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# Clinical relevance of serum angiogenic activity in patients with transitional cell carcinoma of the bladder

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## Abstract

Angiogenesis is essential for tumor growth and progression and is mediated by positive and negative regulators of vessel growth. Since angiogenic mediators found in patient serum have been postulated to reflect the angiogenic potential of a malignant tumor, we investigated the angiogenic activity in the serum of patients with transitional cell carcinoma (TCC). The data were correlated to tumor characteristics and the clinical course of the patients. Eightyone patients with transitional cell carcinoma and 53 control persons were included in the study. Preoperative serum samples were collected and both vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were quantified by ELISA. Additionally, the serum evoked proliferative activity on human umbilical vein endothelial cells (HUVEC) was evaluated. Data were compared to the clinical course of the patients. Serum of tumor patients significantly enhanced the proliferative capacity of HUVEC, compared to cells grown in standard culture medium ($p = 0.0032$), but not when compared to serum from control persons. Serum from patients with superficial TCC and well differentiated tumors induced a significantly higher angiogenic response ($ANG^{hi}$) than serum from patients with poorly differentiated and invasive carcinomas ($ANG^{lo}$; $p = 0.037$). VEGF level of $ANG^{hi}$ serum was $384.22 \pm 247.76$ pg/ml ($n=37$) which significantly differed from mean VEGF level detected in $ANG^{lo}$ serum ($247.72 \pm 211.93$ pg/ml, $n=42$; $p=0.019$). Similarly, mean bFGF levels were $9.58 \pm 5.91$ pg/ml in $ANG^{hi}$ serum versus $5.74 + 3.52$ pg/ml) in $ANG^{lo}$ serum ($p=0.0043$). A negative correlation was established between VEGF/bFGF serum concentration and patient prognosis. The experiments demonstrate a positive correlation between VEGF and bFGF serum level and endothelial proliferation *in vitro*. The inverse relationship between angiogenic activity and tumor stage might disclose information about angiogenesis and tumor progression in TCC.

**Keywords**: transitional cell carcinoma - angiogenesis - stimulation - angiogenic activity

## Introduction

Angiogenesis, sprouting new blood vessels from preexisting capillaries, is essential for tumor growth and progression [1, 2], either by up-regulation of angiogenic stimulators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), or by down-regulating endogenous angiogenesis inhibitors (*e. g.* thrombospondin-1, angiostatin and endostatin) [3].

The majority of bladder transitional cell carcinomas (TCC) are of the superficial type (70%), and usually do not metastasize. The rest are highly invasive tumors with strong metastatic potential [4, 5].

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Though angiogenesis-dependent growth has been documented for several tumor types, conflicting data are available with respect to bladder TCC.

Dickinson *et al.* have reported a positive correlation between tumor stage, TCC progression and tumor microvascular density [6]. Significant differences in VEGF serum level were observed in healthy controls and patients with bladder cancer, which was closely associated with tumor stage, grade, vascular invasion and carcinoma in situ [7]. Another study has demonstrated the association of pre-operative urinary VEGF levels with a risk of recurrence in patients with superficial bladder TCC, although there was no statistical correlation between VEGF levels and tumor stage (Ta or T1), tumor size or tumor grade [8]. Furthermore, Inoue *et al.* have proposed that VEGF and bFGF might serve as significant predictors of disease recurrence in bladder cancer patients [9, 10], contrary to analyses of others who have found no correlation between VEGF and bFGF content in tumor tissue and the ability of this tissue to induce angiogenesis [11].

In the present study the serum levels of VEGF and bFGF in 81 patients with transitional cell carcinoma and 53 control patients were determined. Since these mediators reflect only a limited segment of the angiogenic process we also established an in vitro activity assay to investigate the effect of patient serum on endothelial cell proliferation.

