RESEARCH ARTICLE

Endothelial Cell Proliferation and Vascular Endothelial Growth Factor Expression in Primary Colorectal Cancer and Corresponding Liver Metastases

Balica Amalia Raluca[1], Anca Maria Cimpean[1]*, Andreea Cioca[3], Octavian Cretu[2], Ovidiu Mederle[1], Alexandru Ciolofan[2], Pusa Gaje[1], Marius Raica[1]

Abstract

Background: . Colorectal carcinoma (CRC) is one of the major causes of cancer death worldwide. Data from the literature indicate differences between the proliferation rate of endothelial cells relative to the morphology growth type, possibly due to origin of specimens (autopsy material, surgery fragments) or quantification methods. Vascular endothelial growth factor (VEGF) is a factor that stimulates the proliferation of endothelial cells. It is expressed in more than 90% of cases of metastatic CRC. Aim: The aim of this study was to evaluate the endothelial cell proliferation and VEGF expression in primary tumors and corresponding liver metastases. Materials and Methods: Our study included 24 recent biopsies of primary tumors and corresponding liver metastases of CRC cases. CD34/ Ki67 double immunostaining and RNA scope assay for VEGF were performed. Results: In the primary tumors analysis of VEGFmRNA expression indicated no significant correlation with differentiation grade, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas. In contrast, in the corresponding liver metastases, VEGFmRNA expression significantly correlated with the total number of non-proliferative vessels and total number of vessels. CD34/ Ki67 double immunostaining in the cases with poorly differentiated carcinoma indicated a high number of proliferating endothelial cells in the peritumoral area and a low number in the intratumoral area for the primary tumor. Moderately differentiated carcinomas of colon showed no proliferating endothelial cells in the intratumoral area in half of the cases included in the study, for both, primary tumor and liver metastasis. In well differentiated CRCs, in primary tumors, a high proliferation rate of endothelial cells in the intratumoral area and a lower proliferation rate in the peritumoral area were found. A low value was found in corresponding liver metastasis. Conclusions: The absence of proliferative endothelial cells in half of the cases for the primary tumors and liver metastases in moderately differentiated carcinoma suggest a vascular mimicry phenomenon. The mismatch between the total number of vessels and endothelial proliferation in primary tumors indicate that a functional vascular network is already formed or the existence of some mechanisms influenced by other angiogenic factors.

Keywords: Colon carcinoma - endothelial cell proliferation - metastasis

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Introduction

Normal tissues are characterized by the presence of blood and lymphatic vessels lined by endothelial cells exhibiting a low proliferation rate ranged between 0.1- 3 % of all endothelial cells which turnover daily but this percentage decline with age (Schwartz SM et al., 1973). Tumor blood vessels have an increased endothelial cells proliferation rate of 20-2000 times higher than in normal tissues (Hobson et al., 1984, Zecchin et al, 2015), representing 0.05% from the total number of human tumor proliferating cells (Kendall et al., 1999). Significant differences have been observed concerning endothelial cells proliferation rate between several tumor types, this aspect being already certified in tumors as non inflammatory breast cancer and hepatocellular carcinoma (11% and 35%, respectively) (Colpaert et al., 2003; Kendall et al., 1999; Imura et al., 2004; Quinn et al., 1993).

CRC is one of the major causes of cancer death worldwide, being the third most common diagnosed cancer in men and the second in women (Baena and Salinas, 2015). Metastatic ability of colorectal cancer cells is well certified and it is influenced by heterogeneous factors as individual's age, dietary habits, any complaint of obesity, diabetes, previous history of cancer or intestinal polyps (Rasool et al, 2013) or by histopathologic subtypes

[1]Department of Histology, Angiogenesis Research Center, [2]Department of Surgery,"Victor Babes" University of Medicine and Pharmacy, Timisoara, [3]Department of Pathology, Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania
**For correspondence: ancacimpean1972@yahoo.com*

(Hugen et al, 2014). The liver is the most common target for metastasis in patients with this disease and hepatic metastasectomy increase overall survival rates (Frankel and D'Angelica, 2014). In primary CRC, Vermeulen et al. (1995) have demonstrated that the fraction of cycling endothelial cells was higher in tumor tissue (cell labeling index of 9.9%) as compared with adjacent normal mucosa.

