissues and organs, where local capillaries are formed (Oliver and Detmar 2002). Simultaneously Huntington and McClure suggested an alternative model, the centripetal theory. In their opinion primary lymph sacs arise from mesenchymal precursor cells, independent of the veins and secondarily establish venous connections.

To date, the development of the lymphatic vasculature system has not been ultimately resolved. Recent molecular analyses describe a polarized expression of the homeobox transcription factor Prox-1 in anterior cardinal vein endothelial cells, which is required for specification of lymphatic endothelial cells (LECs). Prox-1 is a master regulator which drives the transcription of a variety of genes whose expression is associated with key LEC characteristics (Tammela, Petrova et al. 2005).

2.2 Structure and function of the lymphatic system

The lymphatic vascular system is a hierarchical network comprising blind-ended capillaries, collecting vessels, lymph nodes, lymphoid organs and circulation lymphocytes. A number of important physiological functions have been described. It maintains fluid homeostasis by absorbing and draining e.g. interstitial fluids, plasma proteins and cells extravasated from blood vessels and returning them back into the blood circulation (Butler, Isogai et al. 2009). Furthermore, the lymphatic system is also known to be an important part of the body´s immunological surveillance system (Wiig, Keskin et al. 2010). Lymphatic vessels are distributed to most organs, with the exceptions of the central nervous system, bone marrow, cartilage, cornea and epidermis. Due to its dual role, fluid absorption and lymph transport, the structure of lymphatic vessels differ from blood vessels (Schulte-Merker, Sabine et al.

2011). Lymph capillaries are characterized by loose intercellular junctions, no or an incomplete basement membrane. The wall of lymphatic endothelial cells (LECs) is joined to the extracellular matrix by anchoring filaments. These filaments help the vessels open and function. Collecting lymphatic vessels consist of pericytes, which reduce lymphatic fluid extravasation and they are surrounded by smooth muscle cells (Figure 1) (Shayan, Achen et al. 2006).

Tumour cells can take advantage of these structural characteristics to promote their dissemination to lymph nodes or other organs by the process of permeation into peritumoural lymphatics. In addition, LECs secrete chemotactic agents, which can attract tumours cells toward lymphatics, such as CCL21, whose receptor (CCR7) is expressed on some tumour cells (Shields, Emmett et al. 2007). Chemokines may mediate the tumour LEC interaction by increasing the interactive surface area (Ji 2006).

