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Circulating Vascular Endothelial Growth Factor Six Months after Primary Surgery as a Prognostic Marker in Patients with Colorectal Cancer

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High preoperative circulating vascular endothelial growth factor (VEGF) is predictive of poor prognosis in patients with colorectal cancer (CRC). However, postoperative circulating VEGF has not yet been evaluated as a prognostic marker in CRC patients. In 318 consecutive patients who had undergone curative resection of primary CRC, the prognostic value of VEGF concentrations in plasma and serum obtained 6 months postoperatively was analysed and the results compared with the prognostic value of postoperative carcinoembryonic antigen (CEA) concentrations in matched serum samples. In univariate analyses, high serum and plasma VEGF (>533 pg/ml and >112 pg/ml, respectively) had no significant ($p = 0.17$ and $p = 0.13$, respectively) impact on overall survival. On the contrary, high serum CEA (>5 ng/ml) was significantly ($p < 0.0001$) correlated to a poor prognosis. Finally, in multivariate analyses, the combination of high serum CEA and high serum VEGF was significantly (hazard ratio 3.0, $p = 0.02$) associated with poor survival compared to high serum CEA and low serum VEGF. It is concluded that 6 months postoperatively serum CEA is a better prognostic marker than corresponding serum and plasma VEGF. However, high serum VEGF within high serum CEA was an even better predictor of overall survival than high serum CEA alone.

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The value of serum carcinoembryonic antigen (CEA) in predicting prognosis of patients with colorectal cancer (CRC) has still not been evaluated in detail (1–3). However, after curative resection, increased serum CEA that returns to a level within the normal range may predict a good prognosis, while sustained high concentrations 6 weeks after surgery are frequently associated with early recurrence (4). Serial determinations of CEA after curative resection of CRC are therefore used to detect recurrent disease (5).

High preoperative serum and plasma vascular endothelial growth factor (VEGF) concentrations may predict poor prognosis in patients with CRC (6). In theory, increased circulating VEGF concentrations should normalize after curative resection of the tumour. Postoperative changes in circulating VEGF may therefore reflect the success of the surgical intervention. However, determination of postoperative circulating VEGF concentrations has not yet been evaluated as a prognostic marker in CRC patients.

The aims of the present study were to evaluate the prognostic value of matched postoperative serum and plasma VEGF concentrations in patients with CRC, and to compare the results with the prognostic value of CEA concentrations in corresponding serum samples.

MATERIAL AND METHODS

Healthy volunteers

The reference ranges of plasma and serum VEGF concentrations in healthy controls were established in a previous study including 50 healthy volunteer blood donors (30 males and 20 females) (6) with a median age of 59 (range 55–65) years.

Patients

The study included 318 (192 males and 126 females) consecutive patients who had undergone curative resection of primary CRC. The median age of the patients at the time of surgery was 68 (33–90) years. All the patients had their primary tumours resected and none had been given chemotherapy or radiotherapy before or after the opera-

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tion. All patients had histologically verified carcinoma localized in the colon or in the rectum and were staged according to the Dukes' classification. The distribution of Dukes' stages according to site of tumour is presented in Table 1. The clinical data included overall survival for all patients, and because all Danes are given a computerized central personal registration number, none of the patients was lost to follow-up. The median follow-up time was 94 months (range 78–108) and 156 patients (49%) died during the observation period. The endpoint for survival analysis was death from all causes.

Sampling of blood

After written informed consent in accordance with the Helsinki II Declaration, preoperative peripheral blood samples were drawn from the patients on the day of their operation, just before skin incision. Six months after surgery, at the routine control in the outpatient clinic, similar blood samples were drawn. The blood samples from the volunteer blood donors were obtained at the time of their routine donation of blood in the blood bank.

In all individuals, blood samples were collected in endotoxin-free silicone-coated tubes (Becton-Dickinson, Mountain View, CA, USA) with EDTA as additive (plasma) or without additive (serum). The plasma samples were centrifuged (3 000 RPM, 4°C, 10 min) immediately after the aspiration, and the plasma was removed and stored at −80°C until analysed. The serum samples were allowed to clot at room temperature for 30 min before centrifugation (3 000 RPM, 4°C, 10 min) and the serum was removed and stored at −80°C until analysed. At the end of the study, complete sets of matched pre- and postoperative serum and plasma samples were available in only 228 out of 318 patients.

