VEGF-C, VEGF-D and Flt-4 in Transitional Bladder Cancer: Relationships to Clinicopathological Parameters and Long-term Survival

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Abstract. *Background: Our aim was to determine the role of the lymphangiogenic markers VEGF-C, VEGF-D and Flt-4 in transitional bladder cancer. Materials and Methods: Archival cystectomy tumor blocks of 286 patients were selected for construction of a tissue microarray (TMA). Paraffin sections were assessed immunohistochemically using polyclonal antibodies against VEGF-C, VEGF-D and Flt-4. Staining results were evaluated semiquantitatively and analyzed for their association with various clinicopathological factors. Results: There was no association of VEGF-C with histopathological parameters or clinical outcome. Patients with VEGF-D overexpression had higher pathological tumor stages (p=0.021) and regional lymph node metastasis (p=0.008). Furthermore, they had a significantly reduced disease-free survival (p=0.042). Overexpression of Flt-4 was particularly present in the subgroup of G3 and G4 tumors (p=0.001) and was associated with a shorter disease-free survival (p=0.041). In multivariate analysis, only tumor stage and lymph node metastasis were independent prognostic parameters. Conclusion: Targeting VEGF-D and Flt-4 could be a useful tool to predict and control progression of bladder cancer.*

Bladder cancer is the fourth most common cancer in men and eighth most common in women. In the United States, an estimated 61,420 new cases of bladder cancer were diagnosed and approximately 13,060 deaths were attributed to bladder cancer in 2006 (1). Despite the drop in smoking rates in the US, the age-adjusted incidence rates for both men and women have stayed the same or risen slightly since the 1980s (2). Transitional cell carcinoma (TCC) is the most common histological type in western countries, with approximately 90% of bladder tumors arising from the urothelium. Metastatic spread to regional lymph nodes is an early step in the systemic dissemination of TCCs and lymph node metastasis is generally associated with poor survival (3).

The mechanisms of lymphangiogenesis in bladder cancer are complex. The best validated signalling system for tumor lymphangiogenesis involves the secreted vascular endothelial growth factors (VEGF)-C and VEGF-D that signal *via* their tyrosine kinase receptor Flt-4 by autophosphorylation. The VEGFs have been shown to promote tumor lymphangiogenesis, the metastatic spread of tumor cells to lymph nodes and, in some cases, distant organ metastasis in multiple animal models of cancer (4). Furthermore, expression of these growth factors correlated with lymph node metastasis in different human cancers (3). Recently, VEGF-D and Flt-4 were reported to be independent prognostic markers in gastric adenocarcinoma and the presence of VEGF-D was associated with lymphatic metastasis in this tumor type (5). It seems that the VEGF-C / -D / Flt-4 signalling system is currently the most attractive target for antilymphangiogenic therapeutics designed to restrict cancer metastasis (6).

There are few reports on VEGF-C, VEGF-D and Flt-4 overexpression in bladder cancer (7-10). Results are controversial and their impact on clinicopathological features is unclear. Therefore this study shall further extend the knowledge of VEGF-C, VEGF-D and Flt-4 in invasive bladder cancer and its association with pathological parameters and prognosis in a large group of patients after cystectomy.

Materials and Methods

Patients. A total of 286 patients treated with radical cystectomy for invasive transitional cell carcinoma of the bladder at our institution were selected from our bladder cancer database (11). Surgery had been carried out between February 1990 and June 2005. The

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median age of patients was 62 years (range 31-81 years; 191 men, 95 women). None of the patients had received neo-adjuvant treatment before cystectomy. Histological slides and formalin-fixed, paraffin-embedded tumor tissue blocks from all 286 patients were obtained from the files at the Institute of Pathology (University of Münster). All slides were reviewed and representative tumor tissue blocks were selected for construction of a tissue microarray (TMA). A database comprising histopathological and clinical data according to the TNM system was created (Table I).