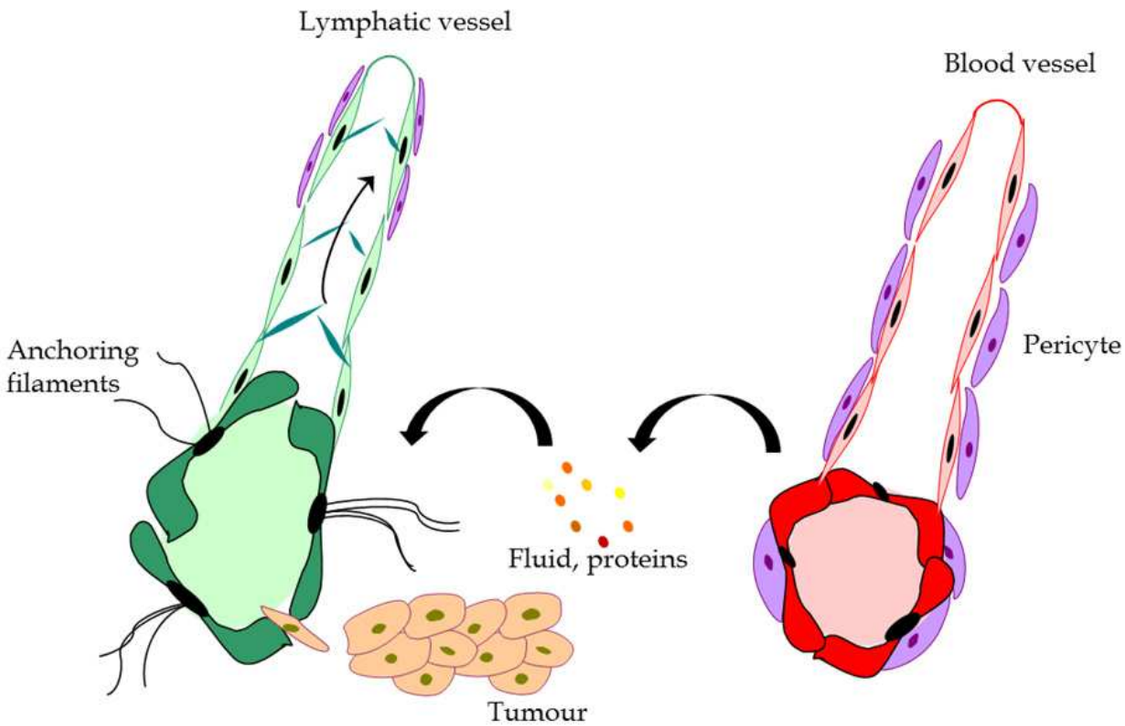
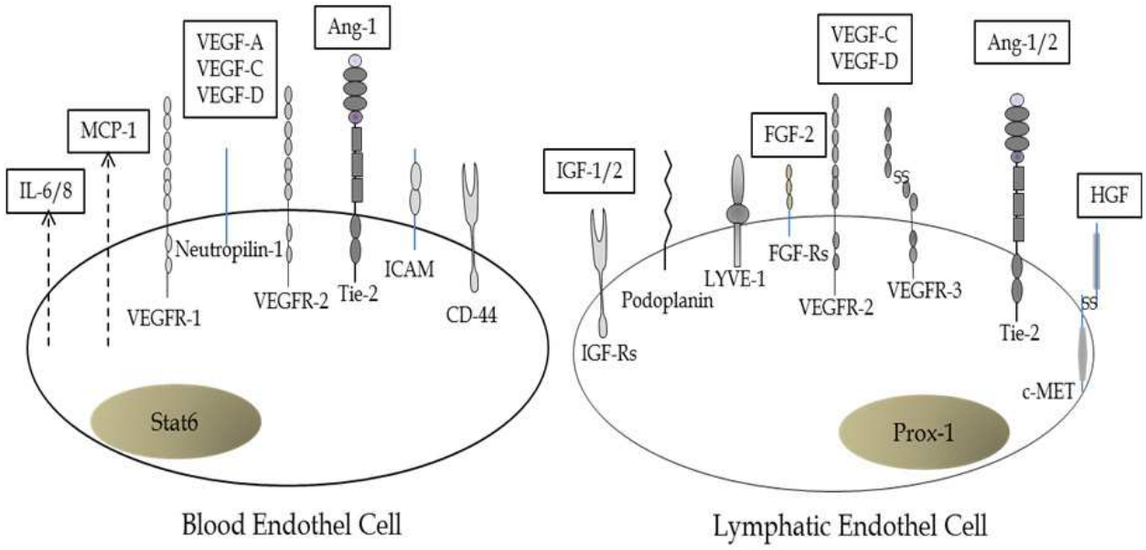


Fig. 1. Structure of lymphatic vessels compared against blood vessels. The initial lymphatics have no or an incomplete basement membrane and no pericytes, which makes them suitable for the uptake of tumour cells. Anchoring filaments attach LECs to the extracellular matrix (ECM) and prevent vessel collapse.

Gene expression profiles of LECs and blood endothelial cells (BECs) have been analysed and compared (Figure 2). The most obvious differences were detected in genes coding for pro-inflammatory cytokines/chemokines and their receptors, cytoskeletal and cell matrix organisation (Saharinen, Tammela et al. 2004). For example Interleukin (IL)-8, IL-6, the chemokine receptor CXCR4, ICAM-1, Integrin α5 are expressed in higher levels in the BECs.



Abbreviations: Stat6 (signal transducer and activator of transcription 6), MCP-1 (monocyte chemotactic protein-1), IL-6/8 (Interleukin-6/-8), ICAM (intracellular adhesion molecule), Ang-1 (Angiopoietin-1), VEGF/ R (Vascular endothelial growth factor/receptor), CD-44 (Cluster of Differentiation 44), IGF-1/2 (Insulin like growth factor 1/2), FGF-2 (Fibroblast growth factor 2), HGF (Hepatocyte growth factor), c-MET (mesenchymal epithelial transition factor), Tie-2 (angiopoietin receptor 2). Note that not all molecular markers are shown.

Fig. 2. Molecular characteristics of BECs and LECs.

3. Lymphangiogenesis

3.1 Lymphangiogenesis and cancer metastasis

Lymphangiogenesis takes place in a variety of physiological and pathophysiological processes, such as embryonic development, regeneration and wound healing on the one hand, and in lymph vascular malformations, inflammation and cancer on the other hand (Witte, Jones et al. 2006). Carcinogenesis is a complex multi-step process and despite the importance, that the lymphatic system provided one of the main routes for cancer progression, little information has been available about the molecular mechanisms by which the tumour cells gain access to the lymph system and are able to spread.

Traditionally, lymphatic metastasis of tumours was considered to be a passive process, where tumour cells metastasized to lymph nodes by utilizing pre-existing lymphatic vessels via open junctions or that lymphatic vessel entry occurred by tumour eroding. The process of new lymphatic formation (lymphangiogenesis) does not occur.

