VEGF-1 Expression in Colorectal Cancer is Associated with Disease Localization, Stage, and Long-term Disease-specific Survival

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Abstract. *Background: Angiogenesis plays an important role in progression of colorectal carcinoma (CRC). Evidence from preclinical and clinical studies indicates that vascular endothelial growth factor (VEGF) is the predominant angiogenic factor in CRC. Indeed, VEGF is expressed in approximately 50% of CRCs, with minimal to no expression in normal colonic mucosa and adenomas. In this study, the expression of VEGF-1 was examined in stage I-IV CRCs to determine its clinicopathological correlates, and association with the response to treatment and disease outcome. Patients and Methods: The present series consisted of tissue samples obtained from 360 patients with stage I, II, III, or IV CRC who had undergone large bowel resection during 1981-1990 at Turku University Hospital. Archival paraffin-embedded CRC tissue samples were used to build up tissue microarray (TMA) blocks and VEGF-1 expression was assessed immunohistochemically using an automated staining system. Three different grading systems were applied to evaluate the expression of VEGF-1. Results: Seventy percent of patients with stage IV CRC had positive VEGF-1 expression, while 50% and 47%, respectively of patients with stage II and III CRC had positive VEGF-1 expression (p=0.005). VEGF-1 expression in the left colon and rectum was significantly higher than that in the right colon (61% vs. 45%, respectively) (p=0.006). Significant statistical correlation (p=0.04) was found between VEGF-1 and 10-year disease-specific survival: patients who died of the disease more frequently had a VEGF-1-expressing tumour than did those who survived for 10 years. Conclusion: Quantification of VEGF-1 expression seems to provide valuable prognostic information in CRC, particularly in selecting those patients at high risk for disease progression who are likely to benefit from adjuvant therapy.*

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Despite the fact that most colorectal cancer (CRC) patients undergo potentially curative surgery and despite the availability of adjuvant chemotherapy, approximately 50% of all patients initially thought to be cured by surgery subsequently relapse and die of their disease (1). Advanced CRC is defined as a disease that is either metastatic or locally advanced and for which surgical resection is unlikely to be curative (2). Once metastasis has occurred, the prognosis is considerably worse, with the 5-year survival rate for advanced CRC being less than 5% (2). For the majority of patients, chemotherapy can improve survival and is the main mode of treatment (3).

The awareness that the growth and spread of tumours are dependent on angiogenesis (4) has opened new avenues for research designed to help us better understand cancer biology and facilitate the development of new therapeutic strategies. The process of angiogenesis consists of multiple, sequential and interdependent steps with several positive and negative regulators of angiogenesis being involved (4). Neoangiogenesis, the formation of new capillaries from pre-existing blood vessels, is essential for tumour development beyond a diameter of 2 to 3 mm^3 (5). This process is mediated by angiogenic cytokines and not only provides tumours with nutrients for growth, but also increases the opportunity for tumour cells to enter the circulation and metastasize (6). The most potent of these cytokines is vascular endothelial growth factor (VEGF-A), a heparin-binding glycoprotein with potent angiogenic, mitogenic and vascular permeability-enhancing activities specific for endothelial cells (6).

In gastrointestinal cancer, several growth factors have been identified that regulate angiogenesis, including pro-angiogenic factors such as VEGF and antiangiogenic factors, *e.g.* thrombospondin (7). Angiogenesis also plays an important

role in the progression of CRC. Evidence from preclinical and clinical studies indicates that VEGF is the predominant angiogenic factor in CRC (8). Indeed, VEGF is expressed in approximately 50% of CRCs, with minimal to no expression in normal colonic mucosa and adenomas. Increased VEGF is significantly correlated with advanced lymph node status and distant metastasis (9, 10). The survival is significantly worse in patients with high levels of VEGF expression as compared to those with weak or no expression (11).

In this study, we examined the expression of VEGF-1 in stage I-IV CRC to determine its clinicopathological correlates and association with the response to treatment and disease outcome.

Patients and Methods

The present series consisted of tissue samples obtained from 360 patients with stage I, II, III, or IV CRC who had undergone bowel resection during 1981-1990 at Turku University Hospital, available for study at the archives of the Department of Pathology. All pertinent clinical and histopathological data of the patients were collected from the patients' case records and are summarised in Table I.

