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ORIGINAL PAPER

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# Tumour vascular endothelial growth factor (VEGF) mRNA in relation to serum VEGF protein levels and tumour progression in human renal cell carcinoma

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**Abstract** Angiogenesis is gaining interest because of its importance in tumour growth and metastasis. Renal cell carcinoma (RCC) is known to be a well-vascularized tumour. The aim of this study was to evaluate the expression of VEGF mRNA and receptor flt-1 mRNA (VEGF R1) in a clinical material of RCCs compared with clinicopathological variables and serum VEGF levels. Total RNA was extracted from snap-frozen tumour tissue obtained from 61 patients. Expression of mRNA for $VEGF_{121}$, $VEGF_{165}$ and flt-1 were analysed using quantitative RT-PCR. Relative VEGF mRNA levels, corrected for corresponding cyclophilin value, were related to stage, grade, RCC type and survival time. Serum $VEGF_{165}$ protein was analysed using a quantitative ELISA. Papillary RCC had significantly lower $VEGF_{121}$ and flt-1 mRNA levels compared with conventional RCC ($p = 0.001$). $VEGF_{121}$ mRNA levels were significantly lower in locally advanced tumours in relation to tumours limited to the kidney and those with metastatic disease ($p = 0.047$ and $p = 0.036$). This statistical difference disappeared when only conventional RCCs were evaluated. No association was found between VEGF mRNA levels and nuclear grade. Patients with lower $VEGF_{121}$ mRNA levels had significantly longer survival time compared with those with higher levels (when adjusted to stage, $p = 0.0097$, log rank test). There was an inverse relation between $VEGF_{165}$ mRNA and serum $VEGF_{165}$ levels. The trend to lower $VEGF_{121}$ mRNA levels in locally advanced RCC indicate that angiogenic activity and degradation might be up-regulated in tumours with a high ability to invade. The association with tumour progression shows that VEGF is a promising angiogenic factor especially important in conventional RCCs. VEGF expression might possibly be of help to identify RCCs susceptible for anti-angiogenic therapies.

**Keywords** Renal cell carcinoma · mRNA · VEGF · flt-1 · RT-PCR · Prognosis

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## Introduction

Renal cell carcinoma is characterized by a variable clinical course. It is therefore of great value to identify the underlying molecular basis of the aggressive biological behaviour of individual tumours. Vascularization is one major prerequisite for tumour growth and progression. Vascular endothelial growth factor (VEGF) is considered to be one of the most important angiogenic factors in tumour angiogenesis [3, 6]. Hypoxia, as a result of tumour growth beyond a critical size, induces release of a complex cascade of growth factors including VEGF. The tumour itself also has an autocrine secretion of endothelial growth factors such as VEGF modulating and stimulating angiogenesis. The effects of VEGF are mediated through flt-1, but the role of this receptor in RCC has not been elucidated [11]. Previous studies have shown that both VEGF mRNA and VEGF protein levels were elevated in tumour compared with normal kidney tissues [27, 28]. Increased levels of VEGF mRNA have been correlated with progression and microvessel density [10, 28] while other studies found no correlation between microvessel density and clinical stage or survival in RCC [19, 25]. In a previous study, Jacobsen et al. demonstrated a significant correlation between serum VEGF levels and stage as well as outcome in patients with RCC [12]. To evaluate

whether VEGF mRNA expression is linked to tumour progression in RCC, the expression of VEGF and flt-1 mRNA were analysed using a competitive quantitative RT-PCR. These results were compared with serum VEGF protein levels and clinicopathological variables.

## Material and methods

### Patients

The study included 61 patients with histopathologically verified RCC, admitted between June 1988 and December 1999. The median age was 63.8 years (mean 65; range 25–85 years). Among the 61 patients, 56 patients were operated with radical nephrectomy, three with partial, and two with combined partial and radical nephrectomy due to bilateral RCC. Staging procedures included physical examination, chest radiography, ultrasonography, and computerized tomography. In patients with suspected vena cava tumour thrombus invasion, cavography or magnetic resonant scanning was performed. Patients with skeletal symptoms or elevated serum alkaline phosphates were assessed with bone scintigraphy. The patients were followed according to a scheduled follow-up program including clinical and radiological examinations. At the last follow-up, 23 patients were alive with a median follow-up time of 69 months, (range, 34–162 months), 32 patients had died of RCC (median survival 15; range, 1–43 months) and 6 had died of other causes (range, 22–72 months).

