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ORIGINAL ARTICLE

The CD34-microvascular density in colorectal cancer patients

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Summary

Purpose: *To investigate the influence of the angiogenesis parameter CD34 microvascular density (MVD) on overall survival of colorectal cancer (CRC) patients.*

Methods: *Thirty-one CRC patients were followed-up for 72 months after curative colorectal operation. Blood vessels measurement was done using the CD34-MVD immunochemistry method, and light microscopy.*

Results: *MVD was inversely correlated with patients' survival. MVD value < 35 proved as independent good prognostic factor, and patients with this value lived during the 72-month follow up after surgery, while a MVD value > 65 was an independent poor prognostic factor and such patients died within 11 months after radical surgery for CRC ($p<0.01$).*

Conclusion: *According to these results, the CD34-MVD seems to be a significant prognosticator of overall survival in CRC patients.*

Key words: angiogenesis, CD34, colorectal cancer, microvascular density, survival

Introduction

Despite recent progress in our knowledge about the development and therapy of CRC, this disease still remains one of the major causes of cancer-related deaths around the world. The prognosis of patients with CRC is affected by various factors at the time of diagnosis, including stage, location of the tumor, gender, age and performance status. It is known that common prognostic factors do not fully predict individual clinical outcomes, especially among patients with TNM stage II and III disease [1]. Therefore, in order to improve clinical care and give an optimal treatment, efforts are being made to identify and study new biological prognostic factors.

Angiogenesis plays an important role in tumor genesis, progression and metastasis. MVD has become the morphological gold standard to assess angiogenesis in human tumors [2]. In the past, there have been various studies on the relationship between vascular density and some tumor parameters such as tumor size, bulky tumor mass, patient survival and metastasis, particularly in tumors of the breast and prostate, melanoma, and large-cell lung carcinoma. In most cases, a direct correlation between MVD and metastasis, and an inverse relationship between MVD and patient survival has been reported [3].

CD34 is a surface glycophosphoprotein expressed on the early lymphohematopoietic stem cells, as well as in cells during early hematopoiesis and on the endothelial cells of small vessels and embryonic fibroblasts [4]. Today, it is widely used for hematopoietic stem cell purification and as a marker of most vascular endothelial cells, including those of capillaries in the majority of normal and neoplastic tissues.

Having in mind the clinical importance of neovascularization in cancer development and metastasis, we investigated the relationship between MVD, as determined by the CD34 marker, and overall survival in CRC patients.

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Methods

This retrospective study included 31 patients diagnosed with CRC and operated with curative intend at the Clinical Hospital Centre of Zemun, Serbia, from 1998 to 2002. The data used for this study were retrieved from the patients' anamnesis, diagnostic results, operative lists and pathology findings.

Dukes classification was used for stage determination.

Inclusion criteria

Included were patients of both genders, aged over 45 years with histologically confirmed CRC, without chemotherapy or radiotherapy before or after the operation, those with R0 resection, and with at least 12 lymph nodes in the resected material.

Exclusion criteria

These included patients suffering simultaneously from CRC and other malignancy (-ies), presence of severe chronic systemic diseases, with hereditary CRC, with history of previous malignancy or previous chemotherapy or radiotherapy. Patients who died immediately after the operation were not included in this study.

The patients were postoperatively followed up for 6 years (72 months).

Tissue preparation and analyses

Tissue samples from the primary tumor were fixed in 10% neutral formalin and embedded in paraffin. A representative portion of a tumor was selected for processing. Five-micron thick sections of tissue were deparaffinized, hydrated, and placed in citrate buffer (pH 6). Samples were then treated with hydrogen peroxide and by pressure boiling in citrate.

The anti-CD34 monoclonal antibody (DAKO-Denmark) was used as primary antibody and the reaction was visualized using the streptavidin/biotin method. The histological sections, immunostained with CD34, were studied using a light microscope at ×400 magnification in order to locate the tumor areas that contained greater concentration of blood vessels (hot spots). For each patient 3 high power fields (3/HPF ×400) views were studied using Leika microscope (Germany).

MVD was expressed as the mean count of CD34 immunostained vessels in each case.

