

# Expression of Vascular Endothelial Growth Factor Can Predict Event-free Survival in Stage II Colon Cancer

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## ABSTRACT

The usefulness of chemotherapy in patients with stage II disease continues to be debated. Biological prognostic factors may allow further insight into the optimal treatment strategy for patients with node-negative disease. Vascular endothelial growth factor (VEGF) seems to be essential for angiogenesis and for the growth of colorectal cancer. Recently, it was shown able to predict disease recurrence in patients with stage II colon cancer. Specimens of surgically resected colon cancer were immunostained for VEGF. Consecutive patients referred to the study institutions were considered eligible for this study. The main inclusion criteria were stage II tumor, sufficient tumor material, and adequate follow-up information. Analysis was performed on 121 patients. The recurrence rate in the patients with VEGF-positive tumors was 50% (18 of 36 patients), which was significantly higher than that observed in patients with VEGF-negative tumors [11.7% (10 of 85 patients);  $P = 0.001$ ]. Also the degree of VEGF immunoreactivity was significantly higher in 28 relapsing patients compared with 93 disease-free patients (mean VEGF score,  $2.84 \pm 0.38$  versus  $0.66 \pm 0.17$ ;  $P = 0.0001$ ). VEGF may be used in a clinical setting to identify patients at high risk for relapse who may benefit from adjuvant treatment including new therapeutic strategies such as monoclonal antibody neutralizing VEGF.

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## INTRODUCTION

In recent years, adjuvant chemotherapy for colon cancer has been found to improve survival for patients with stage III disease, whereas it has not been clearly demonstrated to offer a significant benefit for patients with stage II disease (1–4). However, 20% of patients with stage II colon tumor die because of recurrent disease. The identification of this subgroup may be helpful in selecting patients who may profit most from adjuvant treatments. With regard to this issue, biological and molecular prognostic factors are promising to allow further insights into the optimal treatment strategy for stage II colon cancer (5, 6).

Recently, although several studies have focused on the prognostic significance of angiogenesis in experimental models of colon cancer (7–10), conflicting results were found in colon cancer patients using microvessel count. In fact, in one trial, a low microvessel count predicted a longer survival time, whereas in another study, a better prognosis was predicted by high microvessel count (11, 12). Moreover, a further study assessing the association of tumor angiogenesis with survival in 22 patients with stage II colon cancer produced still more controversial results. Although there was a trend toward a higher frequency of microvessels in patients with longer survival, it was unlikely that microvessel count was an independent prognostic indicator because there was only a small difference in microvessel frequency between patients with widely different survival times (13). On the other hand, Takahashi *et al.* (14) found that vessel count and particularly the expression of VEGF<sup>2</sup> could be useful in predicting disease recurrence in 27 patients with stage II colon cancer.

The possible importance of angiogenesis measured by VEGF expression as a prognostic factor in stage II colon cancer, as well as the controversies and the limited number of patients included in previous studies, prompted us to investigate the role of VEGF in a larger series of patients with stage II colon cancer to identify individuals at high risk for recurrence.

## PATIENTS AND METHODS

Consecutive patients with stage II (pT<sub>3</sub>N<sub>0</sub>M<sub>0</sub>) disease referred to our institutions between 1988 and 1992 were considered eligible for this study. This length of time was chosen to be assured of adequate follow-up.

Patients who received any form of adjuvant chemotherapy, had a familial cancer syndrome, or had another concurrent malignant neoplasm were excluded from the study.

Preoperative examinations had to show no evidence of metastatic disease. All patients were observed for at least 5 years after surgery and routinely studied by diagnostic imaging (com-

<sup>2</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; DFS, disease-free survival; SPF, S-phase fraction.

puted tomography, ultrasonography, or magnetic resonance imaging) twice a year. The type of recurrence was established by diagnostic imaging, cytology, biopsy, or surgery. The following findings were confirmed in all of the patients as of March 1999.

Original tumor tissues were reviewed by a pathologist for histological confirmation.

After this initial review of all available H&E-stained slides of the surgical specimens, one representative paraffin block was selected from each case for further study. The selected blocks were those in which mucosa, invasive edge, and viable tumor were present. Six tissue sections were immunostained for VEGF.