# Methods

## Patient samples

Serum of 81 patients diagnosed with transitional cell carcinoma (mean age 67.4 years, male to female ratio 3:1) and of 53 control persons (healthy volunteers and patients with kidney stones; mean age 42.2 years, male to female ratio 3:1) were collected. Serum from cancer patients was collected before any surgical procedures were undertaken. Within one hour after collection, blood samples were centrifuged at 3000 rpm for 10 min at 4°C. The serum samples were aliquoted and frozen at -20°C until use.

Isolation and cultivation of human umbilical vein endothelial cells (HUVEC). Fresh umbilical cords were obtained from the Department of Obstetrics, University of Frankfurt am Main. The umbilical vein was rinsed with 50ml of PBS without $Ca^{2+}$ and $Mg^{2+}$ (Life Technologies GibcoBRL, Karlsruhe, Germany). Hereafter, the vein was incubated with 2 ml α-chymotrase (Stathmann AG, Hamburg, Germany) for 20 min at room temperature. Subsequently, the vein was flushed with 50ml Medium 199 (Sigma, München, Germany) and the endothelial cells were collected in a 50ml Falcon tube (Becton Dickinson, Heidelberg, Germany). The suspension was centrifuged at 1200 rpm at room temperature for 10 min. and the pellet was resuspended in 5ml Medium 199 containing 2% Hepes buffer, 500IU heparin / 500ml, 0.2% antibiotics, 10% fetal calf serum (Life Technologies), 10% human serum (Deutscher Blutspendedienst, Frankfurt, Germany) and 2ml endothelial cell growth factor (final concentration 20 µl/ml, Life Technologies). The suspension was pipetted into a 10ml Falcon tissue culture flask and allowed to settle for 24h in an incubator at 37°C and 5% $CO_2$. Fresh medium was added on the next day.

## HUVEC cell growth

HUVEC cultures were trypsinized (Trypsin/EDTA, Pan Systems, Aidenbach, Germany) and resuspended in endothelial cell medium. 4000 viable cells, identified by the Trypan-blue test, were transferred into each well of a 96-well plate. The cells were allowed to attach for 24 h in a humidified atmosphere at 37°C and 5% $CO_2$. Subsequently, the wells were washed with PBS (Sigma) and then incubated for 72h with culture medium enriched with serum samples (tumor versus control patients) at a final concentration of 10%. Cell culture medium enriched with 10% bovine calf serum (BCS, Life Technologies) was used as the standard control.

Proliferative activity of tumor cells and HUVEC were estimated by the PicoGreen assay [12]. Cells were washed and dried after 72h. Cells were then digested with papain (0.125 mg protein/ml) for 20h at 60°C. Fluorescent dye PicoGreen (MoBiTec, Goettingen, Germany), which shows high specificity for dsDNA, was then added (1:200 dilution) for 10 min at 20°C. Fluorescence intensity was determined using a computer-controlled fluorescence reader (Cytofluor 2300 plate scanner; Millipore, Eschborn, Germany) at $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 530 nm.

Serum concentrations for bFGF and VEGF: Serum bFGF and VEGF concentrations of patients with transitional cell carcinoma and of control persons were

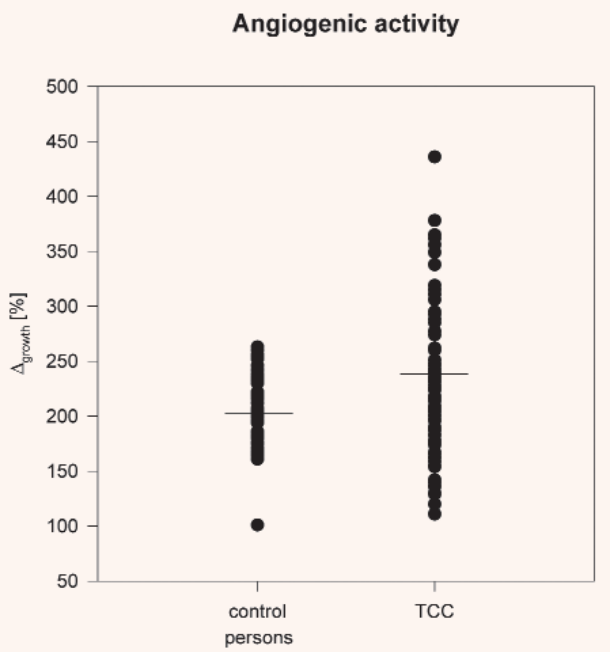


**Fig. 1** Angiogenic activity of serum from control persons versus tumor patients. Y-axis shows endothelial cell growth, given as difference compared to the 100% standard control. Both angiogenic activity of control and tumor patient serum was significantly elevated with respect to the standard control ($p < 0.05$).