### Tumour stage and grade

Tumour staging was performed according to the TNM classification system 1997 [26]. There were 24 patients in stage I–II, 20 in stage III (10 pT3aN0M0 and 10 pT3N1M0) and 17 in stage IV. Histopathological nuclear grading was performed according to Skinner et al. [24] based on the worst grade. Four patients had grade 1, 7 grade 2, 41 grade 3 and 9 patients had grade 4 tumours. RCC type classification was performed according to the Heidelberg consensus conference [15]. Vein invasion was defined as tumour invasion in major renal veins verified microscopically in transverse tissue slices obtained superficially from the renal hilum. The tumour size was measured as the maximum diameter on the surgical specimen or by computerized tomography. The tumour size in largest diameter varied from 30 to 250 mm (median 80 mm).

### Tissue collection and preparation

Tumour and kidney cortex tissue samples were obtained from the surgical specimen. Each sample was divided into three parts. One part of these was snap frozen in liquid nitrogen and stored in −80°C until analysis. Another part was formalin fixed and paraffin embedded for routine morphological examination and immunohistochemical staining and the third part was used fresh for DNA flow cytometry analysis. From nine of the patients, corresponding kidney cortex tissue samples also were analysed.

### Competitive quantitative RT-PCR

Total RNA was isolated from the frozen specimens using the TRIzol method (Life Technologies, Stockholm, Sweden). Total RNA concentrations were measured spectrophotometrically at 260 nm (Lambda 2 UV/VIS, Perkin Elmer, Stockholm, Sweden). The mRNA levels of all isoforms of VEGF, flt-1 (VEGF R1) and cyclophilin were quantified by competitive RT-PCR, as described previously [9]. Primer sequences were designed to quantify all isoforms simultaneously from the human genes of VEGF-A (5′-ATC TTC AAG CCG TCC TGT GTG C-3′ and 5′- TCA CCG CCT CGG CTT GTC ACA T-3′) and flt-1 (5′-AGG AGA GGA CCT GAA ACT GTC TT-3′ and 5′-ATT CCT GGC TCT GCA GGC ATA G-3′). Briefly, 50 ng of total RNA was reverse-transcribed together with appropriate amounts of internal RNA-standards (IS), meaning truncated RNA of VEGF, flt-1 and cyclophilin. Each RNA sample was titrated with three different concentrations of IS for the respective gene (in duplicates). During 30 cycles of PCR (94°C, 30 s; 61°C, 30 s; 72°C, 45 s) templates were competitively amplified with cDNA for corresponding IS. VEGF primers used in the PCR reaction were designed for the simultaneous amplification of all VEGF isoforms. The PCR products were analysed by a laser fluorescence system (ABI PRISM 377 DNA sequencer, Perkin Elmer), and processed by the ABI PRISM GenScan software (Perkin Elmer). Messenger RNA levels were calculated from the linear regression by extrapolation at equivalent templates to IS signals as previously described. The VEGF and flt-1 values were corrected for the corresponding cyclophilin values in each RNA sample and expressed as relative levels (fmol/amol cyclophilin). Results where the double analyses showed a deviation of more than 10% was not used for statistical analysis, but were reanalysed.

### Serum VEGF analysis

Preoperative serum samples were routinely obtained from periferial veins in fasting patients before 10 am and stored at –80°C until analysis. VEGF was analysed using a commercial quantitative immunoassay kit for human $VEGF_{165}$ (Quantikine, Human VEGF immunoassay, R & D Systems, Minneapolis, MN). Briefly, 100 µl of the serum samples diluted in 100 µl buffer solution or serially diluted standard solution (human VEGF) were added to a 96-well microtiter plate precoated with mouse anti-human VEGF monoclonal antibody and incubated at room temperature for 2 h. After washing, 200 µl of the secondary antibody solution, a VEGF-specific polyclonal goat antibody was incubated for 2 h at room temperature. Substrate solution was added and the reaction continued for 25 min. Optical density was determined on a microtiter plate reader (Multiskan MCC/340, Lab Systems, Stockholm, Sweden) at 450 nm assay sensitivity is 9.0 pg/ml and the intra-assay coefficient of variation is 4.5–6.7% according to the manufacturer.

### Statistical methods

For statistical analysis, Mann-Whitney U, Kruskal-Wallis, and Spearman rank correlation tests were used. Survival curves were evaluated with the Kaplan-Meier method, and survival times were compared with the log-rank test. The survival time was measured from date of nephrectomy to date of death, or date of latest follow-up. In all tests, the significance level was set to 0.05, and all tests were two-sided.