VEGF and CEA analyses

The preoperative plasma and serum VEGF data were obtained from an earlier published study (6). Pre- and postoperative VEGF concentrations in patients and in healthy controls were determined using the same commercially available human VEGF quantitative enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA, Cat. No. DVE00) in accordance with the manufacturer's instructions. However, the preoperative serum VEGF concentrations were determined with ELISA kits that had a different batch number from the kits used to analyse the remainder of the plasma and serum samples from patients and controls.

Serum CEA was measured using the Immulite CEA assay (Euro/DPC Ltd., Llanberis, Gwynedd, UK). All analyses were made in duplicate and the mean value was used for statistical calculations. Before analysis, the plasma and serum samples were thawed at room temperature.

Statistical analysis

The SAS® software package (version 8.1; SAS Institute, Cary, NC, USA) was used to manage patient data and for statistical analysis. Plasma and serum VEGF concentrations were dichotomized based on VEGF determination in healthy controls. VEGF was scored as low if less than or equal to the 95th percentile of normal controls (112 pg/ml and 533 pg/ml, respectively) or otherwise scored as high (6). The serum CEA concentrations were scored as low if CEA was less than or equal to 5 ng/ml, or otherwise scored as high (7). The endpoint for survival analysis was death from any cause. The Kaplan–Meier method was used to estimate survival probabilities, and the log-rank test was used to test for equality of strata. Cox's proportional hazards model was used for multivariate analyses. The assumption of proportional hazards was verified graphically. Rank statistics were used to calculate correlation coefficients and to test hypothesis on location. The level of significance was set at 5%.

Table 1

The distribution of Dukes' stage stratified by topographical tumour localization

Dukes' stage	Rectum	Colon	Total
A	32 (10%)	16 (5%)	48 (15%)
B	51 (16%)	96 (30%)	147 (46%)
C	58 (18%)	65 (20%)	123 (39%)
Total	141 (44%)	177 (56%)	318 (100%)

RESULTS

Pre- and 6 months' postoperative VEGF and CEA concentrations

The results of pre- and postoperative VEGF and CEA determinations are summarized in Table 2. In preoperative plasma and serum, there were no significant correlations

Table 2

Pre- and 6 months postoperative plasma VEGF (pg/ml), serum VEGF (pg/ml) and serum CEA concentrations (ng/ml)

	Median	Range	n	p*
Plasma VEGF				
Pre-op.	36	0–567	304	301*
Post-op.	32	2–705	301	0.0004
Serum VEGF				
Pre-op.	231	9–2500	312	Not calculated
Post-op.	346	38–2022	246	
Serum CEA				
Pre-op.	3.0	0.3–428	314	309*
Post-op.	2.0	0.2–3391	312	< 0.0001

Abbreviations: VEGF = vascular endothelial growth factor; CEA = carcinoembryonic antigen.
Wilcoxon's signed rank test for paired data.
The number of pairs* is given above the p-value.