For each of the 286 samples, a representative tumor block was selected as donor block for TMA. Using an H&E-stained slide, two morphologically representative regions were defined for each of the 286 tumor samples. From these regions, cylindrical core tissue specimens (diameter=0.6 mm) were obtained and arrayed precisely into a new recipient paraffin block (20x35 mm) using a custom-built precision instrument (Beecher Instruments, Silver Spring, MD, USA). From the 572 tumor core specimens available, six tissue array blocks were prepared.

Immunohistochemistry for VEGF-C, VEGF-D and Flt-4. The paraffin-embedded tumor tissue blocks were cut into 3-μm slices and mounted on poly-L-lysine-coated glass slides. Tissue slides were dewaxed in xylene, rehydrated in a graded series of alcohol and rinsed in 0.01 M tris buffer (pH 7.3). Immunohistochemical staining for VEGF-C, VEGF-D and Flt-4 was performed in a multistep semiautomatic procedure. Briefly, two polyclonal rabbit antibodies for VEGF-C and Flt-4 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) at a dilution of 1:100 and 1:800, respectively, were incubated for 25 min. For VEGF-D, a polyclonal rabbit antibody (Zytomed, Berlin, Germany) was applied at a 1:100 dilution (25 min) after pretreatment with a steamer for antigen retrieval (Multi-Gourmet, Braun, no. 3216). After washing with phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.3), sections were exposed to biotinylated secondary antibody for 30 min, followed by streptavidin-conjugated peroxidase for another 30 min. Diaminobenzidine was applied as the final chromogen and nuclei were counterstained with haematoxylin to facilitate microscopic assessment. For the negative control, the primary antibody was replaced with PBS. No significant immunohistochemical reaction occurred in the control sections. Specimens were considered positive for statistical evaluation when >25% of carcinoma cells were clearly (moderately or strongly) stained (12).

Quantification. Semiquantitative analysis of staining results from 572 tissue array cores was performed by two investigators blinded to the histopathological data for the corresponding case. Varying numbers of tissue cores were detached, others did not contain a sufficient number of tumor cells; therefore some cases could not be analyzed. For evaluation of VEGF-C, VEGF-D and Flt-4 (all showing a cytoplasmatic and/or nuclear immunostaining expression), intensity of immunostaining was scored semiquantitatively on a four-tiered scale (negative=0, weak=1, moderate=2, strong=3). Samples with a moderate (2+) or strong (3+) immunostaining intensity were defined as having an elevated expression of this marker and thus to be "positive", respectively (12) (Figure 1).

Data analysis. For statistical analysis, the Statistical Package for the Social Sciences for Windows, version 13.0 (SPSS Inc, Chicago, IL, USA) was used. All histopathological parameters were tested for their relationships with staining results by means of cross-tables applying Pearson's χ^2 test and Fisher's exact test. The Kaplan-Meier method was used to derive the cumulative overall and disease-free survival and the log-rank test to compare curves for two or more groups. For multivariate analysis of prognostic factors, a Cox regression analysis was performed. A p-value <0.05 was considered to indicate significant differences between groups.

Table I. *Distribution of pathological and clinical variables in the reported series of bladder cancer specimens (n=286).*

Pathological parameter	n (%)
pT stage[a]	
T1	41 (14.5)
T2	70 (24.8)
T3	133 (47.2)
T4	38 (13.5)
pN stage[b]	
N0	221 (77.3)
N1	34 (11.9)
N2	30 (10.5)
N3	1 (0.3)
cM stage[c]	
M0	272 (95.4)
M1	13 (4.6)
Grade[d]	
G1	6 (2.1)
G2	86 (30.2)
G3	187 (65.6)
G4	6 (2.1)

Information was available in [a]98.6%, [b]100%, [c]99.3%, [d]99.7% of cases, respectively.