**Determination of Tissue VEGF.** VEGF expression was analyzed using a standard avidin-biotin technique. Sections (4- $\mu$ m thick) were deparaffinized in xylene and rehydrated in a graded ethanol series. Specimens were placed in a plastic Coplin jar containing citric buffer and heated three times (5 min each) in a microwave processor. The sections were then left in the Coplin jar at room temperature for 20 min. After an incubation in 3% hydrogen peroxide for 8 min, specimens were covered with normal swine serum for 10 min to reduce nonspecific staining and incubated with a 1:20 dilution of rabbit polyclonal antibody for VEGF (Biogenex, San Ramon, CA) at room temperature for 30 min. The sections were washed with PBS, incubated with a 1:50 dilution of biotinylated swine antirabbit IgG at room temperature for 20 min, and then covered with a 1:100 dilution of streptavidin-biotin-peroxidase complex at room temperature for 20 min. The antibody was localized with 3,3'-diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with light hematoxylin, dehydrated with ethanol, and mounted under a coverslip.

Normal rabbit IgG was substituted for primary antibody as the negative control. For positive controls, normal mucosa known to express VEGF was stained for VEGF.

Slides were then examined under a light microscope and scored independently by two investigators blinded to the clinical data. In each case, the entire section was examined systematically on high-power fields for VEGF immunoreactivity.

Immunohistochemical staining of tumors with this antibody shows primarily a cytoplasmic localization of VEGF protein. Among all immunoreactive cells, only those clearly immunostained were recorded as VEGF positive. The expression of VEGF was assessed according to the percentage of immunoreactive cells of a total of 1000 neoplastic cells (quantitative analysis). Immunoreactivity was graded as follows: (a) positive, more than 10% of carcinoma cells stained; and (b) negative, no detectable staining or less than 10% of carcinoma cells stained (15). Furthermore, the qualitative intensity of staining for VEGF was assessed using a scale of 0–3+, with 0 representing no detectable stain, and 3+ representing the strongest stain (14).

The agreement in VEGF evaluation between the two observers was >95%. In the five cases of disagreement, a final score was determined by consensus after reexamination.

**Statistical Analysis.** The primary end point was event-free survival from the time of surgery until the date of recurrence. DFS curves were computed according to the Kaplan-Meier method; differences in DFS were compared using the log-rank test.

Table 1 Main characteristics of the 121 patients in the study

No. of patients	121
Age (yrs)	
Median	66
Range	41–79
Gender ratio (M:F)	71:50
Colon site of tumor	121
Ascending colon	32
Transverse colon	21
Descending colon	68
Grade	
Well differentiated	32
Moderately differentiated	67
Poorly differentiated	22

The prognostic power of VEGF expression and other pathological variables of the tumors was evaluated by a multivariate analysis (Cox regression model with stepwise selection of variables), considering the SPF, age, gender, tumor size, tumor site, tumor grade, and lymphatic and perineural invasion as variables (16).

## RESULTS

A total of 121 of 124 assessed patients with pT<sub>3</sub>N<sub>0</sub>M<sub>0</sub> colon cancer operated on at our institutions were fully evaluable for the study. Three patients were excluded because of inadequate tumor material. Patient characteristics are summarized in Table 1.

Positive VEGF staining was found in 36 of 121 patients (30%; Fig. 1). The recurrence rate in the patients with VEGF-positive tumors was 50% (18 of 36 patients), which was significantly higher than that observed in patients with VEGF-negative tumors [11.7% (10 of 85 patients);  $P = 0.001$ ].

Also the degree of VEGF immunoreactivity was significantly higher in the 28 relapsing patients than in the 93 disease-free patients (mean VEGF score,  $2.84 \pm 0.38$  versus  $0.66 \pm 0.17$ ;  $P = 0.0001$ ).

These results were also confirmed by applying a Cox regression model to VEGF data. In the final Cox model, the variables that were found to be statistically significant were tumor grade ( $P = 0.01$ ) and SPF determined by flow cytometry ( $P = 0.04$ ); gender, age, tumor size, tumor site, and lymphatic and perineural invasion were excluded because they are not statistically significant ( $P > 0.05$ ).

Fig. 2 shows the DFS curves of the 121 patients with pT<sub>3</sub>N<sub>0</sub>M<sub>0</sub> colon cancer subdivided by VEGF expression (0–1 versus 2–3).