This view has been challenged (Achen and Stacker 2008). The identification of lymphatic specific markers, lymphangiogenic growth factors and their ligand receptor pathways, the

isolation of lymphatic endothelial cells and the development of specific in vitro culture systems in the past decades led to a broader understanding of the molecular mechanisms that control lymphatic metastasis.

Yet there is mounting evidence that lymphangiogenesis does occur in tumours and that it promotes cancer progression. A shift in the balance between lymphangiogenic and anti-lymphangiogenic signalling, like in the process of angiogenesis, might lead to lymphangiogenesis. Therefore a wide range of interactions at the tumour host interface have to take place, which support tumour proliferation, migration and survival. These processes are controlled by growth factors, adhesion molecules, fibroblasts, blood vessels, cytokines and chemo attractants (Figure 3) (Cueni and Detmar 2006; Ji 2006). In the following section the most widely studied molecular mediators of lymphangiogenesis are reviewed.

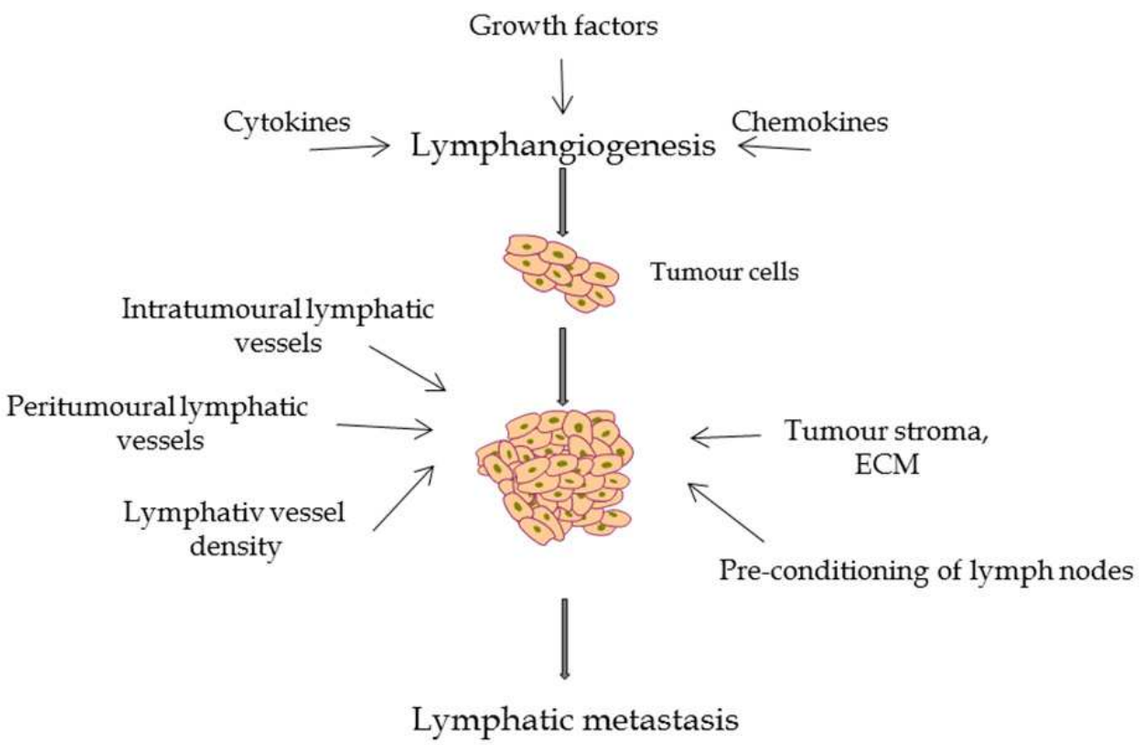


Fig. 3. Schematic overview of processes involved in tumour lymphangiogenesis and metastasis. Growth factors, cytokines, chemokines and tumour stroma contributes to tumour formation, growth, lymphangiogenesis and cancer progress. The tumour stroma consists of fibroblasts, ECM (extracellular matrix), blood vessels, lymphatic vessels and immune cells.

3.2 Molecular players in tumour lymphangiogenesis

3.2.1 Vascular endothelial growth factor

The human VEGF family of growth factors includes VEGF-A, -B, -C, -D and placental growth factor (PIGF). They bind with different specificity to three tyrosine kinase receptors: VEGFR-1 (fms-like tyrosine kinase 1), VEGFR-2 (human kinase insert domain receptor),

VEGFR-3 (fms-like tyrosine kinase 4) and two non-protein kinase co-receptors (neuropilin-1 and neuropilin-2). All VEGFRs have an extracellular binding region containing seven immunoglobulin-like domains (excepted VEGFR-3 who has only 6 domains), a single transmembrane helix and a conserved cytoplasmic domain that contains the catalytic core and regulatory sequences (Lohela, Bry et al. 2009). Activation of VEGFR by its ligands leads to receptor dimerization, autophosphorylation of tyrosine residues and initiation of signalling pathways (Roskoski 2008).