Results

Immunohistochemical overexpression of VEGF-C, VEGF-D and Flt-4. From the 286 patients, VEGF-C, VEGF-D and Flt-4 staining status was available in 261 (91.3%), 262 (91.6%) and 257 (89.9%) cases, respectively. In the missing cases, tissue had been lost during steamer pre-treatment or the core samples did not contain a sufficient number of tumor cells. Staining intensity of VEGF-C, VEGF-D and Flt-4 among different samples varied from complete absence of staining to moderate staining (VEGF-C) and strong staining (VEGF-D, Flt-4). VEGF-C overexpression was identified in 24.1% of cases; overexpression of VEGF-D and Flt-4 was found more often (37.4% and 46.3%, respectively). Normal urothelium was consistently negative for staining. A comprehensive summary of the distribution patterns of VEGF-C, VEGF-D and Flt-4 associated with clinicopathological parameters is shown in Table II.

Association with clinicopathological parameters. VEGF-C overexpression was not associated with tumor stage, lymph node status, distant metastasis or histological grading.

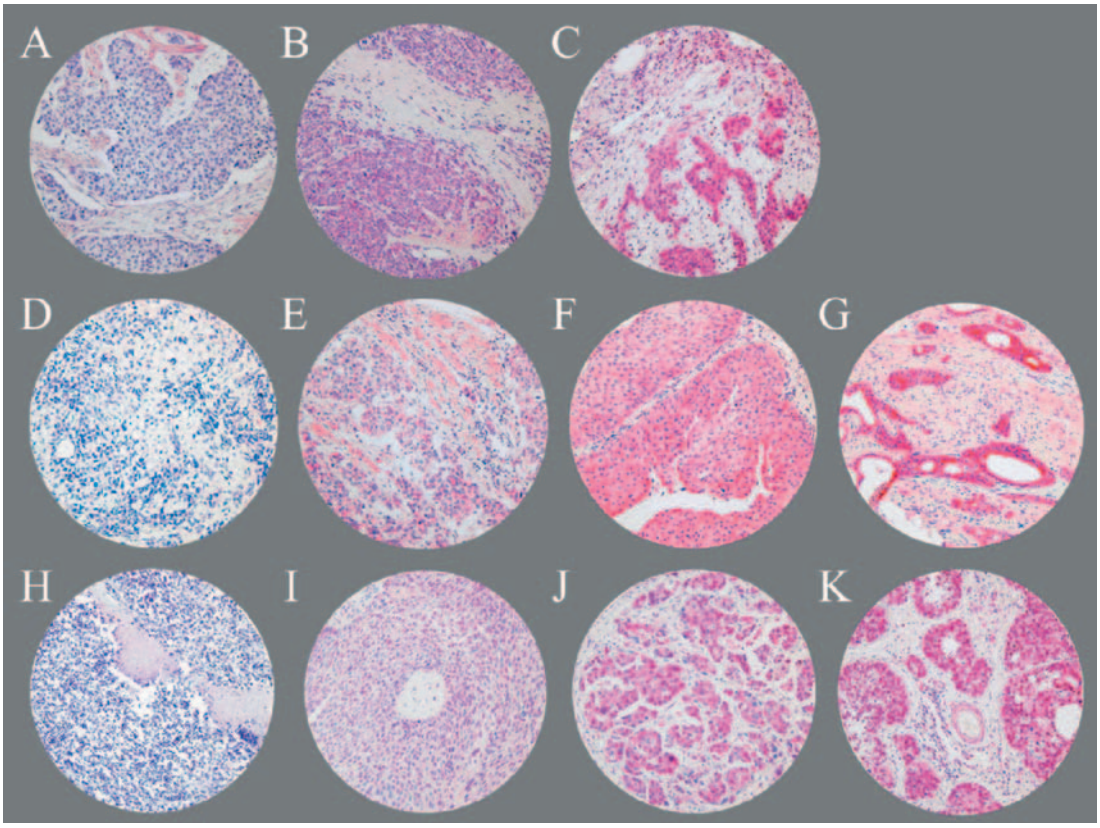

Figure 1. *Representative examples of staining for VEGF-C (A, negative; B, weak; C, moderate), VEGF-D (D, negative; E, weak; F, moderate; G, strong) and Flt-4 (H, negative; I, weak; J, moderate; K, strong) (Figures reduced from original magnification to x20).*

Moreover, there was no association of VEGF-C status with other clinical parameters such as coincidence of an incidental prostate cancer.

