Aim of the study: Evaluation of the relationships between increased expression of VEGF-C (vascular endothelial growth factor-C) and vessel density in the tumour-surrounding stroma, patient survival, and other conventional prognostic factors in patients with pT3-4 colon cancer.

Material and methods: Expression of VEGF-C and vessel density were immunohistochemically assessed in 104 specimens of primary, locally advanced (pT3-4) colon adenocarcinoma after surgical resection.

Results: A significant relationship was found between the expression of VEGF-C and increased vessel density in the tumour-surrounding stroma (p = 0.03). A relationship between VEGF-C expression and location of the tumour in the left side of the colon was also found (p = 0.003). Expression of VEGF-C was likely to occur in well-differentiated tumours. No relationship between patient overall survival and the expression level of VEGF-C in locally advanced colon cancer was observed.

Conclusions: The study results indicate that expression of VEGF-C in cells of locally advanced pT3-4 adenocarcinoma of the colon does not affect the survival time of the patients. Increased expression of VEGF-C is accompanied by a significant increase in vessel density in the pT3-4 tumour stroma. Increased expression of VEGF-C in cancer cells is related to the tumour location in the left side of the colon and better tumour differentiation.

Key words: VEGF-C, vessel density, survival, colon cancer, angiogenesis, lymphangiogenesis.

VEGF-C expression is not a prognostic factor in locally advanced colon adenocarcinoma

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Introduction

One of the most important factors affecting the long-term outcome of colon cancer treatment is the severity of the disease at the time of diagnosis [1]. Improving the treatment results of advanced cases has become a challenge for contemporary medicine.

The discovery of the mediator of angiogenesis, which is VEGF (vascular endothelial growth factor), threw a new light on the biology of neoplastic disease and created the opportunity for antiangiogenic treatment [2].

The VEGF subfamily is made of the following: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and a placenta growth factor – PlGF. Their action is exerted through the following glycoprotein receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4) [3]. Put simply, the ultimate effect of ligand connection on the receptor is the proliferation and migration of arterial, venous, and lymphatic endothelial cells, which result in the formation of new microvessels [4]. VEGF-C, secreted by neoplastic cells, being capable of binding and activating VEGFR-3 and VEGFR-2 receptors, is involved in both angiogenesis and lymphangiogenesis processes. By its activity, VEGF-C increases microvessel density in the tumour stroma [5]. How this process affects the prognosis of patients with resectable invasive colon cancer, penetrating through the colon wall (pT3-4) was the aim of this study.

Material and methods

Paraffin-embedded primary tumour specimens obtained from 104 consecutive patients who underwent surgery for pT3 and pT4 colon cancer (between January 1, 2003 and December 31, 2008) were included in the study. In each case, the local range of the primary tumour resection met the criteria for the radical procedure, regardless of the cancer stage and grade. None of the patients received any neoadjuvant therapy. Patients with synchronous and metachronous colorectal carcinoma or with cancers in other organs were excluded from the study. Local Ethics Committee approval was obtained for the study (NKEBN/142/2008). Women comprised 54.8% (n = 57) of study participants and men 45.2% (n = 47). Participants ranged in age from 32 to 90 years (mean 69.7 years; median 70.5 years). Minimum follow-up period was 66 months. All deaths during the observation period were caused by malignancy. The mean survival time was 57 months, with a median of 68 months.

Table I presents patients' clinical data. The recorded age refers to the time of the surgery. Histological categorisation of the tumour was based on the WHO classification system [6]. All cases included in the study were diag-

nosed as adenocarcinoma. An adenocarcinoma mucinosum was considered poorly differentiated (G3). The colon was divided into the right (R) and left (L) sides. The right side included the caecum, ascending colon, hepatic flexure, and the proximal two-thirds of the transverse colon. The left side included the distal third of the transverse colon, splenic flexure, descending colon, and sigmoid colon. Tumours were classified according to the pTNM system [7].

Immunohistochemistry

Immunohistochemical staining was performed on tissue sections fixed with 4% formaldehyde and embedded in low melting point paraffin. The paraffin-embedded tissue blocks were cut into 5-µm thick sections with a sledge microtome and transferred to silane-coated slides.