Counting of CD34-MVD positive blood vessels was based on the following criteria:

1) Any CD34 positive endothelial cell cluster clearly separated from each other was considered as single countable microvessel.
2) A lumen was not required to identify a vessel.
3) Larger vessels with muscular walls were excluded from counting.
4) Other cell types that may be CD34 positive were not taken into account.

The study was approved by the Ethical Committee of the Clinical Hospital Centre Zemun, Serbia.

Statistical analyses

Descriptive statistics of tumor characteristics included tumor (T) determination, (T1/submucosa, T2/muscularis propria, T3/deeper than muscularis propria and T4/serosa or adjacent organs), lymph node involvement (N), Dukes stage, and MVD determination.

Statistical analyses were carried out using parametric and nonparametric tests: log rank test was used for the analysis of statistical difference between groups with high and low CD34-MVD count values; Wilcoxon test, Mann-Whitney U test were used to assess the independent characteristics of the groups within the same group of CRC patients depending on CD34-MVD values; and Spearman's and Pearson's (parametric and nonparametric) tests were used for analysis of correlation between CD34-MVD and overall survival. The Kaplan-Meier method was used to generate survival curves.

All statistical tests were carried out using the SPSS software, version 15.

Results

Included were 15 male and 16 female patients. The median number of the removed lymph nodes was 29 (range 12-35). The mean number of vessels per hot spot was 46.48 ± 18.95, ranging from 23 to 75.

Detailed patient characteristics are shown in Table 1 and patient survival depending on MVD count in Table 2. Figure 1 shows CD34-MVD values in relation to survival. From Table 1 and Figure 1 it is clear that patients with higher CD34-MVD values had worse prognosis and shorter survival ($p<0.01$).

Comparing the MVD values in the group of patients that survived during the 72-month follow up af-

Table 1. Patient characteristics

Characteristics	*N*	*%*
Gender		
Male	15	48.38
Female	16	51.62
Dukes stage		
A	7	22.58
B	9	29.03
C	12	38.70
D	3	01.09
Lymph node involvement		
N0	18	58.01
N1	4	01.12
N2	9	29.19
T stage		
1	–	–
2	12	38.71
3	16	51.61
Unknown	3	9.68
Grade		
1	8	25.80
2	12	38.70
3	11	35.50
Primary tumor localization		
Caecum	2	6.45
Ascending	2	6.45
Transverse	3	9.68
Descending	3	9.68
Sigmoid	10	32.24
Rectum	11	35.50

Table 2. CD34-MVD count in respect to patient survival

Survival (months)	*0-11*	*12-24*	*36*	*37-60*	*61-72*
CD34-MVD count	65-75	56-59	45	36-39	23-34
Number of patients (%)	10 (32.3)	3 (9.7)	1 (3.2)	3 (9.7)	14 (45.1)

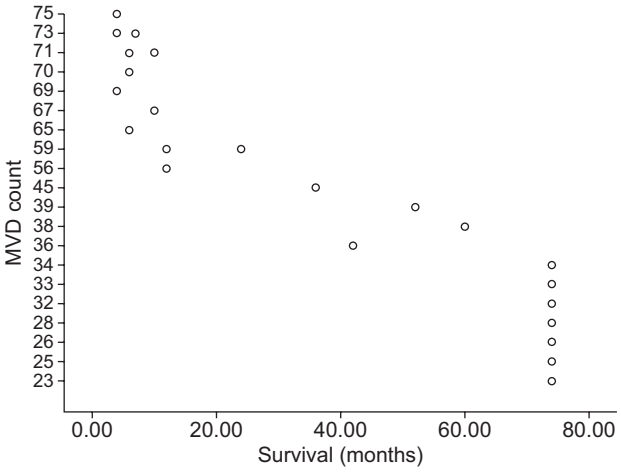

Figure 1. MVD values with respect to colon cancer patient survival followed up to 72 months.

