Evaluation of the Vascular Endothelial Growth Factor (VEGF)-C Role in Urothelial Carcinomas of the Bladder

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Abstract. *Background: Vascular endothelial growth factor-C (VEGF-C) has been associated with angiogenesis, lymphangiogenesis and regional lymph node metastasis and was reported to have an anti-apoptotic and proliferative role. Materials and Methods: An immunohistochemical study was applied to 123 specimens of bladder urothelial carcinoma (BUC) to detect VEGF-C and investigate its clinicopathological and prognostic value. VEGF-C-immunostained BUC specimens (123) were statistically correlated with histological grade and stage, patient overall survival and immuno-expression of Ki-67 and bax proteins. Results: VEGF-C immunopositivity (27/123 BUCs, 22.0%) was inversely correlated with tumor stage and bax immunoexpression ($p=0.007$ and $p=0.032$, respectively). VEGF-C-positive BUCs tended to have better prognosis (univariate analysis). Conclusion: VEGF-C might be associated with an anti-apoptotic phenotype. Our controversial results regarding patient survival suggest that the role of VEGF-C in BUC progression and prognosis remains to be clarified.*

The vascular endothelial growth factor-C (VEGF-C), a new member of the VEGF family, activates VEGF receptor-2 (VEGFR-2, KDR) and VEGF receptor-3 (VEGFR-3, Flt-4) (1). It is believed to be a lymphangiogenic factor in adults, as well as, to a lesser degree, an angiogenic factor (2). Except for the endothelial cells, VEGF-C and its receptors were found to be expressed in cancerous cells themselves, raising the possibility that it may affect cancer growth not only by stimulating lymph/angiogenesis, but also by directly acting on receptors on the cancer cells (3). There are very few reports on the effect of VEGF-C on the basic functions of proliferation and apoptosis in malignant cells (4, 5).

The prognostic significance of VEGF-C was investigated in several forms of human cancer, such as gastric carcinoma (6), colorectal carcinoma (7), non-small cell lung cancer (8), human breast cancer (9, 10), ovarian cancer (11) and pancreatic cancer (12).

To our knowledge, there are only two studies concerning VEGF-C presence in bladder cancer (13, 14). VEGF-C expression in bladder urothelial carcinoma (BUC) was significantly correlated with pelvic lymph node metastasis and poor prognosis (13), and lymphangiogenesis and angiogenesis were suggested to be regulated by VEGF-C and/or VEGF-D (14). Therefore, the precise role of VEGF-C in bladder cancer has not been clearly understood. The aim of the present study was to examine VEGF-C expression along with classic clinicopathological parameters and to investigate any possible anti-apoptotic or proliferative role of VEGF-C in BUC.

Materials and Methods

One hundred and twenty-three patients [mean age at diagnosis 68.99 years (age range, 31-89 years)] with BUC were included in this study. Formalin-fixed tissue samples were obtained from transurethral resections of the bladder or cystectomies. According to the WHO classification, BUCs were graded 1, 2, 3 and local invasion was classified into two groups, pTa-T1 and pT2-4 (15). Patients were observed for survival analysis; the median follow-up was 82.24 months (range 1-193) during which 51 patients had died of BUC.

Immunohistochemistry was used to detect the expression levels of VEGF-C. Immunohistochemical staining for VEGF-C was performed on 4-μm thick formalin-fixed paraffin sections using an avidin-biotin immunoperoxidase technique. Briefly, the sections were dewaxed, rehydrated and treated for quenching of endogenous peroxidase activity. To enhance antigen retrieval, the sections were microwave-treated in 10 mM citrate buffer pH 6.0 at 750 W for 2 cycles of 5 min each. After blocking non-specific binding, sections were incubated overnight at 4˚C with a goat

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Table I. *Relationship between VEGF-C expression and clinicopathological characteristics in 123 patients with urothelial carcinoma of the urinary bladder.*

