Vascular Endothelial Growth Factor-A Expression Is Associated with Subsequent Recurrence in the Liver During Long-Term Follow-Up of Colorectal Cancer Patients in Dukes C

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Abstract

Background  We aimed to determine the subgroup of patients with colorectal cancer, in which expression of vascular endothelial growth factor-A (VEGF-A) affects their prognosis strongly.

Methods  119 paraffin embedded specimens of colorectal cancer were investigated by staining with a monoclonal antibody against VEGF-A as well as basic fibroblast growth factor (FGF2), CD34 and p53, which was compared with the pattern of liver metastasis or recurrence and overall survival.

Results  VEGF-A positive ratio was higher in patients with liver metastasis at diagnosis (45% of 46) or recurrence in the liver during follow-up (39% of 33) than without liver metastasis or recurrence in the liver (16% of 40 patients) (P<0.001). Moreover, the risk of recurrence in the liver during long-term follow-up was significantly increased by VEGF-A production only in patients with Dukes C (N = 46) [odds ratio (OR) = 22.4; 95% confidence interval (CI): 4.6–108], but not in Dukes B (N = 24). In multiple logistic regression analysis using variables of age, gender, expression of FGF2, CD34, p53, stage of lymph node metastasis, and lymph/vascular vessel invasion in pathological specimen, expression of VEGF-A was sole significant factor (OR = 43.5; 95%CI: 4.2–448). In Kaplan-Meier survival curves, 5-year survival in VEGF-A positive and negative patients belong to Dukes C was 68% and 93% (log-rank test: P<0.05), respectively. Hazard ratio of VEGF-A adjusted by age and gender was 4.3; 95%CI: 1.2–15.5.

Conclusions  These results suggest that patients with Dukes C colorectal cancer and VEGF-A positive in tumor specimen may have a higher risk of recurrence in the liver during long-term follow-up.

Key words  Angiogenesis, Pathology, Colorectal, Cancer, Prognosis

Introduction

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer deaths worldwide.1 Approximately, 30% of patients diagnosed with colorectal cancer already have metastasis at first presentation, whose only less than 10% can survive beyond 5 years.1 Recently, for these patients with metastatic colorectal cancer, the addition of bevacizumab, an antibody against vascular endothelial growth factor (VEGF), to fluorouracil-based combination chemotherapy was proved to improve their
Even without metastasis at diagnosis, the prognosis of patients with colorectal cancer is not satisfactory: 25–35% of patients without regional lymph node involvement and approximately 40–75% of patients with positive lymph nodes will not live 5 years after curative resection. Therefore, the next targets of anti-VEGF antibody in addition to standard treatments are these patients without metastasis at diagnosis. However, the relationship between VEGF protein expression and the prognosis of patients with colorectal cancer remains controversial: some have indicated VEGF expression as an independent factor in predicting patients’ prognosis; while others reported no such associations. Therefore, we hypothesized that VEGF may affect the prognosis of a specific subgroup in colorectal cancer patients. In particular, since liver metastasis/recurrence can be a key issue in colorectal cancer, we aimed to determine the subgroup, of which VEGF expression associates with prognosis, by stratifying with either no recurrent (Group I) or subsequent liver recurrence during follow-up (Group II) or liver metastasis at diagnosis (Group III) and further by stratifying with Dukes stages, simultaneously p53 and FGF2 as other known angiogenic factors as well as CD34 as a marker for neovascularisation were also stained for comparison.

**Patients and Methods**

**Patients**

Retrospectively, 33 patients with delayed liver metastasis were selected as cases. For case comparison, 40 patients without liver metastasis and 46 patients with simultaneous liver metastasis were chosen from the list in the department of surgery matched by age within 5 years independent of survival. As a result, this study included 119 patients with colorectal cancer. Patients aged 28 to 80 years (mean ± SD: 58 ± 11 years) at diagnosis and there were more men (N = 82) than women (N = 37). No major complications were observed and all of the patients could be discharged from the hospital. Although 3 patients who moved to different hospitals after discharge were deleted from this study due to a short term follow up period of less than 90 days, all 119 patients were followed up at the outpatient clinic of Jikei University Hospital periodically. The primary endpoint was set as death due to any cause and the secondary endpoint was set as liver recurrence during follow-up. Patients who did not develop to the primary endpoint were counted as censored cases on the final day of the outpatient clinic. Paraffin-embedded specimens and information regarding the pattern of liver metastasis were available for all 119 patients.

