# Increased Serum Levels of Vascular Endothelial Growth Factor in Patients with Renal Cell Carcinoma

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**Neovascularization, an essential event for the growth of solid tumors, is regulated by a number of angiogenic factors. One such factor, vascular endothelial growth factor (VEGF), is considered to exert a potent angiogenic activity, as indicated by immunohistochemical and molecular evidence. In this study we investigated the serum VEGF level (s-VEGF) in patients with renal cell carcinoma (RCC). s-VEGF in peripheral blood samples was analyzed in 40 RCC patients and 40 patients without cancer (controls) using a sandwich enzyme-linked immunoassay. In 20 RCC patients, serum samples were obtained separately from the bilateral renal veins. s-VEGF was also measured before, 4 and 8 weeks after nephrectomy in 11 patients. There were significant differences in s-VEGF between the RCC patients and the controls (207.3±32.9 vs. 71.5±9.1 pg/ml, mean±SE) (P<0.005), between the tumor-bearing renal veins and the contralateral ones (P<0.01), between the pre- and post-nephrectomy situations (P<0.01) and among the various parameters of tumor status such as tumor extent (P<0.001) and existence of metastasis (P<0.001). s-VEGF significantly correlated with the tumor volume obtained by a three-dimensional measurement (r=0.802, P<0.0001). The sensitivity and specificity of s-VEGF at the cut-off level of 100 pg/ml, as determined by the receiver-operating-characteristics curve, were 80.0% and 72.5%, respectively. The results indicate that tumor tissue of RCC liberates VEGF into the systemic blood flow and that s-VEGF is a possible marker for RCC.**

Key words:    VEGF — Serum — Renal cell carcinoma

Neovascularization is essential to the growth and metastasis of solid tumors, and is regulated by various kinds of angiogenic factors.[1] Previous studies have shown that angiogenic factors are involved in neovascularization of renal cell carcinoma (RCC),[2] brain tumor[3] and colon cancer,[4] and the microvessel density in tumor tissue correlates with the malignant potential of some solid tumors.[5–7] Vascular endothelial growth factor (VEGF), a potent angiogenic factor, specifically acts on the endothelial cells, and is involved in the proliferation, migration and permeability of these cells.[8] Recently, overexpression of *VEGF* gene and localization of VEGF protein have been shown in several solid tumors.[9–13] VEGF is  released into the extracellular matrix, acts on target cells via a paracrine mechanism and is partially liberated into the systemic blood circulation.[14] Thus, VEGF level in the circulating blood may reflect the tumor status. This study was carried out in order to determine the serum level of VEGF in patients with RCC, a representative tumor characterized by hypervascularity.

## MATERIALS AND METHODS

Blood samples were obtained before breakfast from the peripheral veins of 40 patients with RCC and 40 control subjects with no evidence of cancer or inflammatory disease. The control subjects consisted of 30 males and 10 females, with a mean age of 46.1±9.2 (SD) years ranging from 33 to 86. Characteristics of the patients are summarized in Table I. No significant difference in age or sex was observed between RCC patients and the controls. Evaluations of clinical status, including TNM classification of UICC,[15] tumor volume and tumor progression, were based on the radiological findings including plain X-ray, RI imaging, CT scan and MR imaging. The sum of tumor volume, including metastatic lesions, in each patient was calculated three-dimensionally from CT scan and/or MR imaging films. Radical nephrectomy was performed on 30 RCC patients, partial nephrectomy on 3 and selective embolization of the renal artery on 4. The remaining 3 patients underwent palliative treatments because of a far advanced stage of the disease. Sampling of the peripheral blood in the 40 RCC patients was performed before initiation of the treatments. In 11 of these patients, blood samples were also obtained 4 and 8 weeks after radical (8

