VEGF, Flt-1, and microvessel density in primary tumors as predictive factors of colorectal cancer prognosis

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Abstract. Angiogenesis in the primary tumor is known to be necessary for tumor progression in adenocarcinomas of the colon. However, whether angiogenesis in the primary tumors of patients with colorectal cancer affects their prognosis has yet to be fully elucidated. The aim of the present study was to assess the association between selected pathoclinical parameters and overall survival of resectable colorectal cancer patients with the expression of angiogenesis-promoting factors, including vascular endothelial growth factor (VEGF) and Fms-like tyrosine kinase receptor (Flt-1), and microvessel density (MVD) in the primary tumor. VEGF and Flt-1 expression were assessed, as well as MVD (with anti-CD34) by immunohistochemistry in 139 archived primary colorectal cancer tissue samples. These results were compared with the overall survival of the patients and potential prognostic pathoclinical parameters. A higher MVD in the tumors expressing Flt-1 (P=0.04) was identified. However, there was no correlation between the pathoclinical parameters of colon cancer and Flt-1 expression, VEGF expression, or MVD in the tumor. Furthermore, the intensity of VEGF expression, Flt-1 expression and tumor MVD did not correlate with the overall survival of the patients. Therefore, although increased expression of VEGF and Flt-1 was correlated with an increased expression of MVD in the primary tumors of resectable colorectal cancer patients, these factors were not correlated with prognostic pathoclinical factors and overall survival.

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Introduction

The heterogeneous course of adenocarcinoma of the colon has prompted the search for new prognostic and diagnostic tools. As a result of this search, angiogenesis in the primary tumor was revealed to be necessary for tumor progression. In addition, the heterogeneity in large intestinal adenocarcinomas has been attributed to the level of angiogenesis. Therefore, an improved understanding of the process of angiogenesis may provide novel anticancer therapies, as well as new prognostic and predictive tools.

In tumors, an autonomous system of blood vessels develops under the strict control of stimulating and inhibiting factors (1,2). A key player at all stages of angiogenesis is the signaling molecule, vascular endothelial growth factor (VEGF). VEGF belongs to the family of platelet-derived growth factors, which includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (3,4). However, the predominant factor promoting the formation and growth of new vessels is VEGF-A (5). VEGF exerts its biological effects through the following glycoprotein tyrosine kinase receptors: VEGFR-1 (also known as the Fms-like tyrosine kinase, Flt-1); VEGFR-2 (also termed fetal liver kinase, Flk-1) and VEGFR-3 (6,7). In particular, Flt-1 and Flk-1 are the two receptors directly involved in the formation of blood vessels (8). Although the role of Flk-1 is well understood (i.e., it transduces the stimulating signal into the cell through the activation of the tyrosine kinase cascade), the role of Flt-1 in the angiogenic process has yet to be properly elucidated.

Flt-1 is involved in both inflammation and carcinogenesis. Expression of Flt-1 is not restricted to vascular endothelial cells. It is also found on cells of hematopoietic lineage (i.e., monocytes and macrophages), where it has a regulatory function. For example, Flt-1 has been shown to be involved in the mobilization of macrophages, and it is able to induce macrophage cytokine secretion (9). Additionally, Flt-1 is expressed on dendritic cells, osteoclasts, pericytes, hepatocytes, trophoblast cells of the placenta (10) and smooth muscle cells (11). With respect to carcinogenesis, activation of Flt-1 may affect tumor development multidirectionally. It contributes to the proliferation and migration of vascular endothelial cells and tumor cells. Furthermore, Flt-1 has been

revealed to support the phenotypic change of cancer cells into mobile units that are capable of migration, a process called epithelial-mesenchymal transition (EMT) (12). Flt-1 is also involved in the preparation of the pre-metastatic niche, i.e., a 'metastasis-friendly' environment. In the first stage of the niche formation, fibroblasts produce and secrete fibronectin, which is a target for migration of the hematopoietic progenitor cells (monocytes and macrophages) released from the bone marrow that contain Flt-1. These monocytes and macrophages are subsequently able to mobilize tumor cells, thereby creating a 'metastasis-friendly' environment (13,14).