The following parameters were recorded in all patients: cancer site (right and left colon by using the middle transverse colon as partition), kinds of surgical resection, Dukes’ stage, number of resected nodes, number of metastatic nodes, tumor size, macroscopic findings of tumor, postoperative complications, and recurrences after potentially curative resections.

All patients and their families were informed about the possible risks and benefits of operations as well as the usage of clinical data for research purposes, and written consent was obtained.

**Pathologic specimens**

Tumor specimens were obtained by surgery. Formalin-fixed, paraffin-embedded specimens of colon cancer were retrieved from the Department of Pathology and processed for conventional histological assessment by hematoxylin and eosin (H&E) staining. Only patients with adenocarcinoma of the colon and rectum confirmed by two or more board-certified pathologists were included. Histologic features of the extent of the lesions, depth of tumor invasion, invasion into lymphatic (Ly) or blood vessels (V), and lymph node metastasis (N) as well as histologic differentiation (well, moderate, or poor) were also evaluated.

**Immunohistochemical staining for VEGF-A, fibroblast growth factor-2 (FGF2), CD34 and p53**

Immunohistological staining was performed by standard methods as follows: 1) Deparaffinize and hydrate sections; 2) Wash in deionized water;
3) Block endogenous peroxidase with 3% hydrogen peroxide for 5 minutes; 4) Wash in deionized water; 5) Block non-specific staining by incubation with 10% porcine serum (Bio-Products, Woodland, CA, USA) in phosphate buffered saline (PBS) for 10 minutes at room temperature; 6) Incubate with diluted primary antibody overnight at 4°C: VEGF-A (A-20: sc-152) that is considered to detect precursor/mature and all kinds of VEGF-A of human (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) at the dilution of 1:50; FGF-2 (Santa Cruz Biotechnology, Inc.) at the dilution of 1:800; CD34 (BD Biosciences. San Jose. California USA) at the dilution of 1:30; p53 mouse monoclonal antibody (Novocastra, Newcastle, UK) at the dilution of 1:100; 7) Rinse in PBS; 8) Incubate with diluted biotinylated antibody (DAKO Cytometion, Glostrup, Denmark) at the dilution of 1:500 for 30 minutes at room temperature; 9) Rinse in PBS; 10) Incubate with diluted peroxidase-conjugated streptavidin (DAKO Cytometion) at the dilution of 1:500 for 30 minutes at room temperature; 11) Rinse in PBS; 12) Incubate with 20 mg 3,3’-Diaminobenzidine, tetrahydrochloride and 20 µl 30% hydrogen peroxide in 100 ml PBS for 5 minutes at room temperature; 13) Wash in water; 14) Counter-stain with hematoxylin for 1 minute; 15) Wash in water; 16) Dehydrate and clear sections; 17) Mount with permount.

Two investigators (K.E. and M.O.) who were blinded to the clinical information of each patient evaluated the staining levels independently, after which discordant evaluations were adjusted by connected microscopes.

Statistical Analysis
Chi-square test and the analysis of variance were used to evaluate the associations between VEGF-A expression and clinicopathologic parameters. Survival curves of the patients were compared using the Kaplan-Meier method and analyzed using log-rank test. Cox proportional hazards models were fitted for multivariate analysis.