Tissue microarray. Archival paraffin-embedded CRC tissue samples were used to build up tissue microarray (TMA) blocks for immunohistochemical (IHC) staining. Areas 1 mm in diameter were chosen and marked in hematoxylin and eosin-stained 4-μm-thick sections under light microscopy. Of all tumours, invasive areas with the lowest degree of differentiation or the highest number of mitoses and of highest cellular atypia were chosen. Wide and homogenous areas (compatible with cellular atypia and poor differentiation) were preferred. To avoid contamination, areas at least 2 mm apart from normal tissue or adenoma were chosen. Necrotic and autolytic areas and areas rich in stromal reaction were discarded. Of the tumours producing abundant intra- or extracellular mucin, invasive areas with the highest number of epithelial cells were chosen. A 1-mm diameter cylinder was cut with a TMA instrument (Beecher Instruments, Sun Prairie, WI, USA) into a new paraffin block. Serial 4-μm sections were then cut from the TMA paraffin blocks. The sections were mounted on ChemMate™ Capillary Gap Plus Slides (Grey) (DAKO). Normal colorectal mucosa was also marked by the pathologist and obtained from tumour blocks adjacent to but at least 2 mm distant from tumour areas of the section. If available, another sample was obtained from the normal colorectal mucosa at both resection margins of the surgical specimen. Lymphatic follicles and hyperplastic and inflamed areas were avoided. To obtain enough mucosa for the TMA, tangentially cut areas were discarded.

Immunohistochemistry (IHC). VEGF-1 expression was assessed immunohistochemically using an automated DAKO Autostainer and purified anti-human VEGF (121, 165, and 189 isoforms), clone VG-1 at dilution 1:150 (Biosite company, Stockholm, Sweden). After staining, the sections were dehydrated in ethanol, cleared in xylene and covered with Mountex and cover-slips.

Evaluation of VEGF expression staining. The expression of VEGF-1 in the tumour tissue and in the surrounding stromal tissue were assessed by two observers (RB & AB) blinded to the clinical data and was weighted according to the expression in the total tumour area. The stromal tissues were invariably negative for VEGF-1, whereas the tumour tissues showed only cytoplamic staining. The cytoplasmic staining was graded into four categories: 0, negative, no detectable staining; 1, weak but still detectable staining; 2, moderate staining, clearly positive but still weak; 3, intense staining. The evaluation of staining of all TMAs was performed with a Nikon light microscope at the magnification of ×40 without associated knowledge of tumour grade, stage or clinical outcome. The typical expression patterns of VEGF-1 are illustrated in Figure 1.

Three different grading systems to evaluate the expression of VEGF were tested; in addition to the 4-grade system described above, two other 2 grade systems were applied: i) negative/weak *vs.* moderate/strong; and ii) negative *vs.* positive. The latter grading system proved to be most useful and was adopted for all statistical calculations.

Statistical analysis. Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 16.0.1 and STATA/SE 10.1). Frequency tables were analysed using the Chi-square test, with likelihood ratio (LR) or Fischer's exact test being used to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95% CI) were calculated where appropriate using the exact method. Differences in the means of continuous variables

Table I. *Clinicopathological characteristics of the patients.*

Characteristic	No. of patients (%)
Gender	
Male	167 (46.4%)
Female	193 (53.6%)
Age (years)	
<65	159 (44.2%)
>65	201 (55.8%)
Histological grade	
I	50 (13.9%)
II	268 (74.4%)
III	42 (11.7%)
Stage	
I	50 (13.9%)
II	201 (55.8%)
III	47 (13.1%)
IV	62 (17.2%)
Localization	
Right colon	115 (31.9%)
Left colon	122 (33.9%)
Rectum	123 (34.2%)
Recurrence during the follow-up	
Yes	125 (34.7%)
No	173 (48.1%)
NA*	62 (17.2%)
Status at the end of follow-up	
Alive	102 (28.3%)
Dead as result of disease	180 (50.0%)
Dead from other cause(s)	78 (21.7%)

*NA: not available.