## Results

### VEGF and flt-1 mRNA expression

All tissue samples expressed mRNA for VEGF and VEGF receptor flt-1. The VEGF 206 and 189 isoforms mRNA were expressed in very low levels and were not further analysed. Conventional RCC had significantly higher $VEGF_{121}$ mRNA and flt-1 mRNA levels compared with papillary RCC ($p = 0.001$ and $p < 0.001$, respectively). For $VEGF_{165}$ mRNA a similar trend was shown ($p = 0.075$). No correlation between $VEGF_{121}$, $VEGF_{165}$ or flt-1 mRNA levels, and nuclear grade was found (Table 1). There was no significant difference in

**Table 1** Relative VEGF and flt-1 mRNA levels, and serum VEGF protein levels in relation to RCC type

| | No. | Mean | Median | Range |
|---|---|---|---|---|
| $VEGF_{121}$ mRNA | | | | |
| Conventional RCC | 46 | 4.52 | 2.59* | 0.22–23.53 |
| Papillary RCC | 8 | 0.84 | 0.21 | 0.10–2.96 |
| Chromophobe RCC | 4 | 5.01 | 2.71 | 0.18–14.45 |
| $VEGF_{165}$ mRNA | | | | |
| Conventional RCC | 44 | 1.92 | 1.48 | 0.11–9.95 |
| Papillary RCC | 8 | 0.61 | 0.43 | 0.10–1.91 |
| Chromophobe RCC | 4 | 1.45 | 0.73 | 0.07–4.29 |
| flt-1 mRNA | | | | |
| Conventional RCC | 45 | 4.79 | 2.08** | 0.15–4.79 |
| Papillary RCC | 9 | 0.46 | 0.15 | 0.04–1.32 |
| Chromophob RCC | 4 | 0.98 | 0.94 | 0.09–1.97 |
| Serum $VEGF_{165}$ protein | | | | |
| Conventional RCC | 41 | 549.08 | 407.15 | 88.31–2261.00 |
| Papillary RCC | 9 | 568.46 | 498.60 | 148.50–2000.0 |
| Chromophob RCC | 3 | 400.14 | 197.70 | 70.43–932.28 |

Relative VEGF mRNA levels were expressed as fmol/amol cyclophilin mRNA while serum VEGF protein levels in pg/ml.
* $p = 0.001$ between conventional and papillary RCC.
** $p < 0.001$ between conventional and papillary RCC.

**Table 2** Relative VEGF and flt-1 mRNA levels, and serum VEGF protein levels in relation to nuclear grade in conventional RCCs

| | No. | Mean | Median | Range |
|---|---|---|---|---|
| $VEGF_{121}$ mRNA | | | | |
| Grade 1 | 3 | 5.08 | 3.64 | 0.68–10.92 |
| Grade 2 | 7 | 6.71 | 4.21 | 0.22–17.47 |
| Grade 3 | 28 | 4.48 | 2.58 | 0.29–23.53 |
| Grade 4 | 8 | 2.52 | 1.92 | 0.30–7.27 |
| $VEGF_{165}$ mRNA | | | | |
| Grade 1 | 3 | 3.25 | 2.51 | 0.35–6.89 |
| Grade 2 | 6 | 3.81 | 1.92 | 0.18–9.95 |
| Grade 3 | 28 | 1.64 | 1.49 | 0.11–6.84 |
| Grade 4 | 7 | 1.14 | 0.83 | 0.12–3.85 |
| flt-1 mRNA | | | | |
| Grade 1 | 2 | 9.13 | 9.13 | 7.00–11.26 |
| Grade 2 | 7 | 4.81 | 3.90 | 0.38–11.27 |
| Grade 3 | 28 | 5.26 | 1.93 | 0.15–48.40 |
| Grade 4 | 8 | 2.59 | 2.06 | 0.16–3.85 |
| Serum $VEGF_{165}$ protein | | | | |
| Grade 1 | 2 | 387.7 | 387.7 | 94.2-681.3 |
| Grade 2 | 6 | 321.8 | 302.3* | 88.3–563.2 |
| Grade 3 | 25 | 494.3 | 425.3 | 116.1-1334.0 |
| Grade 4 | 9 | 915.9 | 567.8 | 215.5–2261.0 |

Relative VEGF mRNA levels were expressed as fmol / amol cyclophilin mRNA and serum VEGF protein levels in pg/ml.
* $p = 0.071$ between grade 2 and grade 4 tumors.

serum VEGF protein levels between the different RCC types ($p = 0.786$, Table 2).