Results
Patients’ characteristics and liver metastasis patterns
After surgery, patients were followed from 126 days to 19 years (median: 5.7 years). According to the pattern of liver metastasis or recurrence, 119 patients were divided into 3 groups: Group I: no metastasis at diagnosis and during follow-up (N = 40; median follow-up: 8.1 years); Group II: no metastasis at diagnosis but liver metastasis emerged during follow-up (N = 33; median follow-up: 5.7 years); Group III: liver metastasis at diagnosis (N = 46; median follow-up: 2.5 years). By these patterns of liver metastasis, 5 year survival curves were compared to find significant differences: Group I: 98%; Group II: 69%; Group III: 39% (log rank test: P<0.0001) (Fig. 1). Associations between patients’ characteristics and these three liver metastasis patterns are shown in Table 1. Invasion of tumor cells into vascular vessel was observed more in Group I.

Protein expressions of VEGF-A, FGF2, CD34 and p53 and liver metastasis pattern
Typical histologic pictures of VEGF-A, FGF2, CD34 and p53 staining of paraffin-embedded specimens are demonstrated in Fig. 2. Although VEGF-A was stained lightly or moderately, the staining pattern was clearly discriminate as positive or negative. In contrast, FGF2 was positive in parts of tumor cells. Vascular cells were clearly stained with CD34. Positive staining pattern of p53 was sufficiently clear to between discriminate positive and negative. The positive ratio of VEGF-A, FGF2, CD34 and p53 was 69 (58%), 53 (45%), 57 (49%), 84 (71%) in 119 patients.
Positive VEGF-A expression was associated with the positive expressions of both CD34 \((P = 0.017)\) and p53 \((P = 0.003)\). Moreover, FGF2 had positive associations with expressions of CD34 \((P < 0.001)\) and p53 \((P < 0.001)\). Similarly, CD34 showed positive association with p53 \((P < 0.001)\). In association with the pattern of liver metastasis, the expression of VEGF-A protein was significant, but not FGF2, CD34 nor p53 (Table 2).

Table 1 Patients’ characteristics by pattern of liver metastasis

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years old): mean ± sd</td>
<td>57 ± 12</td>
<td>57 ± 11</td>
<td>60 ± 10</td>
<td>NS*1</td>
</tr>
<tr>
<td>Male/Female</td>
<td>27 (33%)</td>
<td>24 (29%)</td>
<td>31 (38)</td>
<td>NS*2</td>
</tr>
<tr>
<td>Dukes B/C/D</td>
<td>11/29/0</td>
<td>13/17/3</td>
<td>0/0/46</td>
<td>&lt;0.001*2</td>
</tr>
<tr>
<td>HPN classification*3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H: 0/1/2/3</td>
<td>40/0/0/0</td>
<td>33/0/0/0</td>
<td>0/29/5/12</td>
<td>&lt;0.001*2</td>
</tr>
<tr>
<td>P: 0/1/2/3</td>
<td>40/0/0/0</td>
<td>30/1/1/1</td>
<td>38/6/2/0</td>
<td>NS*2</td>
</tr>
<tr>
<td>N: 0/1/2/3</td>
<td>14/16/7/3</td>
<td>11/16/6/0</td>
<td>9/26/8/3</td>
<td>NS*2</td>
</tr>
<tr>
<td>Vessel invasion*3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphatic: 0/1/2/3</td>
<td>0/34/5/1</td>
<td>1/24/6/2</td>
<td>0/31/9/6</td>
<td>NS*2</td>
</tr>
<tr>
<td>Vascular: 0/1/2/3</td>
<td>2/34/4/0</td>
<td>12/19/1/1</td>
<td>5/32/8/1</td>
<td>0.004*2</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>well/moderate/poor</td>
<td>35/4/1</td>
<td>22/10/0</td>
<td>38/5/2</td>
<td>NS*2</td>
</tr>
</tbody>
</table>

Group I: no metastasis at diagnosis and during follow-up. Group II: no metastasis at diagnosis but liver metastasis emerged during follow-up. Group III: liver metastasis at diagnosis. *1: Statistical difference was analyzed with ANOVA. *2: Statistical difference was analyzed with chi-square test. *3: According to the Japanese classification of colorectal carcinoma.