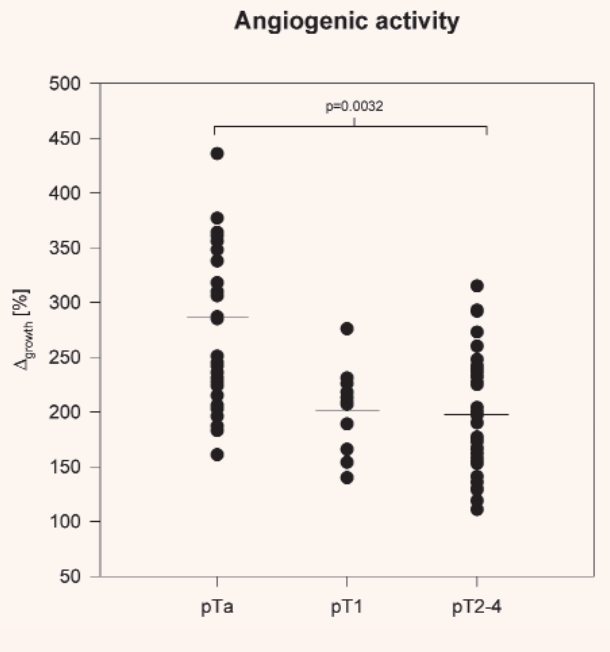


**Fig. 2** Angiogenic activity of serum taken from patients with pTa, pT1 and pT2–4 tumors. Activity of pTa serum was significantly higher than activity of pT1–4 serum. Y-axis indicates endothelial cell growth, given as difference compared to the 100% standard control.

determined using standard 96-well ELISA plates (R&D Systems, Abingdon, UK) according to the manufacturer's guidelines.

### Statistics

Data are presented as median and ranges. All calculations were performed using the double-sided Student's t-test. Results were considered to be significantly different at $p < 0.05$.

## Results

### Cancer patients characteristics

All tumors were histologically evaluated after removal, and both tumor stage and tumor grading were determined according to the TNM classification (UICC, 1987) (Table 1).

### Endothelial cell proliferation assays

Addition of TCC serum to HUVEC led to enhanced HUVEC proliferation (mean: 225.07 ± 66.12% (min 111.27%, max. 435.64%), compared to the standard control which was set to 100%. Serum from control persons evoked a mean HUVEC stimulation of 204.86 ± 30.82% (min 101.66%, max 263.56%), compared to the standard control (100%) (Fig. 1). No difference was observed between volunteers and patients with kidney stones.

The mean endothelial cell stimulatory activity of serum taken from patients with superficial pTa tumors (n = 24) was 270.30 ± 72.32%, compared to the standard control (100%, p=0.0008). Interestingly, the pTa evoked stimulatory activity on HUVEC was significantly higher than HUVEC stimulation triggered by serum of invasive pT2-4 tumors (mean: 197.95 ± 52.43%; p = 0.0032; n = 40). Serum angiogenic activity of early invasive tumors (pT1, n = 12) ranged between serum activity of superficial and invasive pT2-4 tumors (mean: 202.64 ± 38.67%) (Fig. 2).