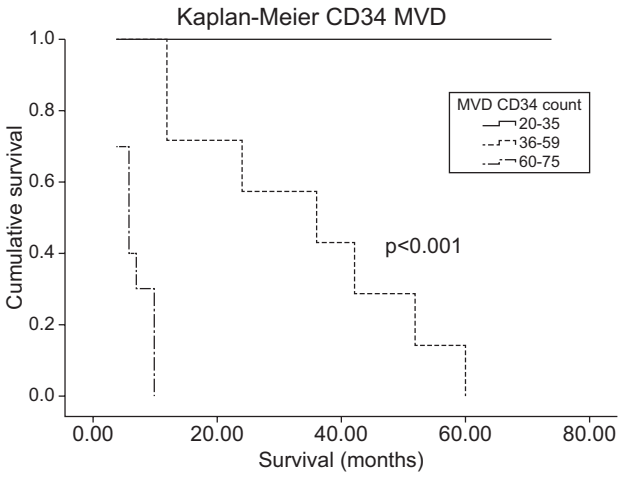

Figure 2. Kaplan-Meier curve in colorectal cancer patients depending on MVD CD34 count during the 72-month follow up.

ter surgery, with the patients who died during follow up, high statistical difference was found (Wilcoxon test, Mann-Whitney U test, $p<0.01$). Correlating all CRC patient survival with increasing CD34-MVD values, significant negative correlation was obtained (2-tailed Spearman's test, $p<0.001$; Figure 2).

In addition, patients that survived during the 72-month follow up had MVD value < 35 (MVD= 29.14±6.1), significantly different from those with MVD value > 65 and with survival time up to 11 months (MVD=70.10 ± 5.0; log rank, $p<0.0001$).

Figure 3 shows photomicrographs of representative hematoxylin-eosin stained (3A) and CD34-MCV immunostained (3B, C, D) sections from several patients.

Discussion

For many years, clinical researchers have tried to identify biological markers that could predict tumor growth, resistance to therapy and recurrence. MVD is

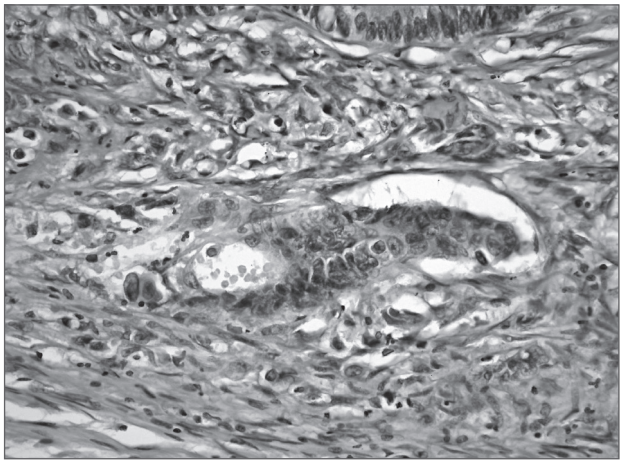

Figure 3A. Colon cancer tissue showing malignant cells invading blood vessels and also clusters of single to few cancer cells (H&E ×400).

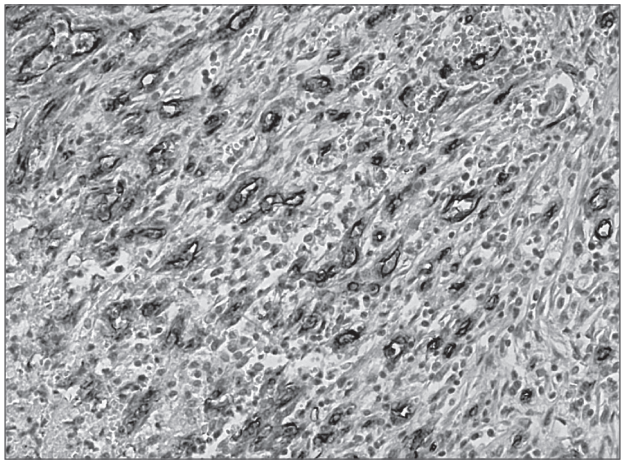

Figure 3B. CD34 immunostained tissue section showing high expression of CD34 in a patient with Dukes C stage (×400).