The immunochemical staining was performed in 104 resected specimens of colon tumours and in 10 normal colon mucosa samples as a control. After deparaffinisation and rehydration with distilled water, the sections were subjected to heat-induced antigen retrieval with a citrate buffer (pH 6.0) at 99°C for 40 minutes in a water bath. Endogenous peroxidase was then blocked by 3% hydrogen peroxide for 10 minutes.

The following primary antibodies were used:
- CD34 – Monoclonal Mouse Anti-Human (Dako, cat. no. M 7165); antibody dilution 1 : 50; incubation at room temperature for 30 minutes; detection for 30 minutes using the EnVision system (Dako, cat. no. K 4011);
- VEGF-C (C-20) – Goat Polyclonal IgG (Santa Cruz Biotechnology, cat. no. 1881); antibody dilution 1 : 200; incubation overnight at 4°C (in refrigerator); detection for 30 minutes using the LSAB system (Dako, cat. no. K 0690).

The sections were visualised by incubation with 3,3'-diaminobenzidine (DAB) for 10 minutes at room temperature then counterstained with Mayer's haematoxylin and mounted with Canada balsam. Between particular stages of the procedure, the sections were rinsed with phosphate buffered saline (PBS) twice for 5 minutes. Microscopic examination was performed using an Olympus CX41.

Cytoplasmic expression of VEGF-C in tumour cells was scored on a scale from 0 to 3, according to the intensity of the staining: no staining (0), weak staining (1+), moderate staining (2+), and strong staining (3+).

In addition, the percentage of cytoplasmic staining of the tumour cells was scored on a scale of 0 to 5: 0% stained (0), 0% > 1% stained (1), 1% > 10% stained (2), 10% > 33% stained (3), 33% > 66% stained (4), and greater than 66% stained (5).

For overall expression of VEGF-C, a total score (TS) was calculated by combining staining intensity (a) and proportion of positively stained cells (b), as follows:

$$TS = \frac{a + b}{8}$$

The equation was based on the scoring system developed and used by Allred et al. for the evaluation of oestrogen and progesterone receptor expression in breast carcinoma [8].

If TS was 5/8 or greater, the expression was considered positive for VEGF-C.

Vascular density was assessed by utilising CD34 monoclonal antibodies according to the method previously described by Weidner et al. [9].

Statistical analysis

Analysis was carried out using the statistical software package STATISTICA (data analysis software system) version 10. www.statsoft.com. StatSoft, Inc. (2011). The Mann-Whitney U test was used to evaluate the relationship between vessel density in the tumour-surrounding stroma and the expression of VEGF-C. The correlation between the expression of VEGF-C and clinicopathological characteristics was assessed using Pearson's Chi-square test. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare differences between survival times in groups. In multivariate analysis, the Cox proportional hazard regression model was used. The statistical significance level was set at $p < 0.05$.

Results

Positive expression of VEGF-C was found in 55 (53%) colon cancers. In 49 (47%) tumours, VEGF-C expression was qualified as negative. Mean microvessel density in a single field evaluated with the method of Weidner et al was 48.5, median 42. A significant relationship between VEGF-C expression and microvessel density in the tumour stroma was demonstrated ($p = 0.03$, U Mann-Whitney test). Table 1 presents the relationships between VEGF-C expression and clinicopathological parameters in the analysed material. Poorly differentiated and mucinous cancers (G3) were

Table 1. Clinicopathological characteristics of 104 patients with colon adenocarcinoma. Relationship between expression of VEGF-C and clinicopathological parameters (Pearson's χ^2 test)

Parameter		No. of patients	VEGF-C (+) vs. (–) p value
Median age – 70.5 years	> 70.5	52	NS
	≤ 70.5	52	
Gender	Female	57	NS
	Male	47	
Tumour localisation in colon	Right-sided	53	0.003
	Left-sided	51	
Histological grade of tumour	G1 + G2	88	0.01
	G3	16	
Spread of primary tumour (T)	T3	91	NS
	T4	13	
Regional lymph nodes (N)	N (–)	60	NS
	N (+)	44	
Distant metastasis (M)	M (–)	88	NS
	M (+)	16	
TNM disease stage	II	56	NS
	III	32	
	IV	16	

NS – not significant

Table 2. The influence of selected clinicopathological parameters on five-year survival