Many factors are involved in the development and progression of liver metastasis. The tumor cell-endothelium interaction influences the organ-specific metastasis. One of the most important angiogenic growth factor is represented by vascular endothelial growth factor (VEGF) (Yu et al. 2012). This factor is involved in the induction of vasodilation, endothelial cells migration, proliferation and vessel assembly (Lamalice et al., 2007; Zhou et al., 2012). The overexpression of VEGF was found to the patients with liver metastasis of colon carcinoma (90.9% of cases) and particularly in well differentiated colon carcinomas (83.3%) (Cao et al. 2009). Data from the literature on endothelial cell proliferation in liver metastases of colon carcinoma shows variable results. Thus, the study of endothelial cell proliferation in relation with the three growth pattern of liver metastasis indicated a significant difference of the proliferating endothelial cells fraction between desmoplastic, replacement (3%) and the pushing growth pattern (11%) (Vermeulen et al. 2001). Eefsen et al. (2012) found elevated proliferation fraction of endothelial cells for the pushing growth pattern, but without a significant difference comparative with desmoplastic or replacement growth pattern.

Based on these considerations, the aim of this study was to evaluate the endothelial cells proliferation and VEGF expression in primary tumors and corresponding liver metastasis.

Materials and Methods

Our study included 24 recent biopsies of primary tumors and corresponding liver metastasis from patients with CRC. Biopsies were fixed in buffered formalin and embedded in paraffin. Sections from each case were stained with hematoxylin-eosin for histopathological diagnosis. Immunohistochemical study included double immunostaining for CD34 and Ki67. Heat-induced epitope retrieval with pH 6.0 solution (Leica Biosystems, Newcastle uponTyne, UK), for 30 minutes was followed by endogenous peroxidase blocking (3% hydrogen peroxide, 5 minutes) and incubation with primary antibody Ki67 (Dako Glostrup Denmark, ready to use, clone MIB-1, 30 minutes). NovoLink Max Polymer Detection System The Leica Biosystems, Newcastle uponTyne, UK, was used as visualization system and 3,3 - diaminobenzidine as chromogen. Immunohistochemical technique continued with endogenous peroxidase blocking with 3% hydrogen peroxide for 5 minutes, incubation with the second antibody CD34 (Dako Glostrup Denmark, mouse monoclonal anti-human, clone 1A4, ready to use, 30 minutes), visualised with Vina Green as chromogen, for 10 minutes (Biocare Medical, LLC, Concord, CA 94520, USA). Nuclear staining was performed with Lille's hematoxylin. The full immunohistochemical procedure was performed with DakoAutostainer Plus (DakoCytomation). The proliferating endothelial cells were defined as cells lining the vessels lumen and coexpressing both CD34 and Ki67. Proliferative and non-proliferating vessels were counted on three consecutive microscopic fields at 200X magnification, in the intratumoral and peritumoral areas. RNA scope assay was performed using RNAScope 2.0 FFPE Reagent Kit (Advanced Cell Diagnostics, Inc., Hayward, CA) according to the manufacturer's instructions. The formalin fixed, paraffin embedded tissue sections were pretreated with heat and protease prior to hybridization with a target probe. A HRP-based signal amplification system was then hybridized to the target probes followed by color development with DAB. VEGF quantification was made according to the following score: score 0 (1 dot/cell), score 1 (1-3 dots/cell), score 2 (4-10 dots/cell), score3 (>10 dots/cell, less than 10% positive cells), score 4 (>10 dots/cell, more than 10% positive cells). Image acquisition and analysis were performed using Nikon Eclipse E 600 microscope and Lucia G software for microscopic image analysis.

The local research ethics committee approved the protocol of the study and informed consent was obtained from all subjects according to the World Medical Association Declaration of Helsinki.

Results

Histopathological evaluation based on routine haematoxylin and eosin method revealed liver metastasis from well (8 cases), moderately (8 cases) and poorly differentiated (8 cases) CRC.

For well differentiated colon carcinoma, in primary tumor, a high proliferation rate with 3 to 15 proliferative endothelial cell in the intratumoral area was noticed. A lower proliferation rate was found in the peritumoral area. In comparison with the primary tumor, in corresponding

Table 1. Values of Proliferating Endothelial Cells According with Grading, Localization, Tumor Area

Grading	Primary tumor		Liver metastasis	
	intratumoral proliferating endothelial cell	peritumoral proliferating endothelial cell	intratumoral proliferating endothelial cell	peritumoral proliferating endothelial cell
well differentiated	52	32	12	4
moderately differentiated	24	10	12	16
poorly differentiated	20	48	38	16

Table 2. VEGF mRNA Expression in Primary Tumors and Corresponding Liver Metastasis