Characteristic	Positive expression of VEGF-C			
	No. of patients	N	%	*P*
Total no.	123	96	78	
Gender				
male	107	24	22.4	1.000[a]
female	16	3	18.8	
Histological grade				
1	24	8	33.3	
2	40	9	22.5	0.261
3	59	10	16.9	
Local invasion				
pTa-T1	71	22	31.0	**0.007[a]**
pT2-T4	52	5	9.6	
Bax expression				
negative (<20% of cancer cells)	42	12	28.6	**0.032[a]**
positive (≥20% of cancer cells)	30	2	6.7	
Ki-67				
negative (<10% of cancer cells)	67	12	17.9	0.363[a]
positive (≥10% of cancer cells)	50	13	26.0	

[a]Fisher's exact test.

polyclonal antibody to VEGF-C (sc-1881), raised against peptide mapping at the carboxy terminus of VEGF-C of human origin (Santa Cruz Biotechnology, CA, USA) at a dilution of 1:60. The sections were incubated in biotinylated horse anti-goat secondary antibody, followed by peroxidase-conjugated avidin-biotin complex (Vectastatin Elite ABC Kit, Vector Lab, Burlingame, CA, USA). 3',3'-Diaminobenzidine tetrahydrochloride was used as a chromogen. Finally, sections were counterstained with Harris hematoxylin and were mounted.

For negative controls, the primary antibody was omitted. As positive controls, formalin-fixed paraffin-embedded sections from normal human placenta were stained for VEGF-C. For statistical reasons, specimens were considered VEGF-C-negative when less than 10% of cancer cells were stained and as VEGF-C-positive when equal to or greater than 10% of cancer cells were stained, as previously described (13).

With regard to VEGF-C immunostaining, statistical correlations were investigated with classic clinicopathological prognostic indicators, patient overall survival and several immunohistochemical markers expression (Ki-67 and bax) (16, 17). The latter data were available from our archives.

Statistics. The relationships between the expression of VEGF-C and clinicopathological parameters and other categorical parameters of interest were evaluated by Fisher's exact test or Pearson's χ^2 test, as indicated. Overall survival distribution curves were assessed by the Kaplan-Meier test and log-rank statistics followed by Cox's proportional hazards regression model.

Table II. *Contribution of parameters of statistical significance to patient overall survival via step-wise forward Cox's proportional hazard regression (HR) model.*

	B	SE	df	Sig.	HR	95% CI for HR	
						Lower	Upper
All cases							
stage	1.002	0.193	1	<0.0001	2.723	1.865	3.975
age	0.051	0.020	1	0.010	1.053	1.012	1.095
Ta-T1							
Ki-67	0.932	0.544	1	0.028	3.297	1.136	9.572
age	0.073	0.035	1	0.037	1.076	1.004	1.152
T2-T3-T4							
age	0.051	0.025	1	0.040	1.052	1.002	1.105

Results

VEGF-C was expressed in tumor cell cytoplasm, faintly in normal urothelial cell cytoplasm (Figure 1A) and also in stromal fibroblasts. Of the 123 BUCs, 27 (22.0%) were VEGF-C-positive, whereas 96 (78.0%) were VEGF-C-negative. In immunopositive cases, a diffuse cytoplasmic staining pattern was observed (Figure 1B, C, D) in the tumor cells. Staining intensity was generally heterogeneous among the positive cells, ranging from moderate reactivity to a dark brown reaction product.

In the total pool of specimens (Table I), Fisher's exact test indicated that VEGF-C immunoreactivity was inversely correlated with tumor pathological stage at a statistically significant level ($p=0.007$). In all patients, VEGF-C reactivity was negatively associated with bax immunoexpression (Fisher's exact test $p=0.032$). VEGF-C expression was unrelated to both classic prognostic factors, *i.e.,* tumor grade (Table I) and Ki-67.

Whether VEGF-C expression directly influenced patient survival was investigated. Kaplan-Meier survival analysis followed by the log-rank test showed that the patients with VEGF-C-positive tumors tended to have better prognosis than those with VEGF-C-negative tumors, however, there were no statistically significant differences ($p=0.1020$). A Cox proportional hazards model was constructed using established prognostic factors and VEGF-C expression. The analysis showed that patient tumor pathological stage ($p<0.0001$) and age ($p=0.010$), but not VEGF-C expression ($p=0.245$), were independent prognostic parameters. Interestingly, when patients with superficial disease (*i.e.,* Ta-T1 BUCs) were separately examined, Ki-67 expression (Cox regression model $p=0.028$) and patient age ($p=0.037$) emerged, as significant independent adverse prognostic factors (Table II).