Fig. 2 Typical positive staining of CD34, p53, bFGF2 and VEGF

Slides were examined by two independent examiners blinded to each other’s work and with no prior knowledge of clinical and pathological parameters. For each colorectal cancer, staining was evaluated at the invasive edge of the tumor. Slides were examined at \( \times 400 \) (40 \( \times \) objective, 10 \( \times \) ocular).
Risk of liver metastasis during follow-up evaluated by VEGF-A expression

Since emerging liver metastasis during follow-up is a strong prognostic factor, next we focused only on patients without liver metastasis at diagnosis (N=73) (Table 3). The risk of delayed liver metastasis was significantly increased by the presence of VEGF-A in tumor tissue in focusing on patients belongs to Dukes C (N=46) alone [odds ratio (OR)=22.4; 95% confidence interval (CI): 4.6–108] (P<0.001), but not in Dukes B (N=24). In multiple logistic regression analysis with age, gender, stages of hepatic, peritoneal and lymph node metastasis, and lymph/vascular vessel invasion in pathological specimen, expression of VEGF-A was the sole significant factor (OR=43.5; 95%CI: 4.2–448) (P=0.002).

Survival of patients evaluated by VEGF-A expression stratified by Dukes classification

Kaplan-Meier survival curves of patients by VEGF-A expression are shown stratified by Dukes B (Fig. 3A), Dukes C (Fig. 3B) and Dukes D (Fig. 3C). Significant difference was detected only in Dukes C (log-rank test: P<0.05), but not in Dukes B and not in Dukes D. In Dukes C, 5-year survival rate in VEGF-A positive and negative patients was 68% and 93%, respectively. The hazard ratio of VEGF-A adjusted by age and gender in Cox regression analysis was 4.3; 95%CI: 1.2–15.5 (P=0.027) in restricting to Dukes C.

Discussion

We demonstrated that VEGF-A production by primary colorectal cancer might be associated with liver metastasis/recurrence. Our data support previous articles suggesting that VEGF may associate with the prognosis of patients with colorectal cancer. Neovascularization is a key process in the growth of solid tumours, as these tumours will not grow beyond a few cubic millimetres unless a vascular network is established. To stimulate neovascularization, the tumor cells produce a variety of angiogenic factors, such as VEGF. Thus, VEGF produced by primary tumor induced neovascularization to make cancer cells shed into the circulation and colonize distant sites as well.
In this study, no association between liver metastasis pattern and other markers: FGF2, CD34 and p53 was detected in multivariate analysis. A predictive value of serum FGF, an angiogenic factor, was not as clear as VEGF, as in our research. CD34 was used to monitor neovascularization around the tumor tissue, which was demonstrated to predict the prognosis of patients with colorectal cancer. Theoretically, increase of micro-vessels detected with CD34 can be a result of VEGF production, thus both have similar meaning regarding the progression of tumor and prognosis of patients.

Mutations of p53 and activation of the Ras/MAPK pathway plays a role in the induction of VEGF expression in human colorectal cancer. This evidence is consistent with our results in that positive VEGF expression is associated with positive expression of both CD34 and p53.

VEGF is reported to associate with the prognosis of patients with Dukes A, B in colorectal cancer. On the other hand, the clinical effectiveness of anti-VEGF on patients with Dukes D colorectal cancer was demonstrated by randomized clinical trial as already mentioned in 'Introduction'. Of interest, an association between VEGF production and liver metastasis was observed only in Dukes C but not in Dukes B and not in Dukes D in this study. Previous morphometric studies of colon cancer suggest that neovascularisation reaches its maximal level early in the malignant process. Indeed, transcription levels of VEGF isoform show an inverse relationship with Dukes stage. Others indicate that a tumor area with a low microvascular count is associated with VEGF expression up-regulated in response to hypoxia, induced by a lack of a functional vasculature. Therefore, we hypothesized that neovascularisation is already established in tumor of Dukes D through VEGF and/or other angiogenic factors and thus production of VEGF may not discriminate the prognosis of patients with this stage anymore.

In conclusion, patients with Dukes C colorectal cancer and VEGF-A positive in tumor specimen may have a higher risk of recurrence in the liver during follow-up.
References