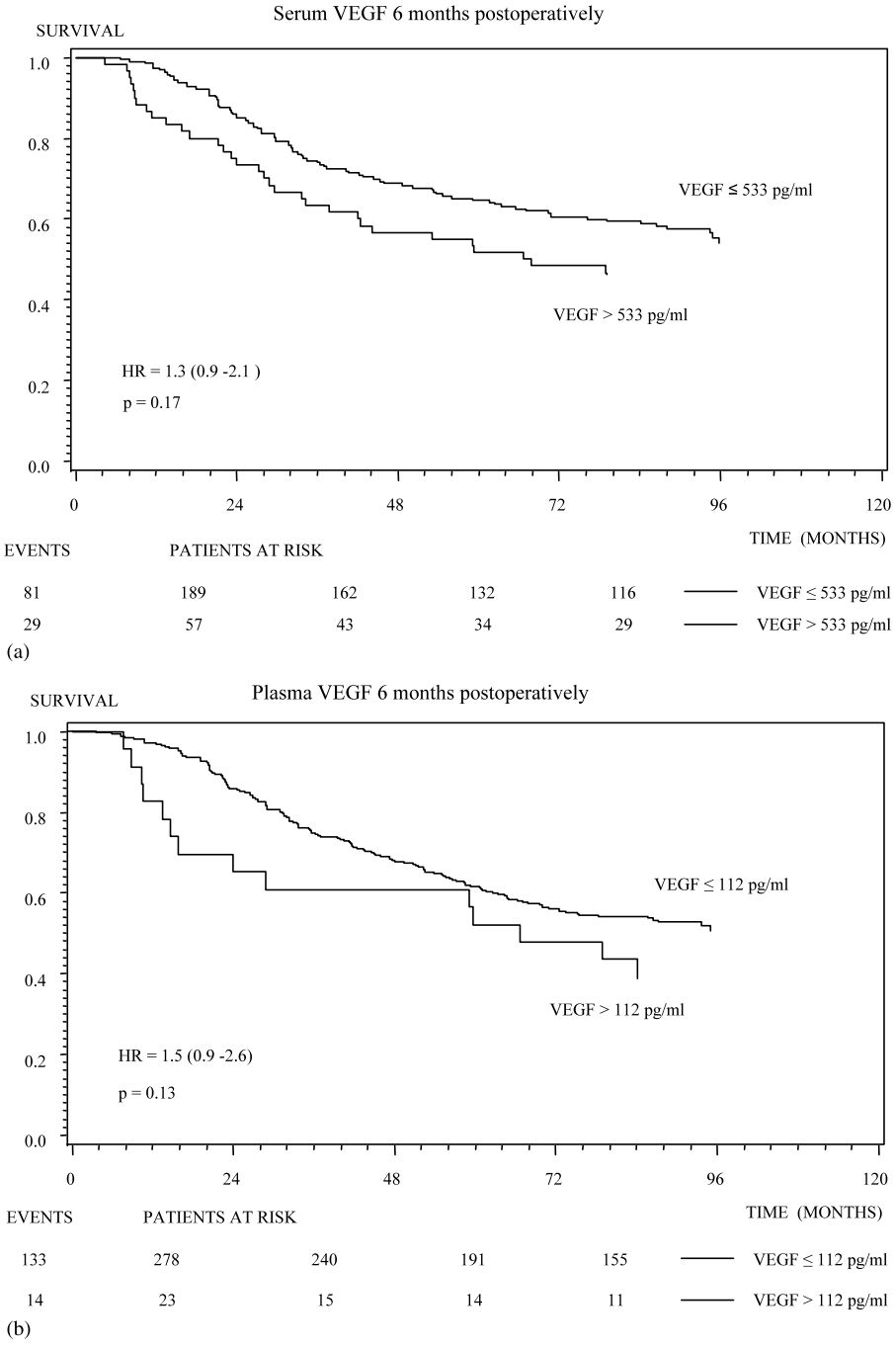

Fig. 1. Survival curves of curative resected colorectal cancer patients dichotomized by the upper limit of the 95th percentile of healthy volunteer blood donors. The number of events (deaths) in each group and the number of patients at risk (survivors) after each 24-month interval up to 72 months is indicated below the curve. a) The two curves represent patients with 6 months postoperative serum vascular endothelial growth factor (VEGF) values below or equal to 533 pg/ml (n = 189, upper curve), and patients with serum VEGF above this level (n = 57, lower curve). b) The two curves represent patients with 6 months postoperative plasma VEGF values below or equal to 112 pg/ml (n = 278, upper curve), and patients with plasma VEGF above this level (n = 23, lower curve).

between age and VEGF concentrations ($r_s = 0.01$, $p = 0.82$, and $r_s = 0.05$, $p = 0.37$, respectively), and no significant difference in plasma or serum VEGF between men and women ($p = 0.51$ and $p = 0.61$, respectively).

In postoperative plasma and serum, there were no significant correlations between age and VEGF concentrations ($r_s = -0.05$, $p = 0.38$, and $r_s = -0.06$, $p = 0.39$, respectively), and no significant difference in plasma or

serum VEGF between men and women ($p = 0.96$ and $p = 0.40$, respectively).