### VEGF and flt-1 expression and stage

When all conventional, papillary, and chromophobe RCCs were included, tumours confined to the kidney (pT1–2N0M0) as well as those with distant metastases (M+) had significantly higher $VEGF_{121}$ mRNA levels than locally advanced RCCs ($p = 0.047$ and $p = 0.036$, Fig. 1a). There was a similar trend for $VEGF_{165}$ mRNA as show in Fig. 1b. However, evaluating conventional RCCs only, the statistical difference in mRNA $VEGF_{121}$ levels between the stages disappeared, although a trend remained (Table 3). In contrast, for serum $VEGF_{165}$ protein levels, locally advanced tumours had higher protein levels than tumours confined to the kidney ($p = 0.008$, Table 1c). There was an inverse nonsignificant relation between serum $VEGF_{165}$ protein and $VEGF_{165}$ mRNA, as shown in Fig. 1d. A significant correlation between $VEGF_{121}$ and $VEGF_{165}$ mRNA levels ($r = 0.744$, $p < 0.001$) as well as between $VEGF_{165}$ and flt-1 mRNA levels ($r = 0.667$, $p < 0.001$) were observed (data not shown).

### Survival

Patients with higher $VEGF_{121}$ mRNA had significantly longer survival time compared with those with lower $VEGF_{121}$ mRNA levels when adjusted to stage ($p = 0.0033$, log rank test). Survival curves for patients with TNM stage III (pT3a-c,N0–1,M0) and stage IV (pT4,M1) are shown in Fig. 2a and b ($p = 0.0141$ and 0.0385, respectively). For $VEGF_{165}$ mRNA and flt-1 receptor mRNA levels, no significant survival information was found.

## Discussion

RCC is a malignancy whose behaviour in individual tumours is difficult to predict. The tumour is characterized by high vascularity, tumour thrombus formation, and frequently necrotic areas indicating association to angiogenesis. Tumour angiogenesis and its mediators have gained interest because of their association with progressive growth and metastatic potential, as observed in a large variety of solid tumours such as lung, breast, urinary bladder and prostate cancer [2, 7]. VEGF has been identified as one of the most important factors for angiogenesis [3]. In this study, it is therefore of great value to identify the underlying molecular basis of the VEGF mRNA and its relation to the biological behaviour of the malignancy.

Tumour microvessel count, which reflects the overall degree of vessel formation, has been shown to correlate with prognosis for patients with localized RCC [1, 20], whereas other studies found no prognostic value [19, 25]. This difference might be explained by the well-known heterogeneity found in RCCs [17] but might also be due to the fact that RCC contains different tumour subtypes based on specific genetic alterations [15]. In conventional RCC, 98% of the tumours have a specific deletion of chromosome *3p*, including the VHL tumour suppressor gene. Recently, it was shown that the VHL tumour-suppressor gene, which usually is mutated in conventional RCC, not only modulates growth factor levels, but also affects the hypoxic responsiveness of the tumour