Primary tumor		Liver metastasis
well differentiated carcinoma	heterogeneous expression, score 3, distinct dots	heterogeneous expression, score 1;
moderately differentiated carcinoma	different intensity of expression in the tumor area, score 4, compact clusters	heterogeneous expression, score 3; expression in the endothelial cells of sinusoides and very rare in the hepatocytes
poorly differentiated carcinoma	low intensity of reaction, score 2, visible dots and small clusters	low expression, score 1, more intense in the adiacent hepatic parenchyma

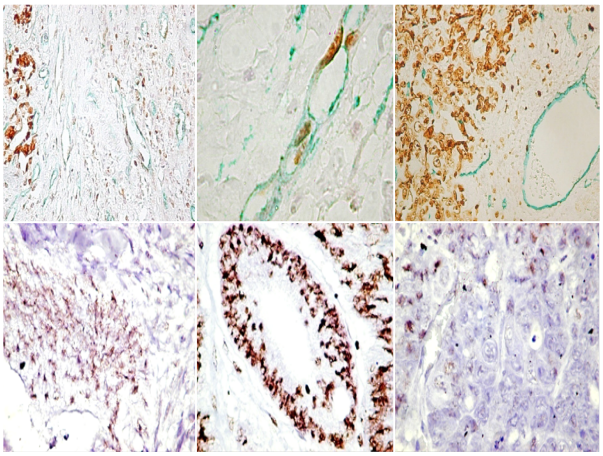

Figure 1. A) High Number of Proliferating Endothelial Cells in the Peritumoral Area and a Low Number in the Intratumoral Area of the Primary Tumor, 20x; B) Intussusception Phenomenon Associate with Endothelial Cell Proliferation, 100x; C) Discontinuous Wall and Proliferating Endothelial Cells in the Centrilobular Vein Adjacent to the Metastasis, 20x; D) VEGFmRNA Expression, Well Differentiated Carcinoma- Score3, 20x; E) VEGFmRNA Expression, Moderately Differentiated Carcinoma- Score4, 20x; F) VEGFmRNA Expression, Poorly Differentiated Carcinoma- Score2, 40x

liver metastasis, a low value was found. Thus, liver metastasis showed values between 1 and 3 for the proliferating endothelial cells in the intratumoral area and the absence of proliferating endothelial cells in the peritumoral area.

The moderate differentiated CRC showed the absence of proliferating endothelial cells in the intratumoral area of half of the evaluated cases, for both primary tumor and liver metastasis. A high rate of proliferating endothelial cells was found in the peritumoral area with close values for primary tumor and corresponding metastasis.

CD34/ Ki67 double immunostaining in the cases with poorly differentiated carcinoma indicated a high number of proliferating endothelial cells in the peritumoral area and a low number in the intratumoral area for the primary tumor (Figure1 A). As a particular aspect, we noticed to these cases the presence of intussusception phenomenon associate with endothelial cell proliferation (Figure 1 B). In the corresponding liver metastasis, the proliferating endothelial cells were present in the peritumoral area and absent in the intratumoral area. The intratumoral blood vessels were small, non proliferative with collapsed lumen. The central vein adjacent to the metastasis showed a discontinuous wall and proliferating endothelial cells (Figure 1 C).

The main values of proliferating endothelial cells and the relations with grading, localization and tumor area are summarized in table 1.

In the intratumoral area of primary tumor, we found a significant correlation between the number of proliferative vessels (CD34+/Ki67+) and intratumoral proliferative endothelial cells (p=0.001). Number of non-proliferative vessels (CD34+/ Ki67-) was significantly correlated with the total number of intratumoral vessels (p=0.001) and with poor differentiated carcinoma. It was noticed that the total number of intratumoral vessels partialy correlate with the differentiation degrees (p= 0.05).

Peritumoral area of primary tumor presented a correlation between non-proliferative vessels and differentiation degrees (moderate and poor differentiated type; p=0.01).

VEGFmRNA expression in primary tumor had variable scores with differentiation degree. In primary tumors, the score distribution was as follows: for well differentiated carcinoma score 3, with heterogeneous expression and distinct dots (Figure 1 D); moderately differentiated carcinoma presented a different intensity of expression in the tumor area, compact clusters and score 4 (Figure 1 E); poorly differentiated carcinoma had low intensity of reaction, visible dots and small clusters with a value of score 2 (Figure 1 F).

Analysis of VEGFmRNA expression in liver metastasis indicated a heterogeneous expression with values from 1 to 3 (Table 2). In the primary tumors no significant correlation between VEGFmRNA expression and pathological type, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas was found. Opposite, in the corresponding liver metastasis, VEGFmRNA expression was significantly correlated with the total number of non-proliferative vessels (p=0.026) and total number of vessels (p=0.036).