Pre- and postoperatively, there were no significant correlations between age and CEA ($r_s = 0.10$, $p = 0.10$ and $r_s = -0.001$, $p = 0.98$, respectively), and no significant difference in CEA between men and women ($p = 0.25$ and $p = 0.42$, respectively).

Pre- to postoperative changes in VEGF and CEA levels

Median plasma VEGF and serum CEA concentrations decreased significantly ($p = 0.0004$ and $p < 0.0001$, respectively) 6 months postoperatively (Table 2) compared with the preoperative levels. The preoperative serum VEGF concentrations (6) were determined with ELISA kits having a different batch number from the kits used to analyse the postoperative serum VEGF concentrations. A significant increase in the postoperative serum VEGF levels was detected compared with the preoperative serum VEGF levels. In order to investigate this shift, 25 preoperative randomly selected serum samples from available samples were reanalysed using the ELISA kits with the new batch number. This re-evaluation showed that the VEGF levels in the 25 preoperative serum samples were significantly ($p = 0.01$) higher when they were determined with the new batch number. In addition, it was shown that there was a strong correlation ($r_s = 0.9$, $p < 0.0001$) between the VEGF concentrations obtained from the two different batches. These results demonstrate a consistent and systematic difference between the two batch numbers. Unfortunately, only a few of the original preoperative serum samples were available, and therefore a complete re-evaluation of all preoperative serum samples was not possible. Statistical evaluation of the differences between pre- and postoperative serum VEGF levels was therefore not done.

Univariate prognostic analyses of postoperative VEGF and CEA concentrations

High postoperative serum and plasma VEGF concentrations (> 533 pg/ml and > 112 pg/ml, respectively) had no significant ($p = 0.17$ and $p = 0.13$, respectively) impact on overall survival (Fig. 1a and 1b). In addition, whether divided into Dukes' stages or in topographical tumour localization (colon/rectum), no significant prognostic impact of high serum VEGF levels was observed in any of the subgroups. Dichotomizing the postoperative serum CEA, using the cut-point 5 ng/ml, high CEA concentrations were significantly ($p < 0.0001$) correlated with a poor prognosis (Fig. 2).

Prognostic analyses of combined postoperative serum CEA and VEGF concentrations

Six months postoperatively, patients with high serum CEA (> 5 ng/ml) and a concomitant high serum VEGF concentration (> 533 pg/ml) showed a significantly ($p = 0.03$) reduced overall survival compared with patients with high CEA (> 5 ng/ml) and low VEGF concentrations (≤ 533 pg/ml) (Fig. 3a). Furthermore, in the subgroups of patients with colon and rectal cancer, combined high serum CEA and VEGF levels significantly ($p = 0.02$) reduced overall