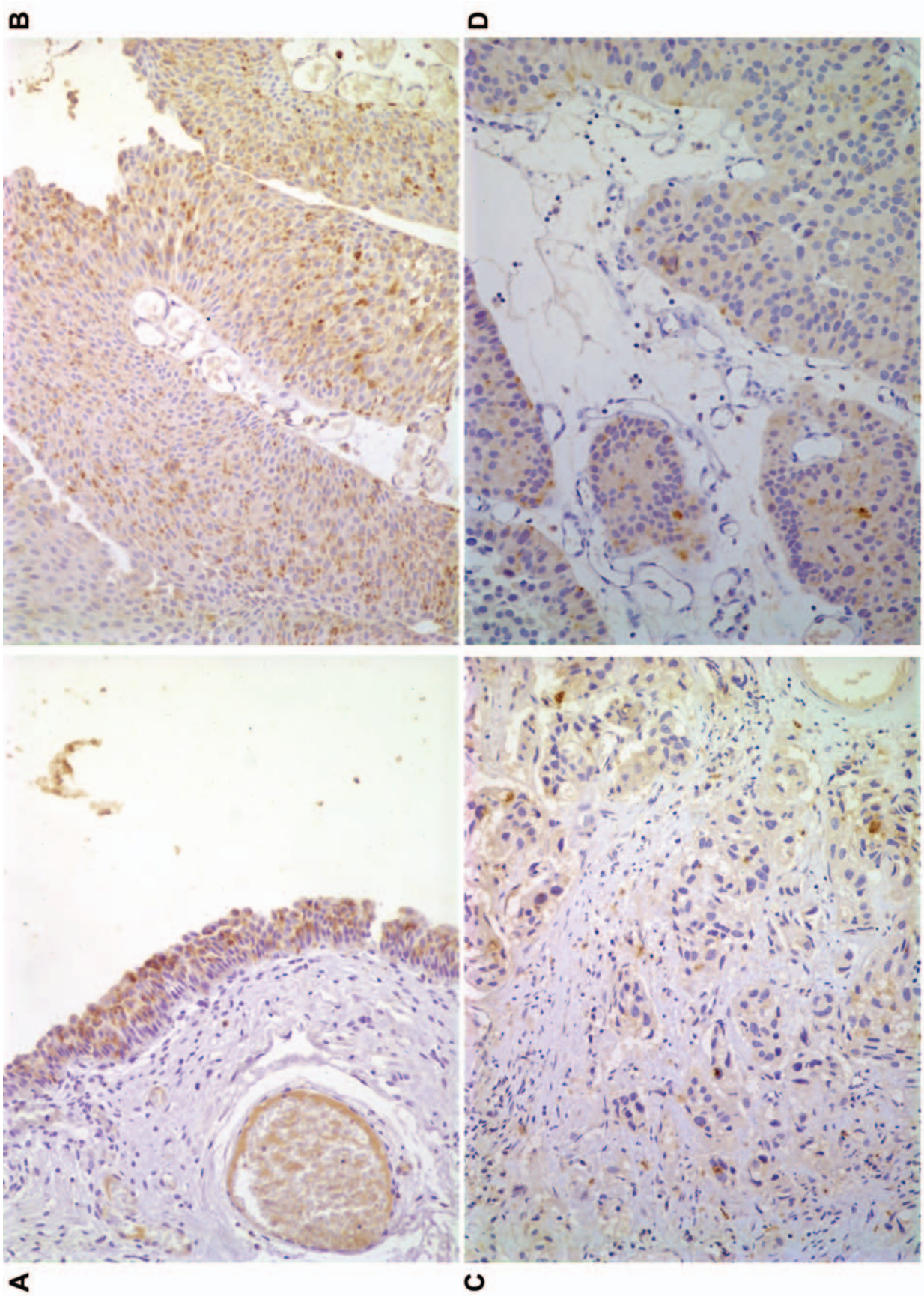

Figure 1. *VEGF-C immunopositivity in the cytoplasm of the normal urothelial cells (A) and in the cytoplasm of the malignant cells of a papillary, non-invasive urothelial carcinoma (UC) (B) and of two invasive UCs (C, D)(X200).*