**Table 1** Documentation of tumor characteristics of the 81 patients included in the study. Tumor classification was performed according to the TNM classification (UICC, 1987). N = number of cases, % = percent of the total of 81 tumor patients, N1-2 / M1 = metastasis to lymph nodes, liver, lungs and bones.

| Tumor characteristic | n | % |
|---|---|---|
| pTa | 24 | 29,6 |
| pT1 | 12 | 14,3 |
| pT2 | 10 | 12,3 |
| pT3 | 23 | 28,4 |
| pT4 | 7 | 8,6 |
| Tis | 3 | 3,7 |
| Tx | 2 | 2,5 |
| G1 | 11 | 13,6 |
| G2 | 31 | 38,3 |
| G3 | 29 | 25,8 |
| G4 | 3 | 3,7 |
| Gx | 7 | 8,6 |
| N1-2 / M1 | 12 | 14,8 |

According to tumor grading, well differentiated tumors (G1, n = 11) demonstrated a significantly higher angiogenic activity compared to moderately and poorly differentiated (G2-4, n = 63) tumors (median: 285.19%; range 181.18 – 435.64; versus 213.19%, range 111.27 - 377.24; p = 0.035) (Fig. 3).

Serum of patients mith metastatic disease did not demonstrate significant differences in endothelial cell stimulation, compared to serum from patients with localized tumors.

## Serum bFGF and VEGF concentration

Patient samples were divided into those with high angiogenic activity ($ANG^{hi}$; defined by HUVEC growth > mean value of all TCC samples) and low angiogenic activity ($ANG^{lo}$; defined by HUVEC growth < mean value of all TCC samples), and VEGF and bFGF serum concentrations were determined in both subgroups. The mean VEGF level of $ANG^{hi}$ serum was 384.22 ± 247.76 pg/ml (n = 37), which significantly differed from the mean VEGF level detected in $ANG^{lo}$ serum (247.72 ± 211.93 pg/ml, n=42; p=0.019) (Fig. 4). The mean bFGF levels was 9.58 ± 5.91 pg/ml in $ANG^{hi}$ serum versus 5.74 ± 3.52 pg/ml in $ANG^{lo}$ serum (p=0.0043) (Fig. 5).

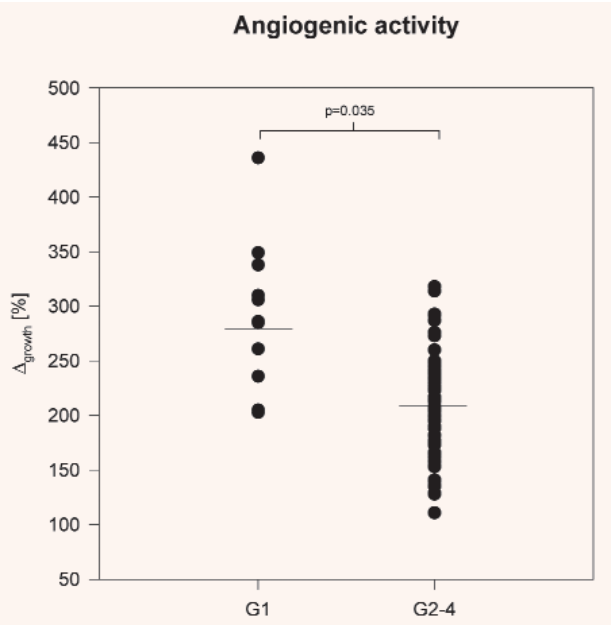


**Fig. 3** Comparative analysis of angiogenic activity of patients with G1 versus G2–4 tumors. Activity of G1 serum was significantly higher than activity of G2–4 serum. Y-axis indicates endothelial cell growth, given as difference compared to the 100% standard control.

## Follow-up studies

A follow-up investigation after 2 years was performed in tumor patients who had the highest (352.3 ± 26.5% HUVEC-growth; n = 10) and the lowest (135 + 10.1% HUVEC-growth; n = 10) serum angiogenic activity (p < 0.01). Mean VEGF in high and low growth groups were 428.1 ± 305.3 and 265.2 ± 168.9 pg/ml, respectively (p > 0.05). Mean bFGF levels in high and low growth groups were 12.3 ± 5.6 and 4.8 ± 1.7 pg/ml, respectively (p < 0.05).