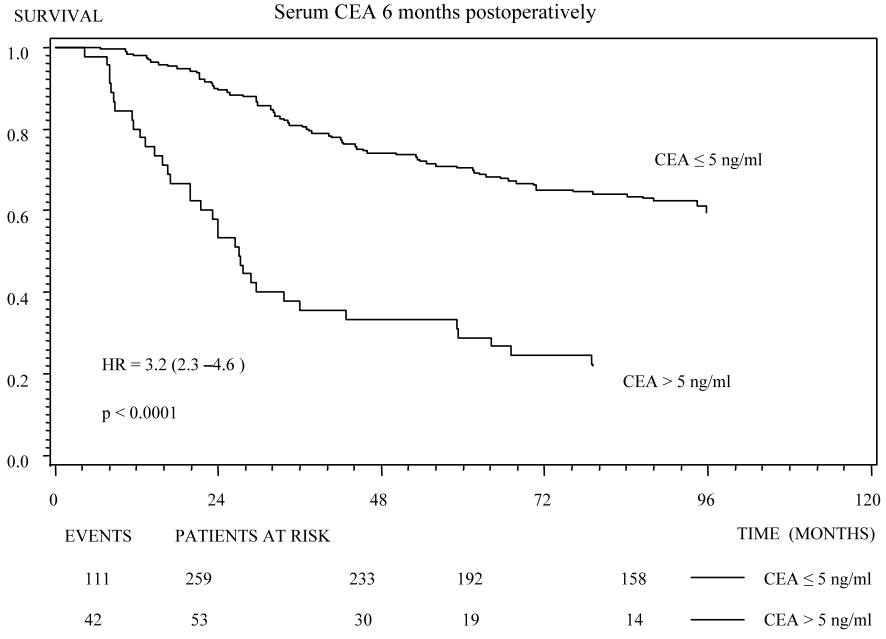

Fig. 2. Survival curves of 312 curative resected colorectal cancer patients. The threshold for elevated postoperative serum carcinoembryonic antigen (CEA) was 5.0 ng/ml. The two curves represent patients with serum CEA values below or equal to 5 ng/ml ($n = 259$, upper curve), and patients with serum CEA above this level ($n = 53$, lower curve). The number of events (deaths) in each group and the number of patients at risk (survivors) after each 24-month interval up to 72 months is indicated below the curve.