Parameter		Five-year survival probability	log-rank *p* value
Expression VEGF-C	(+)	0.62	NS
	(–)	0.50	
Gender	Female	0.65	NS
	Male	0.47	
Tumour localisation in colon	Right-sided	0.55	NS
	Left-sided	0.59	
Histological grade of tumour	G1 + G2	0.56	NS
	G3	0.56	
Spread of primary tumour (T)	T3	0.61	0.02
	T4	0.31	
Regional lymph nodes (N)	N (–)	0.72	0.0002
	N (+)	0.36	
Distant metastasis (M)	M (–)	0.66	< 0.0001
	M (+)	0.06	
TNM disease stage	II	0.77	0.00001
	III + IV	0.33	

NS – not significant

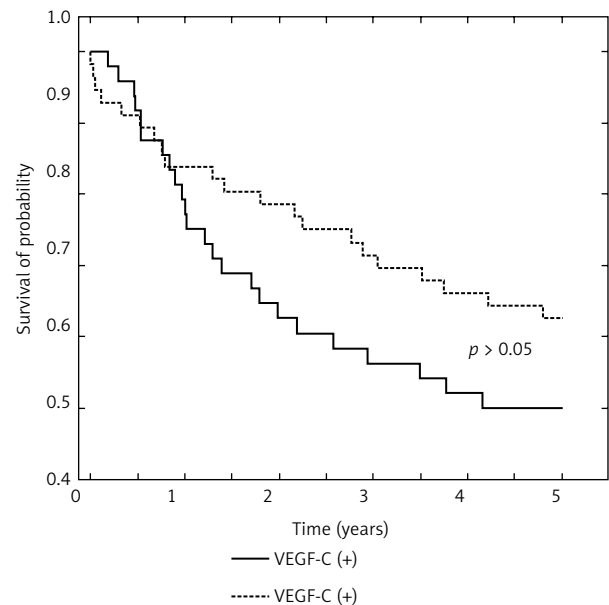**Fig. 1.** Kaplan-Meier 5-year survival curves for VEGF-C (+) and VEGF-C (–) ($p = 0.1$, log-rank test)

located in the right side of the colon in 87% of cases (R vs. L, $p = 0.001$). The results of univariate analysis of survival with respect to chosen clinicopathological parameters are shown in Table 2. VEGF-C expression was not significantly related to five-year survival ($p > 0.05$, Fig. 1). According to Cox multivariate analysis, the presence of distant metastases was the factor independently related to five-year survival ($p = 0.008$, 95% CI: 0.28–1.92, HR 3.02).

Discussion

Immunohistochemical evaluation of VEGF-C expression in tumour cells can be made by either determination of the rate of cells with observed positive reaction or by combination of the positive reaction cells rate and the evaluation of the reaction intensity. The latter, used in cases of colon cancer, indicates 47–68% VEGF-C positive tumours [10–12].

A similar rate of positive VEGF-C reaction was observed in the study concerning oesophageal cancer (51%), where positive reaction in at least 30% of tumour cells was considered as a criterion of positive expression [13]. We accepted similar rules of evaluation of VEGF-C expression in our study, specifying intensity and rate of cells with the presence of the reaction. Hypothetically, increased VEGF-C expression should be accompanied by an increase of microvessel density in the tumour stroma [4, 5]. One of the most common means of microvessel density evaluation is the method described by Weidner *et al.* [9]. This method was also used in our study, and CD34 was applied as a marker of endothelium. A significant relationship between strong VEGF-C expression and increased microvessel density in the pT3-4 tumour stroma was obtained.

Microvessel density in the tumour stroma with positive VEGF-C expression was significantly higher than in cases without such expression ($p = 0.03$, *U* Mann-Whitney test).

Similar results of the relationship between VEGF-C expression and microvessel density in colon cancer stroma with use of CD34 as an endothelial marker have been observed by other authors [14, 15].

The present study reveals significantly higher VEGF-C expression in pT3-4 left-sided (0.003) and in well and moderately differentiated cancers ($p = 0.01$). At the same time pT3-4 poorly differentiated and mucinous tumours were found significantly more frequently in the right side of the colon. Similar results were obtained in another study evaluating simultaneous expression of VEGF-C and VEGF-D in colon pT1-4 cancer cells [16]. Differences in clinical parameters, histological differentiation, and prognosis in cancers located in the left and right sides of the colon are discussed in many papers. It is recognised that right-sided colon tumours are associated with worse prognosis, and they are more frequently poorly differentiated [17, 18].