## DISCUSSION

The selection of patients who are likely to benefit from adjuvant chemotherapy after surgical resection of colon cancer is based prevalently on nodal status. In fact, patients with stage III disease colon cancer have been shown to have a survival benefit from adjuvant chemotherapy, whereas analysis of patients with stage II cancer failed to find a clear survival benefit (1–4). One reason suggested for this apparent difference in activity of adjuvant therapy is that there are so few recurrences in the stage II group that a survival benefit of adjuvant therapy

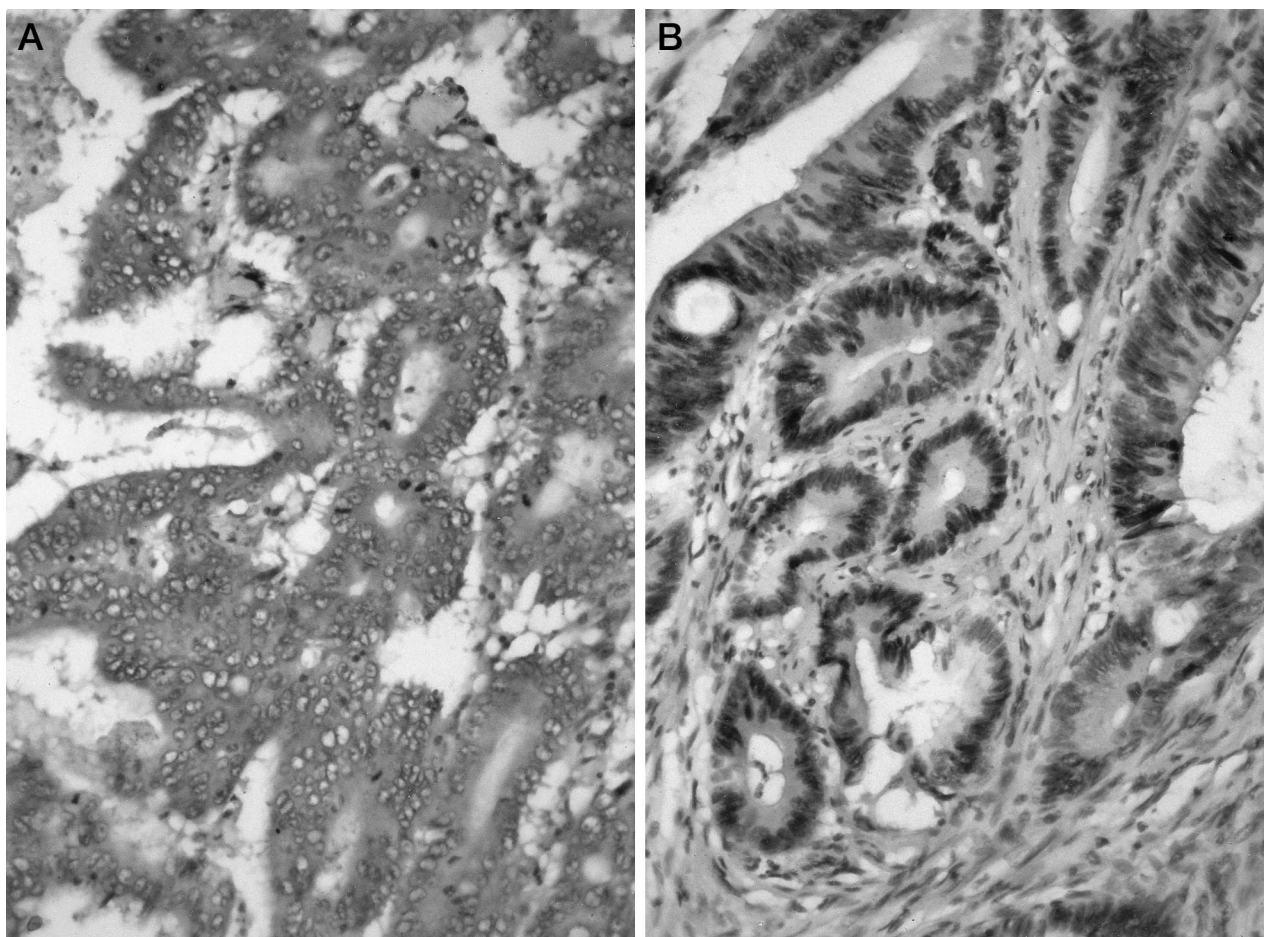


Fig. 1 Positive (A) and negative (B) immunostaining of stage II colon cancers with antibody to VEGF.

can be missed (17). However, it seems necessary to identify the subset of patients with stage II colon cancer who have a worse prognosis (20% of them will present a relapse) and may benefit from an adjuvant therapy.

Investigators have studied many pathological, biological, and molecular markers to more appropriately select patients for adjuvant therapy. Apart from stage, pathological features have not been identified as clinically useful predictors of distant failure in colon cancer (18).