The present study aimed to determine whether pro-angiogenic factors (i.e., VEGF and Flt-1), as well as angiogenesis itself [measured by the microvessel density (MVD)] in the tumor, contribute to the pathology and prognosis of patients with resectable colorectal cancer.

Materials and methods

Patients and tissue samples. The present study was a retrospective study of 139 patients who underwent surgery in the Clinic of Oncologic Surgery, Medical University of Gdansk, Poland, between September 1998 and December 2002. For the immunohistochemistry staining, archived tissue material from primary tumors obtained during surgery was used. The pathoclinical characteristics of the study group are presented in Table I. Patients were not subjected to oncological treatment prior to surgery. The operation met the criteria of R0 resection, i.e., it was locally oncologically radical. The stage of cancer was determined according to the pathological tumor-lymph nodes-metastasis (pTNM) classification system (15). Histological evaluation was based on the World Health Organization classification (16). In all cases, adenocarcinoma was diagnosed, and well (G1), moderately (G2) and poorly (G3) differentiated tumors were differentiated. The minimum follow-up of patients remaining alive was 44 months. The study was approved by the Independent Bioethical Committee for Scientific Research, no. NKEBN/4/005.

Immunohistochemistry. The tissues were fixed in 4% formaldehyde solution, dehydrated with ethyl alcohol, and embedded in low-melting paraffin. Paraffin blocks were subsequently processed and cut on the sledge microtome into 4-μm sections. The sections were routinely stained with hematoxylin and eosin. Tumor fragments without necrosis were selected for the present study. Representative sections were applied on to glass slides coated with 2% silane solution (APES; cat. no. A3648, Sigma-Aldrich, St. Louis, MO, USA). Sections were incubated at 36˚C for 24 h, deparaffinized and rehydrated. The following antibodies were used: Polyclonal anti-Flt-1 (c-17) rabbit antibody (cat. no. sc-316; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal anti-VEGF (A-20) rabbit antibody (cat. no. Sc-152; Santa Cruz Biotechnology), and monoclonal anti-CD34 mouse antibody (cat. no. M7165; Dako, Carpinteria, CA, USA). Determination of antigen expression was performed according to the antibodies' manufacturers' protocols.

Vascular density determination using anti-CD34 antibodies. The slides were subjected to heat treatment in a water bath in Target Retrieval Solution (cat. no. S1700; pH 6.0, Dako) at a temperature of 99˚C for 20 min. Subsequently, slides were cooled at room temperature for 20 min and washed in phosphate-buffered saline (PBS) twice for 10 min. They were subsequently immersed in a 3% solution of hydrogen peroxide for a further 10 min, prior to being washed twice for 10 min in PBS. The primary anti-CD34 antibodies (cat. no. M 7165; Dako) were applied to the slides at a dilution of 1:25, and incubated for 1 h at room temperature. Following incubation, the slides were washed twice in PBS for 10 min. Biotinylated anti-mouse or anti-rabbit linking antibodies (Dako; cat. no. K675) were subsequently applied to the slides, and incubated at room temperature for 30 min. Following incubation, the slides were washed twice in PBS for 10 min prior to streptavidin-conjugated horseradish peroxidase (Dako; cat. no. K675) being applied and incubated at room temperature for 30 min. The slides were washed twice in PBS for 10 min, subsequently immersed in a substrate solution of diaminobenzidine (cat. no. K3468; Dako), and further incubated at room temperature for 10 min. The slides were washed with running water for 10 min, stained with Mayer's hematoxylin (Sigma-Aldrich) for 5 min, and subsequently washed again in running tap-water for 10 min. The procedure ended with dehydration of the preparation, clearing and mounting it with Canada balsam (Avantor Performance Materials Poland S.A., Gliwice, Poland). Finally, in order to assess the MVD in the slides stained for CD34, areas of increased vascularity ('hot spots') were searched at x40 and x100 magnification, according to the procedure described by Weidner *et al* (17). The MVD calculation was performed at x200 magnification in the area of 0.785 mm^2.