Discussion

VEGF-C expression was identified in the cytoplasm of tumor cells and faintly in the cytoplasm of normal urothelial cells. In addition, stromal fibroblasts occasionally expressed VEGF-C. A previous study regarding BUC revealed VEGF-C positivity in tumor cell cytoplasm, but neither in normal cell cytoplasm nor in stromal components (13). The discrepancy between this study and the present one might be ascribed to differences in the preparation of the specimens. After a short review of the literature regarding VEGF-C immunostaining in various carcinomas, we found that although most researchers use the same antibody, others reported VEGF-C positivity in only the tumor cell cytoplasm (6, 8, 9, 12, 18, 19) and others in the tumor cell cytoplasm, normal cell cytoplasm and few stromal elements (3, 11, 20, 21), regardless of the dilution of the antibody.

In the present study, VEGF-C expression in cancer cells was found to be inversely correlated with tumor pathological stage, in the sense that it was detected more often in the superficial (pTa-pT1) than the invasive urothelial carcinomas (pT2-pT4). The direct implication of VEGF-C in invasion, if any, is virtually unknown. However, according to its lymphangiogenic implication, one would have expected VEGF-C protein to be detected more often in invasive than in superficial carcinomas. One possible explanation may be that VEGF-C expression may be an early event in the acquisition of the invasive phenotype by the malignant cell, prior to the invasive expansion. Furthermore, the fact that the normal urothelium in our specimens expressed VEGF-C further supports the possible involvement of this factor in the early stages of the acquisition of the malignant phenotype. However, our finding disagrees with that of Suzuki *et al.*, who found VEGF-C expression to positively correlate with pathological stage in urothelial carcinomas (13).

The patients with VEGF-C-positive tumors tended to have a better prognosis than those with VEGF-C-negative tumors, however there were no statistically significant differences ($p=0.1020$). The univariate analysis in the study by Suzuki *et al.* (13) demonstrated that patients with VEGF-C-positive BUCs had poorer prognosis than those with VEGF-C-negative BUC. The discrepancy between these studies might be ascribed to differences in the number of patients and to the way the specimens were obtained. Our study included 123 BUC patients who underwent transurethral cystectomy, whereas Suzuki *et al.* studied 87 BUC patients who underwent radical cystectomy.

VEGF-C reactivity was negatively linked with bax immunoexpression (Fisher's exact test $p=0.032$). The Bax pro-apoptotic protein resides in the cytosol and induces programmed cell death. This finding supports the anti-apoptotic role that VEGF-C may possess in BUC. According to literature, the VEGF-C/VEGFR-3 (FLT-4) pathway has an anti-apoptotic role (4, 22). Mutated VEGF-C, which binds to VEGFR-3 only and not to VEGFR-2, protected leukemic cells from the apoptotic effects of 3 chemotherapeutic agents (4). Removal of VEGF-C from the medium of uterine microvascular endothelial cell culture caused a marked reduction in cell number due to massive apoptosis (22). Dias *et al.*, in leukemic cells (4) as well as Vacca *et al.*, in plasma cells (5) showed that VEGF-C signaling through VEGFR-3 triggers malignant cell proliferation, a finding which failed to be demonstrated at the tissue level, since VEGF-C expression wasn't correlated with the proliferation marker Ki-67.

In conclusion, the present study suggested that VEGF-C expression in BUC might be an early event in the acquisition of an aggressive phenotype prior to angiogenesis/lymphagiogenesis and lymph node metastasis. In addition, this is the first *in vivo* study to imply that VEGF-C in BUC has an anti-apoptotic role. Ongoing investigation is needed for a better understanding of the VEGF-C/VEGF-D/VEGFR-3(FLT-4) molecular pathway in BUC.

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