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Table I. Clinical and Pathological Findings of 40 RCC Patients

| Tumor volume | | $182.8\pm47.3$ (4.3–1,267.5) ml[a] |
|---|---|---|
| Primary tumor | T1 | 4 (3)[b] |
| | T2 | 25 (23)[b] |
| | T3 | 8 (7)[b] |
| | T4 | 3 (0)[b] |
| Distant metastasis | M0 | 28 (27)[b] |
| | M1 | 12 (6)[b] |
| Pathological grade | G1 | 20 (20)[b] |
| | G2 | 10 (10)[b] |
| | G3 | 3 (3)[b] |

Patients: age $60.8\pm10.8$ (37–83) (mean±SD), M:F=28:12.
a) Mean±SD (range).
b) No. of patients subjected to partial or total nephrectomy.

patients) or partial nephrectomy (3 patients). In 20 patients, blood samples were separately obtained from the bilateral renal veins at the time of venography. Clotted blood samples were centrifuged at $1,100g$ for 10 min, and the sera were stored at $-80°C$ until analysis. Informed consent was obtained from every subject.

For quantitative analysis of VEGF, we used a human VEGF measuring kit (Immunobiological Laboratory Co., Fujioka). Serum samples (100 $\mu$l) were added to 96-well microplates coated with purified anti-human VEGF mouse IgG monoclonal antibody (16F/2E1) and kept at room temperature for 20 h. The plates were washed 7 times in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 100 $\mu$l horseradish peroxidase-labeled Fab fragment solution of anti-human VEGF-1 rabbit IgG antibody was added to each well and incubated at 37°C for 30 min. The plate was washed 9 times in PBS containing 0.05% Tween 20, then 100 $\mu$l of substrate solution containing tetramethylbenzidine and hydrogen peroxide was added to each well and the reaction was terminated by adding 1 $N$ sulfuric acid. Absorbance was measured at 450 nm using a microplate reader (EAR 400, SLT Labinstruments, Austria) and the VEGF content was determined from a standard curve derived from serially diluted recombinant human VEGF (R&D Systems, Minneapolis, MN). The VEGF standard curve was linear at concentrations from 7.8 to 500 pg/ml and the detection limit was 3 pg/ml. VEGF concentration was expressed as mean±SE. The statistical significance of differences was determined using the Wilcoxon rank sum test and the Mann-Whitney test for paired and unpaired samples, respectively. To evaluate differences in the serum VEGF levels and the influence of various clinicopathological factors, ANOVA and the $\chi^2$ test were used, respectively. The criterion of significance was set at $P<0.05$. Sensitivity and specificity were calculated from the receiver-operating-characteristics (ROC) curve.

## RESULTS

The mean serum VEGF level of RCC patients ($207.3\pm32.9$ pg/ml) was significantly higher than that of the controls ($71.5\pm9.1$ pg/ml)($P<0.01$)(Fig. 1). The sensitivity and specificity of serum VEGF at the cut-off level of 100 pg/ml given by the ROC curve were 80.0% and 72.5%, respectively. A serum VEGF level above the cut-off level was found in 29 (72.5%) of the RCC patients and in 8 (20%) of the controls ($P<0.001$). The serum VEGF level in the tumor-bearing renal vein was higher than that in the contralateral vein in 17 (85%) of 20 RCC patients, with a significant difference between the ipsilateral ($244.7\pm22.6$ pg/ml) and contralateral veins ($229.6\pm21.2$ pg/ml) ($P<0.01$). Correlations between the serum VEGF levels and parameters of the tumor status, such as existence of distant metastasis, grade and clinical stage, are shown in Table II. The mean serum VEGF levels of patients with non-invasive (T1–2) and invasive (T3–4) tumors were significantly increased as compared to that of the controls ($P<0.05$ and $P<0.001$, respectively) and a significant difference was also observed between the patients with non-invasive and invasive tumors ($P<0.001$). The mean serum VEGF level in the patients with distant metastasis ($349\pm93.7$ pg/ml) was markedly higher than that in the patients without metastasis ($150.6\pm16.0$ pg/ml)($P<0.001$). A trend of increased serum VEGF levels was seen in the patients with higher-grade tumors. The serum VEGF level was significantly correlated with the tumor volume calculated from the three-dimensional measurement ($r=0.802$, $P<0.0001$) (Fig. 2). A decrease of serum VEGF level was seen within 8 weeks after nephrectomy in all of the 11 patients analyzed, the postoperative VEGF level ($56.3\pm11.0$ pg/ml) being significantly lower than the preoperative one ($156\pm24.8$ pg/ml)($P<0.005$). Seven (78%) of 9 patients with a preoperative VEGF level above the given cut-off level (100 pg/ml) showed a normalization (<100 pg/ml) of VEGF level after nephrectomy (Fig. 3).