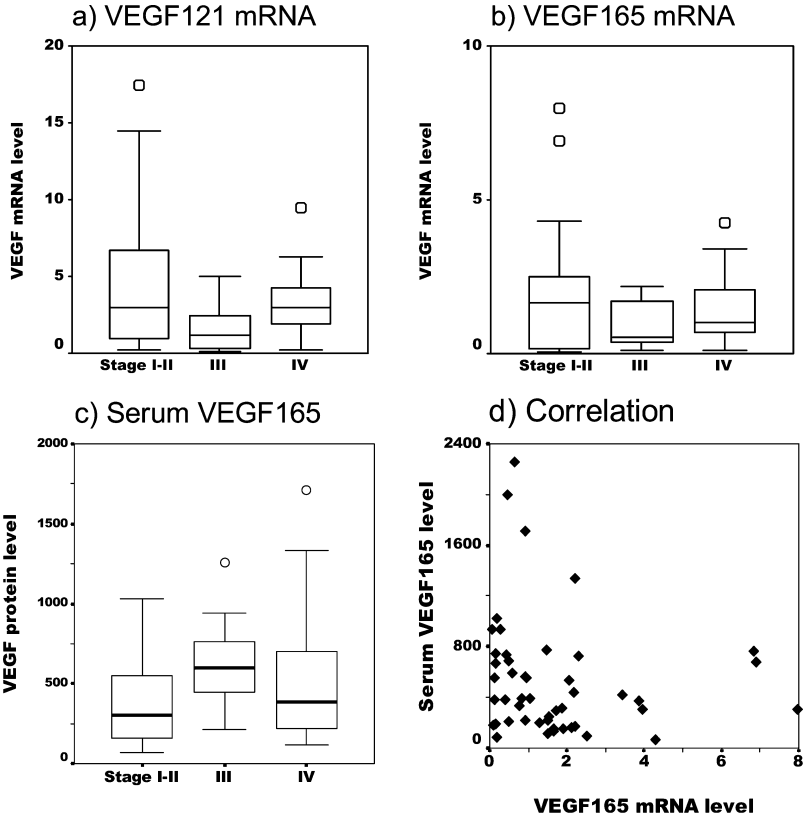


**Fig. 1a–d** Boxplot illustrating VEGF levels in relation to TNM stage in patients with RCC. **a** $VEGF_{121}$ mRNA levels; **b** $VEGF_{165}$ mRNA levels; **c** serum $VEGF_{165}$ protein levels; **d** the correlation between tumour $VEGF_{165}$ mRNA levels (expressed as fmol/amol cyclophilin mRNA) and serum $VEGF_{165}$ protein levels (pg/ml)

**Table 3** Relative VEGF and flt-1 mRNA levels, and serum VEGF protein levels in relation to to 1997 TNM tumor stage in conventional RCCs

| TNM stage | No. | Mean | Median | Range |
|---|---|---|---|---|
| $VEGF_{121}$ mRNA | | | | |
| Stage I–II (pT1–2N0M0) | 18 | 4.70 | 3.50 | 0.22–17.47 |
| Stage III (pT3a-cN0–1M0) | 14 | 2.28 | 1.71 | 0.11–7.96 |
| Stage IV (pTanyN0–1M1) | 14 | 3.69 | 2.96 | 0.18–11.36 |
| $VEGF_{165}$ mRNA | | | | |
| Stage I–II (pT1–2N0M0) | 17 | 2.28 | 1.71 | 0.11–7.96 |
| Stage III (pT3a-cN0–1M0) | 14 | 2.04 | 0.95 | 0.12–9.95 |
| Stage IV (pTanyN0–1M1) | 14 | 1.43 | 0.99 | 0.10–4.24 |
| flt-1 mRNA | | | | |
| Stage I–II (pT1–2N0M0) | 17 | 4.29 | 3.90 | 0.15–11.27 |
| Stage III (pT3a-cN0–1M0) | 14 | 5.74 | 1.46 | 0.16–48.40 |
| Stage IV (pTanyN0–1M1) | 16 | 4.13 | 1.61 | 0.15–37.79 |
| Serum $VEGF_{165}$ protein | | | | |
| Stage I–II (pT1–2N0M0) | 16 | 372.8 | 308.9* | 88.31–1027.9 |
| Stage III (pT3a-cN0–1M0) | 14 | 642.3 | 641.2 | 250.8–1256.5 |
| Stage IV (pTanyN0–1M1) | 13 | 705.8 | 389.0 | 116.10–2261.00 |

Relative VEGF mRNA levels were expressed as fmol/amol cyclophilin mRNA while serum VEGF protein levels in pg/ml.
* $p = 0.008$ between TNM stage I–II and stage III.

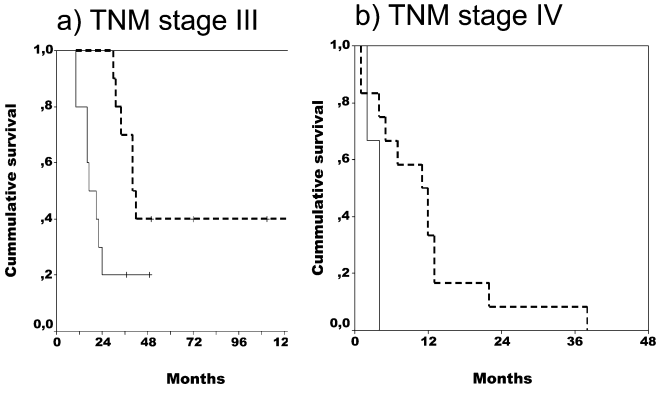


**Fig. 2** Kaplan-Meyers survival curves for: **a**) stage III tumours with $VEGF_{121}$ mRNA levels above than median value (no 10) compared with those with levels below (no 10); **b**) stage IV tumours with $VEGF_{121}$ mRNA levels above the median value (no 12) compared with those with levels below the median level (no 3)