VEGF expression determination using an anti-VEGF antibody. The slides were prepared as described for the anti CD-34 antibodies, except that the primary anti-VEGF antibodies (A-20; cat. no. sc-152; Santa Cruz Biotechnology) were applied at a dilution of 1:100 and incubated for 2 h at room temperature. VEGF expression in tumor cells was evaluated on a two-point scale of the reaction intensity, depending on the resulting color reaction (i.e., 0 for no reaction or a weak reaction, indicating no expression of VEGF, and 1 for an intense reaction, indicating overexpression of VEGF).

Flt-1 expression determination using an anti-Flt-1 antibody. The slides were prepared as described for the anti CD-34 antibodies, except that the primary Flt-1 antibody (cat. no. c-17; Santa Cruz Biotechnology) was applied at a dilution of 1:300 and incubated for 2 h at room temperature. Flt-1 expression in tumor cells was evaluated on a two-point scale of the reaction intensity, depending on the resulting color reaction (i.e., 0 for no reaction, indicating no Flt-1 expression, and 1 for a reaction, indicating Flt-1 expression).

Statistical analysis. The association among VEGF and Flt-1 expression, MVD and overall survival in months, median age, gender, location of the tumor (colon vs. rectum), the extent of tumor infiltration (pT), status of regional lymph nodes (pN), the presence of distant metastases (pM), pTNM staging, and tumor grade (G1-G3) were assessed. Statistical analysis was performed using the data analysis software

Table I. Characteristics of patients in the study group (n=139).

Parameter	No. of patients (%)
Clinical stage according to:	
pTNM	
I	15 (10.8)
II	47 (33.8)
III	48 (34.5)
IV	29 (20.9)
pT feature	
1	2 (1.58)
2	21 (15.1)
3	96 (69.0)
4	20 (14.4)
pN feature	
0	68 (48.9)
1	43 (30.9)
2	28 (20.2)
pM feature	
0	110 (79.1)
1	29 (20.9)
Grade	
G1	21 (15.1)
G2	106 (76.3)
G3	12 (8.6)
Location	
Rectum	61 (43.9)
Colon	78 (56.1)
Gender	
Female	61 (43.9)
Male	78 (56.1)
Age (median, 66 years)	
≥Median	78 (56)
<Median	61 (44)

p, pathological; TNM, tumorlymph nodesmetastasis (staging system).

Table II. The relationship between VEGF or Flt1 expression and the microvessel density in the study group (n=139).

Protein/expression	Median microvessel density in the tumor (microvessels/ field of view)	P-value[a]
Flt1		0.04
Positive expression	30	
No expression	24	
VEGF		ns
Overexpression	30	
No expression	25.5	

[a]P-values were determined using the Mann-Whitney U test. ns, not significant; VEGF, vascular endothelial growth factor; Flt1, Fmslike tyrosine kinase receptor.

system, STATISTICA tools, version 10 (www.statsoft.com; StatSoft, Inc., 2011). To determine the correlation between MVD in tumor stroma and the expression of VEGF and Flt-1, or the association between the MVD and pathoclinical tumor parameters, the Mann-Whitney U and the Kruskal-Wallis analysis of variance (ANOVA) tests were used. Assessment of the correlation between VEGF and Flt-1 expression and tumor pathoclinical parameters was performed using Pearson's χ^2 test. Survival analysis was performed using the Kaplan-Meier method. Differences between survival times in the studied groups were verified using the log-rank test. For all calculations, $P<0.05$ was considered to indicate a statistically significant value. In certain cases, for the purpose of statistical analysis, the groups of evaluated pathoclinical parameters were combined due to their small size (i.e., T1+T2 vs. T3+T4, and stages I+II vs. stages III+IV).