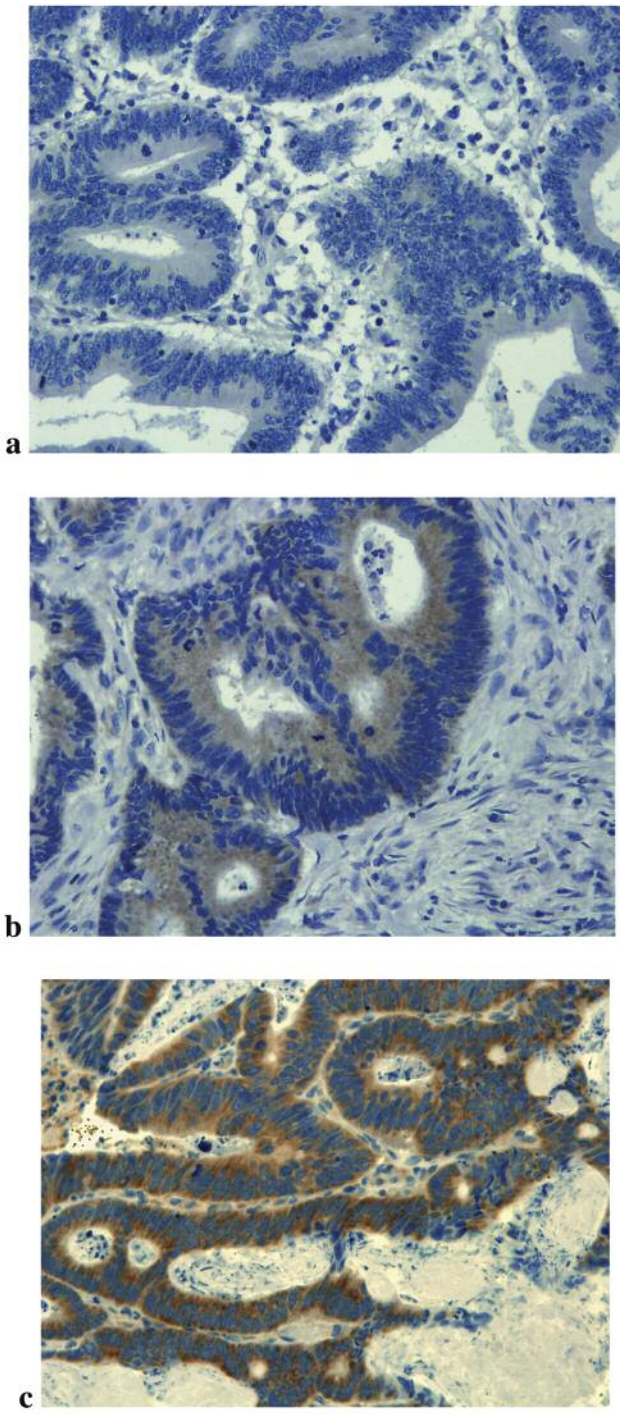

Figure 1. *Different immunohistochemical staining patterns for VEGF-1 in cancer epithelium of colorectal carcinoma. A medium-powered view of adenocarcinoma showing different cytoplasmic VEGF-1 expressions. a, negative; b, moderate; and c, intense expression.*

were analysed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2 and multiple independent samples, respectively. ANOVA (analysis of variance) was only used for deriving the mean values (and their 95% CI) of each individual stratum. Univariate survival analysis for the outcome measures of disease-specific survival (DSS) and disease-free survival (DFS) was based on the Kaplan-Meier method, with log-rank (Mantel-Cox) comparison test. DSS and DFS were calculated based on the time from diagnosis to death (due to disease) and on the time from diagnosis to the appearance of metastatic disease, respectively. In all tests values of $p<0.05$ were regarded as statistically significant.

Results

VEGF and disease localisation. When divided into two categories according to localization as right colon *vs*. left colon and rectum, VEGF expression in the left colon and rectum was significantly more frequent than that in the right colon (61% *vs*. 45%, respectively) ($p=0.006$).

VEGF and tumour stage. Seventy percent (70%) of patients with stage IV CRC had positive VEGF expression compared to 30% who had negative expression, while 50% and 47%, respectively, ($p=0.005$) patients with stage II and III disease had positive VEGF expression. There was no significant correlation with lymph node involvement.

VEGF and treatment response. A significant inverse correlation between VEGF and treatment response was found: VEGF expression was rarer (49%) among patients who showed clinical benefit from treatment [complete response (CR), partial response (PR), and stable disease (SD)] as compared to that (61%) in patients with progressive disease ($p=0.041$).

VEGF and survival. No significant correlation was found between VEGF expression and DFS. However, a significant inverse statistical correlation ($p=0.04$) was also found between VEGF and 10-year DSS, with patients who died of the disease more frequently (61%) having a VEGF-expressing tumour than did those (49%) who survived for 10 years.