VEGF-C, VEGF-D and their ligand VEGFR-3 were the first discovered and most exensively studied lymphangiogenic factors (Baldwin et al. 2002; Nagy et al. 2002). After activation of VEGFR-3 by its ligands, autophosphorylation of tyrosine residues results in binding of the signalling adaptor proteins Shc (adaptor protein p66), Grb-2 (growth factor receptor-bound protein) and in activation of the ERK 1/2 (extracellular signal regulated kinase) signal transduction cascade in a protein kinase C dependent manner and via PI3K-Akt (phosphatidylinositol 3-kinase protein kinase B) signalling cascade (Figure 4). Binding of the adaptor protein CRK 1/2 initiates the MKK4-JNK 1/2 (mitogen-activated protein kinase kinase 4- Jun N-terminal kinase) pathway and results in induction of c-JNK (c-Jun N-terminal kinase) expression. The VEGFR-3 pathway mediates lymph endothelial growth, survival and migration (Wissmann and Detmar 2006).

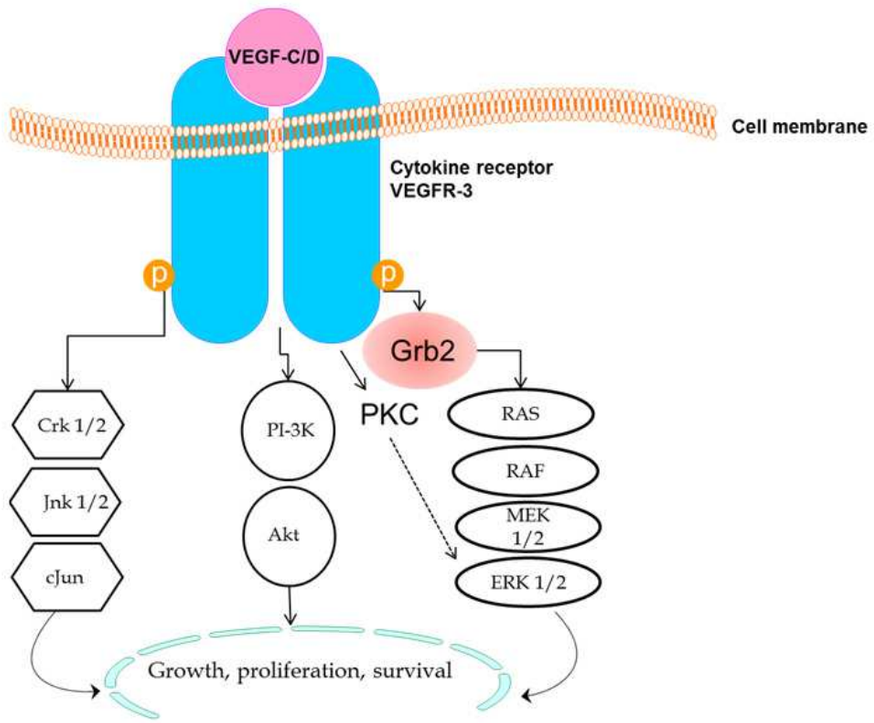


Fig. 4. The VEGF-C and VEGF-D pathways via VEGFR-3. Proteolytic processing, signal adaptor binding and activation of downstream signalling molecules results in lymph endothelial growth, proliferation and survival.

There are several studies, which suggested a correlation between the expression level of VEGF-C and lymph node metastasis (LNM) in e.g. CRC, gastric, prostate, esophageal and

lung cancers (Achen and Stacker 2008). The mechanisms regulating the VEGF-C/VEGF-D expression in tumours are not fully revealed. It is known, that pro-inflammatory cytokines such as tumour necrosis factor (TNF) and Interleukins induce the expression of VEGF-C in tumour cells. The local ECM environment is assumed to trigger different VEGF receptors, resulting in signalling pathways which promote lymphangiogenesis.
In fact, the results of some studies showed that the expression levels of VEGF-D and VEGFR-3 in colorectal carcinoma tissues are significantly higher than in normal tissues (Omachi, Kawai et al. 2007). Furthermore, recent reports have linked the VEGF-C/VEGF-D expression to lymphatic metastasis and poor patient outcome (Nagahashi, Ramachandran et al. 2010; Lin, Lin et al. 2011). Our histopathological examination also revealed that VEGF-C was present in CRC tissue, whereas the surrounding tissue was negative.