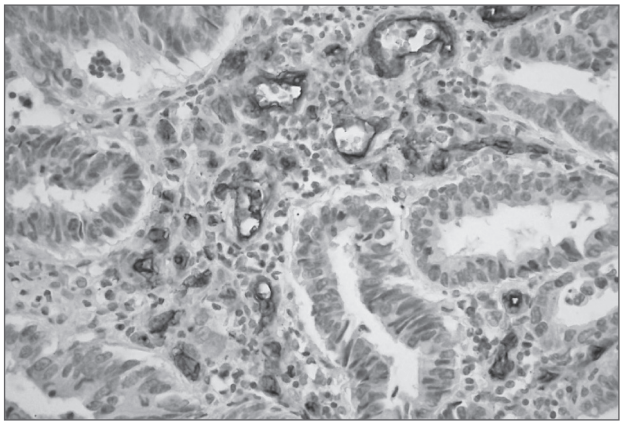

Figure 3C. CD34 immunostained tissue section in a patient with Dukes D stage. Dense area with complete destruction of tissue architecture from colon cancer tissue (×400).

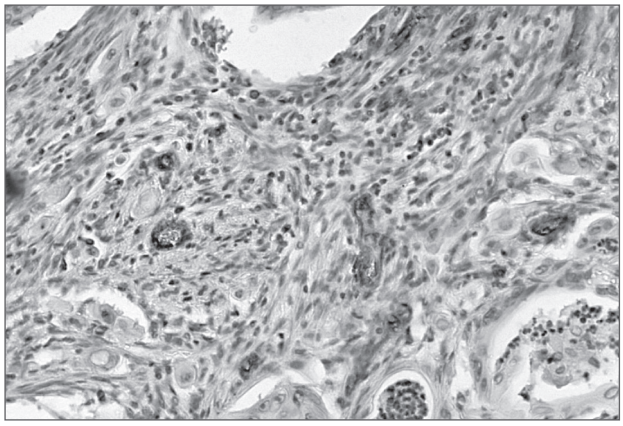

Figure 3D. CD34 immunostained tissue in a patient with CRC that survived 72 months after radical surgery. Image shows small number of blood vessels (×400).

suggested to be a possible prognostic factor in CRC patients [5]. In addition, various methods for the quantification of MVD have been described in the past [5].

In our study, immunohistological staining and determination of CD34 molecule as a marker of neovascularization was used, because we thought it was a reproducible, accurate and cost-effective method for MVD assessment. Some authors postulated that differences in the reported data may be due to the different MVD quantification methods employed, variable immunostaining techniques, and different types of tissue preservation [6]. Our research indicated an association between higher MVD with more aggressive tumor behavior and poorer clinical outcome.

However, some other reports gave conflicting results [7-15]. On the other hand, it has been suggested that in both experimental and clinical models, the increase of MVD, by favoring drug access, could be considered as a predictor of response to chemotherapy and can therefore be associated with even better prognosis. However, there are no definite conclusions of the clinical relevance of MVD in patients with operable CRC treated with anticancer drugs in the adjuvant setting. Also, some authors suggested that there was a probability that a higher MVD induces a greater immune response that could slow tumor growth and dissemination, thus making the MVD a positive prognostic factor.

Our opinion is that, in the future, MVD count could be a very important prognostic factor in CRC patients, especially considering the well-known relationship between tumor angiogenesis and the probability of metastasis. As for the findings that stress the impact of high MVD on chemotherapeutic drug access in cancer tissue, one can speculate that metastasis is a much more important factor that affects prognosis than drug access and/or local immune response. It may be true that a higher degree of neovascularization in a tumor has a large impact on drug availability at the tumor site; however, it may also mean that the tumor would have better access to nutrients from the blood, thus making the tumor tissue more aggressive. Since metastatic disease is the main factor affecting patients' survival we assume that drug access and local immune response are only sporadic factors that cannot overcome the negative impact of high probability of tumor dissemination on prognosis. Our study included 31 CRC cases, and although many previous studies have been carried out on even smaller number of patients, we think that a bigger and even more homogeneous sample might be needed to draw definite conclusions about the prognostic value of MVD.

Altogether, our results suggest that CD34-MVD can be used as a prognostic marker in CRC patients. The CD34-MVD count value below 35 measured by immunohistochemistry can be considered as good prognostic factor for CRC patients. CD34-MVD count value over 65 is a negative prognostic factor, and such patients die within 11 months after radical surgery.

Acknowledgments

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