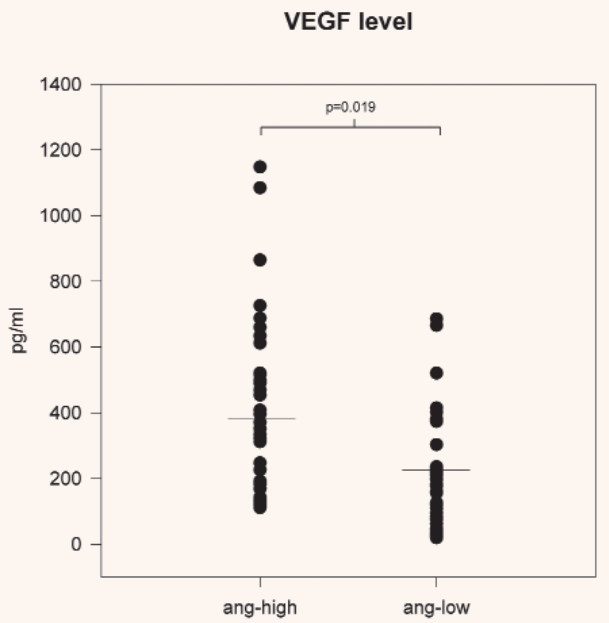


**Fig. 4** Quantification of VEGF in high (ang-high; HUVEC growth > mean value of HUVEC growth induced by all TCC samples) versus low angiogenic activity serum (ang-low; HUVEC growth < mean value of HUVEC growth induced by all TCC samples). VEGF level was significantly enhanced in ang-high serum samples.

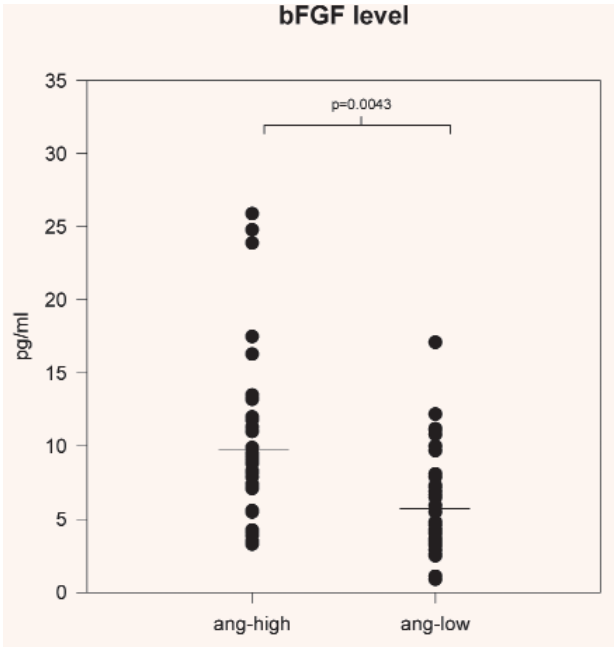


**Fig. 5** Quantification of bFGF in high (ang-high; HUVEC growth > mean value of HUVEC growth induced by all TCC samples) versus low angiogenic activity serum (ang-low; HUVEC growth < mean value of HUVEC growth induced by all TCC samples). bFGF level was significantly enhanced in ang-high serum samples.

Of the ten patients with high angiogenic activity one had metastases at the time of surgery and none developed metastases within the two year follow-up. Two of the patients died within two years after surgery, one of them due to metastatic progression of the carcinoma. Of the ten patients with low angiogenic activity none had metastases at the time of surgery but three developed metastases within two years. All three patients died within two years after surgery. The remaining patients were free of recurrence and progression at the end of the observation period.

## Discussion

Angiogenesis is the process by which tumours provide their blood supply, crucial for growth and progression. Increased expression of angiogenic factors, such as VEGF, bFGF and microvessel density, might identifiy patients who are at high risk of developing metastases. However, the relationship of angiogenesis and disease progression in patients with TCC is not yet clear.