In our experience, a biological characteristic such as SPF has been shown to disclose prognostic differences in stage II colon cancer (5). Also, the molecular characteristics of colon cancer have prompted investigators to use molecular markers as a predictor of recurrent disease. High fractional allelic loss and loss of chromosome fragments 17p and 18 p have been associated with poorer prognosis in patients with Dukes' stage B or C tumors (19). Similarly, Ki-ras mutational status and p53 overexpression were found to be significant prognostic factors in patients with stage II and III colon cancer (20).

Although these findings provide a foundation for predicting patients at risk for recurrent cancer, few clinical laboratories are capable of performing such studies; therefore, it is necessary to develop a reproducible assay or test that can accurately and

easily predict patients at risk for recurrent cancer and can be performed by all laboratories in community hospitals.

A correlation has been demonstrated between angiogenesis measured by microvessel counts and the risk of metastasis in colon cancer. However, angiogenesis assessed by microvessel counts can have a limited value in determining prognosis in individual cases; overlaps and large SDs of microvessel counts related to the presence or absence of other pathological or prognostic features may explain the conflicting results obtained (11, 12, 21).

Several angiogenic factors have been identified. Among these, VEGF seems to play a crucial role in the proliferation and migration of endothelial cells, providing nourishment to the growing tumors and making the tumor cell establish continuity with the host vasculature (22, 23).

Recent studies have shown an association between VEGF expression and tumor aggressiveness in colon cancer. In fact, VEGF expression was found to be higher in patients with metastatic tumors than in those with nonmetastatic tumors (7-9). Takahashi *et al.* (14) demonstrated that VEGF expression in primary cancer is significantly related to time to recurrence in patients with stage II tumors. Because this study was limited to only 27 patients, we believed that the



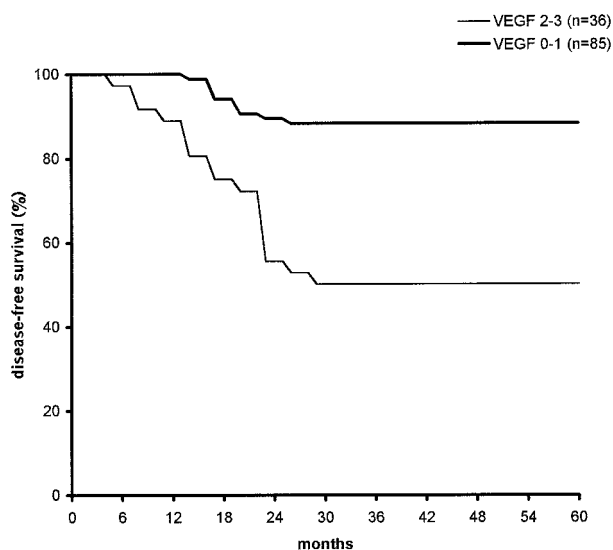


Fig. 2 Relapse-free survival curves of patients with stage II colon cancer subdivided by positive or negative immunostaining for VEGF.

prognostic role of VEGF should be confirmed in a larger series of stage II colon cancer patients before being offered as a routine prognostic factor in clinical practice. Our results on 121 patients with stage II colon cancer confirm that VEGF positivity is associated with a significant reduction in the 5-year DFS rate, which ranges from 90% in patients with negative VEGF expression to 50% in patients with positive VEGF expression.

Technical drawbacks are unlikely to have influenced the results of our VEGF expression analysis. In fact, the procedure and the dichotomization value between positive and negative VEGF expression used in our analysis were as described previously and tested in other studies (14, 15). Furthermore, differences in DFS were present when VEGF expression was assessed by the percentage of positive cells and by the degree of immunoreactivity. This concordant double assessment of VEGF expression should support the validity of our conclusions. However, some caution could be required in the interpretation of these data because of the retrospective nature of our analysis, although only a small number of patients (0.2%) in the consecutive series were excluded. Furthermore, these data may also be affected by the antibody and the staining technique, which are common limitations of all immunohistochemical studies. For these reasons, we believe that our data should be reproduced by prospective studies in other laboratories and/or with other techniques. Nevertheless, these results can be potentially useful not only because they define an unfavorable group of stage II colon cancer patients but also because they allow us to select patients not benefiting from standard adjuvant chemotherapy who could receive new treatment options such as monoclonal antibody or antagonistic molecules neutralizing VEGF, which are now available for clinical trials.

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