Results

Correlation between MVD and VEGF or Flt-1 expression. The average MVD in the patient tissue samples was 30.3 microvessels (median, 27.5 microvessels) in the field of view. MVD ranged from 5-80 microvessels/field of view (standard deviation, 15 microvessels/field of view). Overexpression of VEGF was identified in 73 (52.5%) cases of colorectal cancer in the present study. In the remaining 66 tumors that were analyzed (47.5%), no VEGF expression was detected; nor it was expressed at very low levels. Flt-1 expression was detected in 102 (73%) primary colorectal tumors, whereas 37 (27%) tumors revealed no Flt-1 expression. Subsequently, the correlation between the MVD in the primary tumor and the expression level of VEGF or Flt-1 in the tumor cells was analyzed (Table II). Significantly higher vascular density in tumors with positive expression of the Flt-1 was observed (P=0.04; Fig. 1). Furthermore, a higher MVD in the tumor stroma in tumors with VEGF overexpression was also observed; however, this correlation was not statistically significant (P=0.6).

Correlation between colorectal cancer pathoclinical parameters and MVD, VEGF or Flt-1 expression. No significant correlation was identified between MVD and the pathoclinical parameters of colorectal cancer in the present study ($P>0.05$, according to the Mann-Whitney U and Kruskal-Wallis ANOVA tests). Furthermore, no statistically significant correlation was identified among the pathoclinical parameters of colorectal cancer, including age, gender, location of the tumor, stage or grade, and the intensity of VEGF expression in cancer cells. Similarly, statistically significant correlation was identified between these parameters and Flt-1 expression in the analyzed tumors ($P>0.05$, according to Pearson's χ^2 test). However, a significant correlation between VEGF overexpression in tumor cells and Flt-1 expression was identified. In tumors with Flt-1 expression (n=102), 59 (58%) also revealed overexpression of VEGF, whereas 43 (42%) Flt-1 positive tumors had no VEGF expression (P=0.03, according to Pearson's χ^2 test).

Correlation between overall survival and MVD, VEGF or Flt-1 expression. A survival analysis was performed on the 139 patients included in the present study on the basis of VEGF or Flt-1 expression, MVD, and selected pathoclinical

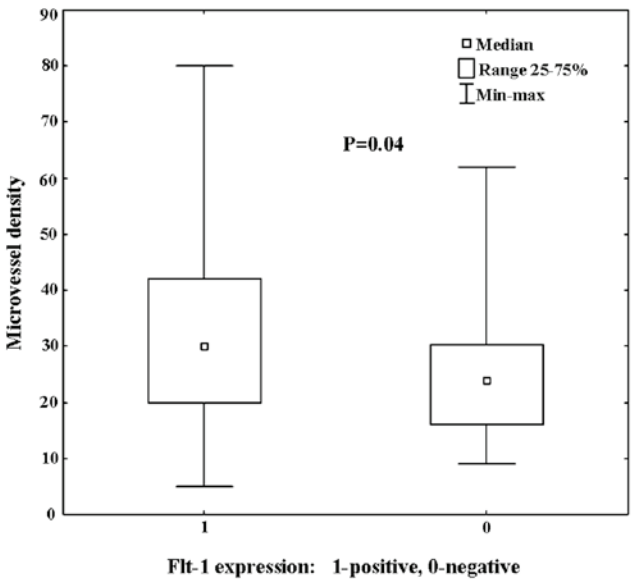

Figure 1. The association between Flt-1 expression and microvessel density in the tumors (n=139). Flt-1, Fms-like tyrosine kinase receptor.

parameters of colorectal cancer (Table III). The intensity of VEGF and Flt-1 expression, and of MVD in the tumor stroma did not correlate with the prognosis in patients with colorectal cancer, as measured by overall survival. Patients with colon cancer had a significantly better prognosis (P=0.03). In addition, patients with metastases in the regional lymph nodes (pN) and distant metastases (pM) had a significantly poorer prognosis (P=0.03 and P<0.01, respectively).