VEGF-D overexpression was associated with higher tumor stages ($p=0.021$). Especially in pT4 tumors, overexpression of VEGF-D was found in 26/36 (72.2%) cases. Furthermore, higher VEGF-D expression was related to higher regional lymph node metastasis ($p=0.008$). Only 67/201 (33.3%) patients without regional lymph node metastasis overexpressed VEGF-D, whereas 31/61 (50.8%) patients with metastatic lymph nodes showed overexpression of VEGF-D. Additionally, this association was even more distinct in the subgroup of patients with lymph node metastasis and poorly differentiated tumors (G3) where 26/40 (65.0%) were VEGF-D-positive ($p=0.001$). Patients with distant metastasis tended to overexpress VEGF-D compared to patients without distant metastasis. However, this difference was not significant ($p=0.183$).

Flt-4 was related to histological grading. Patients with high-grade tumors (≥G3) showed up-regulated Flt-4 expression compared to low-grade tumors (92/172 (53.5%) *vs.* 26/84 (31.0%)) ($p=0.001$). Although there was an increase of Flt-4 overexpression with advancing tumor stage, results were not significant ($p=0.074$). Furthermore, no association was found for Flt-4 with lymph node stage or distant metastasis. Patients with incidental prostate carcinomas in their cystoprostatectomy specimens tended to overexpress Flt-4; however, results were not significant ($p=0.152$).

Survival analysis. All patients were followed up for a minimum of 16 months (median: 63 months, range 16-151 months). The median overall and disease-free survival times were 30 months (95% confidence interval (CI): 23-37 months) and 27 months (95% CI: 17-37 months), respectively.

Using Kaplan-Meier survival analysis and the log-rank test for comparison of survival curves, stratification of patients according to VEGF-C did not reveal any significant differences in disease-free or overall survival times (Figure 2A). Patients with overexpression of VEGF-D had a significantly reduced disease-free survival ($p=0.042$) compared to those without VEGF-D overexpression (Figure 2B). There was no association of VEGF-D overexpression with overall survival ($p=0.282$). Stratifying survival for all