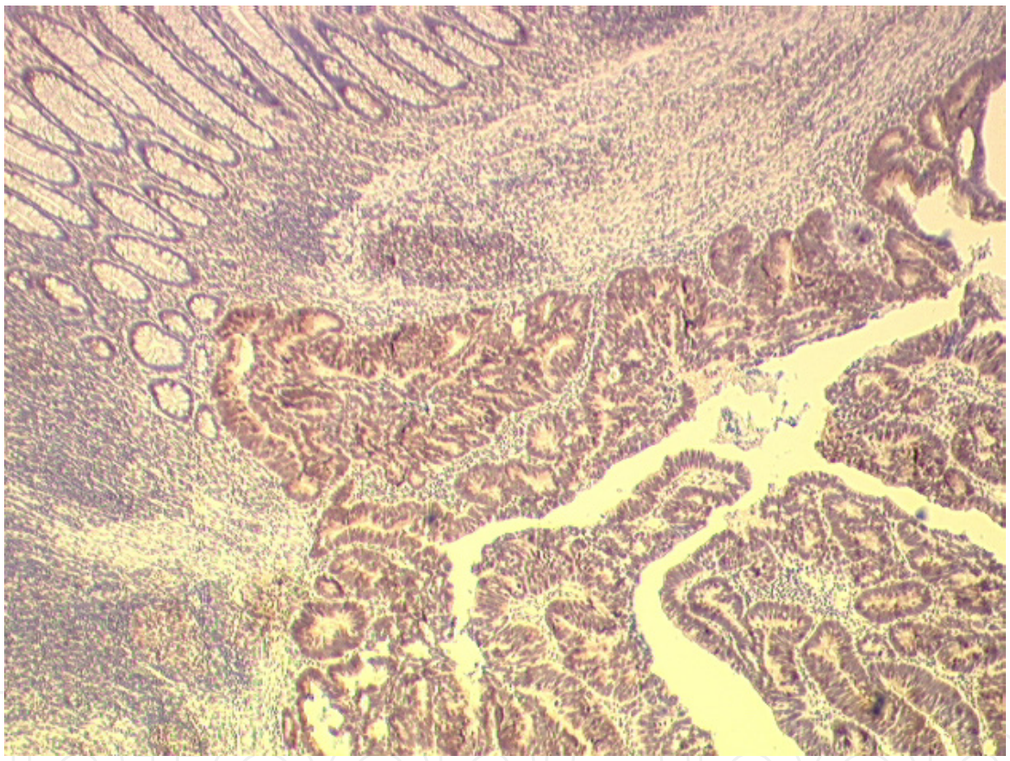


Fig. 5. CRC immunohistochemically staining for VEGF-C expression. The strong VEGF-C expression appears in the colon carcinoma tissue, while the surrounding tissue is negative.

VEGF-A is associated with angiogenesis, but it may also contribute to lymphangiogenesis. During angiogenesis VEGF-A induces proliferation and migration of endothelial cells, protease production and promotes cell survival. Fibroblasts, macrophages and endothelial cells are cells in the tumour microenvironment which are known to secrete VEGF-A. Evidence indicates that transforming growth factor α (TGFα) plays a role in regulating VEGF-A expression. The biological activity of VEGF-A is mainly mediated direct via activating of VEGFR-2 and indirectly by recruiting monocytes and neutrophils, which express VEGFR-1 and produce VEGF-C/VEGF-D.
A number of reports describe in CRC a correlation between VEGF-A expression levels and lymph node metastasis (Sundlisaeter, Dicko et al. 2007).

3.2.2 Prox-1

Prox-1 is a homeobox transcription factor. In several tissues, such as liver and pancreas Prox-1 is an important regulator of cell differentiation and oncogenesis. As mentioned before, the expression of Prox-1 is also essential for the lymphatic development and downstream signalling results in up-regulation of e.g. LYVE-1, VEGFR-3 and other lymphatic endothelial specific molecules.

Prox-1 expression is revealed to be significantly increased in CRC (Parr and Jiang 2003). The precise function must be further clarified.

3.2.3 Podoplanin

Podoplanin is a 38-kDa single transmembrane mucin-type glycoprotein and in normal human tissue it is expressed e.g. by osteoblasts, kidney podocytes and lung alveolar type 1 cells (Cueni, Hegyi et al. 2010). Due to its expression on lymphatic endothelial cells but not on blood vessels it is used as a specific marker for LECs.

Under normal conditions podoplanin is involved in the regulation of the shape of podocytes, LV formation and it is supposed to be involved in platelet aggregation. The expression of podoplanin is regulated by Prox-1 (Raica, Cimpean et al. 2008).