Discussion

Anti-angiogenic therapy has proven beneficial in the treatment of advanced colorectal cancer (18). However, in the present study, no significant correlations were identified between the pathoclinical parameters of colorectal cancer and stromal vascular density. Furthermore, no differences were identified between the vascular density in patients differing in their T, N and M classification, or in their tumor grade. Similarly, no differences were identified in vascular density in the primary tumor with respect to characteristics including age, gender, and tumor location.

Although our results showing no correlation between MVD and colorectal cancer parameters are similar to findings previously reported by British and Japanese researchers (19,20), to date, it has not been conclusively determined whether the MVD in the primary tumors of colorectal cancer affects the prognosis of the disease. Indeed, in esophageal cancer, Kitadai *et al* (21) reported a correlation between increased MVD in the primary tumor, which was assessed with anti-CD34, and poor prognosis and early local recurrence (21). Similarly, in gastric cancer, high vascular density correlated with a poorer prognosis in early (22) and in advanced (23) cancers of the stomach. However, in the case of colorectal cancer, no such conclusive assessments were drawn. Similarly to the present study, other reports have indicated that no correlation exists between MVD in the primary tumor, as assessed with anti-CD34, and patient prognosis (24,25). On the other hand, certain studies have demonstrated that the MVD in the primary tumor of colorectal cancer, which was also assessed using anti-CD34, correlates with a poorer prognosis (26,27). Previous reports have also demonstrated a positive effect of an increased MVD, which was determined using anti-CD31 and antibodies against the von Willebrand factor in the primary tumor of colorectal cancer on prognosis (28,29).

These discrepancies in the effects of MVD on colorectal cancer prognosis may be attributed to the various markers

Table III. Effect of vascular growth factors, microvessel density, and selected pathoclinical parameters of colorectal cancer on overall survival time in the study group (n=139).

Parameter	P-value[a]
Gender	ns
Female	
Male	
Age (median, 66 years)	ns
<Median	
≥Median	
Location	0.03
Colon	
Rectum	
pT feature	ns
T1 + T2	
T3 + T4	
pN feature	0.03
N0	
N+	
pM feature	<0.01
M0	
M+	
Stage	0.01
I + II	
III + IV	
Grade	ns
G1	
G2	
G3	
Flt1	ns
Positive expression	
No expression	
VEGF	ns
Overexpression	
No expression	
Microvessels/field of view	ns
<Median	
≥Median	

[a]P-values were determined using the logrank test. ns, not significant; VEGF, vascular endothelial growth factor; Flt1, Fmslike tyrosine kinase receptor.

that were used to identify the vascular endothelium. In the present study, the anti-CD34 antibody was used due to its high sensitivity and the reproducibility of the obtained results (30). However, anti-CD34 reacts with both active and inactive vascular endothelial cells, and therefore it is not a marker of endothelial cell proliferation. However, it may be used to detect endothelial cells that are 'trapped' in the tumor cells. Another commonly used antibody for determining the vascular density is an antibody raised against the von Willebrand factor (31). However, this antigen is not present in all endothelial cells, and it is also present on platelets. Finally, an appreciable number of studies have used anti-CD31 or anti-CD105 glycoprotein antibodies. The first antibody identifies endothelial cells, although it is also present on certain lineages of leukocytes, whereas the latter only reacts with activated endothelial cells and is therefore a marker associated with proliferation (32,33). Thus, the MVD measurements using these above-mentioned markers are subject to considerable risk of error, depending on the antibodies used. Additionally, the risk increases with the subjectivity of the method for evaluating the MVD (30).