Discussion

Preclinical models confirm the important role of VEGF and angiogenesis in the progression of CRC. In colon cancer cell lines, Kondo and colleagues found that VEGF-expressing tumours had greater vascularity and metastatic potential (both hepatic metastasis and peritoneal dissemination) than control tumours where VEGF was not up-regulated. These results suggest that VEGF stimulates angiogenesis in colon cancer and accelerates the growth of metastatic deposits (12). Evidence from several studies implicates VEGF and angiogenesis as being prognostic factors in CRC, VEGF expression being associated with poor prognosis (13-16). Similarly, a recent meta-analysis

by Des Guetz and colleagues confirmed that VEGF expression is associated with poor overall survival in CRC. This meta-analysis included 27 studies specifically investigating VEGF in CRC and VEGF expression was shown to be significantly correlated with poor overall survival and was a stronger predictor of overall survival than was microvessel density (17).

In the present study, a series of 360 CRCs comprising all stages of disease (and with prolonged post-treatment follow-up) were analysed for VEGF expression using an IHC approach. Several intriguing observations were made, as discussed below. Interestingly, VEGF expression was significantly associated with the localisation of the tumours: VEGF positivity and intensity of its expression were significantly confined to left-sided (distal) disease (p=0.004). This indicates that there might be some basic biological differences between normal right and left colonic segments that could favour malignant transformation through different molecular pathways. Indeed, tumours in the hereditary cancer syndromes such as hereditary non polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) occur predominantly in the proximal (right) and distal (left) colon, respectively. Such differences are probably related to the molecular characteristics of the tumours, with microsatellite instability and methylator phenotypes being associated with right-sided tumours and chromosomal instability with left-sided tumours. We suggest that the higher levels of VEGF expression associated with the distal tumours may be due to the divergent genetic pathways that the left and right-sided CRCs are thought to take, but this remains to be established by future molecular studies (18-20).

VEGF seems to be an indicator of poor prognosis in breast cancer as well and was shown to be correlated with tumour stage (21). Similarly, serum VEGF-A levels have been shown to correlate with disease stage in CRC, with increasing levels being associated with more advanced disease (22-24). Preoperative serum VEGF levels have also been shown to correlate with advanced tumour stage at the time of surgery (25, 26). When measured prospectively in a group of patients undergoing curative resection for CRC, serum VEGF levels were significantly higher in patients who subsequently developed metastases than in those who did not (27-29). Our present observations are fully consistent with these previous reports, confirming that 66.7% of the patients with metastatic disease (stage IV) had VEGF-positive tumours. Furthermore, VEGF expression in stage IV tumours was significantly more intense than in stage II and III disease (p=0.005), implicating a direct relationship between VEGF expression and the stage of the disease; intense VEGF expression is associated with more advanced stage and propensity to develop metastatic disease.

Another important observation in the present study was the close correlation of VEGF expression with the treatment response: a lower proportion of patients who clinically benefited from treatment had VEGF-expressing tumours as compared to those who had progressive disease. (49% *vs.* 61%, respectively, p=0.041) This suggests that VEGF expression in the tumours bears some relationship with the response to treatment in that VEGF-expressing tumours are less likely to respond to therapy.

Finally, VEGF expression seems to be of some prognostic value in CRC as suggested by the present results and some previous data (15, 30). Indeed, Ogata and colleagues (31) studied a series of 342 patients with resected stage II or III CRC, of whom 225 received adjuvant oral fluoropyrimidines and 117 received no further treatment after surgery, reporting that VEGF overexpression had a significantly deleterious effect on DFS. This is similar to the results of another study (32) showing that an increase in blood vessel count and VEGF concentration correlated with progression and metastases of CRC. In yet another study, both overall and DFS were found to be significantly lower in patients with VEGF-positive tumours (33). In the present study, we found a significant (p=0.04) correlation between VEGF expression and 10-year DSS survival, VEGF expression being more frequent (61%) among patients who died of their disease than among the 10-year survivors (49%).

Conclusion

The present results demonstrate a significant difference in VEGF expression ascribable to the localization of CRC, implicating some basic differences in the molecular pathways which lead to malignant tumours in the proximal and distal colon, as also suggested by the divergent expression of other molecular markers (34-36). VEGF expression was clearly accentuated in advanced disease stages, implicating an effect on the propensity to develop a metastatic phenotype. Furthermore, VEGF expression was shown to be rarer among patients who showed clinical benefit of treatment as compared to those who had progressive disease, suggesting that VEGF expression might make the tumour cells more resistant to therapy. Finally, VEGF expression in the primary tumours seems to be associated with less favourable long-term (10-year) survival as compared with VEGF-negative tumours, possibly implicating some differences in the inherent malignancy of CRC that only become manifest after prolonged follow-up.

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