## DISCUSSION

Four isoforms of VEGF are regulated from a single gene by an alternative splicing mechanism, and produce characteristic signal peptides. The isoforms $VEGF_{121}$ and $VEGF_{165}$, having a low affinity for heparin, are thought to be liberated into the blood circulation as they freely diffuse within the adjacent tissue after secretion by VEGF-producing cells.[16] Recent studies revealed an elevated serum VEGF level in patients with several kinds of cancers. Kumar et al. showed that the serum VEGF level in patients with colorectal cancer was significantly elevated and correlated with clinical stage.[17] Takigawa et al. reported that the serum VEGF level in patients with lung
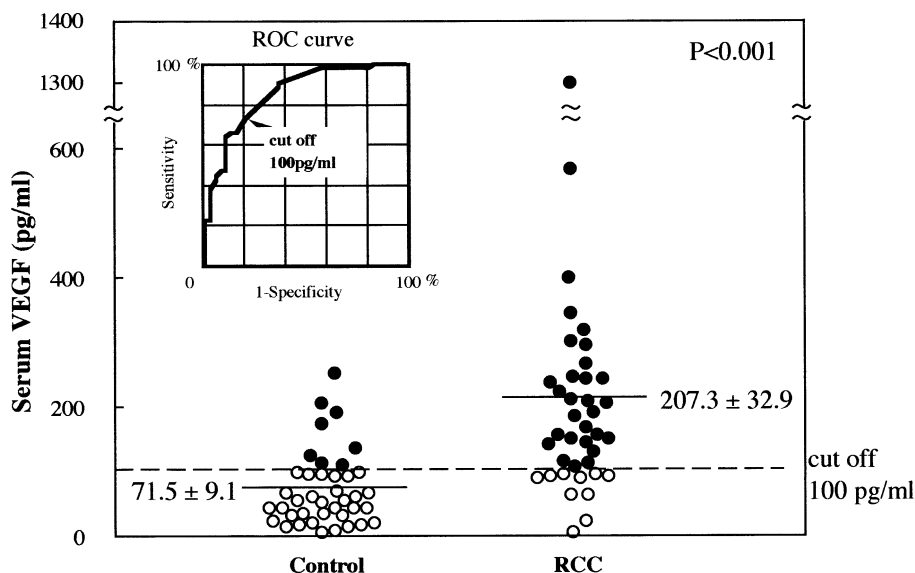
Fig. 1. Serum VEGF levels in 40 patients with RCC and 40 control subjects. There is a significant difference in VEGF levels between the two groups ($P<0.005$). ● and ○ indicate positive and negative cases for VEGF, respectively, when applying a cut-off level of 100 pg/ml with a sensitivity of 80.0% and a specificity of 72.5%. The ROC curve is inserted in the figure.

Table II. Serum VEGF Levels in RCC Patients according to Clinical and Pathological Status

| | Serum VEGF (pg/ml)[a] | Positive/total (positive rate)[b] |
|---|---|---|
| Control | 71.5±9.1 | 8/40 (20.0%) |
| RCC | 207.3±32.9[*] | 29/40 (72.5%)[*] |
| Primary tumor | | |
| T1−2 | 159.8±14.8[**] | 19/29 (65.5%) |
| T3−4 | 356.0±115.5[***] | 10/11 (90.9%) |
| Distant metastasis | | |
| M0 | 150.6±16.0 | 18/28 (66.7%) |
| M1 | 349.3±93.7[†] | 11/12 (90.0%) |
| Pathological grade | | |
| G1 | 134.7±18.6 | 15/21 (71.4%) |
| G2 | 183.6±30.5 | 6/9 (66.7%) |
| G3 | 260.0±38.1 | 3/3 (100.0%) |