[14, 16]. Accumulation of hypoxia inducible factor (HIF), induced by hypoxia or in VHL-mutated tumours in normoxic conditions, will increase mRNA transcription of angiogenic genes such as VEGF [5, 29]. Briegler et al. have found that conventional RCCs were more vascularized than papillary and chromophobe RCCs [3]. These latter RCC types generally have a better prognosis than conventional as previously shown [18]. In the present study, conventional RCC had significantly higher $VEGF_{121}$ mRNA levels and also a trend for increased $VEGF_{165}$ mRNA levels than papillary RCCs. We were also able to confirm previous results showing that conventional RCC, compared with papillary RCC, had significantly higher expression of the VEGF receptor flt-1, known to be induced by hypoxia [8]. It is believed that flt-1 modulates vascular growth by controlling the rate of endothelial cell division and contributes to the structural stability of the newly

developed blood vessels [13]. These results are in line with previous data showing that papillary RCCs, in contrast to the highly vascularized conventional RCCs, frequently were hypovascular [3]. The up-regulation of angiogenic growth factor receptors was more efficient in conventional compared with papillary RCC [8], and that tumour vascularization was associated to VEGF expression [3].

In this study $VEGF_{206}$ and $VEGF_{189}$ mRNA isoforms were expressed in very low quantities compared with $VEGF_{121}$ and $VEGF_{165}$. Five different isoforms of VEGF transcripts have been identified based on numbers of amino acids in the protein chains [21]. The isoforms are produced by alternative splicing from a single gene, and possess different biological activity. It has been shown that three isoforms, $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$, are highly expressed in RCC as well as in normal kidney [23]. Furthermore, biological functions and properties are different between various isoforms [28]. $VEGF_{121}$ is acidic and does not bind to heparin and is secreted in a soluble form. $VEGF_{165}$ is basic and has heparin-binding properties, while $VEGF_{189}$ is even more basic and predominantly binds to the cell surface [4]. We found that $VEGF_{121}$ mRNA was the only isoform that showed a clear association with tumour biology, although there was a significant correlation between $VEGF_{121}$ and $VEGF_{165}$ mRNA as well as with the VEGF receptor flt-1. In contrast, the observations by Tomisawa et al. indicated that $VEGF_{189}$ mRNA isoform was closely associated with angiogenesis, although increased expression of $VEGF_{121}$ and $VEGF_{165}$ isoforms were shown to stimulate the growth of RCC [28].

The present study indicates that VEGF mRNA levels seems to be lower in locally advanced tumours. This indicates that highly proliferative and invasive tumours might express lower levels of VEGF mRNA. These observations are in line with the results by Hemmerlein et al. [10], who found that $VEGF_{(121,\ 165)}$ mRNA was decreased in proliferative active RCCs. Highly proliferating tumours are likely to develop hypoxia, which is a strong stimulus of VEGF. Interestingly, there was an inverse correlation between VEGF mRNA and serum $VEGF_{165}$ levels; however, the reason for this is unclear and needs to be further evaluated. In contrast to the VEGF mRNA levels, serum VEGF levels were higher in tumours with local tumour invasion compared with tumours confined to the kidney, results in accordance with a previous study [12]. In other studies, increased serum levels of VEGF were also demonstrated in patients with locally invasive metastatic RCC [12, 22, 28]. Also, in patients with lung, breast, cervix, ovary, uterus, and colorectal carcinoma, elevated serum levels of VEGF have been observed [2, 7]. It was shown that the concentration of serum VEGF in patients with such tumours is higher in patients with a progressive and metastatic disease than in patients who respond to treatment.

In conclusion, our results showed that VEGF mRNA levels correlated inversely with serum VEGF protein levels. In addition, the trend to lower mRNA VEGF levels in locally advanced tumours indicate that angiogenic activity and metabolism might be up-regulated in tumours with a high ability to spread with metastases. The association between angiogenesis and tumour progression clearly shows that VEGF is an interesting angiogenic factor, with a need for additional characterization of its regulation in vivo. This might be a promising candidate for anti-angiogenic therapy in RCC.

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