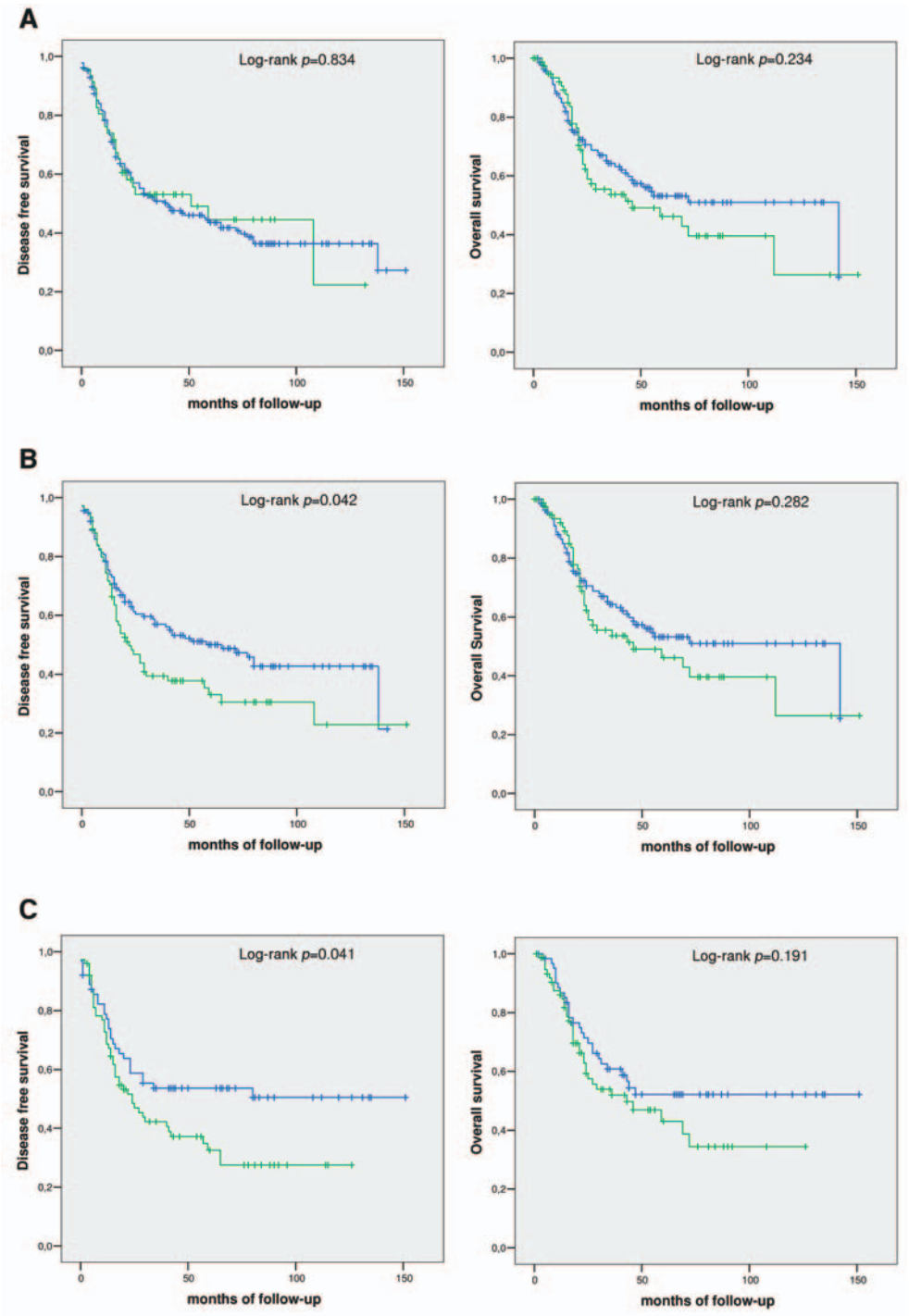

Figure 2. *Disease-free and overall survival in relation to expression of VEGF-C (A), VEGF-D (B) and Flt-4 (high-grade tumors G≥3) (C) (blue line: negative, green line: positive).*

VEGF-D expression levels (0, 1+, 2+ and 3+), we found an improved survival with decreasing VEGF-D expression, although statistical significance was not reached ($p=0.090$). In the subgroup of VEGF-D-positive patients with regional lymph node metastasis, again no significant results regarding disease-free or overall survival were found ($p=0.122$ and 0.220, respectively). Patients with Flt-4 overexpression did not show any significant differences in

Table II. *Expression of VEGF-C, VEGF-D and Flt-4 in association with clinicopathological variables.*