a) Expressed as mean±SE.
b) Positive means above the cut-off level of 100 pg/ml VEGF.
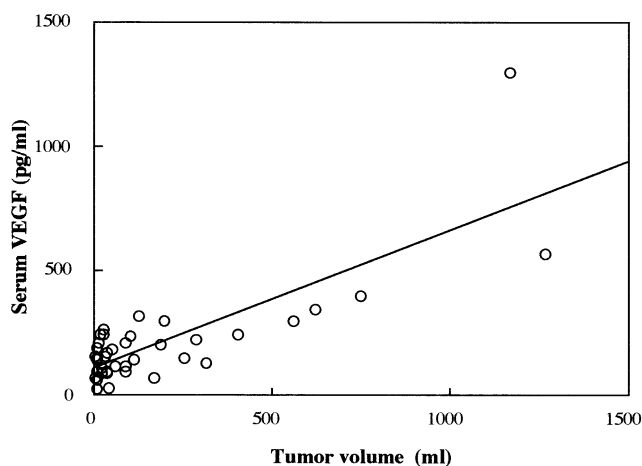* $P<0.005$ vs. control, ** $P<0.05$ vs. control, *** $P\leq0.001$ vs. T1−2, † $P<0.001$ vs. M0.



Fig. 2. Serum VEGF levels and tumor volume. VEGF levels significantly correlate with tumor volume determined by a three-dimensional measurement ($r=0.802$, $P<0.0001$).

cancer was significantly elevated, but there were no significant correlations between the serum VEGF level and pathological or clinical parameters.[18] As for RCC, overexpression of the *VEGF* gene has been observed,[10] but the serum level of VEGF protein has yet to be determined.

In this study, we found that the serum VEGF level in patients with RCC was significantly higher than that in the controls, the trend being more significant in higher stage tumors, and correlated well with the tumor volume. To our knowledge, this is the first document that describes a significant correlation between the serum level of angiogenic
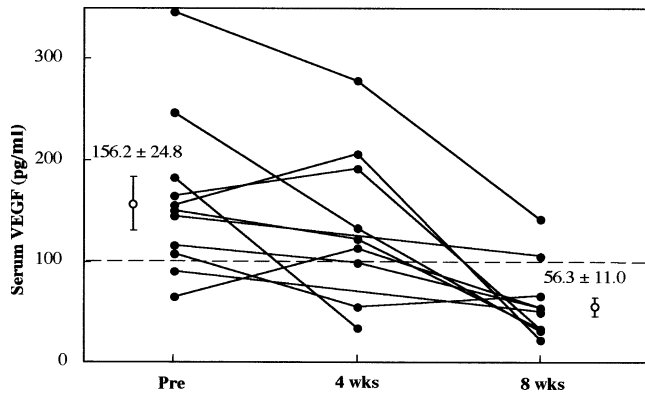
Fig. 3. Serum VEGF levels before, 4 and 8 weeks after nephrectomy. VEGF levels significantly decrease within 8 weeks after nephrectomy ($P<0.005$). Seven of 9 patients with a preoperative VEGF level above the cut-off level of 100 pg/ml show a normalization of serum VEGF (<100 pg/ml) after nephrectomy.

factors and the tumor volume assessed by a three-dimensional measurement. We also found that the VEGF level in the renal vein of the tumor-bearing kidneys was higher than that of the contralateral normal kidneys in 85% of the cases, though the difference was not large, and that the serum VEGF level significantly decreased within 8 weeks after resection of the tumors. These findings indicate the possible utility of VEGF as a biological marker of RCC.

