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.

Original paper

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

Mariusz Szażewski1,2, Wiesław Janusz Kruszewski1,2, Joanna Lakomy1, Maciej Ciesielski1,2, Krzysztof Kawecki1, Jarosław Szefel1,2

1Department of Surgical Oncology, Gdynia Oncology Centre, PCK’s Maritime Hospital in Gdynia, Poland
2Department of Propaedeutic of Oncology, Faculty of Health Sciences, Medical University of Gdansk, Poland
3Department of Pathology, Medical University of Gdansk, Poland

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 mucinoma 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. 18811); 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 of 0 to 5: 0% 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:

\[ \text{TS} = \frac{a + b}{8} \]

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

| 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 1. Clinicopathological characteristics of 104 patients with colon adenocarcinoma. Relationship between expression of VEGF-C and clinicopathological parameters (Pearson’s χ² 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
carcinoma, such as VEGF-C.

In a previously published paper, we analysed simultane-
ously for pT3-4 tumours with present VEGF-C expression
asymptomatically for a long time, and as they progress
evolution as a consequence of accumulation of mutations leads to changes in the bi-
ological profile of the tumour, which may be expressed as a
decrease in expression of some factors secreted by the
tumour, such as VEGF-C.

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

| Parameter                      | Expression VEGF-C | Five-year survival probability | log-rank p value |
|-------------------------------|-------------------|-------------------------------|-----------------|
| Expression VEGF-C             | (+)               | 0.62                          | 0.1             |
|                               | (–)               | 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

Similar results of the relationship between VEGF-C expres-
sion 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 moder-
ately 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 evalu-
ating simultaneous expression of VEGF-C and VEGF-D
in colon pT1-4 cancer cells [16]. Differences in clinical pa-
rameters, 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 respon-
sible 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
symptomatically 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 bi-
ological 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 re-
sults of survival analysis (Fig. 1), which show better prog-
nosis 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 binding they also mediate neovascularisation [5].

The authors declare no conflict of interest.

References

1. Cutsem EV, Oliveira J on behalf of the ESMO Guidelines Working Group: Primary colon cancer: ESMO clinical recommendations for diagnosis, adjuvant treatment and follow-up. Ann Oncol 2009; 20 Suppl. 4: 49-50.

2. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol 2002; 20: 4368-80.

3. Shibuya M. Structure and function of VEGF/VEGF-receptor system involved in angiogenesis. Cell Struct Funct 2001; 26: 25-35.

4. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003; 9: 669-76.

5. Jussila L, Altitalo K. Vascular growth factors and lymphangiogenesis. Physiol Rev 2002; 82: 673-700.

6. Hamilton SR, Aaltonen LA. Pathology and genetics of tumours of the digestive system. IARC Press, Lyon 2000.

7. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC Cancer Staging Handbook 7th edition. Springer. New York 2010.

8. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol 1998; 11: 155-68.

9. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol1993; 143: 401-9.

10. Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. Br J Cancer 2000; 83: 887-91.

11. Onogawa S, Kitadai Y, Tanaka S, Kuwai T, Kimura S, Chayama K. Expression of VEGF-C and VEGF-D at the invasive edge correlates with lymph node metastasis and prognosis of patients with colorectal cancer. Cancer Sci 2004; 95: 52-9.

12. Soumaoro LT, Uetake H, Takagi Y, Iida S, Higuchi T, Yasuno M, Enomoto M, Sugihara K. Coexpression of VEGF-C and Cox-2 in human colorectal cancer and its association with lymph node metastasis. Dis Colon Rectum 2006; 49: 392-98.

13. Matsumoto M, Natsugoe S, Okumura H, et al. Overexpression of vascular endothelial growth factor-C correlates with lymph node micrometastasis in submucosal esophageal cancer. J Gastrointest Surg 2006; 10: 1016-22.

14. Furudoi A, Tanaka S, Haruma K, Kitadai Y, Yoshihara M, Chayama K, Shimamoto F. Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. Oncology 2002; 62: 157-66.

15. Kaio E, Tanaka S, Kitadai Y, Sumii M, Yoshihara M, Haruma K, Chayama K. Clinical significance of angiogenic factor expression at the deepest invasive site of advanced colorectal carcinoma. Oncology 2003; 64: 61-73.

16. SzaJewski M, Kruszewski WJ, Lakomy J, Ciesielski M, Kawecki K, Jankun J, Buzek T, Szelfi J. VEGF-C and VEGF-D overexpression is more common in left-sided and well-differentiated colon adenocarcinoma. Oncol Rep 2014; 31: 125-30.

17. Benedix F, Kube R, Meyer F, Schmidt U, Gastinger I, Lippert H. Comparison of 17,641 patients with right- and left-sided colon cancer:
differences in epidemiology, perioperative course, histology, and survival. Dis Colon Rectum 2010; 53: 57-64.

18. Hansen IO, Jess P. Possible better long-term survival in left versus right-sided colon cancer – a systematic review. Dan Med J 2012; 59: A4446.

19. Nawa T, Kato J, Kawamoto H, Okada H, Yamamoto H, Kohno H, Endo H, Shiratori Y. Differences between right- and left-sided colon cancer in patient characteristics, cancer morphology and histology. J Gastroenterol Hepatol 2008; 23: 418-23.

20. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988; 319: 525-32.

21. Kanazawa T, Watanabe T, Kazama S, Tada T, Koketsu S, Nagawa H. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. Int J Cancer 2002; 102: 225-9.

22. Rindknecht M, Detmar M. Molecular mechanisms of lymph node metastasis. In: Lymphangiogenesis in cancer metastasis. Stacer SA, Achen MG (eds). Springer, New York 2009; 55-82.

23. Hu WG, Li JW, Feng B, et al. Vascular endothelial growth factors C and D represent novel prognostic markers in colorectal carcinoma using quantitative image analysis. Eur Surg Res 2007; 39: 229-38.

24. Jin C, Wang A, Chen J, Liu X, Wang G. Relationship between expression and prognostic ability of PTEN, STAT3 and VEGF-C in colorectal cancer. Exp Ther Med 2012; 4: 633-39.

25. Moreira LR, Schenka AA, Latuf-Filho P, Penná AL, Lima CS, Soares FA, Trevisan MA, Vassallo J. Immunohistochemical analysis of vascular density and area in colorectal carcinoma using different markers and comparison with clinicopathologic prognostic factors. Tumor Biol 2011; 32: 527-34.

26. Martins SF, Garcia EA, Mendes Luz MA, Pardal F, Rodrigues M, Longatto Filho A. Clinicopathologic Correlation and Prognostic Significance of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 Expression in Colorectal Cancer. Cancer Genomics Proteomics 2013; 10: 55-67.

27. Glimelius B, Tiret E, Cervantes A, Arnold D on behalf of the ESMO Guidelines Working Group: Rectal cancer: ESMO Clinical Recommendations for diagnosis, treatment and follow-up. Ann Oncol 2013; 24 Suppl. 6: 81-88.

Address for correspondence
Mariusz Szajewski MD, PhD
Department of Surgical Oncology
Gdynia Oncology Centre
PCK’s Maritime Hospital in Gdynia
Powstania Styczniowego 1
81-519 Gdynia, Poland
e-mail: mszaj@gumed.edu.pl

Submitted: 13.05.2015
Accepted: 19.11.2015