Pathological variable	VEGF-C staining n positive/total (%)	*P*-value	VEGF-D staining n positive/total (%)	*P*-value	Flt-4 staining n positive/total (%)	*P*-value
pT stage		0.843		0.021		0.074
T1	10/33 (30.3%)		14/33 (42.4%)		12/31 (38.7%)	
T2	16/62 (25.8%)		19/63 (30.2%)		27/63 (42.9%)	
T3	25/127 (19.7%)		39/127 (30.7%)		57/124 (46.0%)	
T4	12/36 (33.3%)		26/36 (72.2%)		22/36 (61.1%)	
pN stage		0.655		0.008		0.946
N0	48/200 (24.0%)		67/201 (33.3%)		92/197 (46.7%)	
N1	3/31 (9.7%)		13/31 (41.9%)		12/31 (38.7%)	
N2	12/29 (41.4%)		17/29 (58.6%)		14/28 (50.0%)	
N3	0/1 (0%)		1/1 (100%)		1/1 (100%)	
cM stage		1.000		0.183		0.346
M0	60/249 (24.1%)		92/251 (36.7%)		116/246 (47.2%)	
M1	3/11 (27.3%)		6/10 (60.0%)		3/10 (30.0%)	
Grade		0.701		0.804		0.001
G1	1/6 (16.7%)		1/6 (16.7%)		2/6 (33.3%)	
G2	22/78 (28.2%)		32/80 (40.0%)		24/78 (30.8%)	
G3	37/170 (21.8%)		61/169 (36.1%)		89/167 (53.3%)	
G4	3/6 (50.0%)		4/6 (66.7%)		3/5 (60.0%)	
Incidental PCAa		0.771		0.465		0.152
Yes	18/65 (27.7%)		22/66 (33.3%)		36/66 (54.5%)	
No	26/116 (22.4%)		40/118 (33.9%)		49/116 (42.2%)	

[a]Incidental PCA=Incidental prostate cancer. *P*-values derived from Pearson's χ^2 test and Fisher's exact test

survival curves. In the subgroup of high-grade tumors (G≥3), statistical significance was reached regarding disease-free survival ($p=0.041$) (Figure 2C), which was even greater when expression levels were stratified and calculated according to level ($p=0.030$). Flt-4 positive patients tended to have an impaired survival, although statistical significance was not reached either before stratifying for expression levels ($p=0.141$), or after ($p=0.118$). In the subgroup of patients with incidental prostate cancer in their cystoprostatectomy specimens, no prognostic impact could be seen for positive VEGF-C, VEGF-D or Flt-4 expression according disease-free and overall survival.

Additionally, multivariate Cox-regression analysis was performed (Table III). Both tumor and lymph node stage were independent prognostic parameters ($p=0.026$ and $p<0.001$, respectively), but VEGF-C, VEGF-D and Flt-4 were not.

Table III. *Multivariate analysis of prognostic factors in 286 bladder cancer patients.*

Overall survival, all patients (n=286)			
Prognostic variable	Risk ratio	95% CI	Statistical significance
VEGF-C	1.074	0.645-1.788	0.784
VEGF-D	1.277	0.617-1.548	0.321
Flt-4	1.184	0.625-1.550	0.345
T	1.404	1.042-1.892	0.026
N	1.837	1.392-2.422	<0.001
M	0.396	0.138-1.130	0.083
G	1.093	0.722-1.654	0.675
PCA	1.695	0.984-2.919	0.057

Discussion

The progression of malignant tumors is associated with invasive growth to peripheral tissue and metastasis to lymph nodes through the lymphatics, or metastasis of distant organs by the vasculature. Pelvic lymph node involvement is usually the first sign of metastasis in many bladder cancer cases followed by the metastasis to other structures and organs (13). In this process, tumor cells may produce many factors that promote angio- and lymphangiogenesis in order to obtain a sufficient nutritive supply as well as providing pathways for dissemination (14). A range of experimental studies in animal models demonstrated that the VEGF-C/VEGF-D/Flt-4 signalling axis can promote tumor lymphangiogenesis and the metastatic spread of tumor cells (6). Recent clinicopathological studies have reported that expression of VEGF-C, VEGF-D or Flt-4 is correlated with lymph node

metastasis in a variety of human cancers (3, 15). According to these studies, VEGF-C expression in tumor cells is associated with lymph node metastasis in lung, colorectal and prostate cancer, as well as in melanoma (16). Interestingly, in melanoma, the level of lymphangiogensis as well as the level of VEGF-C expression were associated with the primary tumor's risk of metastasis to sentinel lymph nodes (17). VEGF-D was described as a prognostic marker in colorectal cancer and was associated with lymphatic involvement (18). In ovarian carcinoma, VEGF-D was a predictor of poor outcome (19). In the latest study to date, VEGF-D and Flt-4 were reported to be independent prognostic parameters in gastric adenocarcinoma and the presence of VEGF-D was correlated with lymphatic metastasis in this tumor type (5).