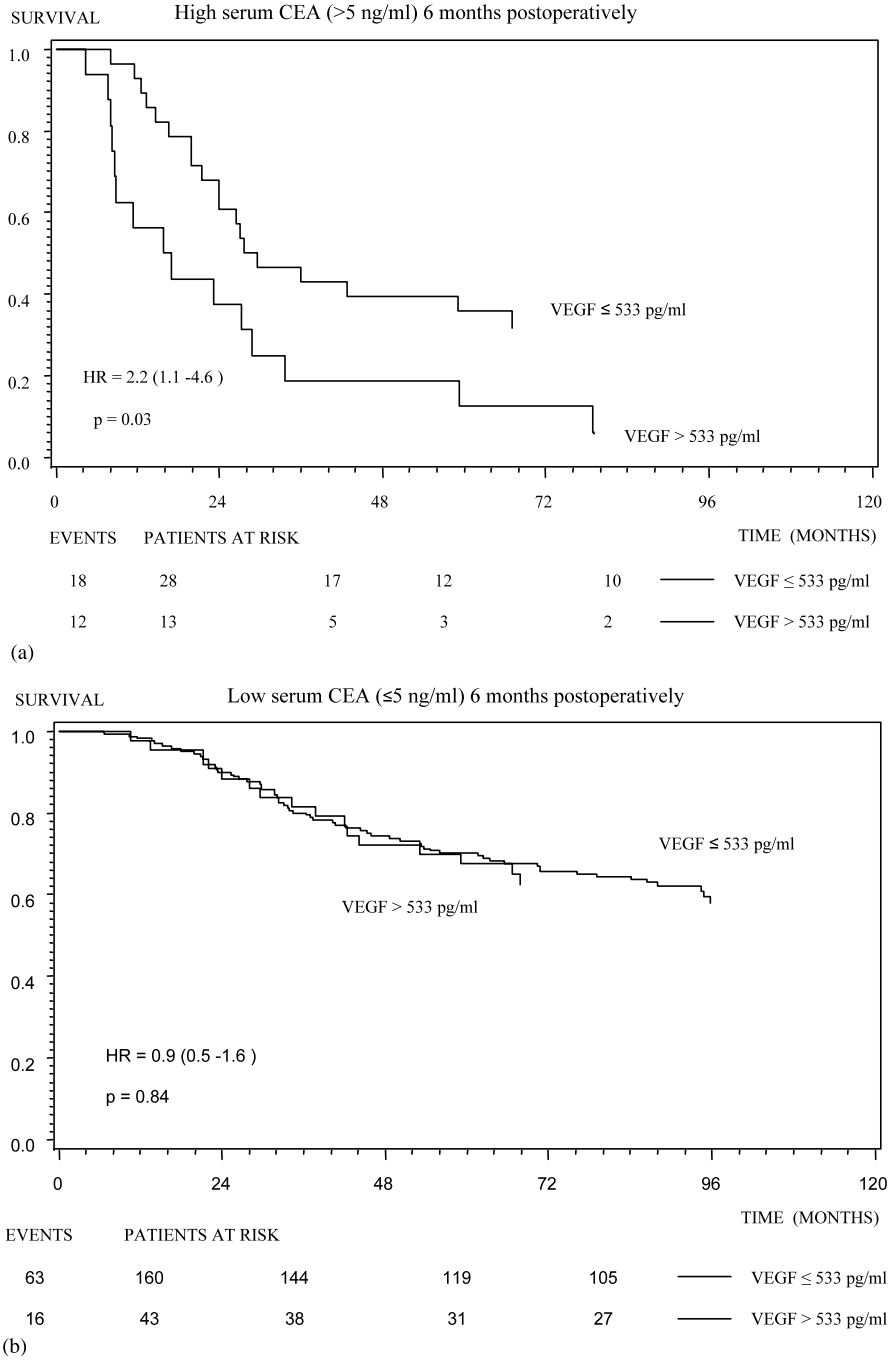

Fig. 3. a) Survival curves of 41 curative resected colorectal cancer patients with high serum carcinoembryonic antigen (CEA) (> 5 ng/ml) 6 months after surgery. The two curves represent patients with serum vascular endothelial growth factor (VEGF) values below or equal to 533 pg/ml (n = 28, upper curve), and patients with serum VEGF above this level (n = 13 lower curve). b) Survival curves of 203 curative resected colorectal cancer patients with low serum CEA (= 5 ng/ml) 6 months after surgery. The two curves represent patients with serum VEGF values below or equal to 533 pg/ml (n = 160), and patients with serum VEGF above this level (n = 43). The number of events (deaths) in each group and the number of patients at risk (survivors) after each 24-month interval up to 72 months is indicated below the curve.

survival in patients with colon cancer (Fig. 4a), while this was not observed in patients with rectal cancer (Fig. 4b). Finally, in patients with low CEA levels ($\leq$ 5 ng/ml) 6 months after surgery, the postoperative serum VEGF level had no additional impact on survival (Fig. 3b).

Multivariate survival analysis

A multivariate survival analysis including postoperative serum CEA and serum VEGF, Dukes' stage, gender, age and topographical tumour localization is presented in Table 3. High postoperative serum CEA levels signifi-