Discussion

Tumors had preferential sites for metastasis. Thus, colon cancer has tendency to give rise to liver metastasis. At the time of the diagnosis, 25% of patients presented liver metastasis.

The addition of Bevacizumab to FOLFOX 4 (oxaliplatin, 5-fluorouracil, and leucovorin) indicated an increased of median overall survival and progression free- survival with 2.1 and 2.6 month (Giantonio BJ et al., 2007; Zhu et al., 2014). No major differences (4.7 and 4.2 month increase) were observed between administration of irinotecan, 5-fluorouracil, leucovorin and placebo treatment with foregoing treatment and bevacizumab (Dirican et al., 2014; Hurwitz et al., 2004).

The fraction of endothelial cell proliferation was described as an important factor used in the evaluation of angiogenesis (Vermeulen et al., 2002; Vermeulen et al., 1996). A study on brain metastases from NSCLC indicated a higher proliferation rate and vascular maturity comparative with primary tumors (Benjamin et al., 1999; Jubb et al., 2011). The mature blood vessels are less sensitive to Bevacizumab than immature vessels and a lower efficacy for these patients was showed.

Three distinctive morphological growth patterns were described for liver metastasis: desmoplatic, replacement and pushing (Vermeulen et al 2001). Recently, these growth patterns seems to have a prognostic significance (Nielsen et al., 2014), growth patterns having a direct correlations with reccurence free survival and other prognostic factors (Eefsen et al, 2015). A high angiogenic activity was found in the pushing growth pattern and a lower one has been noticed in a desmoplastic and replacement growth pattern. Elevated values for endothelial proliferative cells in the pushing growth pattern were found, but without a significant difference comparative to the other two types (Eefsen et al., 2012). We found, in liver metastasis values between 1 and 3 for proliferating endothelial cells for the well differentiated carcinoma, the absence of proliferating endothelial cells in half of the cases of the moderately differentiated colon carcinoma in the intratumoral area.

Our results showed a different proliferative index between the tumor blood vessel endothelium from the tumor core and its peripheral areas. This finding could partially explain the ineffective antiangiogenic and/ or antivascular therapy of tumor angiogenesis in liver metastasis.

Lack of CD34 immunostaining in some blood vessels lined by several Ki67-positive endothelial cells, suggests that liver metastasis tumor blood vessels are more permeable than normal blood vessels. Our study suggests that liver metastasis blood vessels are heterogeneous, do not have the same proliferative status or expression of markers concurrently and may respond in a different way to antiangiogenic therapy

In the present study, the total number of vessels did not correlate with endothelial proliferating cells in the primary tumor. From this, it derived two hypotheses: functional intratumoral vascular network is already formed, vessels are stabilized at the moment of diagnosis or involvement of other angiogenic mechanisms dependent on other growth factors that induce the formation of new vessels. The fact that non proliferating intratumoral vessels correlates with the total number of intratumoral vessels reinforces the idea that nonproliferative vessels are already functional, possibly stabilized.

Tokunaga et al. (1998) demonstrated that expression of VEGFmRNA isoforms was correlated with liver metastasis and poor prognosis in colon carcinoma. Choi et al., (2012) obtained different results: no significant relationship between the expression of VEGF, COX 2 and depth of tumor invasion, lymph node metastasis, vessel invasion, perineural invasion and liver metastasis. On the other hand, it has been showed that low VEGF165b / VEGF total ratio may be a predictive marker for bevacizumab in metastatic colorectal cancer, and individuals with high relative levels may not benefit. Initial studies of the phase III clinical trials of bevacizumab showed no predictive value for total VEGF expression or microvessel density (Bates et al., 2012; Jubb et al., 2006), suggesting that it was not the VEGF levels that determine the outcome.

In our study, no significant correlation between VEGFmRNA and pathological type, proliferative and nonproliferative vessels types, in the intratumoral and peritumoral areas of primary tumors was found. But, for the liver metastasis we noticed a correlation between VEGFmRNA expression and non-proliferative and total number of vessels.

In primary tumors the total number of intratumoral vessels was not correlated with endothelial cell proliferation, which can suggest that intratumoral vascular network is formed, stabilized at the moment of diagnosis. In the primary tumors no significant correlation between VEGFmRNA expression and histopathological type, proliferative and non-proliferative vessels in the intratumoral and peritumoral areas was found compared to corresponding liver metastasis in which VEGFmRNA expression was significantly correlated with total number of non-proliferative and total number of vessels.

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