In this study we investigated the diagnostic value of the serum angiogenic activity in patients with transitional cell carcinoma. The results of this method seem to be a functional and useful approach in the clarification of angiogenic profiles.

Interestingly, serum from patients with superficial (pTa) and well differentiated tumors displayed a significantly higher angiogenic activity than patients with invasive and poorly differentiated tumors. These results were unexpected and do not correspond to the leading academic opinion concerning angiogenesis in connection with tumor aggressiveness and progression. In agreement with our findings, Quentin and coworkers reported that low-stage superficial TCC expressed VEGF mRNA at a significantly higher level than high-stage muscle invasive carcinomas, and low-grade TCC at a higher level than high-grade tumors [13].

Oliveira-Ferrer *et al.* observed silencing of the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) in superficial bladder cancer (pTa) and transitional cell carcinoma *in situ* (pTis) which was coupled to induced angiogenesis via increased expression of VEGF [14].

Based on our in vitro assay, the results indicate that serum angiogenic activity might be a prognostic factor in superficial TCC. Furthermore, the drop of angiogenic activity in the group of pT2–4/G2–4 TCC may represent a crucial event during urothelial carcinogenesis, and possibly indicates an important step in promoting the conversion of bladder cancer from low to high malignancy. Therefore, the detection of angiogenic activity might be a useful "marker" for the diagnosis of the early stages of bladder cancer and for the assessment of tumor progression from a preinvasive to an invasive phenotype.

Angiogenic activity of patient serum significantly correlated with serum levels of VEGF and bFGF in our study. This finding highlights the relevance of pro-angiogenic cytokines in the process of endothelial stimulation and cell growth. Nevertheless, immunohistochemical evaluation of tumor tissue revealed a reduced vascular destabilization and decreased formation of new blood vessels in advanced TCC, suggesting a balance between vessel regression and vascular growth, with a less pronounced vascular remodeling during late phases of urothelial carcinogenesis [14]. Therefore, anti-angiogenic factors should also be considered important regulatory elements in TCC.

In observing the clinical course of patients, we found that 30% of patients with low serum angiogenic activity, free of metastases at surgery, developed distant metastases and died within the follow-up period. None of the patients with high angiogenic activity, free of metastases at surgery, showed progression to metastatic disease. This is in line with the hypothesis presented by Oliveira-Ferrer *et al.* who supposed that VEGF down-regulation preceeds TCC progression from a non-invasive to an invasive phenotype [15]. Larger patient studies should further evaluate whether there is indeed a higher risk of disease progression for patients showing low serum angiogenic activity.

More than 20 years ago, Prout and colleagues observed progressive tumors and distant metastases in a significant number of TCC patients after radical cystectomy [15]. It was argued that tumor dissemination was caused by single tumor cells distributed into the vasculature during the surgical procedure. Growth inhibiting factors, released by the primary tumor, were also speculated to play a role in metastatic progression. In this context, removal of the primary tumor, paralleled by a loss of growth inhibiting cytokines, might trigger the development of secondary tumors. Indeed, the angiogenesis inhibitor angiostatin, produced by the primary tumor, led to the inhibition of metastatic growth, whereas rapid progression became evident after resection of the primary tumor [16]. This model should also be taken into consideration when interpreting our patient data. Possibly, low serum angiogenic activity might be caused by the systemic release of an angiogenesis inhibitor by the primary TCC. After resection of the primary tumor the antiangiogenic activity is lost and metastatic progression begins. Still, this assumption is speculative and needs further investigation.

In summary, our *in vitro* analysis of serum triggered endothelial cell growth might be a valuable tool to reflect tumor progression in TCC patients. Patients characterized by a low serum angiogenic activity seem to be at high risk of developing aggressive and invasive tumors. Therefore, these patients need close follow-up. An enlarged prospective study will further elucidate the prognostic relevance of low serum angiogenic activity in TCC patients.

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