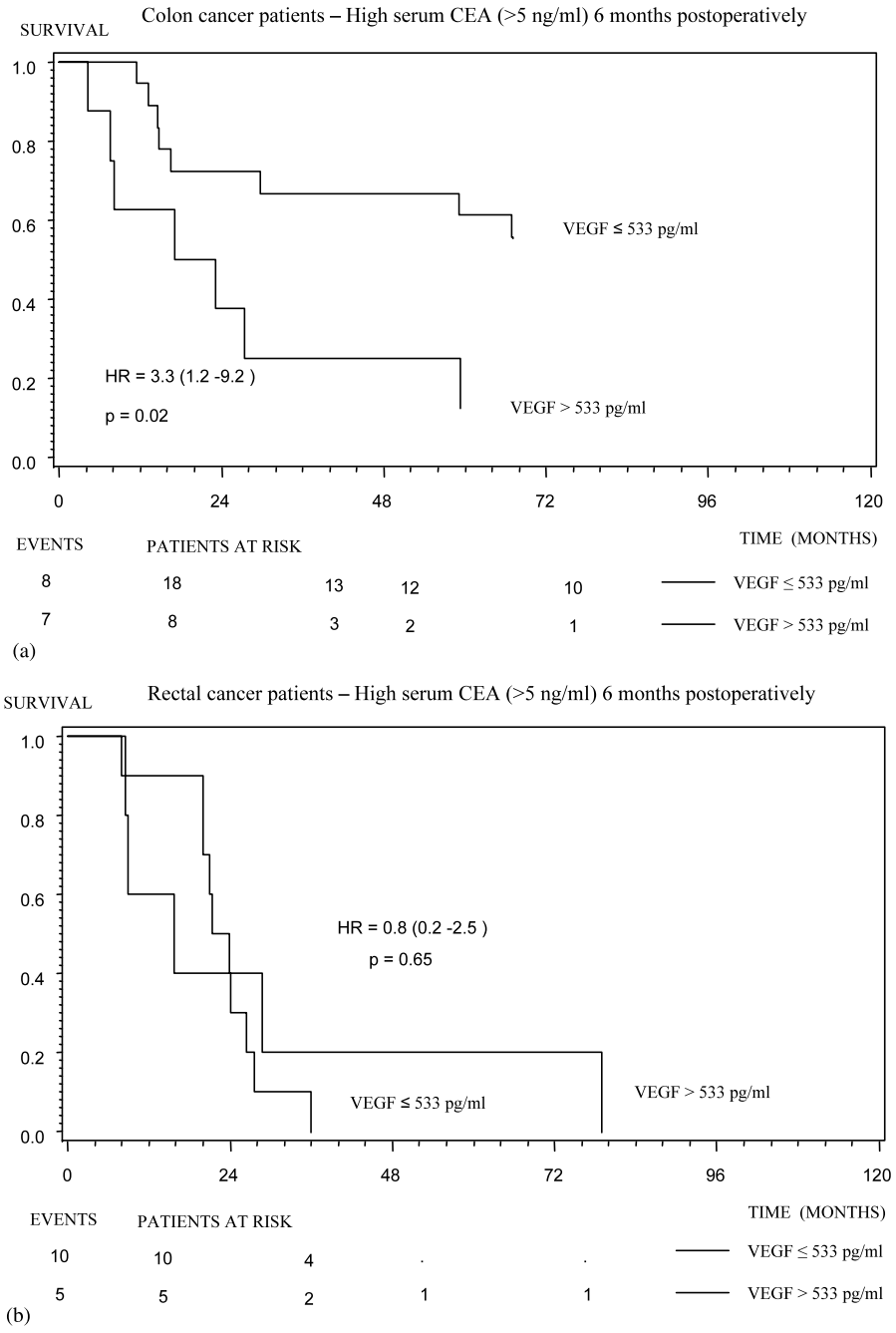

Fig. 4. a) Survival curves of 26 curative resected colon cancer patients with high serum carcinoembryonic antigen (CEA) (> 5 ng/ml) 6 months after surgery. The two curves represent patients with serum vascular endothelial growth factor (VEGF) values below or equal to 533 pg/ml (n = 18, upper curve), and patients with serum VEGF above this level (n = 8 lower curve). b) Survival curves of 15 curative resected rectal cancer patients with high serum CEA (> 5 ng/ml) 6 months after surgery. The two curves represent patients with serum VEGF values below or equal to 533 pg/ml (n = 10), and patients with serum VEGF above this level (n = 8). The number of events (deaths) in each group and the number of patients at risk (survivors) after each 24-month interval up to 80 months is indicated below the curve.

cantly ($p < 0.0001$) reduced overall survival, irrespective of Dukes' stages of disease. Six months postoperatively, the serum VEGF level had no significant ($p = 0.60$) impact on survival in the multivariate survival analyses. However, the combination of high postoperative serum CEA and high postoperative serum VEGF was significantly correlated with a poor prognosis (HR 3.0, 95% CI: 1.2–7.6, $p = 0.02$) compared to high postoperative serum CEA and low postoperative serum VEGF. In contrast, high plasma VEGF within high serum CEA had no additional impact on survival. Finally, localization in the rectum was independently and significantly ($p < 0.0001$) correlated

Table 3

Multivariate survival analysis including postoperative serum CEA, postoperative serum VEGF, Dukes' stage, gender, age and topographical tumour localization

	HR	95% CI	p-value
Dukes' A	1		
Dukes' B	1.9	0.9–4.1	0.10
Dukes' C	5.6	2.7–11.6	< 0.0001
S-VEGF > 533 pg/ml	0.9	0.5–1.5	0.60
CEA > 5 ng/ml	3.0	1.8–5.2	< 0.0001
S-VEGF > 533 within CEA > 5	3.0	1.2–7.6	0.02
Localization (rectum/colon)	2.4	1.6–3.7	< 0.0001
Gender (male/female)	1.8	1.2–2.8	0.005
Age in years	1.04	1.02–1.06	0.0002

Abbreviations: S-VEGF = Serum vascular endothelial growth factor; CEA = serum carcinoembryonic antigen; HR = hazard ratio; 95% CI = 95% confidence interval.

with a poor prognosis compared to localization in the colon.