It has been noted that VEGF liberated into the blood circulation loses its biological activity owing to immediate binding to $\alpha_2$-macroglobulin[19] and that tumor cells generally secrete various cytokines which in turn induce the expression of VEGF.[20] These results imply that the serum VEGF level in the tumor-bearing renal vein does not represent the gross amount of VEGF secreted from the RCC cells, resulting in the marginal difference in serum VEGF levels between the ipsilateral and contralateral renal veins, as observed in the present study. On the other hand, angiogenesis associated with VEGF expression occurs in various physiological events such as wound healing or formation of the endometrium and placenta.[21, 22] An increased serum VEGF level has been observed for more than 3 weeks after traumatic injury.[21] These documents also support our finding that a long period of over 4 weeks was required for normalization of the serum VEGF level after nephrectomy, a surgical trauma associated with wound healing. Finally, the serum VEGF detected in our control subjects may have come from physiological events associated with or without angiogenesis. In either case, however, it has been demonstrated by biochemical and immunohistochemical means that the cancer cells themselves produce VEGF in RCC.[23] Our previous study

showed that tumor tissue had a VEGF mRNA signal intensity of over 3 times that of adjacent normal tissue in 60% of RCCs, and the amount of mRNA expression per tumor volume did not vary in tumors with various characteristics.[10] Consequently the increase of serum VEGF observed in the present study reflects the VEGF secreted from the tumor cells.

The biological significance of the elevated serum VEGF level has not yet been fully clarified. It has been reported that the VEGF concentration in glioma and breast cancer correlates with the microvessel density in tumor tissues, which in turn correlates with tumor progression. In the previous study, we demonstrated a significant correlation between the intensity of *VEGF* gene expression and the microvessel density in RCC.[23] Thus, an elevated serum level may indicate an increased microvessel density and at the same time a malignant potential of individual tumors. Previously, immunohistochemical and serological investigations on gastrointestinal and ovarian cancer revealed a significant correlation between the VEGF status and the patient's outcome.[24, 25] Though the present study could not provide the patient's outcome because of the short follow-up period, the serum VEGF level was well correlated with the extent, histological grade and volume of RCCs. This suggests the possibility that the serum VEGF level could be used as a prognostic parameter for RCC. In fact, we have experienced a few cases in which an increase of normalized serum VEGF level during follow-up was associated with new development of distant metastases (data not shown). Further studies with a larger number of patients and long-term observation are justified to assess the usefulness of serum VEGF level as prognostic and a follow-up marker for RCC.

Fibroblast growth factor-2 (FGF-2), along with VEGF, is another representative angiogenic factor which has been widely investigated in various tumors. Some investigators have noted an elevated serum level of FGF-2 in patients with malignant tumors including RCC and breast cancers.[26, 27] Fujimoto et al.[18] reported that 53.8% of RCC patients showed an increased serum FGF-2 level, which correlated with histological findings such as venous invasion and grade of the tumors. But they did not provide any information on the FGF-2 level of normal subjects or an adequate cut-off level. Although overexpression of FGF-2 has been observed in some cell lines of RCC,[28] it has not been established whether the human RCC tumor tissues express a greater amount of FGF-2 mRNA than the corresponding normal kidney tissues. Immunohistochemical studies have shown that immunoreactivity against FGF-2 is found in both the blood vessel wall and the extracellular matrix in most RCCs, but cytoplasmic FGF-2 is present in only 16% of the tumors.[29] We have also failed to detect overexpression of *FGF-2* gene in RCC tissues and to find any difference in the serum FGF-2 protein levels between

RCC patients and the controls (14.4±1.9 vs. 14.7±3.0 pg/ml; details will be published elsewhere). These findings suggest that the serum FGF-2 level is unsuitable as a tumor marker of RCC.

The present study showed that the serum VEGF level correlated well with the tumor status, indicating tumor extent, volume, clinical stage and existence of metastasis, and significantly decreased after resection of the tumors. We conclude that serum VEGF could be a useful marker for malignant potential of RCC. In addition, considering the recent experimental advances in antiangiogenic therapy for solid tumors,[30,31] serum VEGF could be useful to monitor the therapeutic effect of novel antiangiogenic substances.

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