To date, the role of VEGF-D, VEGF-C and Flt-4 in invasive bladder carcinoma has not been fully elucidated. Some studies in the past two years evaluated lymphangiogenic marker expression with differing results. In 2005, Suzuki *et al.* were the first to describe VEGF-C as an important predictive factor of pelvic lymph node metastasis in bladder cancer patients in a series of 87 cystectomy specimens (8). Miyata *et al.* partly supported these results investigating VEGF-C and VEGF-D expression in 126 patients who had undergone transurethral resections of mainly superficial bladder carcinomas (10). They concluded that lymphangiogenesis influences metastasis-free survival and is regulated by VEGF-C and VEGF-D (10). Zu *et al.* were able to show a significant association of VEGF-C overexpression with a markedly poorer prognosis in a series of 45 patients, and demonstrated that VEGF-C expression was an exclusive independent factor influencing pelvic lymph node metastasis (7). However, at the same time Mylona *et al.* reported VEGF-C in a large series of 123 specimens of transitional cell carcinoma of the bladder to be inversely correlated with tumor stage. In univariate analysis, VEGF-C-positive patients were even associated with a better prognosis, which was contradictory to all former findings (9).

In our study, VEGF-C did not have an effect on clinical outcome either, but VEGF-D was associated with unfavorable tumor stages and reduced disease-free survival. Furthermore, VEGF-D overexpression predicted pelvic lymph node metastasis. Although VEGF-D-positive patients tended to have a shorter overall survival, significant results were not seen. Flt-4 was overexpressed in high-grade tumors and was associated with a reduced disease-free survival. In multivariate analysis, tumor stage and lymph node metastasis were independent prognostic parameters.

Our results suggest that VEGF-D and Flt-4 are linked to lymphatic dissemination and progression of bladder cancer. The association between VEGF-D expression, lymphatic tumor spread and poor prognosis is in agreement with the established clinical observation that lymphatic dissemination is closely related to the clinical outcome of patients with bladder cancer (20). Furthermore, the absence of VEGF-D and Flt-4 expression possibly identifies patients with favorable survival who may therefore need specific therapeutic and/or surveillance consideration. In addition to these pathobiological implications, our results demonstrate that overexpression of VEGF-D and/or Flt-4 in bladder carcinoma is closely associated with differences as to a patient's clinical outcome and therefore should be added to existing diagnostic systems in bladder cancer. Further confirmation to support these findings is required; however, assessment of VEGF-D and Flt-4 patients is a useful approach to establish risk-adapted therapeutic strategies.

Surprisingly, VEGF-C overexpression did not show negative effects on patients' clinical outcome, as described in many previous studies regarding bladder cancer (7, 8, 10). Only in the latest study to date was a positive association between VEGF-C expression and long-term survival seen (9). These contradictory results indicate that the association between VEGF-C as well as its impact on cancer biology and a patient's prognosis are not understood and need further investigation. Our findings for bladder cancer are in line with other tumor entities such as gastric cancer, esophageal and colorectal cancer that also did not show a significant relationship between VEGF-C and survival (21-23).

The VEGFs exist in different molecular isoforms. As yet it is unclear which isoforms are present in bladder cancer and whether differences in the molecular compositions of the VEGFs may be related to different clinical outcomes. Moreover, further clarification is needed regarding the system of proteolytic processing responsible for cleavage of VEGF peptides. This system is little understood and needs further investigation (24). Although we cannot discuss such phenomena with respect to our study population because neither molecular subtypes of VEGF-C, VEGF-D and Flt-4 were examined, nor was proteolysis, we imagine that the answer to these important questions might contribute to future findings regarding the value of the lymphangiogenic markers VEGF-C, VEGF-D and Flt-4. We therefore encourage investigators to assess VEGF isoforms and related proteases in relation to clinical outcomes, which may further extend the diagnostic and prognostic knowledge about these markers.

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