This is explained by the diversities in the process of carcinogenesis of tumours located in the left and right sides of the colon. Probably different genes are responsible for the genesis of cancers in the right side of the colon because phylogenetically different genes are engaged in right colon development [19]. Right colon tumours grow asymptomatically for a long time, and as they progress the rate of poorly differentiated cancers increases [20, 21]. Inhibition of the differentiation process as a consequence of accumulation of mutations leads to changes in the biological profile of the tumour, which may be expressed as a decrease in expression of some factors secreted by the tumour, such as VEGF-C.

This approach to colon cancer biology justifies the results of survival analysis (Fig. 1), which show better prognosis for pT3-4 tumours with present VEGF-C expression (left-sided, moderately and well differentiated tumours). In a previously published paper, we analysed simultane-

ous expression of lymphangiogenic factors (VEGF-C and VEGF-D) in colon pT1-4 cancer cells [16]. Both VEGF-C and VEGF-D are involved in the formation of lymphatic microvessels, and through the capability of VEGFR-2 receptor binding they also mediate neovascularisation [5].

A primary role in lymphangiogenesis is assigned to VEGF-C. VEGF-D is only to support the process as a strong activator. That is why in the literature we can find more publications concerning VEGF-C and its relationships to potential prognostic factors, time to relapse, or overall survival [22]. However, the findings are inconsistent. Hu et al. analysed 69 cases of colorectal adenocarcinoma (33 rectal cancers) and demonstrated a significant relationship between high VEGF-C expression and shorter overall survival time [23]. Similar relationships were demonstrated by Soumaoro *et al.*; however, the authors did not confirm that VEGF-C expression was an independent prognostic factor [12]. Jin *et al.* analysed 68 colorectal cancers, revealing a significant relationship between VEGF-C expression and five-year survival. The probability of five-year survival was significantly lower for patients with positive VEGF-C expression [24]. Conversely, the analysis of VEGF-C expression and of the relationship between the expression and prognosis performed by Akagi *et al.* revealed no relationship between VEGF-C expression and long-term results of treatment [10].

Moreira *et al.* analysed VEGF-C expression in 60 specimens of colorectal tumours. The authors did not demonstrate relationships between the expression and cancer potentially prognostic factors or overall survival [25]. Similarly, in another study performed on over 350 colon cancers, relationships between VEGF-C expression and overall survival, also determined separately for particular TNM stages, were not demonstrated [26].

Taking into account both the superior role of VEGF-C in the process of lymphatic microvessel formation and its role in the angiogenesis, we decided to reanalyse our material with regard to locally advanced (pT3-4) and only colon tumours in patients with a longer time of follow-up (median 85 months vs. 41 months).

The authors of numerous studies concerning VEGF-C expression in large intestine cancer often combine colon and rectal cancer together, and the groups are relatively small [23, 24]. As clinicians, we perceive significant differences between these cancers, including ways of treatment and prognosis [1, 27]. That is why only primary colon tumours were included in the current analysis. We assumed that increasing depth of invasion is associated with increasing risk of metastases and worse prognosis. Together with a longer follow-up period this might increase the chances of revealing a potential relationship between VEGF-C expression and prognosis, if such a relationship exists.

We confirmed a clear dependence of well-recognised prognostic factors such as depth of invasion, presence of lymphatic, or distant metastases and pTNM stage on patient prognosis in the analysed material. There was no relationship between VEGF expression in colon cancer cells and overall survival. VEGF-C expression was not associated with lymph nodes status or the presence of distant metastases.

Our findings suggest no prognostic utility of VEGF-C expression evaluation in pT3-4 primary colon cancer.

In conclusion, VEGF-C expression in locally advanced (pT3-4) colon cancer cells does not affect patients' overall survival time. Intense VEGF-C expression is associated with a significant increase in microvessel density in the tumour stroma. Localisation in the left side of the colon and a higher histological grade of the tumour are associated with increased VEGF-C expression in cancer cells.

The authors declare no conflict of interest.

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