Since podoplanin expression is up-regulated in a number of different carcinomas such as vascular tumours, mesotheliomas and in squamous cell carcinomas, it is suggested that podoplanin is involved in carcinogenesis (Yamanashi et al. 2009). In addition, recent data in numerous of squamous cell carcinomas indicated that podoplanin is expressed at the invasive edge (Wicki and Christofori 2007). Podoplanin might favour tumour invasion via

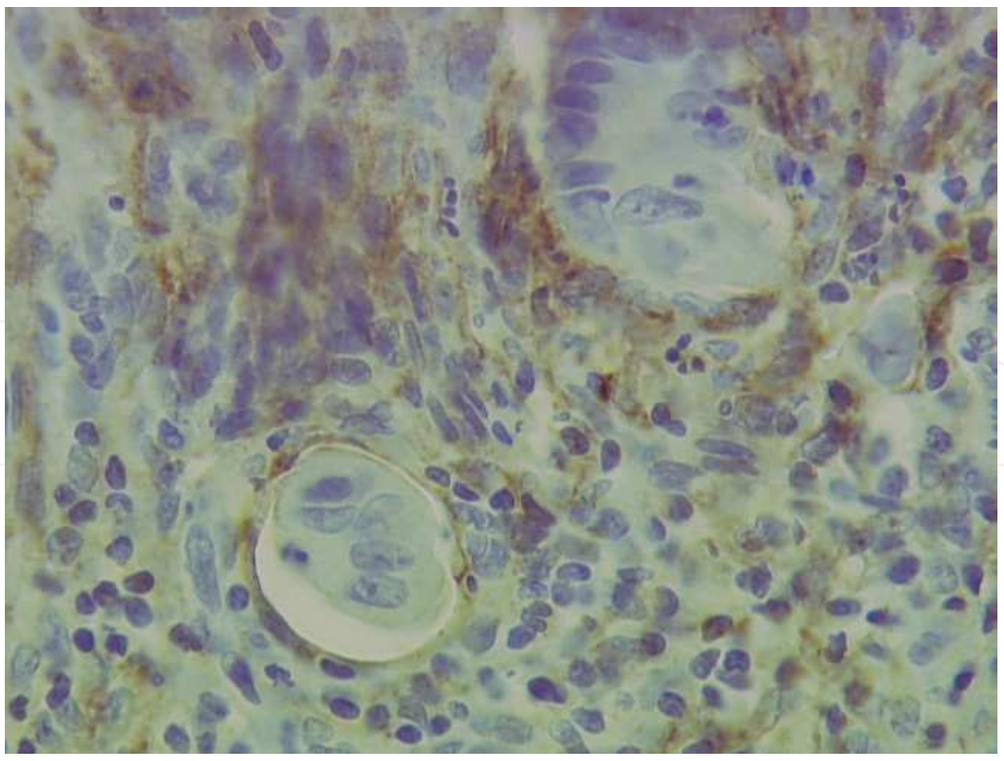


Fig. 6. Immunohistochemical detection of podoplanin positive lymphatic vessels, filled with cancer cells, in CRC.

its ability to remodel the cytoskeleton (Cueni, Hegyi et al. 2010). About the role of podoplanin in CRC little information is available. Lu et al. revealed, that the expression of podoplanin was significantly higher in patients with lymph node metastasis than in those without metastasis (Lu, Yang et al. 2007). While the group of Yamanashi suggested, that a positive podoplanin expression in stromal fibroblasts in patients with CRC is a significant indicator for a good prognosis (Yamanashi, Nakanishi et al. 2009). Figure 6 shows a lymphatic vessel stained with podoplanin and filled with a tumour cell embolus

3.2.4 LYVE-1

LYVE-1 is a homologue of the blood vascular endothelium specific hyaluronan receptor CD 44 and accordingly a member of the Link protein family. CD44 is directly involved in leucocyte migration (Jackson 2009). Lyve-1 is one of the most specific and widely used lymphatic endothelial markers (Hirakawa 2011). During embryogenesis it is expressed in cardinal vein endothelium and is involved in vascular development. On LECs LYVE-1 is expressed on the luminal and ab-luminal surface and functional studies demonstrated that it is able to act as an endocytic receptor for hyaluronan (Al-Rawi, Mansel et al. 2005). Hyaluronan is an important component of the extracellular matrix with versatile features for the interaction of cells during embryogenesis and woundhealing. LYVE-1 is also expressed by sinusoidal endothelial cells in the liver, spleen and by macrophages. Its exact function remains unclear.