DISCUSSION

The present study shows that, 6 months after surgery, the serum CEA concentration is a better prognostic marker than corresponding serum and plasma VEGF concentrations. However, it was also shown that the combination of high postoperative serum CEA and high postoperative serum VEGF significantly predicted a poorer prognosis than high serum CEA and low serum VEGF. This finding is in agreement with a recent study, indicating that the combination of preoperative serum CEA and VEGF significantly increases the preoperative diagnostic sensitivity (8).

In theory, increased circulating VEGF concentrations should normalize after successful surgical resection of a malignant tumour. The change in the postoperative circulating VEGF concentration might therefore reflect the successfulness of the surgery. In patients with oesophageal (9) and ovarian cancer (10), serum VEGF levels decreased significantly following tumour resection (3 months and 4 weeks postoperatively, respectively). However, in patients with CRC, the postoperative circulating VEGF concentration has not yet been evaluated as a prognostic marker. In our study, both plasma VEGF and serum CEA levels decreased significantly postoperatively. Therefore, one might also expect serum VEGF levels to decrease after successful surgery. Unfortunately, owing to the described consistent and systematic difference between the two different ELISA batch numbers, pre- and postoperative serum VEGF levels were not compared, and therefore we are not able to draw any conclusions on this matter from the present study.

Is it of any interest to determine postoperative circulating VEGF concentrations in order to make clinical decisions regarding patients with CRC? The circulating VEGF level may have prognostic impact in patients with CRC, but other circulating markers such as CEA (5), tissue polypeptide antigen (TPA) (11) and CA 19-9 (12, 13) seem to be better predictors of patient outcome. However, distinctive characteristics separate VEGF from other circulating tumour markers, which may favour the application of VEGF. Firstly, VEGF is a specific growth factor for vascular endothelial cells, and plays a decisive role during tumour vascularization (14). Secondly, in animal experiments, inhibition of VEGF or VEGF signal transduction impairs tumour-associated neovascularization (15). Thirdly, anti-VEGF drugs including neutralizing anti-VEGF antibodies (16) and VEGFR targeting agents (17) are currently being evaluated as postoperative adjuvant treatment in humans (18–20). During investigation of anti-VEGF therapy, changes in circulating VEGF concentrations should obviously be evaluated. However, the applicability of circulating VEGF concentrations to predict or monitor the response of anti-VEGF therapy has not yet been proven.

It is well established that VEGF is a key mediator of tumour vascularization. However, recently it was indicated that VEGF affects other biological functions that may be unfavourable for cancer patients. In several reports it has been suggested that increased VEGF activity influences anti-tumour immunity by impairing maturation of dendritic cells (DCs) (21). DCs are potent antigen-presenting cells critical for the induction of anti-tumour immunity (22). Thus, impaired maturation of DCs because of increased postoperative circulating VEGF concentrations may cause ineffective tumour-antigen presentation, impaired immune system recognition, and inadequate destruction of recurrent tumours (23).

It has been suggested that tumour angiogenesis is associated with recruitment of bone-marrow-derived circulating endothelial precursor cells (CEPs) (24). VEGF receptors are expressed on CEPs, and initiation of VEGFR signal transduction is critical for activation and recruitment of these cells (25). In angiogenic defective mice with impaired VEGFR-driven recruitment of CEPs, it was shown that transplantation of donor VEGFR mobilized stem cells or wild-type bone marrow restored tumour angiogenesis in implanted tumours (24). In addition, subsequent inhibition of VEGF receptors completely ablated tumour growth (24). Thus, increased circulating VEGF concentrations after surgery for CRC may facilitate recruitment of bone-marrow-derived CEPs, and may ultimately lead to increased vascularization of remaining tumour tissue or micrometastases.

Pre- and perioperative concentrations of a variety of circulating angiogenic molecules seem to have prognostic value in cancer patients (26). Determination of circulating VEGF concentrations either alone or in combination with determination of other circulating angiogenic molecules including angiogenin (27), bFGF (28) and endostatin (29)

may therefore be reliable surrogate markers of angiogenic activity that may provide prognostic or predictive information not afforded by conventional clinicopathologic parameters.

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Appendix A

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