The expression of two growth factors, VEGF and Flt-1, was studied, and, as anticipated, a positive correlation between Flt-1 expression and increased MVD in the tumor was demonstrated. There was also a clear trend for a similar association between VEGF expression and increased MVD. However, no correlation was identified between VEGF expression and the potential prognostic pathoclinical parameters describing patients with colorectal cancer. By contrast, the majority of reports concerning colorectal cancer have indicated that the overexpression of VEGF is associated with poor patient prognosis (34). Furthermore, this association between VEGF expression and poor prognosis was consistent for groups where only colon cancer was evaluated, as well as for those where patients with rectal and colon cancer were evaluated together. On the other hand, several reports have corroborated the observations presented in the current study that there is no link between VEGF overexpression and prognosis in colorectal cancer (34).

When interpreting the results in the present study, it should be kept in mind that VEGF was only assessed in the primary tumor. As the primary tumor is undergoing dynamic growth, there may be multiple modes of angiogenesis occurring. Indeed, tumors frequently use more than one strategy to acquire vessels, depending on the tumor stage and grade. Additionally, VEGF expression is not an easy parameter to determine. Generally, immunohistochemical methods using antibodies are used to identify VEGF, and its presence is measured by the intensity of a color reaction, which may also occur, for example, in damaged cells (35). However, there is no uniform, standardized method for assessing the level of VEGF expression. Indeed, the intensity of the color reaction as a measure of VEGF expression may be presented on different scales. In the present study, the analyses were simplified to a two-point scale, i.e., the lack of a reaction, or a weak positive reaction, indicated the lack of VEGF expression, whereas a strong positive reaction indicated that VEGF was overexpressed. Indeed, the prognostic value of VEGF assessment is a topic of debate due to the ambiguous, and often contradictory, research findings (36).

Tumor heterogeneity and inadequacies of the immunohistochemical methods employed may also have affected the assessment of Flt-1 expression and its correlation with the pathoclinical parameters of colorectal cancer reported in the present study. Although EMT occurs in the tumor itself and is involved with tumor cells, the formation of a pre-metastatic niche involving Flt-1 occurs in the place where, subsequently, metastasis from the original site will develop, and is formed by precursor cells migrating from the bone marrow. Therefore, the expression of Flt-1 should also be determined in cells of the liver, lungs and in other regions, as well as the primary tumor (37). Inflammatory cells expressing Flt-1 are similarly dispersed, which are conducive to immune tolerance to cancer. Thus, the large dispersion of cells expressing Flt-1 in the body, and the diverse involvement of Flt-1 in the process of tumorigenesis, indicate that its expression in the tumor alone does not reflect the actual role of Flt-1. In a group of 58 patients with colon cancer and 10 patients with colorectal adenoma, no differences in Flt-1 expression were identified between adenoma and carcinoma patients, or, as reported in the present study, between the particular tumor stages (38). It was hypothesized that Flt-1 expression in the primary tumor should favor metastasis, therefore leading to a poorer prognosis. However, in our study group, no such correlations were identified. Indeed, no correlation between Flt-1 expression and the markers of poor prognosis in patients (i.e., the presence of metastasis, or a shorter survival following surgery) were observed. On the other hand, in several publications associated with colorectal cancer, marked Flt-1 expression was a marker of poor prognosis in patients. In a study on 91 patients with colon and rectal cancer, high Flt-1 expression correlated with the shorter post-operative survival of patients with clinical stage II and III cancer (39). In another study of 140 patients with colon cancer, Flt-1 overexpression was predictive of early local recurrence (40). It is worth noting, however, that immunohistochemical methods are not perfect, and, as mentioned above, the diverse role of Flt-1 and its dispersion throughout the body may have led to an inadequate assessment of its pathoclinical role in colorectal cancer. Such inadequacies may explain the significant differences in the results achieved by various investigators.

In conclusion, in the present study an increased expression of VEGF and Flt-1 receptor was shown to be associated with increased MVD in the primary tumor in resectable colorectal cancer. However, neither the vascular density in the primary tumor, nor the expression of VEGF and Flt-1, correlated with potentially prognostic pathoclinical factors and overall survival in resectable colorectal cancer.

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