3.2.5 Hepatocyte growth factor

Hepatocyte growth factor (HGF) belongs to the plasminogen-prothrombin gene superfamily. C-Met, the HGF receptor is a tyrosine kinase receptor and composed of an extracellular α chain and a transmembrane β chain. HGF activity has been reported to play a role in embryogenesis and organogenesis (Lee et al. 2010).

HGF is also supposed to be a potent lymphangiogenic factor. In this context HGF is involved in proliferation, migration, and tube formation of LECs (Cueni and Detmar 2006). The HGF downstream pathway is mediated via ERK 1/2 and PI3K and resulted in cell growth and inhibition of apoptosis. In many solid tumours c-Met is differently expressed. Novel investigations in CRC revealed an over expression of HGF and c-Met, and increased expression is associated with advanced disease stage and poor outcome (Kammula, Kuntz et al. 2007; Organ, Tong et al. 2011).

3.2.6 Fibroblast growth factors

The fibroblast growth factor family consists of structurally related ligands and four receptors (FGFR-1, FGFR-2, FGFR-3, FGFR-4), which consist the classical receptor tyrosine kinase structure: a extracellular Immunglobulin-like domain, a transmembrane domain and a intracellular tyrosine kinase domain, which initiated downstream signalling. The FGFs are involved in multi biological processes such as proliferation, survival, migration and differentiation during organogenesis and in adult life. A deregulation in human cancer has been found e.g. in breast cancer, prostate cancer, bladder cancer and cancer of the lung (Wesche, Haglund et al. 2011). FGF-2 is able to induce angiogenesis and lymphangiogenesis. Recent studies suggest that in LECs lymphangiogenic signalling is mediated through the

Akt-mammalian target of rapamycin (mTOR)-p70S6 kinase pathway (Matsuo, Yamada et al. 2007).

3.2.7 Angiopoietins

Ang-1 and Ang-2 are the more intensive analysed members of the angiopoietin family. Both bind to the Tie-2 receptor, which is expressed on the surface of LECs. The expression of Ang-1 and Ang-2 differs in human tissue. While Ang-1 is widely expressed in adult tissues, where it promotes vessel maturation and stabilization, Ang-2 expression occurs during vascular remodelling and via acting in conjugation with VEGF-A Ang-2 is supposed to be a stimulator of angiogenesis (Makinen, Norrmen et al. 2007).
About the role and function of the angiopoietins Ang-1/Ang-2 in lymphangiogenesis little information is known. Ang-1 is involved in LEC proliferation and lymphatic vessel sprouting. From analysis of pancreatic cancer, we know that Ang-2 drives lymphatic metastasis via a Tie-2 dependent manner and in a Tie-2 independent manner through enhancing the capacity of tumour cells for adherence to endothelial cells (Schulz, Fischer et al. 2011).

3.2.8 Insulin like growth factors

The insulin like growth factor system consists of the ligands insulin, insulin like growth factor 1 (IGF-1) and insulin like growth factor 2 (IGF-2) and acts via four receptors: the insulin receptor (IR), the type I IGF receptor, the type II IGF receptor and the hybrid IR/IGF-1R receptor. The IGF-IR receptor consists of two α and two β chains. IGF-IR ligand binding induces multiple downstream signal transduction pathways such as the MAPK, ERK and PI3-K pathway. It is well known, that IGF family members are frequently expressed in many solid tumours like CRC and breast cancer (Werner, Roberts et al. 1996; Reinmuth, Liu et al. 2002). In addition IGF-1R contributes to cancer development by regulation cell proliferation, differentiation and by preventing apoptosis. Other researchers investigated that IGF-1 and IGF-2 induce lymphangiogenesis (Bjorndahl, Cao et al. 2005) in a VEGFR-3 independent signalling pathway.

3.2.9 Chemokines

The chemokines, are a super family of chemotactic cytokines. They are key regulators of leukocyte, endothelial and epithelial cell migration and play a functional role in embryogenesis. Chemokines are low molecular weight proteins with cysteins at well conserved domains. According to the position of the cystein residue 4 chemokine subfamilies (CXC, CXC_3, CC, C) have been identified so far. The chemokine CXCL12 is supposed to be involved in lymphogenesis via its receptor CXCR4 and CCL21 mediates homing of lymphocytes and migration of dendritic cells into lymphatic vessel.
Nonetheless, it has been reported that chemokines and their receptors are expressed in a variety of human cancers such as melanoma, breast cancer, gastric cancer or prostate cancer (Hoon, Kitago et al. 2006). Recent findings about the direct role of chemokines in LNM in CRC, suggested an involvement of CXCR3, CXCL12/CXCR4 and CCL21/CCR7 (Kawada and Taketo 2011; Singh et al. 2011; Raman et al. 2010). In addition CXCL12 is supposed to be a prognostic factor for local recurrence and liver metastasis and CXCR4 expression was significantly positive in CRCs with high tumour stage and LNM.
Taken together, Table 2 summarizes factors which are involved in lymphangiogenesis.

Factor	**Function during lymphangiogenesis**
VEGF-C/VEGF-D via VEGFR-3	Growth factor/receptor: proliferation, migration, survival
VEGF-A via VEGFR-2	Activating VEGF-C/VEGF-D/VEGFR-3 signaling pathway
Prox-1	Transcription factor: LEC identity
Podoplanin	Cell motility
LYVE-1	Hyaluronan receptor
HGF	Growth factor: proliferation, migration, tube formation of LECs
FGF	LEC migration, proliferation
Ang-1/2	Growth factor
IGF	Growth factor: proliferation, differentiation and preventing apoptosis
Chemokines CCL21	Lymphocytes homing

Table 2. Molecules which are involved in lymphangiogenesis

3.3 Lymphatic vessel density and tumour progression

Since, microvessel density (MVD), a parameter for the ability of angiogenesis in tumours, is a prognostic marker in numerous cancers, the quantification of lymphatic vessel density (LVD) is of growing interest. Screening the literature, the prognostic significance of LVD in tumours remains controversial (Royston and Jackson 2009). Some studies reported that high LVD was associated with lymph node metastasis and patient outcome, while others could not confirm these findings (Gao, Knutsen et al. 2009). Furthermore, there is a debate about the dominant role of intratumoural vs. peitumoural lymphatic vessels. By some researchers it has been demonstrated that LVD in the intratumoural areas but not in peritumoural areas were associated with lymph node metastasis and poor outcome. Others reported that LVD in peritumoural areas was correlated to advanced tumour stage (Longatto-Filho, Pinheiro et al. 2008). In patients with colorectal cancer, a significant correlation between the number of intratumoural and peritumoural lymphatic vessels with the occurrence of lymph node metastases was evaluated (Matsumoto, Nakayama et al. 2007). These findings again underline the hypothesis of active lymphatic vessel formation within the tumour. These new lymphatic vessels may facilitate the drainage of tumour cells to regional lymph nodes.

3.4 Future perspectives

Further characterization of the exact molecular pathways which are involved in lymphatic metastasis is needed and essential for the development of new forecast estimates and

individually oriented therapies. Gene expression profiling by microarray technique, which allows the investigation of thousands of differentially expressed genes, provide therefore a promising tool to further clarify the molecular signature for lymphatic metastasis in CRC. These signatures can provide new players which are responsible for lymphatic metastasis or identify patients with a high risk for the development of lymph node metastases. New treatment targets could be evaluated and high risk patients can be selected for individual treatment regiments. (Croner, Peters et al. 2005; Croner, Fortsch et al. 2008; Croner, Schellerer et al. 2010).

4. Conclusion

Metastasis via tumour cell invasion into lymphatic vessels and lymph nodes is a common feature of various carcinomas. Our knowledge of the mechanisms controlling lymphatic metastasis has increased significantly in the last decades since the identification of LEC specific markers such as LYVE-1, podoplanin and Prox-1. The visualisation of the lymphatic system led to a new understanding of tumour activities involved in lymphatic vessel differentiation and growth. Take together, lymphangiogenesis is a complex multi step process, which is regulated by numerous molecular players and additional studies are needed to devise new and more efficient strategies against CRC.

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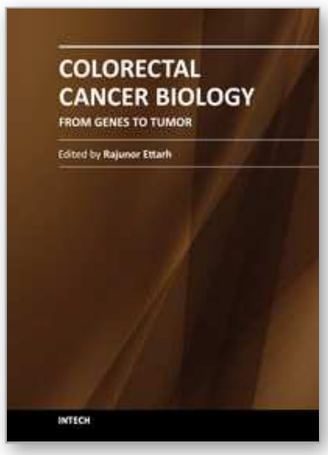

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Colorectal cancer is a common disease, affecting millions worldwide and represents a global health problem. Effective therapeutic solutions and control measures for the disease will come from the collective research efforts of clinicians and scientists worldwide. This book presents the current status of the strides being made to understand the fundamental scientific basis of colorectal cancer. It provides contributions from scientists, clinicians and investigators from 20 different countries. The four sections of this volume examine the evidence and data in relation to genes and various polymorphisms, tumor microenvironment and infections associated with colorectal cancer. An increasingly better appreciation of the complex inter-connected basic biology of colorectal cancer will translate into effective measures for management and treatment of the disease. Research scientists and investigators as well as clinicians searching for a good understanding of the disease will find this book useful.

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